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The effects of healthy ageing on glial cells in the rat spinal cord dorsal horn

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List of abbreviations 3

Abstract 5

1. Introduction 5
1.1 The clinical problem: The epidemiological impact of pain 5
1.2 Not all pain sensations are created equal 6
1.3 How are noxious inputs processed in healthy young individuals? 7
1.4 The central nervous system 9
1.5 The immune system 15
1.6 Ageing: changes in spinal glial cells 20
1.7 Summary of aged-related changes in the spinal cord 25
1.8 Hypothesis and aims 26

2. Methods 26
2.1 Rats 26
2.2 Immunohistochemistry 27
2.3 Image collection 29
2.4 Image analysis 30
2.5 Data analysis 31

3. Results 32
3.1 MEA recordings have no effect on glial cell activation in the DH of young-adult rats 32
3.2 Healthy ageing has an effect on the expression of glial cell markers in the DH 33
3.3 Healthy ageing has an effect on the expression of neuronal cell markers in the DH 38

4. Discussion 40
4.1 Optimisation of tissue selection and analysis 40
4.2 Effects of ageing on spinal cord microglia 41
4.3 Effects of ageing on GFAP labelling of astrocytes 41
4.4 Effects of ageing on spinal cord neuronal cells 42
4.5 Mechanisms associated with a decrease in cells 43
4.6 MEA recordings reveal age-related changes in DH network 44
4.7 Limitations of the study 45
4.8 What does this mean? 46
4.9 Future directions 47

5. Conclusions 48

6. Acknowledgements 49

7. References 49

8. Bibliography 63

List of abbreviations

AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AQP4	Aquaporin-4
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BSCB	Blood-spinal cord barrier
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
Cy5	Cyanine 5
DAPI	Diamidino-2-phenylindole
ddH2O	Double distilled water
DH	Spinal cord dorsal horn
FITC	Fluorescein
GABA	Gamma-aminobutyric acid
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
IASP	International Association for the Study of Pain
IBA1	Ionised calcium-binding adapter molecule 1
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL-1	Interleukin-1
Jak/STAT	Janus kinase/Signal transducer and activator of transcription
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MEA	Multi-electrode array
MGI	Mean grey intensity
mGluR	Metabotropic glutamate receptor
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NeuN	Neuronal Nuclei
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF	Nerve growth factor
NK1	Neurokinin 1
NMDA	N-methyl-D-aspartic acid
PAG	Periaqueductal grey
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PI3k/Akt	Phosphoinositide 3-kinase/Protein kinase B
PNS	Peripheral nervous system
RT	Room temperature
RVM	Rostroventromedial medulla
SEM	Standard error of the mean
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Abstract

Ageing leads to greater susceptibility to pain states, but little is known about how changes in glial cells may contribute to this. The aim of the study is to determine how healthy ageing affects glial cells in the key pain processing hub of the spinal cord dorsal horn, through comparing phenotypic features of microglia and astrocyte cell types and their distribution. Lumbar segments L5-6 spinal cord from aged (18–24 months, *n*=4, male), naïve young-adult (2–3 months, *n*=4, male), and post-multielectrode array young-adult (2–3 months, *n*=3, mixed sex) Sprague-Dawley rats were collected and processed using immunohistochemistry directed against glial cells. Our results indicate that with healthy ageing comes decreased cell numbers within the dorsal horn, and also an alteration in activation states. There is a significant reduction in IBA1+ cells with ageing, and a significant increase in area covered by astrocytes with ageing even though the mean grey intensity for both IBA1 and GFAP were not significantly different. Therefore, we conclude that glial cells do undergo changes as a part of healthy ageing. There was an impact on the number of animals and number of experiments conducted due to the COVID-19 pandemic.

1. Introduction

1.1 The clinical problem: The epidemiological impact of pain

This thesis explores the effect of ageing on pain processing. This was achieved by studying the mechanisms of pain within the dorsal horn (DH) of the spinal cord, a key region involved in pain processing, with a focus on the contribution of glial cells. To understand the changes that take place and how these influence pain processing in the elderly, it is important to have a clear account of these processes in healthy young adults.

The International Association for the Study of Pain (IASP) defines pain as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (IASP Taxonomy Working Group, 2011). The association also states that chronic pain is “pain which persists past the normal time of healing”, which can extend anywhere from one to six months, with the clinical point of diversion being three months. To add further complexity to producing a clear view of pain processing, risk factors exist that can influence the development of chronic pain, such as psychological, biological, demographic and clinical variables (Mills *et al*., 2019).

1.1.2 Pain across the globe

Looking at a global picture of pain, musculoskeletal disorders, mainly lower back pain, are a leading contributor to the development of disabilities and are also the longest lasting of all types of pain over a 15 month time period (IHME, 2021). At a minimum, 10% of

the global population experiences chronic pain, with some countries reaching figures of up to 50% across the population (Fayaz *et al.,* 2016). As of June 2021, the global population stands at 7.9 billion, which suggests that a minimum of 790 million people are currently living with chronic pain. Furthermore, the expectation is that the actual number may not only be higher than this, but is also likely to be growing (Fayaz *et al.,* 2016).

1.1.3 Pain in the UK

A study conducted by Fayaz *et al.* in 2016 reported that just under 28 million adults within the United Kingdom (UK) experience chronic pain (42.8% of the total UK population). Of the adults who are living with chronic pain, 10.4–14.3% stated that they experienced moderate to severe chronic pain to the point of it becoming disabling (Mills *et al*., 2019). In terms of demographics, chronic pain is 3 times more likely in the over 65 age band (public health survey, 2017), and based on data collected by the Office of National Statistics (2018), 18.3% of the population of the UK were over 65 years of age, and this number is projected to increase to 24.2% by 2050.

1.1.4 Pain in sub-populations

Data collected across 42 countries showed that up to 30% of young adults between the ages of 18 and 39 years experience some form of chronic pain (Mills *et al*., 2019). When controlled for age, the male population globally reports slightly higher incidences of chronic pain at 468 per 100,000, whereas the female population reports figures of 389 per 100,000 (IHME, 2021).

The relationship between age and chronic pain is difficult to measure due to differences in pain thresholds and tolerance levels among patients, alongside the impact of work and labour. However, there is a common link between increasing of age and levels of chronic pain (González-Roldán *et al*., 2020), although the incidence of dementia and other cognitive diseases increases with age, also impacting the ability to conduct pain-related research among older participants (Mills *et al*., 2019). A pooled data set revealed a trend showing increasing prevalence of chronic pain in older adults, with 14.3% of the 18–25 age group reporting chronic pain compared to the 62% in the 75+ age group (Fayaz *et al.,* 2016).

1.2 Not all pain sensations are created equal

Pain can be classified as either acute or chronic. Acute pain is short-lasting, sharp and intense, whereas chronic pain, following the earlier IASP definition, “persists past the normal time of healing”. Within this, there is nociceptive pain, which is “actual or threatened

damage to non-neuronal tissue and is due to the activation of nociceptors", and affective pain, which covers the "emotional" aspect of the original IASP definition of pain.

The pathological basis for pain also defines various subsets. Neuropathic pain is brought on by "a lesion or disease of the somatosensory system" such as trauma, diabetes, viral infections or chemotherapy, and can either be central or peripheral; it is recorded as a burning or tingling sensation (IASP Taxonomy Working Group, 2011). Inflammatory pain stems from post-injury tissue damage, arthritis, or another immunoreaction from various sources such as bites; it is characterised by redness, heat, swelling, loss of function and hypersensitivity (Vasko, 2009; IASP Taxonomy Working Group, 2011).

Building on this nomenclature, there are various symptoms/manifestations of pain. Allodynia is "pain due to a stimulus that does not normally provoke pain", and is therefore an increased pain response to a non-noxious stimulus (IASP Taxonomy Working Group, 2011). Where there is "increased pain from a stimulus that normally provokes pain" it is known as hyperalgesia, with the opposite being analgesia, the "absence of pain in response to stimulation which would normally be painful" (IASP Taxonomy Working Group, 2011). Whilst sensitisation is an "increased responsiveness of nociceptive neurones to their normal input, and/or recruitment of a response to normally sub threshold inputs", and can be either central or peripheral. Central sensitisation can manifest via reductions in threshold levels due to the recruitment of Aβ-fibres, increased responsiveness of the DH, and also an expansion of receptive fields.

1.3 How are noxious inputs processed in healthy young individuals?

1.3.1 The peripheral nervous system

The peripheral nervous system (PNS) is a collection of both sensory and motor nerves. Sensory neurones transduce peripheral events into action potentials and convey them to the central nervous system (CNS), and the PNS can be subdivided into different fibre types (Figure 1) (Crossman & Neary, 2020). There are a number of primary afferent fibre types, with three major classes of primary afferent fibres within the somatosensory system for responding to pain and touch sensations specifically: Aβ-fibres, Aδ-fibres, and C-fibres (Hatfield, 2014).

1.3.2 Aβ-fibres

Aβ-fibres are thickly myelinated with a diameter of 6–12 um, and therefore have the quickest condition velocity of the three fibre types at 35–75 m/s (McMahon *et al.*, 2013; Mihailoff & Haines, 2018). They are low threshold mechanosensitive fibres and respond to touch and pressure sensations, making them both proprioceptive and mechanoreceptive

fibres (Koop & Tadi, 2021). Of all the nociceptive fibres, Aβ-fibres make up around 18–65%; in rats specifically the figure is around 50% (Djouhri & Lawson, 2004). These fibre types mainly respond to light touch, pressure and vibration, although they do respond to some pain post-injury. This change in response is also known as a phenotypic switch. A phenotypic switch is when the Aβ-fibres which usually respond to light touch and vibration, switch their phenotype to that of nociceptive fibres seen with both the C-fibres and Aδ-fibres (Neumann *et al.*, 1996). This in turn increases and exaggerates the nociceptive sensations and is due to the expression of substance P from the Aβ-fibres (Neumann *et al.*, 1996).

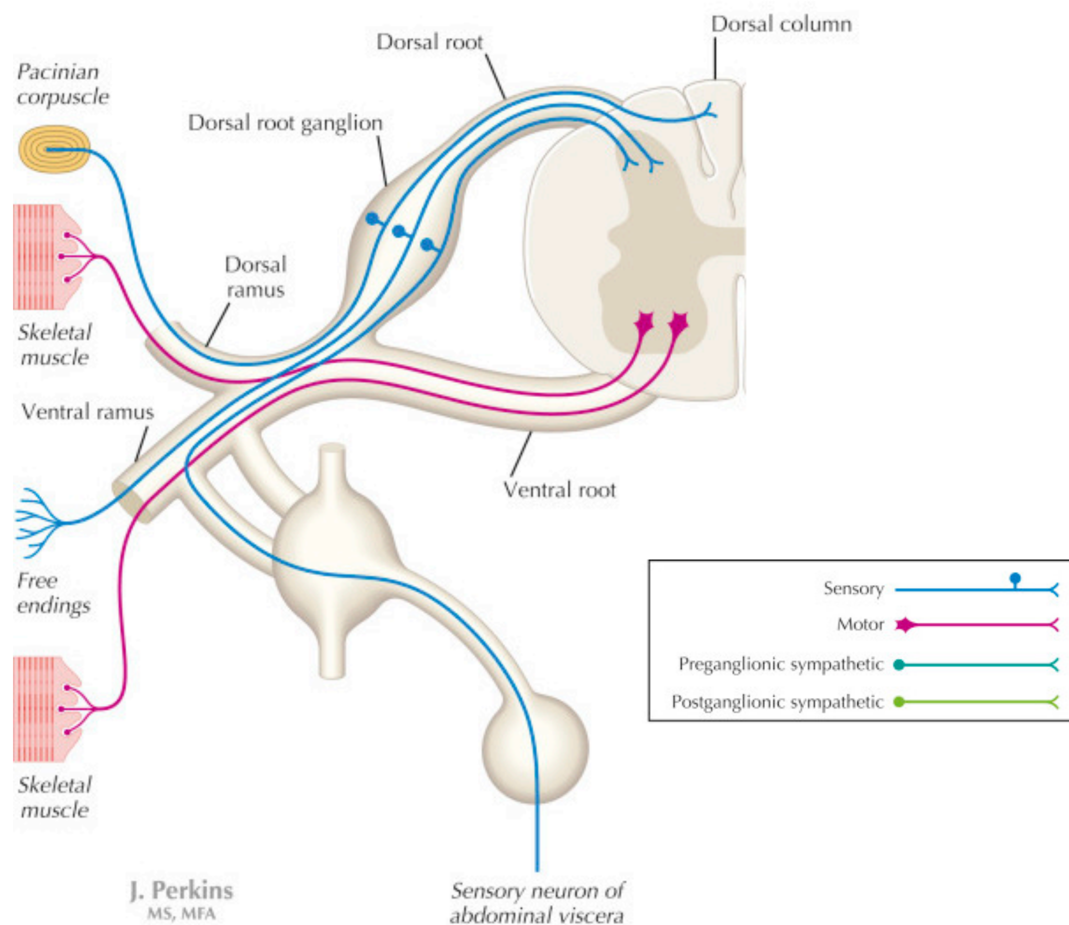


Figure 1 | <u>Afferent and efferent nerve interactions in the dorsal horn</u>

A cross section drawing of both the afferent and efferent nerve interactions with the DH which processes somatosensory inputs. Of particular interest to this study are the sensory Aβ-fibres, Aδ-fibres, and C-fibres, seen in blue (Hatfield, 2014; Felten *et al.*, 2016).

<u>1.3.3 Aδ-fibres</u>

Aδ-fibres are thinly myelinated with either low or high threshold, depending on type. They are mechanosensitive and thermosensitive, mainly processing acute pain information (Koop & Tadi, 2021). The two sub-types of Aδ-fibres are type I and type II. They have a similar diameter of 1–5um, but type I fibres have a conduction velocity of 25–55 m/s and type II have a conduction velocity of 10–30 m/s (McMahon *et al*., 2013; Mihailoff & Haines, 2018). Type I Aδ-fibres are known to be involved with the initiation and establishment of hyperalgesia, and type II are the first to respond to heat stimuli (McMahon *et al*., 2013).

1.3.4 C-fibres

C-fibres are unmyelinated and have the smallest diameter of 0.2–1.5 um, and so have the slowest conduction velocity at 0.5–2 m/s (McMahon *et al*., 2013; Mihailoff & Haines, 2018). They are polymodal, responding to mechanical, chemical, and thermal stimuli (Koop & Tadi, 2021). Much like the Aδ-fibres, C-fibres are composed of nociceptive and thermoreceptive fibre types. They respond to noxious temperature, itching, significant pressure, and due to the fact that they have the highest activation threshold of the fibres but the slowest conduction velocity, they are responsible for slow-onset long-lasting pain (Koga *et al*., 2005). It is also useful to note that while peptidergic C-fibres express substance P and tyrosine receptor kinase A, they are regulated by nerve growth factor (NGF). Non-peptidergic C-fibres express glial cell-derived neurotrophic factor (GDNF) and are also regulated by GDNF (Queme *et al.,* 2020). Although, Queme *et al.'s* (2020) recent study using groups of 3–8 week old mice highlights how an increase in GDNF can increase sensitivity to pain for the primary afferents and increase pain-related behaviours.

1.4 The central nervous system

1.4.1 The spinal cord

Following a noxious stimulus, nociceptors, thermoreceptors and chemoreceptors are part of the primary afferent fibres, so the action potential is generated in these fibres, which then go on to synapse with second order neurons in the spinal cord dorsal horn (first order neurones). From here the second order neurons in the DH, which include projection neurons, which receive input from primary afferent fibres as well as intrinsic neurons of the DH, then pass this signal on to higher brain regions, such as the parabrachial nucleus. Noxious information is passed along second order neurones through the medulla oblongata and synapse in the thalamus of the brain. Third order neurones then carry this information to the primary somatosensory cortex to be processed.

The spinal cord is the first part of the CNS to receive somatosensory information, receiving both noxious and non-noxious inputs and playing an essential role in the

integration and modulation of sensory inputs (McMahon *et al*., 2013). As shown in Figure 2, the grey matter of the spinal cord contains neurones and glia and is surrounded by white matter (Purves *et al*., 2001). The sensory fibres branch from the spinal cord out toward the rest of the body to allow complete innervation of the target regions, these are known as dermatomes (McMahon *et al*., 2013).

In rodents, the lumbar L4–6 region of the spinal cord receives inputs from the hindlimb and is the focus of the majority of studies of somatosensory processing. The dermatome for the lumbar region receives information through the femoral nerve, the obturator nerve and the sciatic nerve (Felten *et al*., 2016).

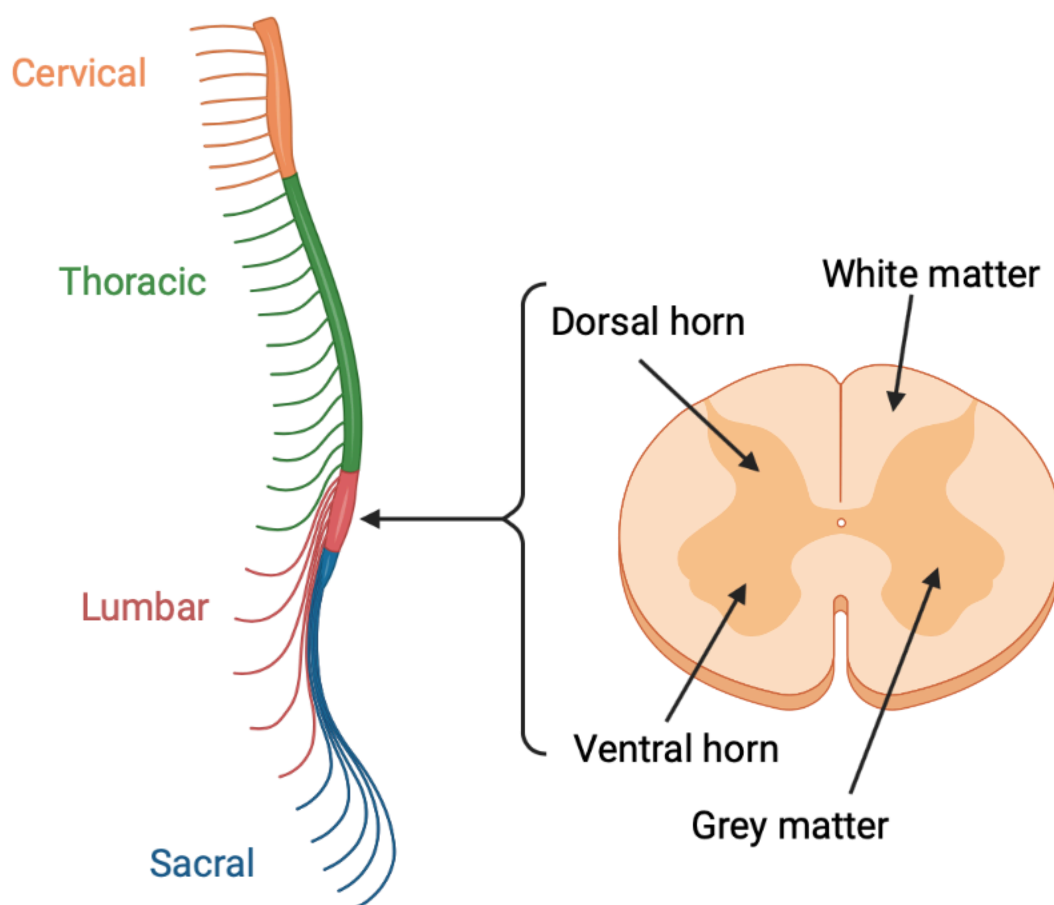


Figure 2 | <u>The spinal cord and cross section of the lumbar region</u>

This diagram highlights the major regions of the spinal cord. The cervical region connects to the brain stem, with the thoracic, lumbar and then sacral region following sequentially. Although there are some regional differences in the size and shape, all regions contain both white and grey matter and a similar overall organisation (Purves *et al.*, 2001). For this research paper the DH in the lumbar region of the spinal column is the region of interest. Diagram created with BioRender.com (2021)

1.4.2 The dorsal horn

In 1952, Rexed segmented the DH of the cat into 6 laminae based upon the size and density of neurones, and this has since been developed further in other species including the rat (Figure 3). Lamina I and IIi/o, also known as the superficial DH, are the main target areas for nociceptive primary afferent fibres and contain a higher volume of smaller cells. The substantia gelatinosa is used to refer to lamina II and this area is also the primary site for the termination of C-fibres within the DH after receiving descending input from the brain (Hunt & Mantyh, 2001). The deeper DH lamina V also receives direct input from nociceptive primary afferent fibres, and so respond to noxious stimuli (McMahon *et al*., 2013). These deeper DH laminae contain larger neurones, and also receive non-noxious inputs from myelinated afferents, and it is also the primary site for glycinergic inhibitory neurone termination which plays a key part in the descending pathway (Foster *et al*, 2015). Lamina VI has smaller, but more regularly arranged cells, with the primary afferents terminating in this region are A-fibres, some of which carry nociceptive input (McMahon *et al*., 2013; Peirs *et al*., 2020).

