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Up-Regulation of Iba-1 and GFAP in the Acidic Saline Model of Chronic Widespread Musculoskeletal Pain: A Pilot Study Examining the Relationship Between Gliosis and Hyperalgesia

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Abstract

Chronic widespread musculoskeletal pain is a major cause of disability. Pro-inflammatory glial cell activity, also known as gliosis, has been associated with the development and maintenance of hyperalgesia within several forms of chronic pain, yet its role in the pathophysiology of chronic widespread musculoskeletal pain has not been clearly established. The aim of this study was to quantitatively compare the expression of glial cells in the acidic saline (AS) model of chronic widespread musculoskeletal pain with controls and determine whether a relationship exists between glial cell expression and pain response. Activation markers of astrocytes (GFAP) and microglia (Iba-1), detected via immunohistochemistry, were quantitatively analyzed in the lumbar spinal cord and thalamus ventrobasal complex. Hyperalgesia was determined using electronic algesiometer measurement of cutaneous mechanical withdrawal threshold. Five out of twenty-one (24%) AS animals developed bilateral hyperalgesia. The expression of Iba-1 and GFAP in the L5 and L6 spinal cord was found to be significantly increased in AS animals relative to controls, regardless of behavioural pain response ($p<0.05$). When sub-grouped according to pain response, only hyperalgesia responders showed increased lumbar Iba-1 expression ($p<0.05$ versus control). In contrast, both hyperalgesia responders and non-responders had increased lumbar GFAP expression ($p<0.05$ versus control). This study provides evidence of spinal cord gliosis in an animal model of chronic widespread musculoskeletal pain and suggests a positive correlation between lumbar microglia expression and hyperalgesia. This novel finding establishes a possible pathophysiological link between reactive gliosis and hyperalgesia in chronic widespread musculoskeletal pain disorders.

Keywords: Gliosis; Glia; Microglia; Astrocyte; Hyperalgesia; Musculoskeletal pain

Introduction

Glial cells of the CNS, including microglia and astrocytes, are known to exhibit functional and morphological changes in response to noxious stimuli and, through crosstalk with neurons, have been implicated in the development and maintenance of central sensitization and hyperalgesia [1,2]. These pathologic changes, known as gliosis, may include cell proliferation, migration, release of pro-inflammatory mediators (i.e., cytokines, chemokines and neuropeptides) and enhancement of synaptic concentrations of excitatory amino acids (i.e., aspartate and glutamate) [3,4]. Gliosis has been found to play a role in a variety of experimental pain models and clinical disease states and, as such, pharmacologic agents targeting activated glial cells have been investigated for potential therapeutic application both in research models [5-8] and clinical trials [9-15]. However, the contribution of gliosis to chronic widespread musculoskeletal pain disorders has not been clearly established.

Chronic widespread musculoskeletal pain is a cardinal feature of fibromyalgia syndrome (FMS) and a leading global cause of disability. The underlying pathophysiology is thought to involve peripheral and central sensitization coupled with abnormalities in central nervous system (CNS) pain processing [16,17]. Clinical research has identified elevated cerebrospinal fluid (CSF) concentrations of glutamate, substance P, interleukin-8, brain-derived neurotropic factor and nerve growth factor in FMS patients, suggesting that neuro-inflammatory processes may be at play within the CNS [18-21].

One of the few research models of chronic widespread musculoskeletal pain is the acidic saline (AS) model, developed by Sluka and colleagues [22]. In this model, two unilateral injections of acidic saline into the gastrocnemius muscle of male Sprague-Dawley rats, administered 5 days apart, produces bilateral hyperalgesia beginning 24 hours following the second injection and persisting for 4-5 weeks [22]. It is established that a portion of AS animals do not develop mechanical hyperalgesia and the rate of hyperalgesia response to the AS model in previous reports is greatly varied [23-25].

Several factors involved in the development of hyperalgesia in the AS model have been previously identified. Firstly, excitatory amino acid transmission in the CNS has been found to play a critical role. Within the rostroventral medulla (RVM), enhancement of descending facilitative glutamatergic transmission has been shown to promote the development of hyperalgesia via N-methyl-D-aspartate (NMDA) glutamate receptors [26-28]. Within the lumbar spinal cord, increased concentrations of glutamate and aspartate have been detected in the dorsal horn 90 minutes following the second acidic saline injection [29,30]. The effect was abolished upon pre-treatment with cobalt chloride, a non-specific calcium channel blocker, thereby demonstrating the involvement of calcium-induced glutamate release immediately following the second injection [30]. At 1 week following the second injection, increases in glutamate, but not aspartate, were

detected within the lumbar spinal cord dorsal horn [30] and blockade of both NMDA and non-NMDA glutamate receptors at this time point significantly diminished hyperalgesia responses [31]. As noted by the authors, astrocytes may contribute to the observed increases in dorsal horn glutamate by participation in calcium-induced vesicular release of excitatory amino acids, through glutamate-induced glutamate release upon α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate glutamate receptor activation, and via pathological alterations in glutamate re-uptake capacity through the high affinity glutamate transporter GLT-1 [30].