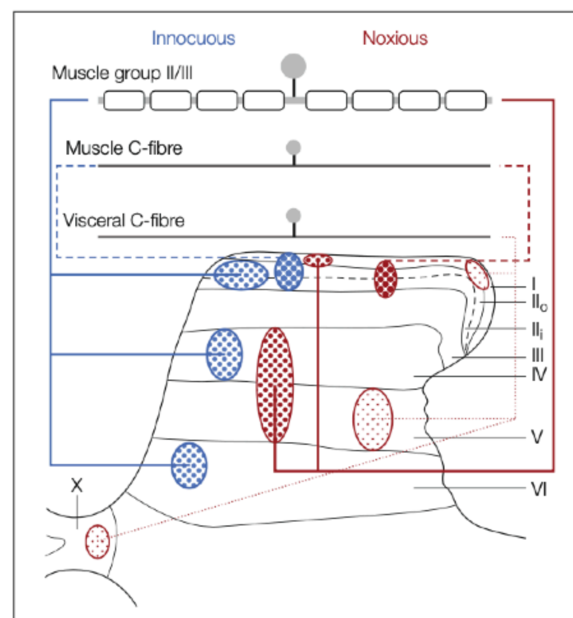


Figure 3 | Layout of the lamina segments of the dorsal horn within the spinal cord

This diagram highlights the segmental division of the DH into its laminae including the termination sites of the afferent fibre types. It highlights both the innocuous and noxious termination sites of the afferent Aβ-fibres, Aδ-fibres, and C-fibres through from lamina I to VI (McMahon *et al*., 2013).

The major neurotransmitter at all primary afferent synapses is glutamate, so all are excitatory. Some Aδ- and C-fibres, only the peptidergic population, release peptide co-transmitters such as substance P and CGRP. Glutamate interacts with ionotropic AMPA, kainate, and NMDA receptors to induce action potentials in second order neurons, but also metabotrophic (mGluRs) (Hunt & Mantyh, 2001) which have slower acting effects, in combination with peptide actions at G protein-coupled receptors (GPCRs) such as NK1R (substance P) and CL1R/RAMP1 (CGRP).

The mechanisms of pain generation and control have been the subject of many reviews (Hunt & Mantyh, 2001; Stucky *et al*., 2001; Stein *et al*., 2009). Studies of the population of cells within the rat DH show that 43% are neurones, 35% are oligodendrites, 13% are astrocytes, and 9% are microglia (Leisengang *et al*., 2020). The majority of neurones are excitatory, but inhibitory neurons, are also part of the pain pathway, use gamma-aminobutyric acid (GABA) and/or glycine as neurotransmitters. A study using male mice reported that of the DH lamina I-III cell neurones, 24–38% express GABA and glycine and therefore are inhibitory neurones (Polgár *et al*., 2013).

The number of neurones changes across the lamina segments of the DH, as shown in a study conducted on adult male Sprague-Dawley rats (Polgár *et al*., 2005). In a 150um2 ROI in 60um thick sections, a range of 22–77 neurones were counted in lamina I, for lamina II the range is far higher at 110–227 neurones present, and across lamina III the range is 85–172 neurones bilaterally (Polgár *et al*., 2005).

Imaging approaches have also been used to study the locations and distributions of the glial cells within the DH. Once microglia are activated, they are mainly found in the medial side of the DH, an area innervated by the Aδ-fibres responding to nociceptive stimuli (Tsuda, 2016). The highest number of astrocytes were shown to be located towards the superficial lamina of the DH, then the intermediate DH, and finally the lowest number of astrocytic cells were within the ventral DH (Meneses *et al*., 2017).

The cross-talk between glial cells and neurones is termed neuroimmune communication, and it is a major factor in the development and maintenance of ongoing pain (Dodds *et al*., 2016). When glial cells become activated they can alter signalling at excitatory neuronal synapses, therefore increasing the overall excitability of the DH and so contributing to increased pain signalling (Dodds *et al*., 2016). Both glial cells and neurones have similar receptors and ion channels along with transporters, also expressing cytokines and neurotransmitters that are similar (Gwak *et al.,* 2012).

1.4.3 Ascending pathways

The two main pathways in the CNS are the spinoparabrachial pathway commencing in lamina I of the DH, which is predominantly involved in the general unpleasant aspects of

pain (affective emotional responses), and the spinothalamic pathway which encodes the specific location and intensity of the pain information from the deeper neurones within lamina V. Within the spinothalamic tract, the main fibres that carry the information are Aδ-fibres and C-fibres (Gebhart & Schmid, 2013). Therefore this ascending pathway is mainly responsible for slow and lasting pain, along with thermal nociception (Al-Chalabi *et al.*, 2021). It is also thought to be important in the development of chronic and central pain due to inflammatory responses triggering hyperexcitability within surrounding neurones (Wasner *et al.*, 2008).

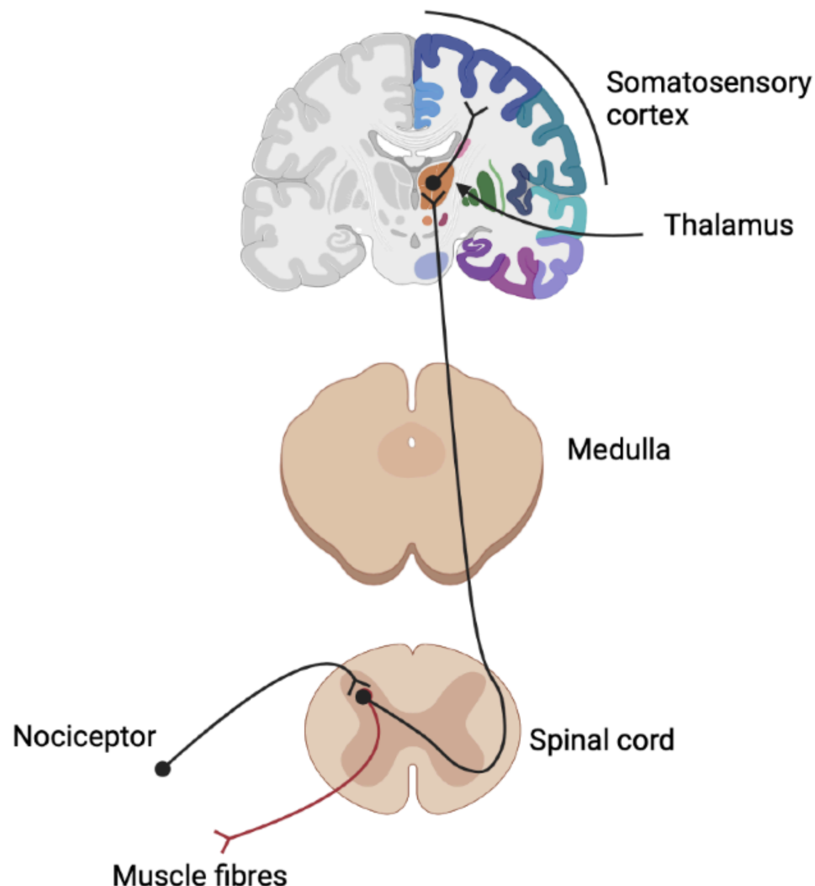


Figure 4 | <u>The ascending pathway</u>

The route of the ascending pain pathway from the nociceptor along the first, second and third order neurones to the somatosensory cortex (Gebhart & Schmidt, 2013). The route of the withdrawal reflex is shown in red from the first order neurone to back to the muscle fibres. Diagram created with Biorender.com (2021)

The spino-parabrachial pathway travels from the spinal cord up to the parabrachial area in the caudal midbrain. Specifically, it starts within the superficial laminae of the DH before becoming the lateral spino-parabrachial tract along the spinal cord (Gebhart & Schmidt, 2013). A more recent study by Browne *et al.* (2021) found that depending on which lamina the lateral spino-parabrachial tract commences from within the DH, the features of

the projection neurones change electrophysiologically and morphologically resulting in varying information being relayed to the lateral spino-parabrachial tract. Some of these differences include varying action potential frequencies and varying levels of receptors for NK1, meaning that this pathway can contribute to both acute and chronic pain (Browne *et al.*, 2021). Supporting this finding, a paper by Barik *et al.* (2021) states that the spinal projection neurones that also express NK1 receptors relaying to the spino-parabrachial tract are necessary for the onset of pain related behaviours and are stimulated by persistent pain.

As well as activating the ascending pain pathways, a noxious stimulus can also trigger a withdrawal reflex, also referred to as the monosynaptic reflex arc, from the DH to the ventral horn, which leads to the activation of motor neurones. This feedback loop is also highlighted in Figure 4 as the red motor neurone relaying information back to the muscle fibres; it is a monosynaptic or simple reflex.

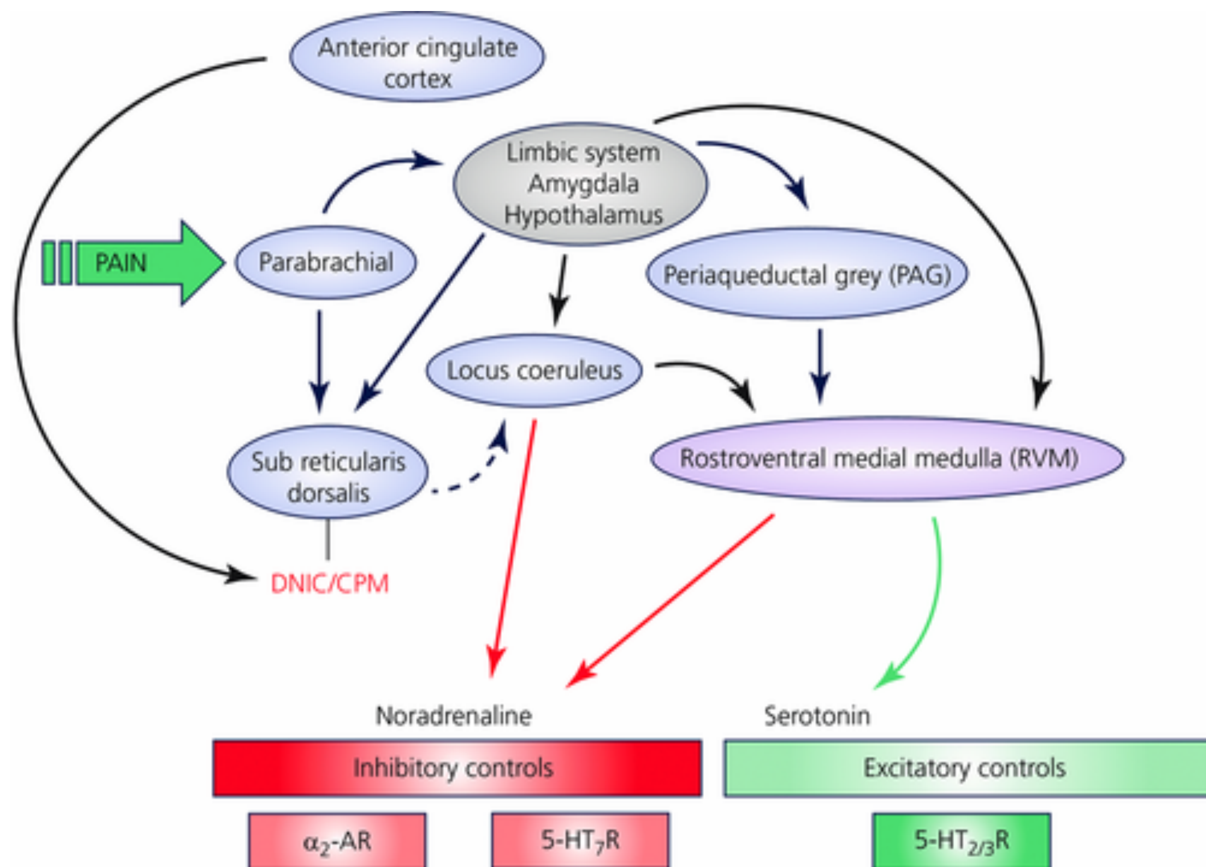


Figure 5 | <u>The regions of the brain involved in the pain pathways</u>

Starting with the pain input to the parabrachial pathway and how it leads to the amygdala and hypothalamus, then on to the periaqueductal grey and the rostoventral medulla to either create inhibitory or excitatory descending controls. Also highlighted is the involvement of the anterior cingulate cortex in the modulation of descending pain. (Bannister & Dickenson, 2017).

1.4.3 Descending pathway

The descending pathway can also inhibit or increase the nociceptive sensation by modulating DH excitability, which is known as descending control or descending facilitation, respectively (Hunt & Mantyh, 2001). The descending pathway originates within the mid-brain and brainstem, such as the periaqueductal grey (PAG). Within the forebrain are structures such as the anterior cingulate cortex (ACC), hypothalamus and the amygdala, with information from these regions being able to output directly to the descending pathways independently of the rostroventromedial medulla (RVM), as is shown in Figure 5 (Bannister & Dickenson, 2017; Chen *et al*., 2018). The RVM can be found near the midline and is part of the medulla, and can either inhibit or facilitate nociceptive sensations (Ossipov *et al*., 2014). Projections from these brain regions to the spinal cord DH, modulate pain sensations with recent studies suggesting that alterations in signalling in these pathways contributes to maintenance of chronic pain (Ossipov *et al*., 2014: Lv *et al*., 2019).

C-fibre inputs are the main targets for descending inhibition in adults, whereas A-fibre inputs are the main targets for descending inhibition in young rats, which suggests that the maintenance of chronic pain may begin with the descending pathway and why chronic pain is more prevalent within the aged demographic (Koch & Fitzgerald, 2014; Heinricher *et al*., 2008). OFF- and ON-cells within the RVM are also involved in the descending inhibition and facilitation, respectively, mediating bidirectional descending control (Heinricher *et al*., 2008). Due to this the RVM has been noted to be one of the major areas of modulating descending nociception (Chen & Heinricher, 2019). The RVM along with the locus coeruleus, located within the brainstem, both impacting descending control, means that inhibition is the tonic state within healthy adults, and facilitation develops from the switching of OFF-cells within the RVM to ON-cells (Khasabov *et al*., 2015).

1.5 The immune system

So far, the focus has been on the role of neuronal cells within pain pathways, but other cells types are also involved in pain processing and modulation. Glial cells can be subdivided into four categories: oligodendrites, ependymal cells, microglia, and astrocytes (Jäkel & Dimou, 2017). Glial cells are more prolific than neurones within the CNS, serving to support synapses and signalling of neurones, directly contributing to electrical signalling via gliotransmission (Purves *et al*., 2001).

Injury or other noxious stimuli can lead to non-neuronal cells undergoing phenotypic changes, involving alteration in morphology and expression of key genes involved in a variety of processes including inflammation. This can trigger the release of both pro- and anti-nociceptive signal molecules, which target nociceptors. Altered pain responses can either be due to changes to sensory processing centrally or to the nociceptive fibre activity

being abnormal, with both reasons leading to altered pain states (McMahon *et al*., 2013). In chronic pain states, the afferent fibres which terminate in the DH release neurotransmitters that activate pathways and receptors which results in inflammation and neuropathy, along with altering plasticity and other aspects of neural circuits, all of which leads to sensitisation and altered pain responses (Cheng, 2010).

The structural plasticity and reorganisation is affected by collateral sprouting which is the growth of axons into surrounding areas. This takes places at various locations within the somatosensory pain circuits with a direct link between the number of synapses on the lamina II neurones and hypersensitivity (Kuner & Flor, 2016). The neurotrophic factor NGFs is released from immune cells as the body's natural response to injury or infection and has a role in pain (Kelleher *et al*., 2017).

1.5.1 Microglia: Function and phenotype

Microglia have thin, dynamic processes which monitor the surrounding area for housekeeping and protective purposes (Hickman *et al*., 2018). Microglial cells move into the CNS via the pial membrane (McMahon *et al*., 2013). The function of microglial cells includes the removal of cellular debris after injury or normal cell turn-over, and the release of signalling molecules such as cytokines (Purves *et al*., 2008). Although they offer protection from injury via mediating host defence against infection or metastatic tumours based in the CNS (Hickman *et al*., 2018), it is established that their role in pain processing contributes to altered pain responses.

A recent study highlighted that stimulation of microglia leads to increased C-fibre activation (Yi *et al*., 2021). Microglial activation leads to high frequency stimulation-induced long-term potentiation (LTP) at the C-fibre synapses, which may be a mechanism by which pain states become chronic (Zhou *et al*., 2019). The study by Zhou *et al.* used young adult (8–12 week old) male adult rats, and showed that microglia have a role not only in the development of chronic pain but also in the maintenance of chronic pain states.

Microglia contribute to the hyperexcitability of the excitatory neurones, but also to pain memory (Ward & West, 2020). Structural modifications mediated by microglia have recently been linked to the onset and maintenance of pain states, and have been implicated in the loss of inhibition, abnormal sprouting of afferent fibres, and supraspinal and dendritic remodelling, seen in Figure 6 (Ward & West, 2020). However, after nociceptive stimulation microglia reduce the release of inhibitory molecules and increase the release of pro-inflammatory and excitatory molecules, which further drives pain states leading to the onset of chronic pain (Inoue & Tsuda., 2018).

Microglia undergo microgliosis upon nerve injury and can differentiate into either the harmful, classical activation state M1, or the protective, alternative activation state M2

(Popiolek-Barczyk & Mika, 2016). The physiological changes from the resting to the activated microglial form are characterised by a reduction in cellular processes branching out randomly within the DH, and they become ameboid in shape and thus more motile, with increased proliferation, increased antigen expression and secretion of pro-inflammatory molecules (Kolos & Korzhevskii, 2020). Microglia have an early and rapid response (minutes to hours) to even minor pathological changes within the CNS (Ji *et al*., 2016).

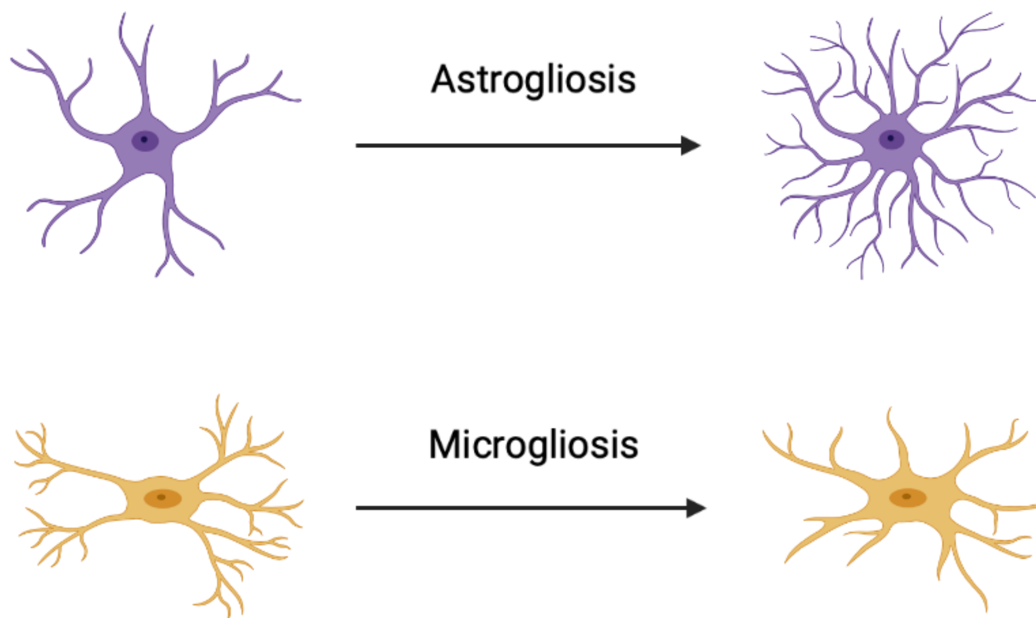


Figure 6 | <u>The morphological progression of astrocytes (top, purple) and microglia (bottom, yellow)</u>

After activation from a noxious stimulus, the microglia and astrocytes undergo morphological changes. For the microglia there is a reduction in cellular process number, and they become ameboid in shape (Kolos & Korzhevskii, 2020). For the astrocytes their cellular projections extend to touch one another (Gaudet & Fonken, 2018). Diagram created with BioRender.com (2021)

Once microglial cells have been activated, the signalling cascades that are triggered include the janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), the mitogen-activated protein kinase (MAPK) pathway, and also the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway (Popiolek-Barczyk & Mika, 2016). Microglia communicate via neuro- and gliotransmitters, cytokines such as tumour necrosis factor-a and -b (TNFa/b) and chemokines, along with glutamate and growth factors such as insulin-like growth factor 1 (IGF-1), NGF and also brain-derived neurotrophic factor (BDNF) (Matejuk & Ransohoff, 2020).

With regard to neuronal excitability, BDNF has a crucial role in the signalling from activated microglial to DH neurones and leads to neuronal excitation via N-methyl-D-aspartic acid (NMDA) receptors, causing an upward shift in synaptic excitation and ultimately an increase in action potential firing rate (Inoue & Tsuda, 2018). NGF increases the expression of BDNF in axons that innervate lamina I and II (Kelleher *et al*., 2017). There is evidence that the microglial release of TNFa regulates the activity of neurones within the DH, especially in inflammatory responses to pain or infection (Klapal *et al*., 2016).