The cAMP pathway in the lumbar spinal cord has also been examined and found to mediate the acute phase of hyperalgesia in the AS model, whereas modulation of the pathway had no effect on the pain response 1 week following the second injection [32]. Jasper et al. demonstrated that administration of the microglia inhibitor minocycline immediately prior to the first but not the second injection inhibited the development of hyperalgesia [24]. In contrast, Ledeboer et al. found no effect of various intrathecal treatments targeting glial cell activation (fluorocitrate, plasmid interleukin-10 DNA, and interleukin-1 receptor antagonist) administered 12-26 days following the second acidic saline injection [33]. The involvement of gliosis within this experimental model therefore remains inconclusive. The aim of the present investigation was to quantitatively compare the expression of Iba-1 and GFAP in the AS model with controls and to assess the relationship between Iba-1 and GFAP expression and mechanical hyperalgesia.

Materials and Methods

Monoclonal goat anti-GFAP, was purchased from Santa Cruz Biotechnology, Dallas, USA (number sc-6171). Monoclonal rabbit anti-Iba1 was purchased from Wako Chemicals, Richmond, VA, USA. Alexa Fluor® 647 donkey anti-rabbit IgG and Alexa Fluor® 488 donkey anti-goat IgG were purchased from Abcam, Cambridge, UK (respective catalogue numbers ab150075 and ab150129). Normal donkey serum (NDS) was obtained from Millipore Sigma, USA (number D9663). Other reagents were obtained from different sources as indicated.

Animals and experimental grouping

The experimental protocol was approved by the University of British Columbia Animal Care Committee (protocol number A14-0334). Eight week-old male Sprague-Dawley rats (Charles River, Saint-Constant, QC, Canada) weighing 250–325 g at the time of the first acidic saline injection were used for the experiment. Animals were housed in an animal care facility having a 12-hour dark (6:00 p.m.–6:00 a.m.) and 12-hour light cycle and allowed *ad libitum* access to standard rodent food and water. Prior to beginning the experiment, animals were acclimatized to the animal care facility for seven days. This experiment was conducted in two independent cohorts of animals (n=25 total). The first cohort consisted of n=12 AS animals sacrificed at 1,3,7 and 10 days following the second pH 4.0 injection for histology (n=3 per time point) and n=4 healthy animals sacrificed within the same time range as the AS group. In order to obtain data from a greater number of hyperalgesia responder animals, the experiment was repeated in a second cohort of n=9 AS animals sacrificed on days 1,7 and 10 (n=3 per time point).

Acidic saline model

The AS model was carried out as per the previously published protocol [22]. Briefly, animals allocated to the AS group received a 100 μL intramuscular injection of pH 4.0 saline into the right gastrocnemius muscle ("injection 1") and a second identical injection after a 5 day interval ("injection 2") [22]. To perform the injection, animals were placed in an anesthesia chamber and received a continuous flow of 4% isoflurane in oxygen (3 L/min). When the animal's righting reflex was lost and the respiratory rate reached approximately 68 breaths per minute, the animal was removed from the chamber and given a reduced level of isoflurane (2.5% in oxygen at 3 L/min) through a custom-made nose cone. The injection was administered via a 27 gauge needle and the animal was allowed to recover from anaesthesia in a recovery cage before being returned to its home cage. The injection was prepared by adjusting preservative-free 0.9% Sodium Chloride for Injection USP (No. 04888010, Hospira, Saint-Laurent, Canada) to pH 4.0 (+/- 0.1 pH) using 0.1N HCl with subsequent filtration through a 0.22 μm polyethersulfone membrane (Millipore, Burlington, USA).

Mechanical hyperalgesia

Paw withdrawal threshold (PWT) measurements were conducted in a sensory testing room separate from the animal housing facility. Measurements were taken between the hours of 11:00 a.m. and 1:00 p.m. Prior to every PWT measurement, animals were allowed to acclimatize to the testing room in their home cage for one hour, followed by a ten-minute acclimatization period within the measurement stand (15 × 15 × 25 cm lucite cubicle with mesh flooring). Using an electronic von Frey meter with rigid tips (IITC Life Sciences, Woodland Hills, USA), gradually increasing force was applied to the plantar surface of the hindpaw until the animal exhibited a paw withdrawal response. The maximum force applied in grams was recorded. Three measurements were taken from each hindpaw with the mean of the three measurements reported as the paw withdrawal threshold. Prior to collecting experimental data, animals were taken through the measurement protocol on two separate occasions for training.