1.5.2 Astrocytes: Function and phenotype

In a healthy state, astrocytes have minimal proliferation and non-overlapping domains of processes (Sofroniew & Vinters, 2010). One of the main functions of astrocytes is the maintenance of neuronal signalling via homeostasis of the chemical environment (Purves *et al*., 2008). They have star-like processes that extend from a cell body into the surrounding grey matter, but they can change morphology in response to injury or infection to the CNS or spinal cord, as seen in Figure 6 (Escartin *et al*., 2021). Under these conditions they can modulate synaptic plasticity, which has a role within pain states as mentioned earlier (Ji *et al*., 2006).

Astrocytes make up around 20–40% of the total number of glial cells within the CNS, and they are “coupled” via gap-junction protein complexes; both these and the non-overlapping domains alter during the reactive transition, known as astrogliosis, to become reactive astrocytes (Ji *et al*., 2019). During the control condition, astrocytes have the ability to sequester glutamate regulating nociceptive excitability under normal conditions. Evidence suggests that during chronic pain reactive astrogliosis occurs due to altered levels of glutamate and glutamine, along with pro-inflammatory cytokines such as TNFa (Ji *et al*., 2019). Synaptic glutamate is taken up by astrocytes as part of homeostasis under normal conditions (Tang *et al*., 2021); when astrocytes become activated this glutamate balance is altered and the levels of glutamate increase in neuronal synapses, which can lead to activation of NMDA receptor-mediated activity within the lamina I and II neurones (Chiang *et al*., 2012).

Figure 6 highlights the progression of glial cell activation within the spinal cord within a pain state. After a noxious stimulus astrocytes undergo reactive astrogliosis, which causes a change in function and gene expression and triggers the astrocytes to differentiate into pro-inflammatory A1 astrocytes and neuroprotective A2 astrocytes (Li *et al*., 2019). The reactive astrocyte becomes hypertrophic and there is an upregulation of glial fibrillary acidic protein (GFAP) (Sofroniew & Vinters, 2010; Refolo & Stefanova, 2019). Once the astrocytes become activated and undergo phenotypic changes the astrocytic projections extend to

touch one another, compared to the unreactive state where astrocytes form a lattice-like structure with no direct contact (Gaudet & Fonken, 2018).

Key observations of microglia and astrocytes in the healthy young-adult

	Microglia	Astrocytes
Function	Remove cellular debris after injury or normal cell turn-over Release signalling molecules such as cytokines Create protection from injury via mediating host defence	Maintenance of neuronal signalling through homeostasis Change BBB permeability once activated
Morphology	Thin, dynamic process Reduction in cellular processes once activated	Star-like processes Astrocytic projections extend to touch one another once activated
Activation process	Upon nerve injury or infection	Upon noxious stimuli
Signalling cascades post-activation	Jak/STAT pathway NF-kB MAPK pathway PI3K/Akt pathway TNFa/b Chemokines Glutamate IGF-1 NGF BDNF	NF-kB TNF-a Glutamate GFAP IL-1b NMDA

Table 1 | <u>Microglial and astrocytic functions, morphology, and activation</u>

This table highlights and compares features of microglia and astrocytes in both normal and reactive states in the healthy, young spinal cord. (Liu *et al*., 2020: Cekanaviciute & Buckwalter, 2016: Matejuk & Ransohoff, 2020: Hickman *et al*., 2018: Popiolek-Barczyk & Mika, 2016).

Neuroinflammation arising as a result of reactive astrocytes causes various physical changes, such as an upregulation of the transcription factor nuclear factor kappa beta (NF-kB) which enhances expression of mainly proinflammatory genes. GFAP positive cell

proliferation prevents axonal outgrowth but also impairs the function of the blood brain barrier (BBB), which is also further impacted by the increase of interleukin-1beta (IL-1b) (Cekanaviciute & Buckwalter, 2016).

In some conditions, both astrogliosis and microgliosis contribute equally to heightened pain states, but in others, such as chemotherapy-induced neuropathic pain, astrogliosis shows a marked increase whereas microgliosis is limited (Ji *et al*., 2019). It is also important to highlight the impact of gender on the reactivity of microglia; studies of inflammatory pain have found that microglia inhibitors are only functional in rodents (Ji *et al*., 2019). To counter this, during adulthood females have increased microglial activation whereas males experience this increased activation during early life (Han *et al*., 2021). However, the authors of this study do note that these differences could also be due to differences in hormone expression as well as environmental factors.

Toll-like receptor-4 (TLR4) is expressed on microglia within the spinal cord and the receptor is unregulated in microglia via noxious stimuli, reducing nerve injury and inducing hypersensitivity due to TLR4's affinity with the immune cells related to pain processing (Lacagnina *et al*., 2017). This process is specific to males, as it was not seen in female mice, and is thought to be related to both the higher testosterone levels present in males and differences within the immune system involvement with pain, despite similar expression of TLR4 in females and males (Lacagnina *et al*., 2017; Mogil. 2020). Once activated, the pro-inflammatory M1 microglia activate A1 astrocytes through IL-1 and TNF-a in a positive feedback loop, whereas the neuroprotective M2 communicates with A2 (Liu *et al*., 2020). Table 1 summarises the literature on glial cells within the healthy adult spinal cord, including function, morphological changes and consequences of activation.

1.6 Ageing: changes in spinal glial cells

Increased activation and reactivity of glial cells is linked to an increase in neurodegeneration (Bennett & Viaene, 2021). Aged microglial cells show slower initial reaction time to injury, but once activation has occurred it is sustained for a longer period of time (Lee *et al*., 2015). A study conducted by Parkinson *et al.* (2016) investigated the effects of ageing in the spinal cord in relation to cholesterol homeostasis. They discovered that there is an increase in activated microglia and astrocytic cells with increasing age, and suggested that an increase in cholesterol homeostasis may contribute to the altered function of glial cells within the ageing spinal cord.

Along with this increased cholesterol homeostasis, there are also marked increases in the presence of lipofuscin. Lipofuscin is an auto-fluorescent pigment which consists of cellular waste material such as protein, lipids, and metals, and is considered to be the result of oxidisation (Moreno-García *et al*., 2018). Microglia are important for the removal of

lipofuscin and accumulate these waste products, and so it was originally considered that lipofuscin is a product of ageing. However, a study by Kushwaha *et al.* (2018) suggests that this accumulation of pigment within the microglia may actually contribute to ageing. In addition to this, a study by Ma *et al.* (2013) highlighted a link between the levels of lipofuscin and the activation states of microglia, suggesting that increasing age leads to increased lipofuscin and thus an increase in reactive microglia. A recent study by Piekarz *et al.* (2020) using wild-type mice at three ages (young, 2–8 months; adult, 16–18 months; aged, 24–28 months), suggests an increase in cellular senescence, changes in blood-spinal cord barrier (BSCB) permeability and a change in metabolites with increasing age, all of which contribute to increased nociceptive pathway excitability and neuronal losses.

The changes in BSCB permeability are interesting as this is already associated with spinal cord sensitisation following injury, and leads to an increase in infiltration of T cells into the spinal cord, which plays a role in the onset and maintenance of neuropathic pain (Montague-Cardoso & Malcangio, 2021). The action of T-cells is more specifically defined by type of injury or illness and the sex of the animal (Laumet *et al*., 2019). Therefore, an alteration of BSCB permeability with age may have similar effects. An increase in cellular senescence stems from oxidative stress, and ultimately leads to an increase in pain hypersensitivity (Ding *et al*., 2017). Supporting this is evidence that an increase in endogenous products, such as nicotinamide adenine dinucleotide phosphate (NADPH), angiotensin II, and myeloperoxidase (MPO), along with the effects of exogenous inputs, such as radiation, pollution, and even cooking, results in an increase in oxidative products such as free radicals. It is suggested that these endogenous processes increase with ageing and therefore so do free radicals and so oxidative products leading to increased oxidative stress (Liguori *et al*., 2018).

In a study monitoring structural changes in rat brains at 3 months and 30 months, a decrease in microglial number but also slower motility of the microglial processes, and a reduced response time to noxious stimuli was reported with ageing (Vaughan & Peters, 1974). It has been suggested that the levels of particular TLRs associated with the ionised calcium-binding adapter molecule 1 (IBA1) found in activated microglia are similar to the levels of these specific TLRs within aged microglia, as reported in a study on rats at 3 months, 10 months, 17 months and 28 months (Letiembre *et al*., 2007). This is supported by a study conducted by Mayhew *et al.* (2019), which found that aged microglia had increased expression of pro-inflammatory cytokines and growth factors in comparison with DH microglia from young animals, with changes in GABAergic signalling, synaptic plasticity and LTP all also identified within the ageing spinal cord. This study compared young male mice (3–5 months) to aged male mice (24–32 months), and further suggested that microglia within the aged DH are more ramified and exhibit slower reaction times to injury in aged animals.

With regards to astrocytes, Jyothi *et al.* (2015) suggested changes in astrocytic morphology in aged humans (88 years of age), reporting shortening and widening of astrocytic processes in comparison to those of younger subjects (28 weeks gestation) (Figure 7). Research conducted by Clarke *et al.* (2018) also highlights the increase in GFAP expression with age within a comparative mouse model, comparing 9.5-month-old mice and 24-month-old mice, and indentifying a concomitant increase in astrocyte reactivity, including the toxic A1 astrocyte types mentioned earlier.

Both of these age comparison studies indicate a morphological and phenotypic change in both astrocytes and microglia, with ageing leading to increased reactivities. The effects of microglial senescence include a reduced capacity to maintain a standard homeostasis within the spinal cord, along with increased cell body size and IBA1 protein expression (Toedebusch *et al*., 2020).

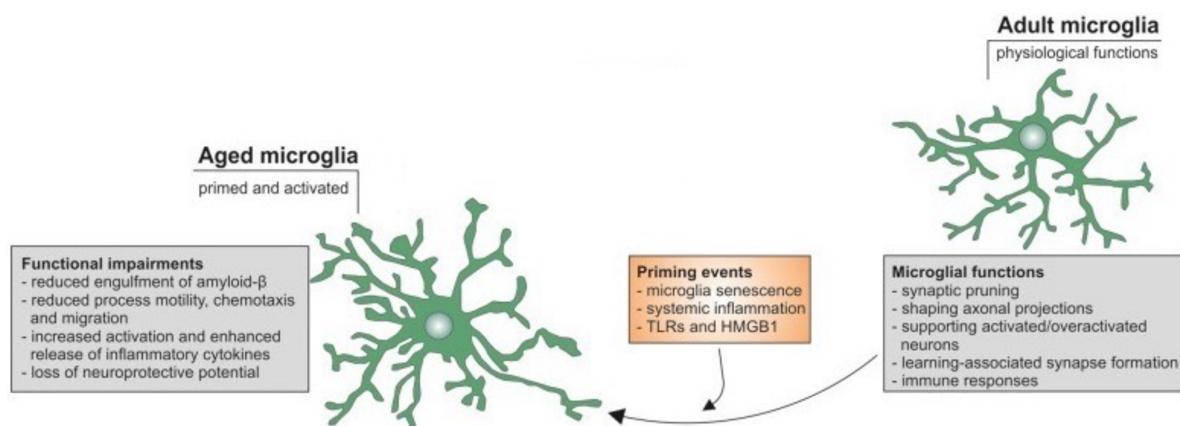


Figure 7 | Development of adult microglia to aged microglia

This diagram highlights the phenotypic and functional developments of adult microglial cells into aged microglial cells. It highlights the events that take place such as cellular senescence of microglia and the release of TLRs, along with the functional changes that cannot take place or are altered in the aged microglial cell (Spittau, 2017).

Aquaporin-4 (AQP4) expression is also seen to increase with age (Owasil *et al.,* 2020), but a report by Valenza *et al.* (2020) indicated that AQP4, IBA1 and GFAP expression were indeed all higher in aged mice (18 months) compared to adult mice (2–3 months), along with reduced clearances of artificially injected waste products within the spinal cord. The AQP channels in astrocytes play a key role in the glymphatic system, which is an efficient waste clearance system of metabolites and soluble proteins in the CNS via perivascular channels and formed by astrocytic cells, and clearance activity of this shows a marked decrease in relation to ageing (Figure. 7) (Jessen *et al*., 2015).

It is also known that glial cells play many roles in neuronal pathways. Astrocytes surrounding individual synapses regulate neurotransmission via uptake of neurotransmitters from the synaptic cleft, but also play a role in synaptic connectivity. Microglial cells also play a major role in regulation of synapses via synaptic pruning. (Um, 2017; Shimizu *et al*, 2022). As mentioned before, GABAergic signalling is altered upon activation of microglia. This, therefore, can impact the pain sensations via reduced inhibitory synapse function, GABAergic signalling, implying that much like the alterations seen with the activation of microglia, the inhibitory pain pathway is impacted due to reduced inhibitory synapse function, increasing pain states (Rozycka & Liguz-Lecznar, 2017; Comitato & Bardoni, 2021). GABA is not the only transmitter involved in inhibitory synapse signalling, glycine also plays a role. The majority of intrinsic neurones terminate within the deep lamina of the dorsal horn, which is the area associated with chronic, lasting pain as mentioned earlier with C-fibres maintaining chronic pain states (Foster *et al*, 2015).

1.6.1 Effects of ageing on pain

What is currently known about ageing humans, is the increase in the incidence of chronic pain (Fayaz *et al.,* 2016). This prevalence increases from 20% in the adult population up to 62% in the over 75 age group with reports of both reduced sensation and increased pain sensitivity in this older age group (Gao et al, 2022). There are limited preclinical studies on the mechanisms behind this change, and of those published to date there is limited consistency and agreement in results (Geltmeier & Fuchs, 2021). The work conducted in this thesis was performed to begin to address some of these inconsistencies within pain research and so it is important to first explore various papers that monitor changes in pain sensation in young-adult versus aged rats to fully understand the effects of ageing on pain.

A review paper from 2000 (Gagliese & Melzack), collected a series of studies that specifically looked at changes in nociception and pain related behaviours with ageing in the rat. Gagliese & Melzack first collect studies and results looking into changes in simple reflexive behaviours. The authors do highlight that not all the studies looking into simple pain behaviours in naïve animals have linear results and also the tests employed for this are simply based upon phasic stimulation rather than chronic pain models. However, the second part of the review collects models of tissue injury and inflammation, and neuropathic pain which is more in keeping with tonic pain. There were differing results between the papers with some showing reduced pain sensitivity and some with increased sensitisation. On top of this, various mechanisms were used to analyse the differences between ages and pain, such that different strains and ages of rats were used, some used mechanical instead of thermal stimuli or vocalisation responses. Mainly to note, is how many papers use a small number of animals per cohort, therefore reducing the strength of their results. Therefore the

overall conclusion from this, supports the notion that there is limited consistencies between papers and limited preclinical studies.

In the past two decades, some additional studies have investigated age-related differences in pain sensitivity and the development of chronic pain in rats. Geltmeier & Fuchs (2021), used behavioural tests to study pain thresholds (mechanical and thermal paw withdrawal thresholds) in young adult (3-6 months) and aged (>20 months) Sprague-Dawley rats, in the presence and absence of inflammation-induced joint pain. Interestingly, whilst control aged and young-adult rats had similar paw withdrawal thresholds, aged rats showed a smaller decrease in both mechanical and thermal thresholds following injection of an inflammogen into the knee joint, suggesting less susceptibility to arthritis-induced pain. The reported increases in pain affects despite reduced evoked pain thresholds reflects differential peripheral and central changes in aged rat pain processing, showing that both need to be considered when interpreting the data.

Bordner *et al.* (2011), examined how ageing affects cognition and locomotion identified increased anxiety-like behaviour in aged (18-24 months) versus young adult (6 months) mice. They identified increased anxiety-like behaviour and reduced locomotor activity in aged mice, which was associated with an increase in proinflammatory markers. This anxiety-like behaviour is particularly interesting given the well established link between the onset of anxiety and depression and increased responses to pain in chronic pain states (Burston, J.J. *et al,* 2019). Although they did not look specifically at glial cells, given the role of glia in modulating inflammation in neural circuits, it is possible that increased glial activation could have a role to play.

Further looking into the effects of inflammatory pain and ageing in mice, Ogbonna *et al* (2015), compared three age groups of mice: 3, 15 and 22 months. The also used monosodium iodoacetate as a model for osteoarthritis, intraplantar zymosan as a model for inflammatory pain were injected into the knee with corresponding saline controls. They conclude reduced in pain responses with induced osteoarthritis and ageing such as increased weight-bearing asymmetry along with reduced early-phase mechanical hypersensitivity, and further conclude that osteoarthrtis-induced microgliosis was reduced within the aged cohort which supports that both central and peripheral inflammatory responses decrease with ageing.

Alongside behavioural measures of pain, such as paw withdrawal thresholds, it is useful to use electrophysiological methods to enable direct sampling of neuronal activity in key sites such as the DH. This provides complementary data which can reveal neuronal mechanisms underlying altered behaviour. Anatomical techniques, such as IHC, can subsequently be used to determine how particular cell types or molecules of interest contribute to these changes, collectively allowing to develop the knowledge and understand the links behind the changes in pain.

1.7 Summary of aged-related changes in the spinal cord

The healthy aged spinal cord preclinical data, exhibits various changes that could contribute to a link between ageing and increased incidence of chronic pain states. However, reports in the literature are inconsistent, and there are multiple mechanisms at play. Table 2 highlights the main contributors to pain states and how they change within the aged DH compared to the healthy adult DH.

An increase in glial cell activation has been reported with healthy ageing, both in terms of number of cells and degree of activation, with individual cells also becoming less motile once activated meaning less interaction with synapses, affecting the LTP of inhibitory synapses and thus decreasing descending inhibition (Mayhew *et al*., 2019). There is an increase in GABAergic signalling which can either enhance or reduce pain sensation, depending on which cell types and pathways are involved (Mayhew *et al*., 2019). Impaired cholesterol homeostasis and an increase in lipofuscin are also commonly reported in neural tissues with ageing. Both mechanisms are related to impaired clearing of waste within the spinal cord, which could contribute to increased pain mechanisms due to affecting the clearance abilities of astrocytes (Piekarz *et al.,* 2020). For example, impaired clearance of waste can lead to an increase in production of oxidative molecules (Liguori *et al*., 2018), which further exacerbates glial cell activation thereby spinal excitability (Ding *et al*., 2017). Finally, BSCB barrier function is reportedly impaired the ageing spinal cord. This further exacerbates existing endogenous and exogenous processes driving increased spinal excitability and contributing to the susceptibility to chronic pain.

Key observations of ageing	Reference
Increased activation of glial cells	Piekarz *et al.* (2020)
Increased Lipofuscin	Kushwaha *et al.* (2018)
Increased GABAergic signalling	Mayhew *et al.* (2019)
Impaired cholesterol homeostasis	Parkinson *et al.* (2016)
Increase in cellular senescence and BSCB permeability	Ding *et al*. (2017)
Decrease in microglial motility	Jyothi *et al.* (2015)

Table 2 | Changes that are noted with ageing which may affect pain

This table shows a summary of the changes that are reported and which are therefore hypothesised to impact pain states in ageing spinal cord. Of particular interest is the increased glial cell activation, and microglial reactivity.

All of these factors contribute to increased spinal excitability, which can result in the central sensitisation which underlies many forms of chronic pain, therefore directly or indirectly contributing to the behavioural differences previously discussed (Bordner *et al,* 2011; Geltmeier & Fuchs, 2021).

1.8 Hypothesis and aims

Ageing leads to greater susceptibility to pain states, and it is understood that the function of the spinal cord changes with age. Although much is known about the changes taking place in the spinal cord in early life compared to adult spinal cord, the changes in the aged spinal cord are less well defined, particularly the changes in glial cells in the DH. The aim of this study is to determine how healthy ageing affects glial cells in the key pain processing hub of the DH, by comparing phenotypic features of both cell types and their distribution. In identifying these changes in glial cells, the objective is to investigate whether activation of glial cells is increased the aged rats compared to the healthy young-adult rats. The rat is used as a translationally relevant model, as similarities in the physiology and pathology aid inferences about changes in the human spinal cord (Kjell & Olson, 2016).

Hypothesis: there is a change in both the morphology and distribution of microglial and astrocytes within the DH with ageing, which likely impacts upon pain processing in aged rats.

2. Methods

2.1 Rats

All rats used in this study were of the Sprague-Dawley strain. Young-adult rats (2–3 weeks, *n*=7) were purchased from Charles River UK. Aged rats (18–20 months, *n*=4) were supplied by Eli Lilly, who had housed them from weaning. Young-adult rats were housed in groups of 4 in individually ventilated cages, aged rats were housed individually or in pairs in open-topped cages. All animals had *ad libitum* access to standard rat chow and drinking water with a 12 hour light/dark cycling in a temperature and humidity controlled environment. All studies were carried out in accordance with the UK Home Office Animal (Scientific Procedures) Act (1986), under the project licence number PB3DA999F.

Aged rats were first used for electrophysiological studies, and tissues were collected after this unrelated research was completed. Young-adult rats were either naïve (*n*=4, male) or first used for electrophysiological recordings (*n*=3, mixed sex). All of the aged animals were utilised for in vivo spinal multi-electrode array recordings (MEA) prior to tissue collection. This procedure involves anaesthetising the animal, surgically exposing the lumbar spinal cord via laminectomy, and inserting a 16-electrode array into the lumbar DH to record

local field potentials and neuronal firing across the full extent of the DH (Greenspon *et al,* 2018). DH responses to a battery of innocuous and noxious mechanical and electrical stimulations of the hindpaw were assessed over a period of hours, after which the animals were killed via anaesthetic overdose and fixed neuronal tissues collected.

Animals were killed via anaesthetic overdose, and transcardially perfused with saline followed by 4% paraformaldehyde (PFA). Lumbar spinal cord was collected and post-fixed for 24 hours in PFA, washed in PBS, then cryoprotected in PBS containing 30% sucrose and 0.05% sodium azide. 40um sections of L5–6 spinal cord were cut on a freezing microtome (Leica SM2010 R sliding microtome, Leica Biosystems), and stored free-floating in phosphate buffer containing 0.05% sodium azide.

The COVID-19 pandemic and associated restrictions on normal working practice limited the number of experiments I was able to complete within the year, and therefore the amount of data it was possible for me to collect. Some of the set-backs I experienced were: delayed animal and reagent delivery, reduced access to the lab, including experimental training, leading to increased time for acquisition and optimisation of the IHC protocol, and reduced access to imaging facility.

2.2 Immunohistochemistry

6–8 spinal sections per animal, were immunolabelled for markers of microglial (IBA1), astrocytes (GFAP) and neuronal cells (NeuN), via established IHC protocols. Sections were first washed thoroughly to remove sucrose, and then antigen retrieval was performed via incubation in citrate buffer (10mM with 0.05% Tween20) 90 degrees Celsius for 20 minutes in a water bath (Optima GD100 series stirred, Grant Instruments: Cambridge). The sections were removed from the water bath and allowed to cool for 5 minutes, then placed into corresponding marked wells, and washed twice with PBS for 5 minutes each. All fluorescents and antibodies for each study are shown in Tables 3 and 4.

Non-specific binding was blocked via incubation in 10% horse serum (H0146, Sigma-Aldrich: Missouri, USA) in PBS, with 0.3% Triton X-100 for tissue permeabilisation (1 hour, room temperature (RT)).

To explore expression of glial cell markers, sections were incubated with a mixture of primary antibodies including rabbit anti-GFAP (1:1000, Z0334, Dako: Denmark) and goat anti-IBA1 (1:1000, NB100-1028, Novus Biologicals: Colorado, USA), 4°C overnight. For the Neuronal Nuclei (NeuN) experiment, the primary antibodies were replaced with mouse anti-NeuN primary antibody (1:1000, MAB377, MilliporeSigma: Massachusetts, USA). All other protocol steps remained the same. Control incubations omitting primary antibodies were run in parallel to assess tissue auto-fluorescence and non-specific secondary antibody background labelling.

Primary antibodies used for immunohistochemistry

Study	Cell type	Target	Species	Supplier	Catalogue number	Dilution	Specificity
Pilot and main	Microglia	IBA1	Goat	Novus Biologicals	NB100-1028	1:1000	Using IHC with primary antibody omitted controls, there was reduced IBA1 signalling (Kwok *et al.*, 2021)
Pilot and main	Astrocytes	GFAP	Rabbit	Dako	Z0334	1:1000	Using GFAP knockout mice, there was no positive labelling of GFAP (Eng *et al.*, 2000)
Neuronal cell	Neuronal cells	NeuN	Mouse	Millipore Sigma	MAB377	1:1000	Using NeuN to monitor neuronal cells, primary antibody omitting controls contained no NeuN signalling (Krzisch *et al.*, 2015)

Table 3 ǀ The primary antibodies used for the immunohistochemistry

This table summaries the primary antibodies used for each IHC study for this research paper, including the supplier, dilution used and the specificity.

Sections were washed thoroughly in PBS (5x5 mins) and then incubated with the appropriate secondary antibody combinations. For the glial labelling experiment, the secondary antibodies were donkey anti-rabbit 647 (A-31573, Invitrogen: Massachusetts, USA) and donkey anti-goat 488 (A11055, Invitrogen), for the neuronal labelling experiment the secondary antibody was donkey anti-mouse 647 (A31571, Invitrogen), all 1:500 dilution in 5% horse serum in PBS, two hours at RT.

The sections were washed thoroughly again (PBS, 3x5 mins). To enable quantification of total cell counts and delineation of laminar boundaries, cell nuclei were then

labelled via brief incubation with diamidino-2-phenylindole (DAPI) in PBS (1:500 dilution, 10 mins RT, B1098-5, 76331-610, VWR Avantor: Pennsylvania, USA). Excess DAPI was removed via washing (PBS, 3x5 mins).

The PBS was removed from the wells and replaced with ddH2O before mounting. All sections were mounted onto gelatinised slides, air-dried for 10 minutes and cover-slipped using Fluoromount aqueous mounting medium (SLCD9458, Sigma-Aldrich: Germany). After 30 minutes the slides were sealed with clear varnish.

Fluorescent secondary antibodies used for immunohistochemistry

Raised in	Raised against	Fluorescence channel	Supplier	Catalogue number	Dilution
Donkey	Goat	488	Invitrogen	A11055	1:500
Donkey	Rabbit	647	Invitrogen	A-31573	1:500
Donkey	Mouse	647	Invitrogen	A31571	1:500

Table 4 | <u>The fluorescent secondary antibodies used for the immunohistochemistry</u>

This table summaries the corresponding, fluorescent secondary antibodies used for each IHC study for this research paper, including species, fluorescence channels and catalogue numbers.

<u>2.3 Image collection</u>

Slides were imaged on a 200M microscope (Carl Zeiss AG: Germany) using Micro-Manager 1.4 software (Edelstein *et al*., 2014). DAPI, Fluorescein (FITC), and Cyanine 5 (Cy5) filter sets were used. Regions of interest (ROI) were selected from the superficial DH using 10x0.3, 20x0.8 and 40x1.3 NA objective lenses and a CoolSNAP MYO camera. Figure 8 below shows the individual channels of DAPI, FITC, and Cy5 for DAPI, IBA1 and GFAP labelling. The splitting of channels allows for easier analysis of cell numbers and distribution. Both standard labelled and secondary only control sections were imaged to highlight natural differences between individuals within the same group, but also individuals across groups. This allowed for a subtraction of the background fluorescence to be removed from the data sets, and also highlighted the difference between the lipofuscin and cell labelling within the aged sections.

40x images were used with a field of view selected to include the superficial DH but omit the white matter border, preventing brightly labelled white matter astrocytes obscuring labelling of astrocytes in the grey matter.

2.4 Image analysis

Images were analysed via ImageJ (Schindelin *et* al., 2012) by a blinded investigator. For each animal, one slide containing 6 sections was imaged with a minimum of 3 sections imaged per slide. On superficial DH was imaged per section, and mean data calculated for each animal for comparison between age groups. For 20x images, all analyses were performed on a fixed-size ROI box (150umx150um), 40x image sets used the entire field of view. In 20x images, which contained highly auto fluorescent white matter and blank areas, the ROI was aligned individually for each image over the superficial DH grey matter under the white matter border, to ensure consistency of the region measured over all images. Total cell counts, and labelling intensity for each marker were monitored as the primary outcomes. DAPI positive nuclei were quantified to provide measures of total cell counts and cell density in both the standard condition sections and also the secondary antibody only sections. IBA1+ cells and NeuN+ were individually counted within the ROI to determine proportional cell populations in the 20x image sets. Assessment of fluorescence intensity and labelling area was also performed for DAPI, IBA1, GFAP and NeuN. The mean grey intensity (MGI) for each marker was calculated using ImageJ (Schindelin *et al.*, 2012), in 20x images within specific ROIs, this was also conducted on the secondary antibody only control sections for comparisons. Image acquisition settings were identical for all images that were compared.

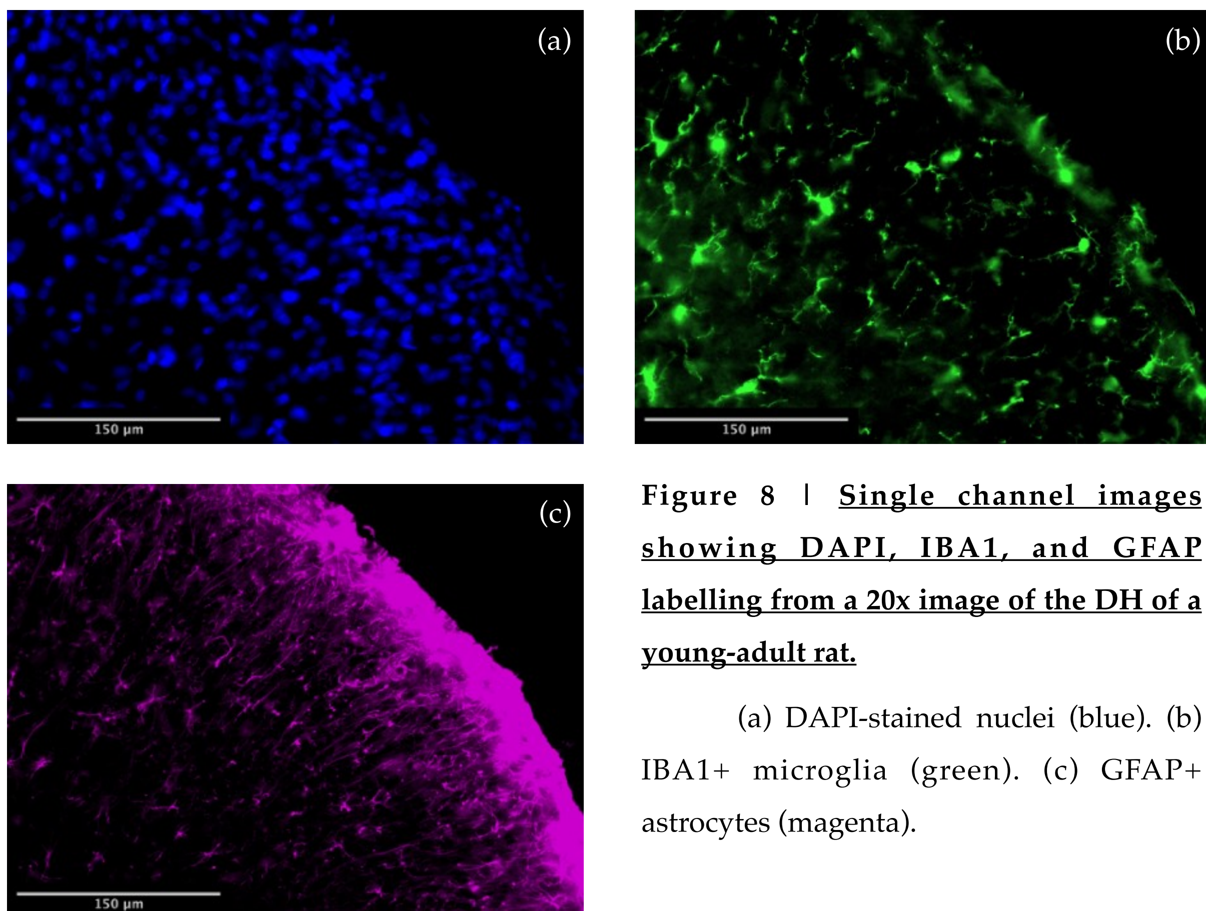


Figure 8 | <u>Single channel images showing DAPI, IBA1, and GFAP labelling from a 20x image of the DH of a young-adult rat.</u>

(a) DAPI-stained nuclei (blue). (b) IBA1+ microglia (green). (c) GFAP+ astrocytes (magenta).

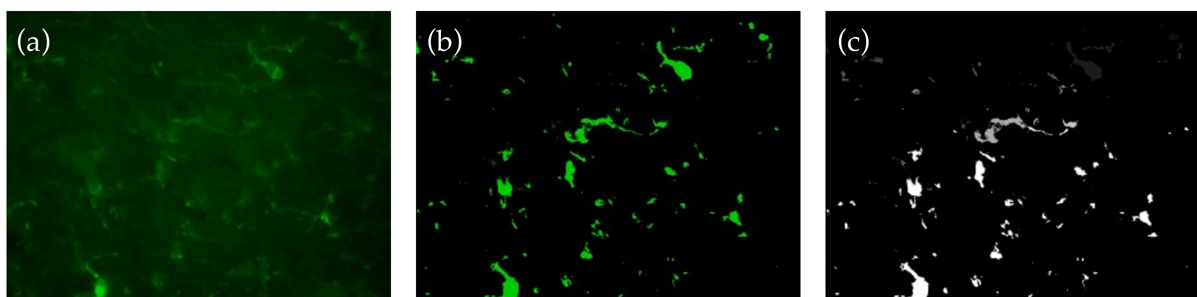


Figure 9 | Progression of an image to obtain the area covered data

All images are of the same 40x image of an adult rat DH for the main study of young-adult rat vs aged rat. These images show the progression from a standard, one channel image (c, left), then to a thresholded image using "Triangle ignore black" (b, middle), and then the count mask (c, right) showing all structures analysed.

For the area covered by IBA1 and GFAP, the 40x image set was used. Area measurements were performed on binary images produced by auto-thresholding algorithms in ImageJ. A subset of representative images was selected and all available auto-thresholding methods trialled to determine which optimally separated specific signal from background for each channel. Auto-thresholding was used over set thresholding for optimisation of binary images. This allowed for accurate and non-bias results across all images set to the same threshold. "Shanbhag thresholding ignoring black" was used on GFAP, and "Triangle thresholding ignoring black" used on IBA1. This step can be seen in Figure 9, where the original image (9a) is thresholded to create a binary image (9b). From here the "analyse particles" function was used to measure the total area of positive labelling for each marker after thresholding. All particles were counted. Count masks were created to be able to visually inspect the thresholding was identifying the correct structures, this final step is seen in Figure 9c. The data recorded were total labelling area and percentage labelling area.

2.5 Data analysis

The data collected were analysed with Prism 9.2 (GraphPad). Separate files were used for each experiment and for 10x, 20x and 40x images. Dataset normality was first tested with the Shapiro-Wilk normality test assuming Gussian distribution of data. If the data passed the normality test, a 2-way ANOVA was used to analyse grouped data, with Šídák's multiple comparisons post-hoc test to compare each cell mean between the age groups. If some or all of the data did not pass the Shapiro-Wilk normality test, then parametric unpaired T-tests were run comparing the two age/treatment groups for the same cell type. Two-tailed T-tests were used due to having a non-directional hypothesis. If there were obvious outliers in the data then a Grubb's test was run to determine whether the data from

that individual could be removed before analyses were run. Some data sets were normalised between the young-adults and aged rats so that more thorough comparisons could be made. The mean values for each test are stated with their corresponding standard error of the mean (SEM). The value for significant differences is set to <0.05 or >95% confidence. Significant differences are denoted on graphs as follows: * p<0.05, ** p<0.01, *** p<0.001.

3. Results

3.1 MEA recordings have no effect on glial cell activation in the DH of young-adult rats

Aged tissue is a valuable resource and so to maximise the data collected from each animal, aged rats were used for electrophysiological recordings prior to tissue collection for anatomical work. These were MEA recordings and involved the insertion of electrodes into the spinal cord and activation of the tissue to record neuronal activities, therefore the activation of surrounding glial tissue prior to tissue collection could have an effect on the glial changes monitored in the young-adult versus aged rat study. A separate cohort of naïve young-adult rats were used to specifically compare glial activation states in naïve young-adult rats and post-MEA young-adult rats. IBA1+, GFAP+ and DAPI+ cell counts, cell percentages, and MGI readings were collected and analysed for this study. Two representative images (Figure 10), show the naïve group versus the post-MEA recording group, also for comparison are the secondary antibody only control sections. Cell count, cell percentage and MGI data are provided in Figure 11.

To determine the effect of MEA recordings on the expression of glial cell markers. MGI values were measured for GFAP, IBA1 and DAPI labelling (Figure 11a). The mean and SEM values for DAPI for naïve rats were 1863±195.9 and for the equivalent post-MEA group of rats was 1432±88.5. For IBA1, MGI values for the naïve group were 809.8±28.6 and for the post-MEA group 516.3±252.6. Finally, for GFAP, the MGI values for the naïve group were 1240±57.8 and for the post-MEA group 1190±177.7. There were no significant differences between the two age groups.

The number of both DAPI+ cells were comparable between naïve rats (98.0±3.6) and the post-MEA group (79.3±10.5). There were no significant differences between the two groups (Figure 11b).

The number of IBA1+ cells were expressed as a percentage of the number of DAPI+ cells within the same individual to determine the proportion of total cells which were microglia for the naïve and post-MEA groups. The mean percentage and SEM were as follows: naïve group 18.3%±1.6%, and for the post-MEA group 22.6%±2.3%. There were no statistically significant differences between the two groups (Figure 11c).

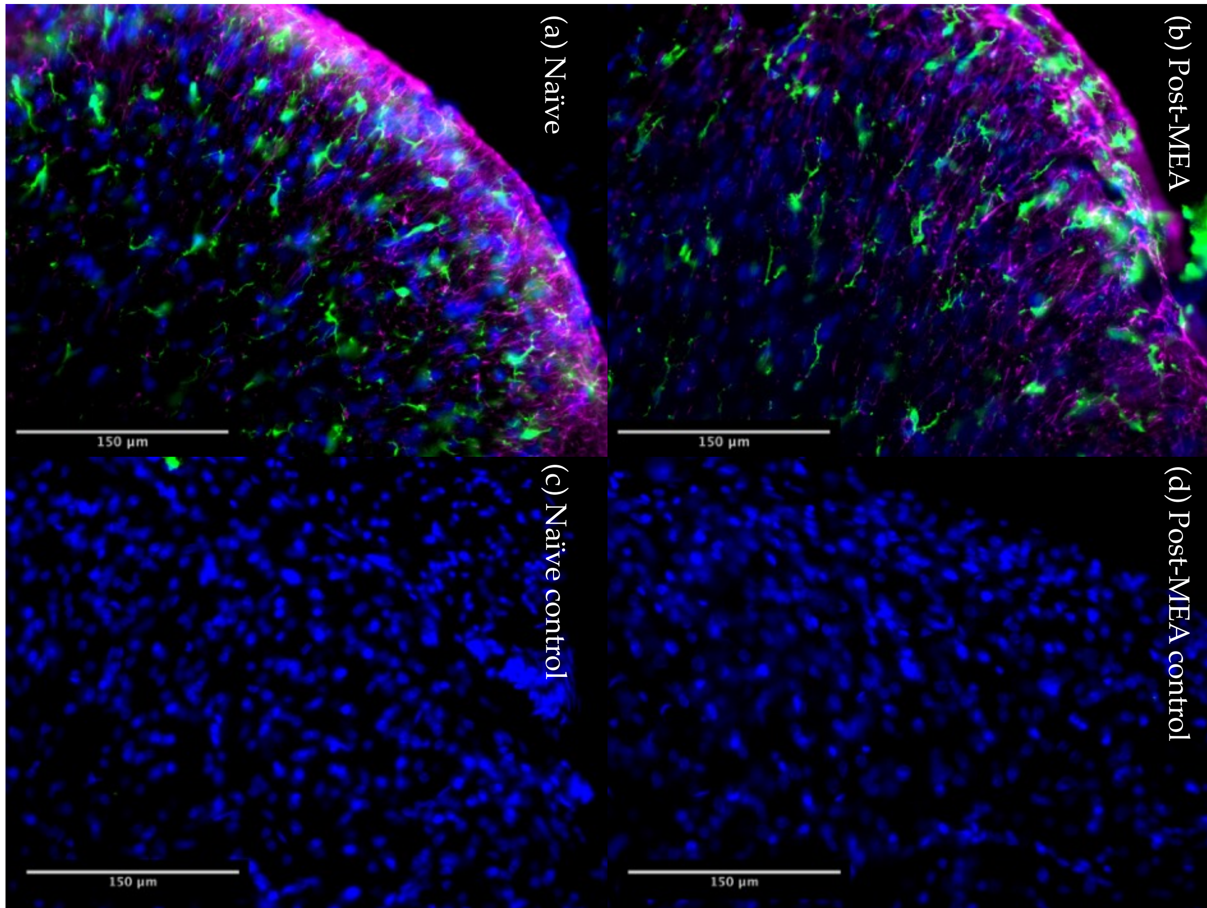


Figure 10 | Example images of the dorsal horn from the naïve rat vs post-MEA rat, both young-adult

Probed sections using IHC techniques to label for glial markers, label conditions on images, microglial IBA1 (green), and astrocytic GFAP (magenta). The sections were then counter-stained using DAPI (blue), to highlight nuclei.

Composite images (20x magnification) of the DH of a naïve young-adult rat in the top left (a) and post-MEA recording young-adult rat in the top right (b). Also shown are the corresponding secondary only controls for each condition (naïve bottom left (c), post-MEA bottom right (d)). Scale bar=150um.

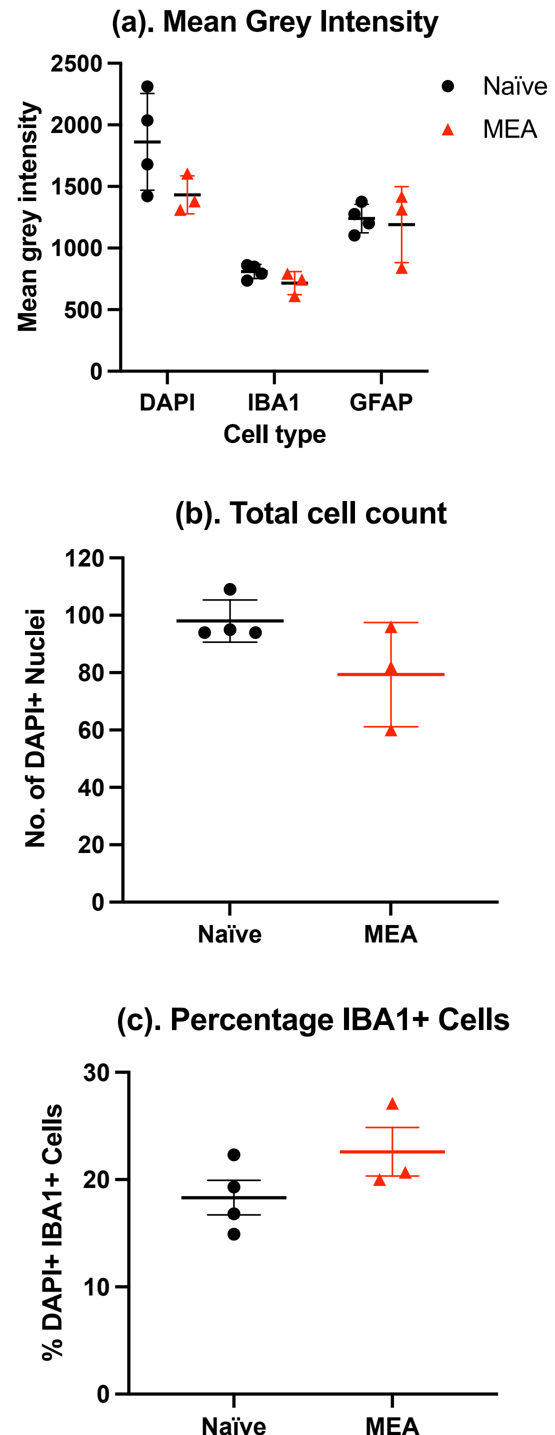
3.2 Healthy ageing has an effect on the expression of glial cell markers in the DH

Glial cell morphology, number and fluorescence intensity were measured for IBA1, GFAP and DAPI in sections from young-adult rats and aged rats collected following MEA recordings. Cell counts and MGIs were measured for IBA1+ and GFAP+ cells. Figure 12 shows representative images, also for comparison are the secondary only antibody control sections from the young-adult and the aged cohorts. Also presented are group data for cell count, cell percentage and MGI in Figure 13.

Figure 11 | Comparison of cell populations in the dorsal horn between naïve rats (*n*=4, male) and rats which underwent MEA recordings in the spinal cord dorsal horn (*n*=3, mixed gender):

Black circles represent individuals from the naïve group and red triangles represent individuals from the MEA group. (a) MGI data for DAPI, IBA1 and GFAP within a region of interest frame on a 20x image. There were no significant differences between the groups. The data analysed via 2-way ANOVA and then Šídák's multiple comparisons test. (b) Cell count of DAPI+ cells within a region of interest on a 20x image. There were no significant differences between the naïve and post-MEA groups. Data analysed via a two-tailed T-test. (c) The percentage of DAPI+ cells that are also IBA1+ cells within a region of interest on a 20x image. There were no significant differences between the two groups. The data for this was analysed by using a two-tailed T-test.

MEA recordings do not impact upon markers of microglia or astrocytes in the DH of the spinal cord.



The average MGI for DAPI was 2173±211.3 for the young-adult group and 2090±145.6 for the aged group. The average MGI values for IBA1 was 268.4±135.3 for the young-adult group and 243.0±72.9 for the aged group. Finally, the average MGI for GFAP was 1548±149.4 for the young-adult group and 1554±106.1 for the aged group. There were no significant differences for DAPI, IBA1 or GFAP between the two age groups (Figure 13a).

The total area was measured on binary 40x images for IBA1+ and GFAP+ labelling, image processing is shown in Figure 14. For IBA1, the mean and SEM values were 969.6±135.5 for the young-adult and 653.6±215.5 for the aged group. For GFAP, there was a

statistically significant increase (p=0.0067) between the area covered in the young-adult group (10,580±1094.1) compared to the aged group (13,789±534.0). The results from this analysis can be seen in Figure 13b.

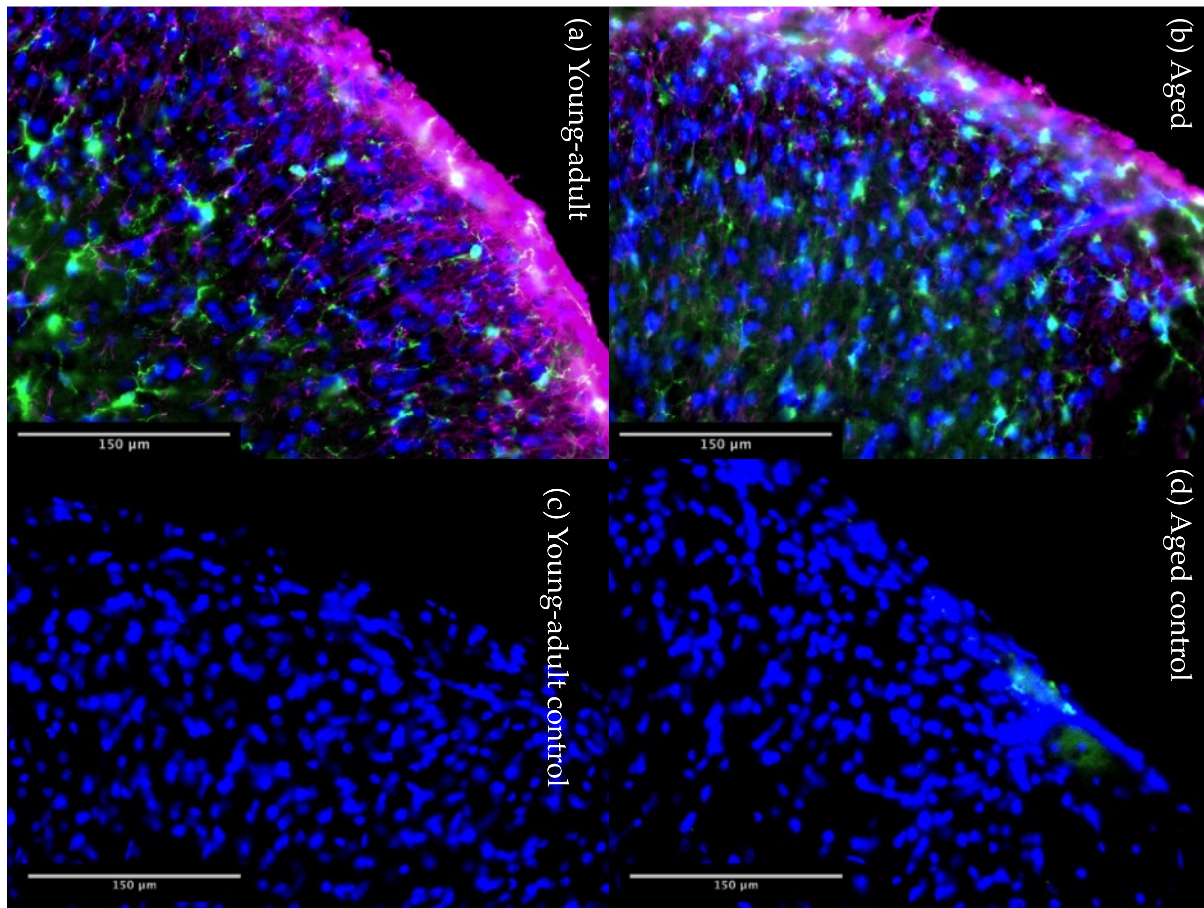


Figure 12 | Example images of the dorsal horn from the young-adult rat vs aged rat

Probed sections using IHC techniques to label for glial markers, label conditions on images, microglial IBA1 (green), and astrocytic GFAP (magenta). The sections were then counter-stained using DAPI (blue), to highlight nuclei.

Composite images (20x magnification) of the DH of a young-adult rat in the top left (a) and an aged rat in the top right (b). Also shown are secondary only controls for each condition: young-adult rat in the bottom left (c), and aged rat in the bottom right (d). Scale bar=150um.

The number of DAPI+ cells was comparable between the young-adult group (48.6±4.2) and for the aged group (44.9±4.9). There were no significant differences between the aged and young-adult groups (Figure 13c). Due to this we were able to normalise the IBA+ cell data to the DAPI+ data between the age groups to allow for a stronger comparison.

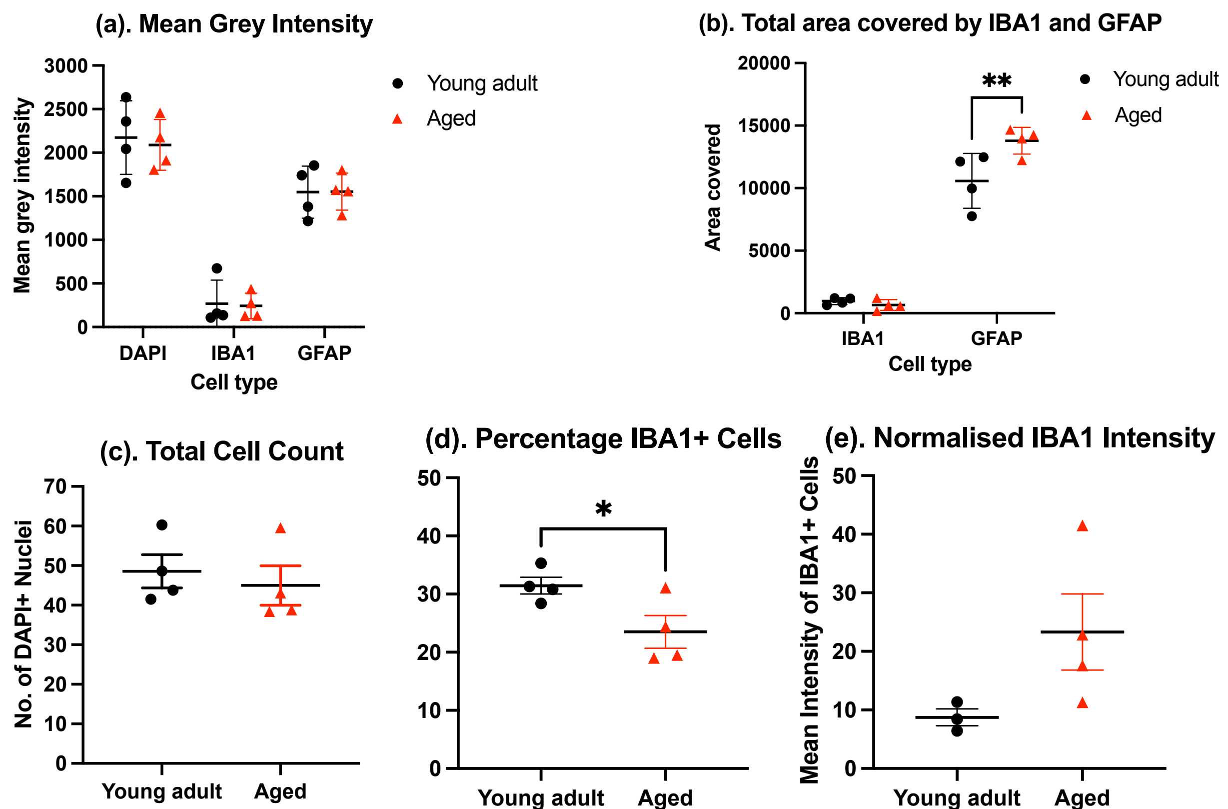


Figure 13 | <u>Comparison of glial cell populations in the spinal cord dorsal horn between young-adult rats (2–3 months, *n*=4, male) and aged rats (18–24 months, *n*=4, male):</u>

Black circles represent individuals from the young-adult cohort and red triangles represent individuals from the aged cohort for all graphs. (a) MGI values for DAPI, IBA1 and GFAP within a region of interest on a 20x image. There were no significant differences between the young-adult and aged groups. Data analysed via 2-way ANOVA and then Šídák's multiple comparisons test. (b) Area covered by GFAP or IBA1 within a region of interest on a 40x image. There was an in the increase of GFAP area coverage from young-adult rats up to aged rats of statistical significance (p= 0.0067). Data was analysed via 2-way ANOVA and then Šídák's multiple comparisons test. (c) Cell count of DAPI+ cells within a region of interest frame on a 20x image. There were no significant differences between the two groups. Data was analysed via a T-test. (d) The percentage of DAPI+ and IBA1+ cells within a region of interest on 20x images. There was a statistically significant difference between the young-adult and aged rats showing a decrease in the percentage of IBA1+ cells (p=0.0453), post t-test. (e) The normalised IBA1 intensity. There was not a significant difference between the two groups.

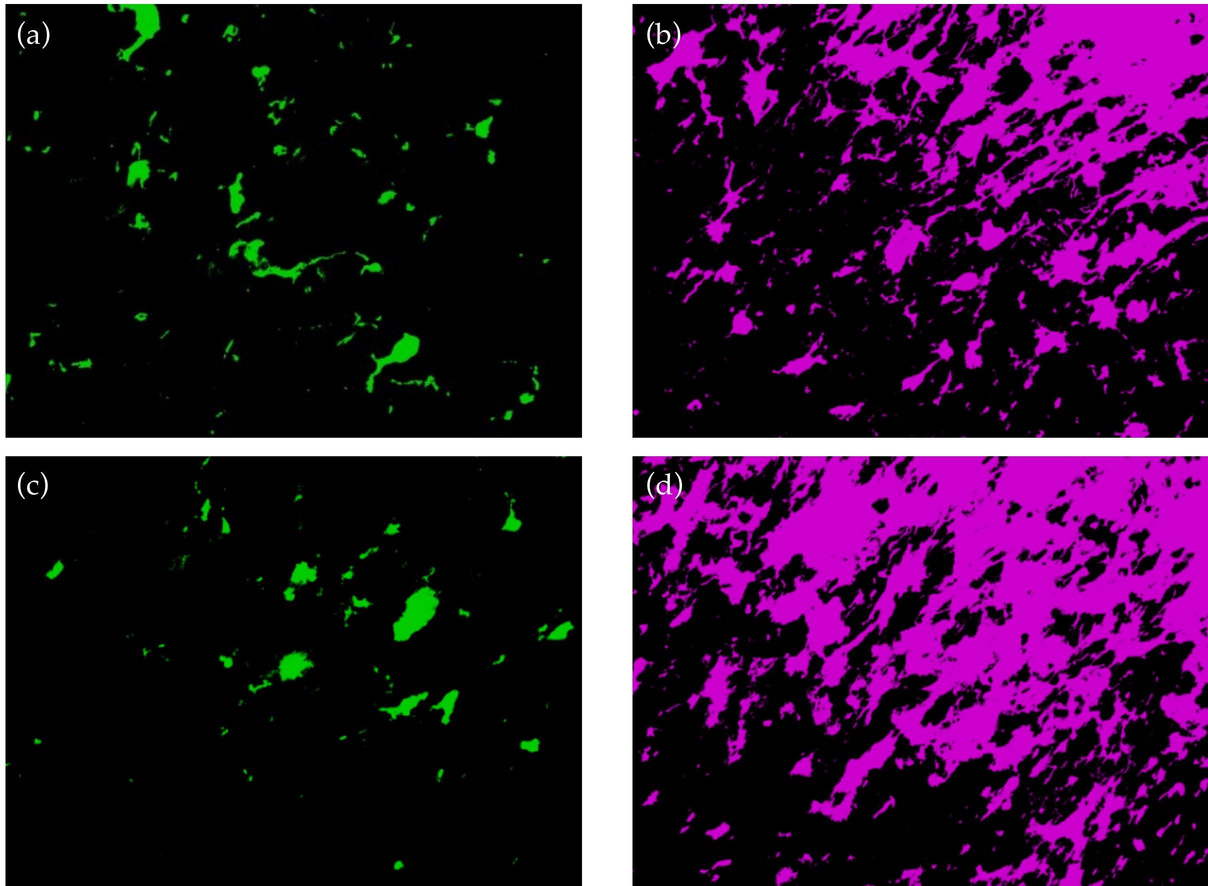


Figure 14 | Representative images from a young-adult rat (top) and an aged rat (bottom)

Fluorescence IHC using antibodies against IBA1+ (green) and against GFAP+ (magenta). Images are from a young-adult rat DH (a, b) and from an aged rat DH (c, d). The region of interest was adjusted on the camera and angled to be just within the DH excluding the white matter border. Notice the similarity in coverage for the left images showing IBA1 (a, c), but how the cells in the young-adult are smaller and of greater number, whereas the cells within the aged are larger, rounder and fewer in number. The right images showing GFAP (b, d) also reveal that the labelling areas for GFAP+ astrocytes are far larger and the processes are reaching further into the DH within the aged individual.

The percentage of DAPI+ cells that were also IBA1+ was analysed. The mean value for the young-adult group was 31.5%±1.4% and statistically significantly higher than the value for the aged group 23.5%±2.8% ($p=0.0453$) (Figure 13d).

Due to numbers of microglial cells varying between fields of view, IBA1 labelling intensity was normalised to IBA1+ cell counts to provide a measure of the average labelling intensity of individual microglial cells within both the young-adult rats and the aged rats. An outlier in the young-adult group was identified using Grubb's test, and removed from the analysis. There were no statistically significant differences.

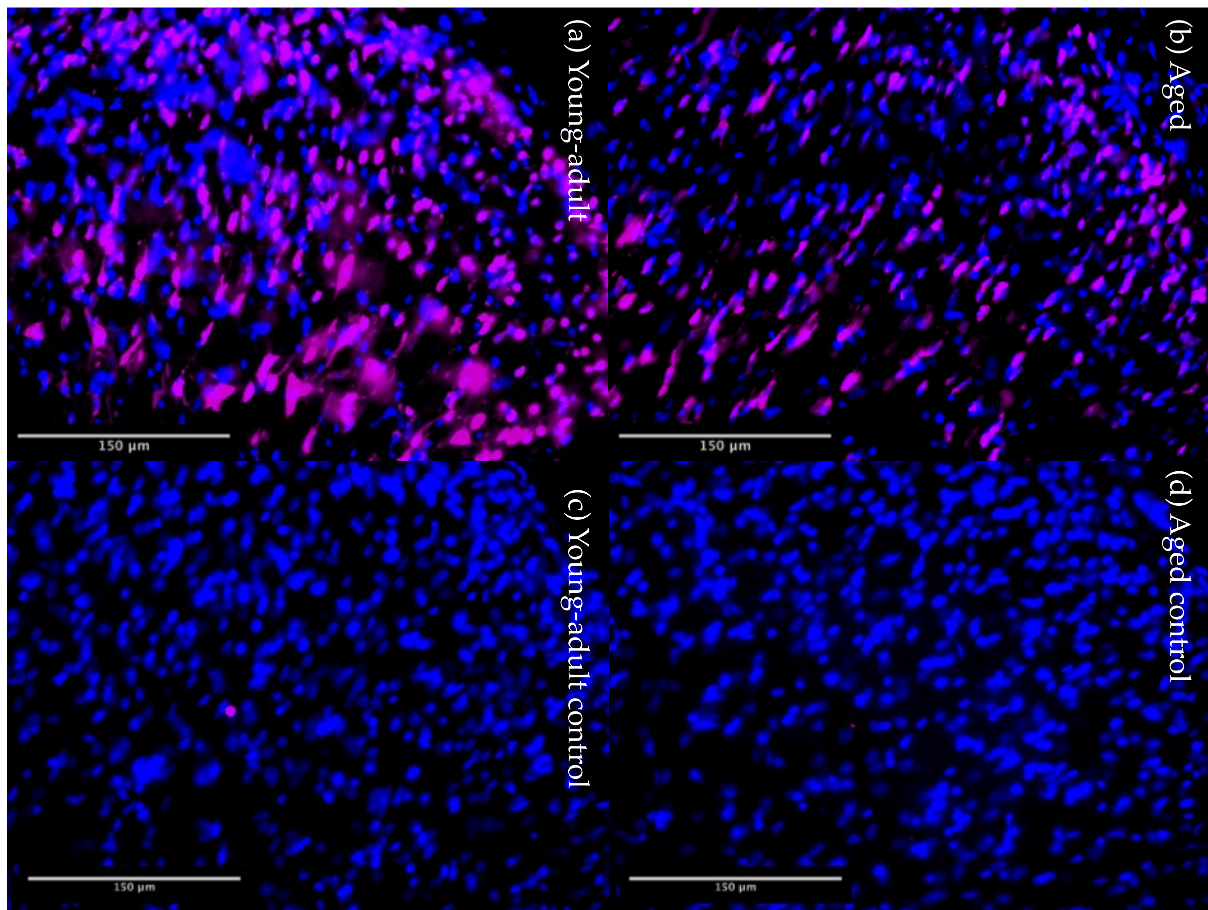


Figure 15 | Example images of the dorsal horn from the young-adult rat vs aged rat

Probed sections using IHC techniques to label for glial markers, label conditions on images, NeuN+ Neuronal cells (magenta). The sections were then counter-stained using DAPI (blue), to highlight nuclei.

Composite images (20x magnification) of the DH of a young-adult rat in the top left (a), and an aged rat in the top right (b). Also shown are secondary only controls for each condition: young-adult rat in the bottom left (c), and aged rat in the bottom right (d). Scale bar=150um.

3.3 Healthy ageing has an effect on the expression of neuronal cell markers in the DH

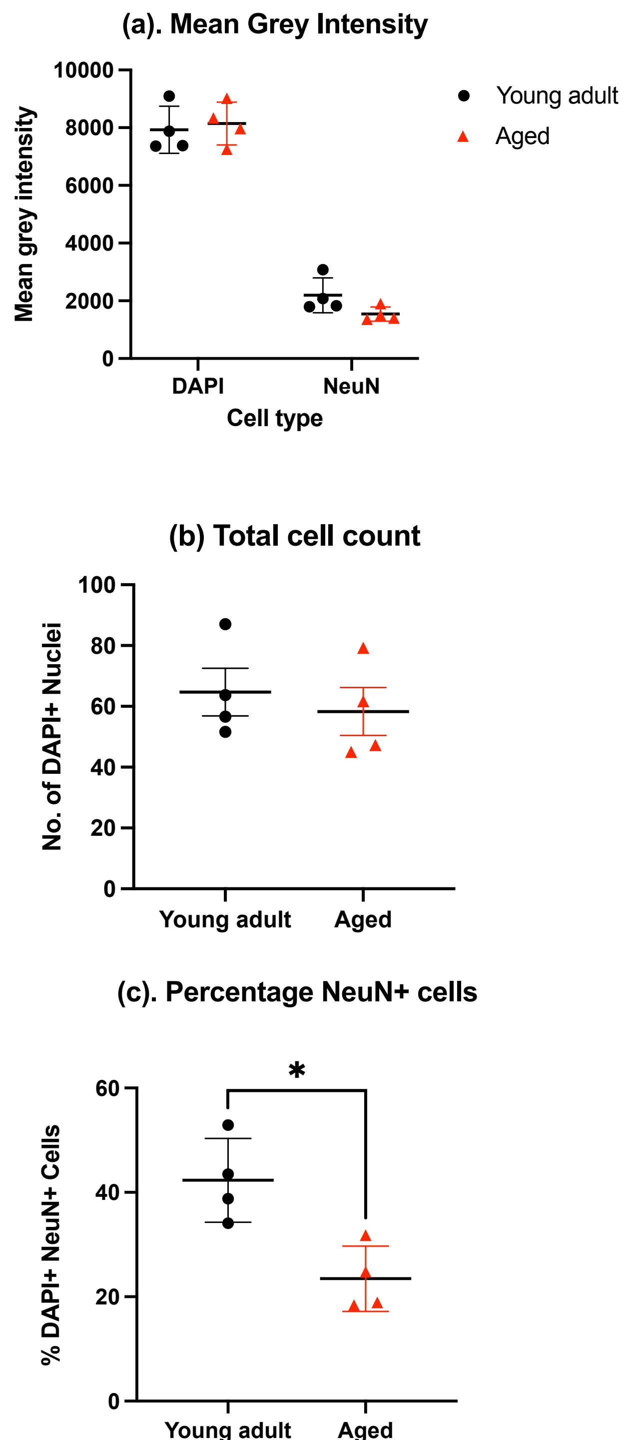
An additional study was run to quantify neuronal cell density within the DH to allow for further analysis of changes within the DH that may take place as a part of healthy ageing and thus lead to increased pain states. Figure 15 shows composite images at 20x magnification, also for comparison are the secondary antibody control sections. Cell count, cell percentage and MGI group data are provided in Figure 16.

The MGI mean value for DAPI for the young-adult group was 7929.0±407.8 and for the aged group they was 8146.0±368.9. The MGI values for NeuN for the young-adult group

was 2195±302.1 and for the aged group they was 1546±122.8. There were no significant differences between the two groups (Figure 16a).

Figure 16 | Comparison of neuronal cell populations in the spinal cord dorsal horn between young-adult rats (2–3 months, *n*=4, male) and aged rats (18–24 months, *n*=4, male):

Black circles represent individuals from the young-adult cohort and red triangles represent individuals from the aged cohort for all graphs. (a) MGI values for DAPI and NeuN within a region of interest on 20x images. There were no significant differences between the young-adult rats and the aged rats. Data analysed via 2-way ANOVA with Šídák's multiple comparisons test. (b) Cell count of DAPI+ cells within a region of interest on 20x images. There were no significant differences between the two groups. The data was analysed via two-tailed T-test. (c) The percentage of DAPI+ cells that are also NeuN+ cells within a region of interest on 20x images. There was a statistically significant difference between the young-adult group and the aged group with a decrease with healthy ageing (p=0.0286). Data was analysed via a two-tailed T-test.



The mean number of DAPI positive nuclei in the young-adult group was 64.7±7.8, whilst in the aged group the values were 58.3±7.9. There was not a significant difference between the two groups, as is shown in Figure 16b.

The relative proportion of neuronal (NeuN+) cells was calculated as a percentage of the number of DAPI+ cells within the same individual. The mean percentages for the young-

adult group were 42.3±4.0% and for the aged group the mean percentages were 23.5±3.1%. There was a statistically significant decrease between the two groups (p=0.0286), as is seen in Figure 16c.

4. Discussion

The major findings of this study were that in healthy aged male rats aged 18 months there was a significant decrease in the number of IBA1+ microglia in the DH, accompanied by an increase in the DH area covered by GFAP+ astrocytes along with a decrease in the number of NeuN+ cells, indicating a loss of neurones compared to young-adult matched male rats. Here we compare our data with that of matched studies within the spinal cords monitoring glial cells and neuronal cells. These data highlight the effects that ageing has on the predominant populations of cells present in the dorsal horn of the spinal cord. Interestingly this study identified a significant reduction in IBA1+ cells in aged rats alongside a significant increase in area covered by astrocytes with ageing.

4.1 Optimisation of tissue selection and analysis

The study of aged rats is hindered by both the availability of animals and the housing for long periods of time. In order to use aged rats in this study, it was necessary to make use of a cohort with prior experimentation using MEA recordings of evoked responses of spinal cord neurones. The MEA method involves anaesthesia, surgery, and temporary implantation of an electrode array, all of which could cause local inflammation and therefore glial cell activation. To quantify any potential impacts from the MEA recordings, a study was undertaken to compare cell populations between young-adult naïve rats and a matched age group of rats used for MEA recordings, which also underwent exposure to anaesthesia and stimulation of the hind paw.

Overall, the data revealed negligible effects of MEA recordings on glial cell number, with no statistically significant differences for any of the data analyses comparing naïve rats to post-MEA recording rats. These data indicate no effects of MEA recording method upon the glial cell number, ruling out any overt direct effects of the MEA protocol prior to tissue collection on the cell populations in the aged rats following MEA recordings.

This finding corroborates earlier work by Lind *et al*. (2012), which also suggests that the implantation of electrodes into rat brains causes little to no aggravation of the tissue response and limited glial scarring. However, Gulino *et al.* (2019), suggest that there can be local physical damage caused by the insertion of electrodes which leads to neural degeneration and an inflammatory response to a foreign object. The Gulino study also builds on the idea that physical damages can lead to a loss of blood flow and therefore reduce oxygen and nutrient supply to the local area of insertion. This loss of oxygen and nutrient

supply in turn could then dampen the inflammatory response from glial cells within the spinal cord. Therefore, whilst there are many potential mechanisms by which MEA recording methods may alter the local area of insertion, evidence suggests these are unlikely to have a significant impact on the glial cell population. On this basis, we were able to proceed to analysis of the spinal cords from the aged rats following MEA recordings.

4.2 Effects of ageing on spinal cord microglia

For the young-adult versus aged rat study, the comparison of microglial cell counts as a percentage of DAPI+ cells revealed a significant reduction in the percentage of IBA1+ cells in the DH of the spinal cord in the aged rats, compared to the young-adults. However, analysis of the MGI for IBA1 did not reveal any difference between the two groups, suggesting that the morphology of the microglia may differ between the two age groups.

Previous work by Von Leden *et al*. (2017) quantified IBA1 in spinal cord of naïve 3 month old (*n*=4) and 12 month old (*n*=4) male Sprague-Dawley rats. They reported increased microglial IBA1 expression at 12 months compared to 3 months age. Although the study by Von Leden *et al*. (2017) quantified their microglial presence differently to our experiments, the similar outcomes permits a positive comparison. Both Toedebusch *et al*. (2020) and Valenza *et al.* (2020) show that the activation of microglia leads to increased expression of IBA1 proteins. Additionally, a report by Hovens *et al*. (2014) conducted an experiment on young naïve rat microglial activation within the spinal cord. They highlighted the percentage of activated microglial cells to be around 30%, which matches the percentage data collected from our young-adult group data for percentage of IBA1+ microglial cells to DAPI+ cells, and so the robustness of our results were partially corroborated by Hovens *et al*. (2014). In our data, we found a statistically significant decrease in the number of IBA1+ cells with healthy ageing, however little to no change in the IBA1 expression as monitored through the MGI and also little to no change in the area covered by IBA1+ cells with healthy ageing. In the context of the surrounding literature, our results suggest that although a decrease in microglial cells with ageing is likely, there is an increase in the expression of the IBA1 protein. This can be deduced through IBA1 expression being maintained across the age groups with a decrease in the number of cells. This implies that the expression per cell has increased, and due to increased expression of IBA1, we can deduce that there might be an increase the reactivity of microglial cells.

4.3 Effects of ageing on GFAP labelling of astrocytes

There was a significant increase in the dorsal horn area covered by GFAP labelling in the aged rats compared to the young-adult rats. Astrogliosis leads to increased GFAP protein expression (Sofroniew & Vinters, 2010; Refolo & Stefanova, 2019) and so various

studies have used GFAP as a surrogate marker for identifying astrocytic cells. The GFAP expression levels recorded from the MGI between the young-adult group and the aged group were not statistically significantly different. Along with a statistically significant increase in area coverage supporting the notion that there was an increase in the astrocytic processes size with healthy ageing. This suggests that whilst astrocytic processes did increase in the area coverage within the dorsal horn region. We do know that cellular processes lengthen naturally with healthy ageing, however perhaps this data suggests that these cells did undergo astrogliosis, which explains the increase in area covered, however as we removed the substantia gelatinosa region from our analysis, perhaps the increase in GFAP expression is instead seen within the astrocytic bodies rather than processes. Another point to mention which would improve future experiments, is the data collection to gather the number of astrocytes. This project was only able to collect 2D images which can only present area covered by GAFP+ cells, whereas a 3D image would be able to separate out these cells for easier analysis of GFAP expression, growth and number.

Previous studies have established a link between astrogliosis and the area covered by astrocytic processes (Escartin *et al*., 2021). The consequences of increased astrocytic processes within the DH: reduced clearance of waste materials and reduced homeostatic maintenance of the chemical environment (Tang *et al*., 2021). This reduced homeostasis then causes an increase in pro-inflammatory markers which may increase cellular firing (Purves *et al*., 2008) potentially effecting neuronal cells within the DH (Brenner, 2014). In chronically high GFAP expression levels reduced homeostasis can even become fatal (Brenner, 2014). Additionally, reactive astrocytes are known to be able to modulate synaptic function and plasticity (Ji *et al*., 2006). This alteration in synaptic function, once GFAP expression has increased, could lead to LTP and altered pain states due to increased excitation (McCall *et al*., 1996). With an expansion of astrocytic processes along with the astrocyte-synapse interaction being dynamic, one single activated astrocyte can contact increased numbers of synapses further affecting the activation states within the DH region (Chung *et al*., 2015). Furthermore, the translational relevance of our results have been corroborated by a study conduced on human tissue highlighted the GFAP expression within the spinal cord and the results mirror the results found in this study both with expression levels just below 15,000 AU within a similar region of interest size (Yang & Wang, 2015).

4.4 Effects of ageing on spinal cord neuronal cells

There was a statistically significant decrease in the percentage of NeuN+ to DAPI+ cells in the aged rats compared to the young adults rats. The numbers of NeuN+ cells in the young-adult rats was comparable to an earlier report by Sardella *et al*. (2011). However, there were no statistically significant changes in the MGI values for NeuN between the two age groups. The NeuN marker highlights the neuronal nuclear antigens within the nuclei of

neurones within the CNS (Gusel'nikova & Korzhevskiy, 2015), which was why it was chosen for our study.

Neuronal cells are affected by the activation states of the surrounding glial cells (McCall *et al*., 1996), which could potentially lead to a loss in neuronal cell numbers within the DH with ageing. The neurones in the spinal cord are both project neurones and interneurones and although we did not identify the neurone type, it is plausible that the loss of neurones reflects a loss of the smaller more vulnerable interneurones. As many interneurones are inhibitory, the consequences of a loss of inhibitory interneurones could lead to facilitated pain responses (McMahon *et al*., 2013).

The data sets from this final study monitoring the neuronal cells within the DH, indicate the effects of healthy ageing on pain states. There was a statistically significant reduction in NeuN+ cells with healthy ageing for cell percentage, having a p-value of less than 0.05. This difference implies that neuronal NeuN was still being produced at the same rates with healthy ageing. However, there were no statistically significant changes of the MGI values, and so little to no change within the expression of NeuN between the two age groups. This data from our study suggests that a decrease in the number of neuronal cells with ageing is likely, and due to no significant differences in the MGI values that there is a similar level of NeuN+ expression within the DH. Overall, the data shows us that there was a statistically significant decrease in the percentage of NeuN+, with a similar expression of NeuN MGI, indicating that there is an increase expression per cell ratio with ageing. Which in turn suggests that the expression of the NeuN marker increases per cell with healthy ageing, altering pain sensations.

From this data we have collected the number, area and intensity of NeuN+ cells. To further this, the study could group these neurones into excitatory interneurones and inhibitory interneurones, giving a further idea of how the changes with ageing would affect the pain sensations. Inhibitory interneurones are GABAergic and glycinergic, and so to identify the number, area and intensity of inhibitory interneurones, further IHC tests could be run to signal for GABA and glycine expression. However, it is also possible to differentiate inhibitory and excitatory neurones during MEA, where tonic firing indicates inhibitory interneurones with delayed firing indicating excitatory interneurones (Mayhew *et al*, 2019).

4.5 Mechanisms associated with a decrease in cells

The decreases in cell number are likely to reflect increased rates of cellular senescence, which is known to increase with age (Ding *et al*., 2017). This is further supported and even shown that the pathway for cellular senescence also leads to greater autofluorescence levels seen within microglia, astrocytes and neuronal cells and so supports the data collected in this study (Sikora *et al*., 2021). The onset of the senescence pathway is

also linked to oxidative stress, and both endogenous means (such as NADPH, angiotensin II, and MPO) along with the effects of exogenous inputs (such as radiation, pollution, and cooking) having a direct link with an increase in pain hypersensitivities, directly affecting the ageing population (Ding *et al*., 2017; Liguori *et al*., 2018). Cellular senescence and increased BSCB permeability not only affects the glial cells along with the number of neuronal cells (Piekarz *et al.,* 2020).

Cascades such as the Jak/STAT pathway, NF-kB, MAPK pathway, PI3K/Akt pathway, TNFa/b, chemokines, glutamate, IGF-1, NGF, and BDNF can be triggered by activated microglia, and some are contributors to cellular apoptosis or senescence (Popiolek-Barczyk & Mika, 2016; Matejuk & Ransohoff, 2020).

Similarly, there are cascades which can respond to activated astrocytes and these include NF-kB, TNF-a, Glutamate, GFAP, IL-1b, and NMDA, again these cascades are also contributors to the cellular senescence pathways (Cekanaviciute & Buckwalter, 2016). Increased neuronal excitability leads to greater glutamate release but the astrocytes are in an increased activation state and less likely to take up the glutamate. Higher levels of glutamate can also cause neuronal senescence (Ji *et al*., 2019; Tang *et al*., 2021; Chiang *et al*., 2012). Although as stated, activation and increase in expression can also lead to further senescence through expression cascades in microglia and astrocytes, it is still unclear as to whether there is an increase in astrogliosis.

To build on this study monitoring changes in glial cells, also monitoring the changes in signalling mechanisms which are related to a decrease in cells would help explain the results collected. This could be done through measuring gene expression of inflammatory pathways spoken about above which affect the glial cell number, this is known as transcriptomics. Conducted in both young-adults and aged rats, comparing the up- or down-regulation of these pathways, done through microarrays, RNA sequencing, or qPCR (Cedeno *et al*, 2021; Rallis *et al*, 2020).

4.6 MEA recordings reveal age-related changes in DH network

Some of these animals underwent MEA electrophysiology prior to tissue collection, and as mentioned earlier, MEA is a useful tool to gather data on the excitability of the DH. This data for the young-adult post-MEA cohort was collected and analysed as part of a separate study which was published in 2018 (Greenspon *et al*), the same techniques used for this publication were also used for the more recent aged animal testing: anaesthetising the animal, surgically exposing the lumbar spinal cord via laminectomy, inserting a 16-electrode array into the lumbar DH. As the animals for this study did not undergo any drug treatment, only the healthy animals can be compared however, responses to a battery of innocuous and noxious mechanical and electrical stimulations of the hindpaw were assessed

over a period of hours. MEA recordings from the full DH network in response to a range of mechanical and electrical stimuli reveal altered responses in aged rats compared to young adults: reduced A-fibre response to low intensity, electrical stimuli within the aged rats (0.5mA) potentially suggesting higher A-fibre activation thresholds, and therefore impaired somatosensation; increased C-fibre thresholds in response to high intensity, electrical stimuli (5mA) potentially suggesting higher pain thresholds; along with shorter, smaller responses overall in response to mechanical stimuli (6g, 15g, and 26g). The data supports impaired somatosensation and increased pain thresholds with ageing in these animals, although it should be noted that there is no corresponding behavioural data. When considering this data in comparison to glial cells, we do know that glial cells interact with synapses and therefore increased glial activation can lead to reduced homeostasis therefore affecting inhibitory synapses. If inhibitory synapses are altered and so function less, then even a smaller response in the A-fibres will lead to an increased response to stimuli felt.

4.7 Limitations of the study

Through the data collected there have been little to no differences between the MGI between the cohorts for each signalling channel, which may reflect a lack of sensitivity or an increase in cell to intensity ratio with ageing. Both increased activation states and build up of auto-fluorescent substances, which could be due to a build up of fatty deposits, can be the cause behind the similar intensities considering the reduction in cell numbers. Increased activation states lead to increased protein expression of the IBA1, GFAP and NeuN proteins, and therefore an increase in activation states directly affects the intensities.

Changes in cellular morphology and activation states could be caused by a number of factors such as changes in BSCB permeability, which increases with age, allowing more substances access to the spinal cord and potentially increases in the activation states of glial cells and potentially neuronal cells too (Piekarz *et al.,* 2020). Lipofuscin also increases with age and is linked to the increased permeability of the spinal cord barrier. However, astrocytes and microglia are responsible for clearing cellular debris such as lipofuscin, and so with the increase in activation of microglia, the cellular levels of lipofuscin increase (Kushwaha *et al.,* 2018). As stated earlier, lipofuscin is an auto-fluorescent substances could also be contributing to increased MGI levels. With the reduction in glial cells as the animal ages but the MGI levels remaining constant it would imply that intensity per cell increases with ageing however, this could not necessarily mean that the glial cells are more reactive with ageing, but rather the glial cells contain higher levels of these auto fluorescent substances.

A study conducted by Stewart *et al.* (2020) focuses on these differing inflammatory profiles in relation to the presence of estrogens and estradiol, which affect B and T cells, and macrophages, along with the presence of testosterone which is seen to effect the TLR-4

pathway. Due to this we only studied males with any gender differences in pain processing and immune cell responses likely to have an impact upon ageing as well.

Due to the relative difficulties in gaining access to the aged rats, and the primary purpose of the study being the MEA study prior to tissue collections, group sizes were naturally small. Therefore, a potential limitation to this study is n number per group, with only 4 animals for the aged and naïve young-adult groups, and 3 for the post-MEA group. It could be argued that increased numbers would confirm trends. On top of this, it is known that males and females respond differently to nociception and with the post-MEA young-adult group being mixed gender this may cause the results to be non-comparable between the groups (Stewart *et al,* 2020). In addition to this mixed gender discrepancy between the groups, there is also the addition of some having undergone MEA testing prior to tissue collection and then a naïve group.

Although, the rat versus human is useful for an initial study such as this one, this could be improved so the results are more translational. Stated earlier, it is shown that the spinal cord physiology of the rat and human are comparable (Kjell & Olson, 2016), however behavioural data from human participants could potentially improve the prediction of rat data into human reality. This could be done through a study on healthy young-adult humans against healthy aged humans, which would match the two groups of rats used for this study. This data could then be compared to that on the young-adult versus aged rat data, if there are similarities, it could be deduced that the rat IHC data would suitably predict the glial cell changes in humans. To further this, experiments could be conducted using human samples, again, corroborating the results from the changes seen in the rats to be translated into changes predicted in human healthy ageing.

4.8 What does this mean?

Neurotrophic factors, phenotypic switches, collateral sprouting, disinhibition, glutamate receptors, activation of descending pathways, lipofuscin, GABAergic signalling, cholesterol homeostasis and BSCB permeability can all lead to chronic pain states (Chen, 2010; Mayhew *et al*., 2019: Piekarz *et al.,* 2020: Liguori *et al*., 2018: Lee *et al*., 2015: Ding *et al*., 2017).

Once microglia and astrocytes become reactive, there is an increase in pro-inflammatory markers being released (Popiolek-Barczyk & Mika, 2016; Matejuk & Ransohoff, 2020), along with a reduction in waste being cleared (Kushwaha *et al.,* 2018). Not only do some of the pro-inflammatory markers lead to cellular senescence, but they can also affect the function of neurones either directly through decreased clearance of glutamate (Ji *et al*., 2019; Tang *et al*., 2021; Chiang *et al*., 2012), or indirectly through the modulation of neuronal synapses leading to LTP and an increase in chronic excitation of C-fibres (Todd, 2010). What

this highlights for this study, is microglia, astrocyte, and neuronal cell data collected can be linked. Therefore, the increase of pain sensation cannot be determined by simply one of these cell types but as a collective impact instead. It highlights the impact of a loss of glial and neuronal cells on the function and pain sensation of the DH, along with the increased expression of IBA1, GFAP, and also NeuN and how their interaction with each other to result in heightened excitability of synapses which ultimately effect the ascending and descending pathways. The results of this study also highlight the microglial-astrocytic cross talk that results in increased reactivity of these glial cells. The overall outcome of pain states in the ageing population, is an increase in the onset of chronic pain due to alterations in the function and morphology of microglia, astrocytes and neuronal cells.

4.9 Future directions

Considering the limitations of this study, future work and directions for research could be aimed at monitoring alternate causes for glial cell and neuronal cell changes and activation states with healthy ageing, completing the research again with a larger number of animals. Also looking at the effects on female rats as well as male rats as a comparative study but as separate groups. On top of this, there could be aims to monitor how these changes might lead to further medical implications impacting chronic pain states, along with potential treatments and preventions.

A particular point to highlight is the statistically significant decrease in percentage of both microglia and neurones in the aged rats compared to the young-adults, but no change in the total cell number or MGI. A way to currently explain this phenomenon would be to say that the labelling intensity per cell could increase, meaning whilst cell numbers decrease the MGI would remain constant indicating cellular activation. Alternately to activation of these cells, there could be an area increase with healthy ageing due to lipofuscin build up, again explaining the results we have collected without the cells becoming activated. To build on this there could potentially be more astrocytes in the aged adults as we have not performed a cell count for astrocytes but a large increase in GFAP area coverage was seen, or perhaps the astrocytic processes increased in size without a change in astrocytic cell numbers. The other cell types that could be involved would need further investigation and analysis of the cellular behaviours within healthy ageing in the spinal cord.

Therefore, to identify the cause of the decrease in IBA1+ cells with healthy ageing but little to no change in IBA1 expression or area coverage, future work could involve monitoring levels of pro-inflammatory markers or other signs of activated microglia being present. To identify the cause of the increase length of astrocytic processes, future work could involve monitoring levels of pro-inflammatory markers or other signs of astrocyte activation to determine a more accurate cause for our contradicting data sets. Finally, to identify the cause of the decrease in the neuronal cells with the maintenance of the NeuN+

MGI data, future work could involve the monitoring and analysis of this relationship and how these neuronal cells could be affected by the reactive glial cells which would further expand the data on how pain states are increased with healthy ageing.

To build on the cells we monitored, the changes of the ascending and descending pathways also affect pain states for the ageing population, and should not be excluded from pain research. This study focuses solely on glial and neuronal cells to be taken into account with the larger picture of pain processing within the body. All aspects and these findings need to be considered in the wider context of pain mechanisms and not solely the immediate changes in glial cells within the dorsal horn. Further research could be based upon the wider areas of pain mechanisms to noxious stimuli.

What is missing from our data is the relationship between the microglia and astrocytes in relation to the neuronal cells, it can only be implied that there is a link between the activation of the glial cells and the effects on the neuronal cells, particularly when researching the effects of healthy ageing on these cells and how this impacts pain states in the elderly.

Finally, although some of the rats used had undergone MEA tests prior to tissue collection, not all did and these tests were done a while in advance to these IHC tests being conducted. What data was available for these animals was useful in exploring the link initially between DH excitability and DH glial cells, however for the improvement of discovering the link, all animals should undergo MEA tests to monitor spinal cord excitability, and then the IHC should be conducted sooner to the tissue collection so the data is more comparable and reliable.

5. Conclusions

Everyday many people within the elderly demographic experience pain with it developing into chronic pain for no known reason, with plenty of literature documenting this healthcare crisis and many more researching the causes behind chronic pain. The data presented within this study supports the notion and other contemporary studies that a decrease in cell numbers and an increase in cell activation states of both glial cells and NeuN+ cells within the DH of the rat model leads to this increase in chronic pain recorded in the healthy aged population.

The changes seen in the data sets collected indicate that with healthy ageing comes decreased microglia, astrocyte and neuronal cell numbers within the DH however, there were no significant differences int MGI values. Although there could be many causes to the reasons for these significant differences in cell numbers, it all comes down to cellular senescence. When looking at the similar MGI levels across the ages, again it can be due to many causes however, it all points to an increase in activation state and protein expression

within both glial cells and neuronal cells. Both of these can support the notion that with age comes increased pain states and thus chronic pain.

This highlights the effects that the reactivity states of glial cells and also neuronal cells have on the development of pain, and the role these cells play within DH pain processing and maintenance of pain.

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7. References

Al-Chalabi, M., Reddy, V., & Gupta, S. (2021) *Neuroanatomy, Spinothalamic tract.* Online: Treasure Island, FL: StatPearls Publishing.

Bannister, K., & Dickenson, A. H. (2017) The plasticity of descending controls in pain: translational probing. *J Physiol,* **595**(13): 4159-4166

Barik, A., Sathyamurthy, A., Thompson, J., *et al.* (2021) A spinoparabrachial circuit defined by Tacr1 expression drives pain. *eLife,* **10**: e61135. https://doi.org/10.7554/eLife.61135

Bennett, M. L., & Viaene, A. N. (2021) What are activated and reactive glial and what is their role in neurodegeneration? *Neurobio Dis,* **148**: 105172. https://doi.org/10.1016/j.nbd.2020.105172

Bordner, K. A., Kitchen R. R., Carlyle, B., *et al*. (2011). Parallel declines in cognition, motivations, and locomotion in ageing mice: association with immune gene up regulation in the medial prefrontal cortex. *Exp Gerontol*, **46**(8): 643-659. https://doi.org/0.1016/j.exger.2011.03.003

Brenner, M. (2014) Role of GFAP in CNS injuries. *Neurosci Lett,* **565**: 7-13. https://doi.org/10.1016/j.neulet.2014.01.055

Browne, T. J., Smith, K. M., Gradwell, M. A., *et al*. (2021) Spinoparabrachial projection neurones form distinct classes in the mouse dorsal horn. *PAIN,* **162**(7): 1977-1944. https://doi.org/10.1097/j.pain.0000000000002194

Burston, J.J., Valdes, A. M., Woodhams, S. G., *et al*. (2019) The impact of anxiety on chronic musculoskeletal pain and the role of astrocyte activation. *PAIN,* **160**(3): 658-669. https://doi.org/10.1097/j.pain.0000000000001445

Cedeno, D. L., Kelley, C. A., Chakravarthy, K., *et al*. (2021) Modulation of glia-mediated processes by spinal cord stimulation in animal models of neuropathic pain. *Front Pain Res,* **2**: 702906. https://doi.org/10.3389/fpain.2021.702906

Cekanaviciute, E., & Buckwalter, M. S. (2016) Astrocytes: Integrative Regulators of Neuroinflammation in Stroke and Other Neurological Diseases. *Neurothera,* **13**(4): 685-701. https://doi.org/10.1007/s13311-016-0477-8

Chen, T., Taniguchi, W., Chen, Q., *et al*. (2018) Top-down descending facilitation of spinal sensory excitatory transmission from the anterior cingulate cortex. *Nat Comms,* **9**: 1886. https://doi.org/10.1038/s41467-018-04309-2

Chen, Q., & Heinricher, M. M. (2019) Descending control mechanisms and chronic pain. *Curr Rheumatol Rep,* **21**: 13. https://doi.org/10.1007/s11926-019-0813-1

Cheng, H. T. (2010) Spinal Cord Mechanisms of Chronic Pain and Clinical Implications. *Curr Pain Headache Rep,* **14**(3): 213-220. https://doi.org/10.1007/s11916-010-0111-0

Chung, W., Allen, N. J., & Eroglu, C. (2015) Astrocytes control synapse formation, function, and elimination. *Cold Spring Harb Perspect Biol*, **7**(9): a020370. https://doi.org/10.1101/cshperspect.a020370

Clarke, L. E., Liddelow, S. A., Chakraborty, C., *et al*. (2018) Normal ageing induces A1-like astrocyte reactivity. *PNAS,* **115**(8): E1896-E1905. https://doi.org/10.1073/pnas.1800165115

Comitato, A., & Bardoni, R. (2021) Presynapsic inhibition of pain and touch in the spinal cord: From receptors to circuits. *Int, J, Mol, Sci,* **22**: 414. https://doi.org/10.3390/ijms22010414

Crossman, A. R., & Neary, D. (2020) *Neuroanatomy: An Illustrated Colour Text, Sixth Edition.* Online: Elsevier. Chapter 3

Dagnino, A. P. A., & Campos, M. M. (2022) Chronic pain in the elderly: mechanisms and perspectives. *Front Hum Neurosci,* **16**: 736688. https://doi.org/10.3389/fnhum.2022.736688

Djouhri, L., & Lawson, S. N. (2004) Abeta-fibre nociceptive primary afferent neurones: a review of incidence and properties in relation to other afferent A-fibre neurones in mammals. *Brain Res Rev,* **46**(2): 131-145. https://doi.org/10.1016/j.brainresrev.2004.07.015

Dodds, K. N., Beckett, E. A. H., Evans, S. F, *et al*. (2016) Glial contributions to visceral pain: implications for disease ethology and the female predominance of persistent pain. *Transl Psychiatry,* **6**: e888. https://doi.org/10.1038/tp.2016.168

Domenichiello, A. F., & Ramsden, C. E. (2019) The silent epidemic of chronic pain in older adults. *Prog Neuropsycopharmacol Biol Psychiatry*, **93**: 284-290. https://doi.org/10.1016/j.pnpbp.2019.04.006

Edelstein, A. D., Tsuchida, M. A., Amodaj, N., *et al*. (2014) Advanced methods of microscope control using μManager software. *J Biol Methods,* **1**(2): e10. https://doi.org/10.14440/jbm.2014.36

Eng, L. F., Ghirnikar, R. S., & Lee, Y. L. (2000) Glial fibrillary acidic protein : GFAP-thirty-one years (1969-2000). *Neurochem Res,* **25**: 1439-51. https://doi.org/10.1023/a:1007677003387

Escartin, C., Galea, E., & Verkhratsky, A. (2021) Reactive astrocyte nomenclature, definitions, and future directions. *Nat Neurosci,* **24**: 312-325. https://doi.org/10.1038/s41593-020-00783-4

Fayaz, A., Croft, P., Langford, R. M., *et al.* (2016) Prevalence of chronic pain in the UK: a systematic review and meta-analysis of population studies. *BMJ Open*, **6**: e010364. https://doi.org/10.1136/bmjopen-2015- 010364

Felten, D. L., O'Banion, M. K., & Maida, M, S. (2016) *Netter's Atlas of Neuroscience: Third Edition.* Philadelphia, PA: Elsevier. Chapter 9, 10.

Foster, E., Wildner, H., Trudeau, L., *et al*. (2015). Targeted ablation, silencing, and activation establish glycinergic dorsal horn neurons as key components of a spinal gate for pain and itch. *J Neuron*, **85**(6): 1289-1304. https://doi.org/10.1016/j.neuron.2015.02.028

Gagliese, L., & Melzack, R. (2000) Age differences in nociception and pain behaviours in the rat. *Neurosci Biobehav Rev,* **24**:843-854.

Gao, Y., Mei, C., Chen, P., Chen, X. (2022) The contribution of neuro-immune crosstalk to pain in the peripheral nervous system and the spinal cord. Inter Immunopharma, **107**: 108700.

Gaudet, A. D., & Fonken, L. K. (2018) Glial Cells Shape Pathology and Repair After Spinal Cord Injury. *Neurothera,* **15**: 554-577. https://doi.org/10.1007/s13311-018-0630-7

Gebhart, G. F., & Schmidt, R. F. (2013) *Encyclopaedia of Pain, 2013 edition*. Berlin, Germany: Springer. Page 228-268

Geltmeier, M. K., & Fuchs, P. N. (2021). Evaluating the impact of age and inflammatory duration on behavioural assessments of nociception. *J Neurosci Let*, **756**: 135966. shpts://doi.org/10.1016/j.neulet.2021.135966

González-Roldán, A. M., Terrasa, J. L., Sitges, C., *et al.* (2020) Age-Related Changes in Pain Perception Are Associated With Altered Functional Connectivity During Resting State. *Front Aging Neurosci*, **12**: 116. https://doi.org/10.3389/fnagi.2020.00116

Greenspon, C. M., Battell, E. E., Devonshire, I. M., *et al*. (2018) Lamina-specific population encoding of cutaneous signals in the spinal dorsal horn using multi-electrode arrays. *J Physiology,* **597**(2): 377-397. https://doi.org/10.1113/JP277036

Gulino, M., Kim, D., Pané, S., *et al*. (2019) Tissure response to neural implants: the use of model systems toward new design solutions of implantable micro electrodes. *Front Neurosci,* **13**: 689. https://doi.org/10.3389/fnins.2019.00689

Gusel'nickova, V. V., & Korzhevskiy, D. E. (2015) NeuN as a neuronal nuclear antigen and neurone differentiation marker. *Acta Naturae,* **7**(2): 42-47.

Gwak, Y. S., Kang, J., Unable, G. C., *et al.* (2012) Spatial and temporal activation of spinal glial cells: role of gliopathy in central neuropathic pain following spinal cord injury in rats. *Exp Neurol,* **234**(2): 362-372. https://doi.org/10.1016/j.expneurol.2011.10.010

Han, J., Fan, Y., Zhou, K., *et al*. (2021) Uncovering sex differences of rodent microglia. *J Neuroinflammation,* **18**: 74. https://doi.org/10.1186/s12974-021-02124-z

Hatfield, L. A. (2014) Neonatal pain: What's age got to do with it? *Surg Neurol Int,* **5**(Suppl 13): S479-S489. https://doi.org/10.4103/2152-7806.144630

Heinricher, M. M., Tavares, I., Leith, J. L., *et al*. (2008) Descending control of nociception: specificity, recruitment and plasticity. *Brain Res Rev,* **60**(1): 214-225. https://doi.org/10.1016/j.brainresrev.2008.12.009

Hickman, S., Izzy, S., Sen, P., *et al.* (2018) Microglia in neurodegeneration. *Nat Neurosci*, **21**: 1359-1369. https://doi.org/10.1038/s41593-018-0242-x

Hovens, I. B., Nyakas, C., Schoemaker, R. G. (2014) A novel method for evaluating microglial activation using ionised calcium-binding adaptor protein-1 staining: cell body to cell size ratio. *Neuroimmunol Neuroflamm,* **1**: 82-88.https://doi.org/10.4103/2347-8659.139719

Hunt, S. P., & Mantyh, P. W. (2001) The molecular dynamics of pain control. *Nat Rev Neurosci,* **2**: 83-91. https://doi.org/10.1038/35053509

Inoue, K., & Tsuda, M. (2018) Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. *Nat Rev Neurosci,* **19**: 138-152. https://doi.org/10.1038/nrn.2018.2

Institute for Health Metric and Evaluation (IHME). *Findings from the Global Burden of Disease Study 2017.* Seattle, WA: IHME, 2021.

International Association for the Study of Pain (IASP) Taxonomy Working Group (2011). *Classification of Chronic Pain, Second Edition (Revised)*. Seattle. WA: IASP Press. Introduction: The Present Classification

Jäkel, S. & Dimou, L. (2017) Glial Cells and Their Function in the Adult Brain: A Journey through the History of Their Ablation. *Front Cell Neurosci*, **11**: 24. https://doi.org/10.3389/fncel.2017.00024

Jessen, N. A., Munk, A. S. F., Lundgaard, I., *et al.* (2015) The Glymphatic System - A Beginner's Guide. *Neurochem Res,* **40**(12): 2583-2599. https://doi.org/10.1007/s11064-015-1581-6

Ji, R., Chamessian, A., & Zhang, Y. (2016) Pain Regulation by Non-neuronal Cells and Inflammation. *Sci,* **354**(6312): 572-577. https://doi.org/10.1126/science.aaf8924

Ji, R., Kawasaki, Y., Zhuang, Z., *et al.* (2006) Possible role of spinal astrocytes in maintaining chronic pain sensitisation: review of current evidence with focus on bFGF/JNK pathway. *Neuron Glia Biol,* **2**(4): 259-269. https://doi.org/10.1017/S1740925X07000403

Ji, R., Donnelly, C. R., & Nedergaard, M. (2019) Astrocytes in chronic pain and itch. *Nat Rev Neurosci,* **20**: 667-685. https://doi.org/10.1038/s41583-019-0218-1

Jyothi, H. I., Vidyadhara, D. J., Mahandevan, A., *et al*. (2015) Ageing causes morphological alterations in astrocytes and microglia in human substantia nigra pars compacta. *Neurobiol Age,* **36**(12): 3321-3333. https://doi.org/10.1016/j.neurobiolaging.2015.08.024

Kelleher, J. H., Tewari, D., & McMahon, S. B. (2017) Neurotropic factors and their inhibitors in chronic pain treatment. *Neurobiol Dis,* **97**(B): 127-138. https://doi.org/10.1016/j.nbd.2016.03.025

Khasabov, S. G., Malecha, P., Noack, J., *et al*. (2015) Activation of rostral ventromedial medulla neurons by noxious stimulation of cutaneous and deep craniofacial tissues. *J Neurophysiol,* **113**(1): 14-22. https://doi.org/10.1152/jn.00125.2014

Kjell, J., & Olson, L. (2016) Rat models of spinal cord injury: from pathology to potential therapies. *Dis Model Mech,* **9**: 1125-1137. https://doi.org/doi: 10.1242/dmm.025833

Klapal, L., Ingelhorst, B. A., & Dietzel-Meyer, I. D. (2016) Changes in neuronal excitability by activated microglia: differential Na+ current up regulation in pyramid-shaped and bipolar neurones by TNF-a and IL-18. *Front Neurol*, **7**: 44. https://doi.org/10.3389/fneur.2016.00044

Koch, S. C., & Fitzgerald, M. (2014) The selectivity of rostroventral medulla descending control of spinal sensory inputs shifts postnatal from A fibre to C fibre evoked activity. *J Physiol,* **592**(7): 1535-1544. https://doi.org/10.1113/jphysiol.2013.267518

Koga, K., Furue, H., Rashid, M. H., *et al*. (2005) Selective activation of primary afferent fibres evaluated by sine-wave electrical stimulation. *Mol Pain,* **1**: 13. https://doi.org/10.1186/1744-8069-1-13

Kolos, E. A., & Korzhevskii, D. E. (2020) Spinal Cord Microglia in Health and Disease. *Acta Naturae,* **12**(1): 4-17. https://doi.org/10.32607/actanaturae.10934

Koop, L. K., & Tadi, P. (2021) *Neuroanatomy.* Treasure Island, FL: StatPearls Publishing. Sensory Nerves.

Krzisch, M., Temprana, S. G., Mongiat, L. A., *et al*. (2015) Pre-existing astrocytes from functional perisynaptic processes on neurones generated in the adult hippocampus. *Brain Struct Funct,* **220**(4): 2027-2042. https://doi.org/10.1007/s00429-014-0768-y

Kuner, R., & Flor, H. (2016) Structural plasticity and reorganisation in chronic pain. *Nat Revs Neurosci,* **18**: 20-30. https://doi.org/10.1038/nrn.2016.162

Kushwaha, S. S., Patro, N., & Patro, I. K. (2018) A Sequential Study of Age-Related Lipofuscin Accumulation in Hippocampus and Striate Cortex of Rats. *Ann Neurosci,* **25**(4): 223-233. https://doi.org/10.1159/000490908

Kwok, C. H. T., Kohro, Y., Mousseau, M., *et al*. (2021) Role of primary afferents in Arthritis induced spinal microglial reactivity. *Front Immunol,* **12**: 626884. Https://doi.org/10.3389/fimmu.2021.626884

Lacagnina, M. J., Watkins, L. R., & Grace, P. M. (2018) Toll-like receptors and their role in persistent pain. *Pharmacy Ther,* **184**: 145-158. https://doi.org/10.1016/j.pharmthera.2017.10.006

Laumet, G., Ma, J., Robison, A. J., *et al.* (2019) T cells as an Emerging Target for Chronic Pain Therapy. *Front Mol Neurosci,* **12**: 216. https://doi.org/10.3389/fnmol.2019.00216

Lee, S., Wu, Y., Shi, X., Q., *et al.* (2015) Characteristics of spinal microglial in aged and obese mice: potential contributions to impaired sensory behaviour. *Immun Ageing,* **12**: 22. https://doi.org/10.1186/s12979-015-0049-5

Leisengang, S., Nürnberger, F., Ott, D., *et al.* (2020) Primary culture of the rat spinal dorsal horn: a troll to investigate the effects of inflammatory stimulation on the afferent somatosensory system. *Euro J Physiol,* **472**: 1769-1782. https://doi.org/10.1007/s00424-020-02478-y

Letiembre, M., Hao, W., Liu, Y., *et al*. (2007) Innate immune receptor expression in normal brain ageing. *Neurosci,* **146**: 248-254. https://doi.org/10.1016/j.neuroscience.2007.01.004

Li, T., Chen, X., Zhang, C., *et al*. (2019) An update on reactive astrocytes in chronic pain. *J Neuroinflammation,* **16**: 140. https://doi.org/10.1186/s12974-019-1524-2

Lind, G., Gällentoft, L., Danielsen, N., *et al*. (2012) Multiple implants do not aggravate the tissue reaction in rat brain. *PLOS One,* **7**(10): e47509. https://doi.org/10.1371/journal.pone.0047509

Liu, L., Liu, J., Bay, J., *et al*. (2020) Interaction of Microglia and Astrocytes in the Neurovasular Unit. *Front Immunol,* **11**: 1024. https://doi.org/10.3389/fimmu.2020.01024

Lv, Q., Wu, F., Gan, X., *et al*. (2019) The involvement of descending pain inhibitory system in electroacupuncture induced analgesia. *Front. Integr Neurosci,* **13**: 38. https://doi.org/10.3389/fnint.2019.00038

Ma, W., Coon, S., Zhao, L., *et al*. (2013) A2E accumulation influences retinal microglial activation and complement regulation. *Neurobiol Age,* **34**: 943-960. https://doi.org/10.1016/j.neurobiolaging.2012.06.010

Matejuk, A., & Ransohoff, R. M. (2020) Crosstalk Between Astrocytes and Microglia: An Overview. *Front Immunol,* **11**: 1416. https://doi.org/10.3389/fimmu.2020.01416

Mayhew, J. A., Callister, R. J., Walker, F. R., *et al*. (2019) Aging alters signalling properties in the mouse spinal dorsal horn. *Mol Pain,* **25**: 1744806919839860. https://doi.org/10.1177/1744806919839860

McCall, M. A., Gregg, R. G., Behringer, R. R., *et al*. (1996) Targeted election in astrocyte intermediate filament (GFAP) alters neuronal physiology. *Proc Natl Acad Sci USA,* **93**(13): 6361-6. https://doi.org/10.1073/pnas.93.13.6361

McMahon, S., Koltzenburg, M., Tracey, I., *et al*. (2013) *Wall & Melzack's Textbook of Pain, sixth edition.* Philadelphia, PA: Saunders, an imprint of Elsevier Ltd. Chapters 1, 4, and 5

Meneses, C. S., Müller, H. Y., Herzberg, D. E., *et al*. (2017) Immunofluorescence characterisation of spinal cord dorsal horn microglial and astrocytes in horses. *Peer J,* **5**: e3965. https://doi.org/10.7717/peerj.3965

Mihailoff, G. A., & Haines, D. E. (2018) *Fundamental Neuroscience for Basic and Clinical Application, fifth edition.* Philadelphia, PA: Elsevier Ltd. Chapter 24

Mills, S. E. E., Nicholson, K. P., & Smith, B. H. (2019) Chronic pain: a review of its epidemiology and associated factors in population-based studies. *Br J Anaesth,* **123**(2): 273-283. https://doi.org/10.1016/j.bja.2019.03.023

Mogil, J. S. (2020) Qualitative sex differences in pain processing: emerging evidence of a biased literature. *Nat Revs Neurosci,* **21**: 353-365. https://doi.org/10.1038/s41583-020-0310-6

Montague-Cardoso, K., & Malcangio, M. (2021) Changes in blood-spinal cord barrier permeability and neuroimmune interactions in the underlying mechanisms of chronic pain. *Pain Rep,* **6**(1): e879. https://doi.org/10.1097/PR9.0000000000000879

Moreno-García, A., Kun, A., Calero, O., *et al*, (2018) An Overview of the Role of Lipofuscin in Age-Related Neurodegeneration. *Front Neurosci,* **12**: 464. https://doi.org/10.3389/fnins.2018.00464

Neumann, S., Doubell, T. P., Leslie, T., *et al*. (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurones. *Nat,* **384**: 360-364. https://doi.org/10.1038/384360a0

Office of National Statistics (ONS). (2019) *Overview of the UK population: August 2019*. Accessed online: January 2021 https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/articles/overviewoftheukpopulation/august2019#the-uks-population-is-ageing

Ogbonna, A. C., Clark, A. K., & Malcangio, M. (2015) Development of monosodium acetate-induced osteoarthritis and inflammatory pain in ageing mice. *AGE,* **37**: 54. https://doi.org/10.1007/s11357-015-9792-y

Ossipov, M. H., Morimura, K., & Porreca, F. (2014) Descending pain modulation and chronificiation of pain. *Curr Open Support Palliat Care,* **8**(2): 143-151. https://doi.org/10.1097/SPC.0000000000000055

Owasil, R., O'Neill, R., Keable, A., *et al.* (2020) The Pattern of AQP4 Expression in the Ageing Human Brain and in Cerebral Amyloid Angiopathy. *Int J Mol Sci,* **21**(4): 1225. https://doi.org/10.3390/ijms21041225

Parkinson, M. G., Dayas, C. V., & Smith, D. W. (2016) Perturbed cholesterol homeostasis in aging spinal cord. *Neurobiol Aging,* **45**: 123-135. https://doi.org/10.1016/j.neurobiolaging.2016.05.017

Peirs, C., Dallel, R., & Todd, A. J. (2020) Recent advances in our understanding of the organisation of dorsal horn neurone populations and their contribution to cutaneous mechanical allodynia. *J Neur Trans,* **127**: 505-525. https://doi.org/10.1007/s00702-020-02159-1

Piekarz, K. M., Bhaskaran, S., Sataranatarajan, K., *et al.* (2020) Molecular changes associated with spinal cord aging. *Gero Sci*, **42**: 765-784. https://doi.org/10.1007/s11357-020-00172-6

Polgár, E., Durrieux, C., Hughes, D. I., *et al*. (2013) A quantitative study of inhibitory interneurones in laminae I-III of the mouse spinal dorsal horn. *PLoS One,* **8**(10): e78309. https://doi.org/10.1371/journal.pone.0078309

Polgár, E., Hughes, D. I., Arham, A. Z., *et al*. (2005) Loss of neurones from laminas I-III of the spinal dorsal horn is not required for development of tactile allodynia in the spared nerve injury model of neuropathic pain. *J Neurosci,* **25**(28): 6658-6666. https://doi.org/10.1523/JNEUROSCI.1490-05.2005

Popiolek-Barczyk, K., & Mika, J. (2016) Targeting the Microglial Signaling Pathways: New Insights in the Modulation of Neuropathic Pain. *Curr Med Chem,* **23**(26): 2908-2928. https://doi.org/10.2174/0929867323666160607120124

Public Health England (PHE). *Chronic pain in adults 2017; Health survey for England.* London, UK: PHE, 2021

Purves, D., Augustine, G. J., Fitzpatrick, D., *et al*. (2001) *Neuroscience, second edition*. Sunderland, MA: Sinauer Associates, Inc. Chapters 1 & 12

Purves, D., Augustine, G. J., Fitzpatrick, D., *et al*. (2008) *Neuroscience, fourth edition*. Sunderland, MA: Sinauer Associates, Inc. Chapter 1 & Appendix

Queme, L. F., Weyler, A. A., Cohen, E. R., *et al.* (2020) A dual role for peripheral GDNF signalling in nociception and cardiovascular reflexes in the mouse. *PNAS,* **117**(1): 689-707. https://doi.org/10.1073/pnas.1910905116

Rallis, A., Navarro, J. A., Rass, M., *et al*. (2020) Hedgehog signalling modulates glial proteostasis and lifespan. *J Cell Rep,* **30**: 2627-2643. https://doi.org/10.1016/j.celrep.2020.02.006

Refolo, V., & Stefanova, N. (2019) Neuroinflammation and Glial Phenotypic Changes in Alpha-Synucleinopathies. *Front Cell Neurosci,* **13**: 263. https://doi.org/10.3389/fncel.2019.00263

Rozycka, A., &Liguz-Lecznar, M. (2017) The space where ageing acts: focus on the GABAergic synapse. *Age Cell,* **16**(4): 634-643. https://doi.org/10.1002/cne.1335

Sardella, T. C. P., Polgár, E., Watanabe, M., *et al*. (2011) A quantitative study of neuronal nitric oxide synthase expression in laminae I-III of the rat spinal dorsal horn. *Neurosci*, **192**(6-2): 708-720. https://doi.org/10.1016/j.neuroscience.2011.07.011

Schindelin, J., Arganda-Carreras, I., Frise, E., *et al*. (2012) Fiji: an open source platform for biological-image analysis. *Nat Methods*, **9**(7): 676-682. https://doi.og/10.1038/nmeth.2019

Shimizu-Okabe, C., Kobayashi, S., Kim, J., *et al*. (2022). Developmental formation of the GABAergic and glycincergic networks in the mouse spinal cord. *Int J Mol Sci*, **23**(2): 834-856. https://doi.org/10.3390/ijms23020834

Sikora, E., Bielak-Zmijewska, A., Dudkowska, M., *et al.* (2021) Cellular senescence in brain ageing. *Front Ageing Neurosci,* **13**: 646924. https://doi.org/10.3389/fnagi.2021.646924

Sofroniew, M. V., & Vinters H. V. (2010) Astrocytes: biology and pathology. *Acta Neuropathol*, **119**(1): 7-35. https://doi.org/10.1007/s00401-009-0619-8

Spittau, B. (2017) Aging Microglia - Phenotypes, Functions and Implications for Age-Related Neurodegenerative Diseases. *Front Aging Neurosci,* **9**: 194. https://doi.org/10.3389/fnagi.2017.00194

Stein, C., Clark, J. D., Oh, U., *et al*. (2009) Peripheral mechanisms of pain and analgesia. *J Brain Res Rev*, **60**(1): 90-113. https://doi.org/10.1016/j.brainresrev.2008.12.017

Stucky, C. L., Gold, M. S., & Zhang, X. (2001) Mechanisms of pain. *PNAS*, **98**(21): 11,845-11,846. https://doi.org/10.1073/pnas.211373398

Stewart, A. N., MacLean, S. M., Stromberg, A, J., *et al.* (2020) Considerations for Studying Sex as a Biological Variable in Spinal Cord Injury. *Front Neurol,* **11**: 802. https://doi.org/10.3389/fneur.2020.00802

Tang, J., Bair, M., & Descalzi, G. (2021) Reactive astrocytes: critical players in the development of chronic pain. *Front Psychiatry,* **12**: 682056. https://doi.org/10.3389/fpsyt.2021.682056

Toedebusch, C. M., Garcia, V. B., Snyder, J. C., *et al*. (2020) Lumbar spinal cord microglial exhibited increased activation in ageing dogs compared with young adult dogs. *Gero Science,* **42**(1): 169-182. https://doi.org/10.1007/s11357-019-00133-8

Tsuda, M. (2016) Microglia in the spinal cord and neuropathic pain. *J Diabetes Investig,* **7**(1): 17-26. https://doi.org/10.1111/jdi.12379

Um, J. W. (2017) Roles of glial cells in sculpting inhibitory synapses and neural circuits. *Front, Mol, Neurosci,* **10**: 381. https://doi.org/10.3389/fnmol.2017.00381

Valenza, M., Facchinetti, R., Steardo, L., *et al*. (2020) Altered Waste Disposal System in Aging and Alzheimer's Disease: Focus on Astrocytic Aquaporin-4. *Front Pharmacol,* **10**: 1656. https://doi.org/10.3389/fphar.2019.01656

Vasko, M. R. (2009) Inflammatory pain. *Encyclopaedia of Neuroscience.* Hiedelberg, Berlin: Springer. Inflammatory pain.

Vaughan, D. W., & Peters, A. (1974) Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. *J Neurocytol*, **3**: 405-429. https://doi.org/10.1007/BF01098730

Von Leden, R. E., Khayrullina, G., Moritz, K. E., *et al*. (2017) Ages exacerbates microglial activation, oxidative stress, inflammatory and NOX2 gene expression, and delays functional recovery in a middle-aged rodent model of spinal cord injury. *J Neuroinflamm,* **14**: 161. https://doi.org/10.1186/s12974-017-0933-3

Ward, H., & West, S. J. (2020) Microglia: sculptors of neuropathic pain? *R Soc Open Sci,* **7**: 200260. https://doi.org/10.1098/rsos.200260

Wasner, G., Lee, B. B., Engel, S., *et al*. (2008) Residual spinothalamic tract pathways predict development of central pain after spinal cord injury. *Brain,* **131**(9): 2387-2400. https://doi.org/10.1093/brain/awn169

Yang, Z., & Wang, K. K. W. (2015) Glial fibrillary acidic protein: from intermediate filament assembly and gloss to neurobiomarker. *Trands Neurosci,* **38**(6): 364-374. https://doi.org/10.1016/j.tins.2015.04.003

Yi, M., Liu, Y. U., Umpierre, A. D., *et al*. (2021) Optogenetic activation of spinal microglia triggers chronic pain in mice. *PLOS Biol,* **19**(3): e3001154. https://doi.org/10.1371/journal.pbio.3001154

Zhou, L., Peng, J., Xu, Y., *et al*. (2019) Microglia are indispensable for synaptic plasticity in the spinal dorsal horn and chronic pain. *Cell Rep*, **27**(13): 3844-3859. https://doi.org/10.1016/j.celrep.2019.05.087

8. Bibliography

Alles, S. R. A., Odem, M. A., Lu, V. B., *et al*. (2021) Chronic BDNF simultaneously inhibits and unmasks superficial dorsal horn neuronal activity. *Sci Rep*, **11**: 2249. https://doi.org/10.1038/s41598-021-81269-6

Askew, K., & Gomez-Nicola, D. (2018) A story of birth and death: Insights into the formation and dynamics of the microglial population. *Brain Behav Im,* **69**: 9-17. https://doi.org/10.1016/j.bbi.2017.03.009

Benarroch, E. E. (2009) Astrocyte-neurone interactions: Implications for epilepsy. *Neurology,* **73**(16): 1323-1327. https://doi.org/10.1212/WNL.0b013e3181bd432d

Borzuola, R., Giombini, A., Torre, G., *et al*. (2020) Central and Peripheral Neuromuscular Adaptations to Ageing. *J Clin Med,* **9**(3): 741. https://doi.org/10.3390/jcm9030741

Chen, G., Zhang, Y., Qadri, Y. J., *et al*. (2018) Microglia in Pain : Detrimental and Protective roles in pathogenesis and resolution of pain. *Neuron,* **100**(6): 1292-1311. https://doi.org/10.1016/j.neuron.2018.11.009

Chiang, C. Y., Sessle, B. J., & Dostrovsky, J. O. (2012) Role of Astrocytes in Pain. *Neurochem Res,* **37**: 2419-2431. https://doi.org/10.1007/s11064-012-0801-6

Coull, J. A. M., Boudreau, D., Bachand, K., *et al*. (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurones as a mechanism of neuropathic pain. *Nat*, **242**(6951): 938-942. https://doi.org/10.1038/nature01868

Deuis, J. R., Dvorakova, L. S., & Vetter, I. (2017) Methods used to evaluate pain behaviours in rodents. *Front Mol Neurosci,* **10**: 284. https://doi.org/10.3389/fnmol.2017.00284

Ding, R., Sun, B., Liu, Z., *et al.* (2017) Advances oxidative protein products cause pain hypersensitivity in rats by inducting dorsal root ganglion neurones apoptosis via NADPH oxidase 4/c-Jun N-terminal kinase pathways. *Front Mol Neurosci*, **10**: 195. https://doi.org/10.3389/fnmol.2017.00195

Diógenes, M. J., Costenla, A. R., Lopes, L. V., *et al.* (2011) Enhancement of LTP in aged rats is dependent on endogenous BDNF. *Neuropsychopharm*, **36**: 1823-1836. https://doi.org/10.1038/npp.2011.64

Halsey, A. M., Conner, A. C., Bill, R. M., *et al*. (2018) Aquaporins and Their Regulation after Spinal Cord Injury. *Cells,* **7**(10): 174. https://doi.org/10.3390/cells7100174

Harry, G. J. (2013) Microglia during development and ageing. *Pharm Thera*, **139**(3): 313-326. https://doi.org/10.1016/j.pharmthera.2013.04.013

Hoheisel, U., Chacur, M., Treede, R. D., *et al*. (2019) Action potentials of dorsal horn neurones in a rat model of myositis: a study employing intracellular recordings in vivo. *J Neurophys*, **122**(2): 632-643. https://doi.org/10.1152/jn.00338.2018

Ironside, J. W., & Smith, C. (2019) *Underwood's Pathology: Seventh edition.* Philadelphia, PA: Saunders, an imprint of Elsevier Ltd.

Jackson, T. P., Stabile, V. S., & McQueen, K. A. K. (2014) The global burden of chronic pain. *Am Soc Anaes,* **78**: 24-27.

Jutzeler, C. R., Huber, E., Callaghan, M. F., *et al*. (2016) Association of pain and CNS structural changes after spinal cord injury. *Sci Rep,* **6**: 18534. https://doi.org/10.1038/srep18534

Kang, W., & Hébert, J. M. (2011) Signalling Pathways in Reactive Astrocytes, a Genetic Perspective. *Mol Neurobiol,* **43**(3): 147-154. https://doi.org/10.1007/s12035-011-8163-7

Kauppinen, A., Paterno, J. J., Blasiak, J., *et al*. (2016) Inflammation and its role in age-related macular degeneration. *Cell Mol Life Sci,* **73**: 1765-1786. https://doi.org/10.1007/s00018-016-2147-8

Kohro, Y., Matsuda, T., Yoshihara, K., *et al.* (2020) Spinal astrocytes in superficial laminae gate brainstem descending control of mechanosensory hypersensitivity. *Nat Neurosci,* **23**: 1376-1387. https://doi.org/10.1038/s41593-020-00713-4

Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway maps database. (2021) *Cellular senescence - Reference pathway.* Accessed online : January 2021. https://www.kegg.jp/kegg-bin/show_pathway?org_name=map&mapno=04218&scale=1.0&image=map04218_org_2021020423152810377 8.png&nocolor=&show_description=hide

Larson, C. M., Wilcox, G. L., & Fairbanks, C. A. (2019) The study of pain in rats and mice. *Am Assoc Lab Ani Sci,* **69**(6): 555-570. https://doi.org/10.30802/AALAS-CM-19-000062

Lattke, M., & Wirth, T. (2018) Astrocyte - Physiology and Pathology. London, UK: IntechOpen. Chapter 2.

Liguori, I., Russo, G., Curcio, F., *et al*. (2018) Oxidative stress, ageing, and diseases. *Colin Interv Ageing,* **13**: 757-772. https://doi.org/10.2147/CIA.S158513

Mazzone, G. L., Mohammadshirazi, A., Aquino, J. B., *et al*. (2021) GABAergic mechanisms can redress the tilted balance between excitation and inhibition in damaged spinal networks. *Mol Neurobiol.* https://doi.org/10.1007/s12035-021-02370-5

Okazaki, T., Kanchiku, T., Nishida, N., *et al*. (2018) Age-related changes of the spinal cord: A biomechanical study. *Exp Ther Med,* **15**(3): 2824-2829. https://doi.org/10.3892/etm.2018.5796

Palmer, A. L., & Ousman, S. S. (2018) Astrocytes and Aging. *Front Aging Neurosci*, **10**: 337. https://doi.org/10.3389/fnagi.2018.00337

Robillard, K. N., Lee, K. M., Chiu, K. B., *et al*. (2016) Glial Cell Morphological and Density Changes Through the Lifespan of Rhesus Macaques. *Brain Behav Immun,* **55**: 60-69. https://doi.org/10.1016/j.bbi.2016.01.006

Sandkühler, J. (2007) Understanding LTP in pain pathways. *Mol Pain*, **3**: 9. https://doi.org/10.1186/1744-8069-3-9

Suter, R. M., Wen, Y., Decosterd, I., *et al*. (2007). Do glial cells control pain? *Neuron Glia Biol,* **3**(3): 255-268. https://doi.org/10.1017/S1740925X08000100

Todd, A. (2007) Nociceptive circuitry in the spinal cord. Schmidt, R., Willis, E (Des) Encyclopaedia of pain. Springer: Heidelberg, Berlin. https://doi.org/10.1007/978-3-540-29805-2_2748

Todd, A. J. (2010) Neuronal circuitry for pain processing in the dorsal horn. *Nat Revs Neurosci,* **11**: 823-836. https://doi.org/10.1038/nrn2947

Wanke, E., Gullo, F., Dossi, E., *et al*. (2016) Neuron-glia cross talk revealed in reverberating networks by simultaneous extracellular recording of spikes and astrocytes' glutamate transporter and K+ currents. *J Neurophysiol,* **116**(6): 2706-2719. https://doi.org/10.1152/jn.00509.2016