CNS tissue collection and immunohistochemistry

Animals underwent transcardiac perfusion using heparinized 0.9% NaCl followed 4% paraformaldehyde in phosphate-buffered saline (PBS). The lumbar spinal cord, collected via laminectomy, and brain were post-fixed for 4-5 hours in 4% paraformaldehyde. After post-fixing, the tissues were transferred to 30% w/v sucrose aqueous solution and stored at 4°C until the time of sectioning. Thirty-five μm-thick free-floating sections were collected from the L5 and L6 spinal cord (transverse sections) and brain region passing through the thalamus (sagittal sections) using a vibratome. Immunohistochemistry treatments took place in 24-well cell culture plates, all in PBS solutions. Sections underwent blocking in 5% v/v normal donkey serum (NDS) at room temperature for one hour followed by overnight incubation in primary antibodies monoclonal goat anti-GFAP (1:1000) and monoclonal rabbit anti-Iba-1 (1:1000) prepared in 1% v/v NDS. Following three washes with 0.1% v/v Tween-20® in PBS (PBST), sections were incubated with Alexa Fluor® 647 donkey anti-rabbit IgG (1:1000) and Alexa Fluor® 488 donkey anti-goat IgG (1:1000) in 1% v/v NDS. Following three washes in TBST, tissue sections were mounted on poly-L-lysine coated microscope slides. Antibody concentration

optimization and secondary antibody control tests were performed prior to the experiment.

Histology imaging and analysis

Histology images were acquired using an Aperio FL® digital pathology scanner (Leica Biosystems Buffalo Grove, IL, USA). All images were captured using identical, optimized settings. Spinal cord analysis was performed using Halo® software (Indica Labs, Albuquerque, NM, USA). For the analysis of microglia expression, the percent area of each tissue section having an immunofluorescence signal above a minimum threshold of 0.205 fluorescence units was recorded. Astrocyte expression was analyzed by using Halo® software to partition the superficial dorsal horn area into 2500 μm^2 square tiles within a manually selected sample region. Thirty percent of the tiles were then randomly removed by the software in order to reduce selection bias of the sample region. Within the remaining 70% of tiles, the total number of cells was counted and then divided by the number of tiles, giving the average number of cells per 2500 μm^2 dorsal horn tissue. Five tissue sections each from the L5 and L6 lumbar spinal cord were analyzed per animal. Glial cell expression in the ventrobasal complex (VBC) region of the thalamus was analyzed by counting the total number of each microglia and astrocytes within the VBC, bilaterally, within each tissue section. Five tissue sections were analyzed per animal.

Statistical analysis

Statistical analyses were performed in R version 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria). Paw withdrawal threshold data was analyzed by one-way ANOVA for differences between experimental days followed by Tukey's HSD test for post-hoc analysis. Following this analysis, an animal was considered to be a hyperalgesia responder if two criteria, similar to those published previously [24], were met, bilaterally. The first criterion required that the animal's PWT on day 1 and beyond was below 51.6 g. This value is the lower limit of the 95% confidence interval around the mean PWT in control animals. The second criterion required that the animal's average PWT on day 1 and beyond decreased by at least 10 g, relative to its baseline PWT. For glial cell expression analysis, differences between the control and disease model groups were assessed using Welch's t-test for Iba-1 expression and the student's unpaired t-test for astrocyte cell count. Subgroup analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. For all statistical tests, p-values less than 0.05 were considered statistically significant.

Results

Incidence of bilateral hyperalgesia

Of the twenty-one animals subjected to the AS model protocol, a total of five were classified as bilateral hyperalgesia responders, according to our criteria, yielding a 24% response. Grouped according to the day of sacrifice following the second pH 4.0 injection, three animals in the Day 3 group and two animals in the Day 1 group met bilateral hyperalgesia responder criteria (Table 1). As shown in Figure 1, analysis of pooled data from bilateral hyperalgesia responder animals revealed significant reductions in both ipsilateral and contralateral hindpaw withdrawal thresholds, following the second pH 4.0 injection.

Time Point Group	Cohort 1	Cohort 2
Day 1	0/3	2/3
Day 3	3/3	-
Day 7	0/3	0/3
Day 10	0/3	0/3

Table 1: Number of AS animals classified as bilateral hyperalgesia responders out of total animals per group.

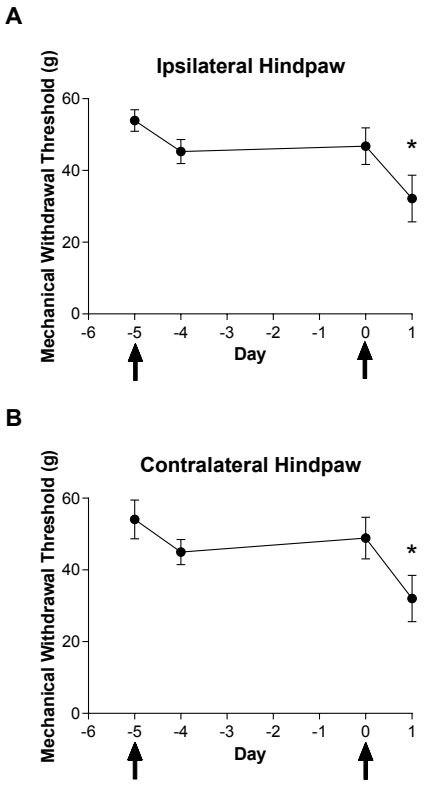

Figure 1: Pooled ipsilateral (A) and contralateral (B) hindpaw withdrawal thresholds from AS animals classified as bilateral hyperalgesia responders (n=5). Mechanical withdrawal thresholds were assessed thirty minutes prior to injection on injection days. Arrows indicate injection days. Data points represent mean ± SD. g =grams. * =P<0.05 vs. all other experimental days.

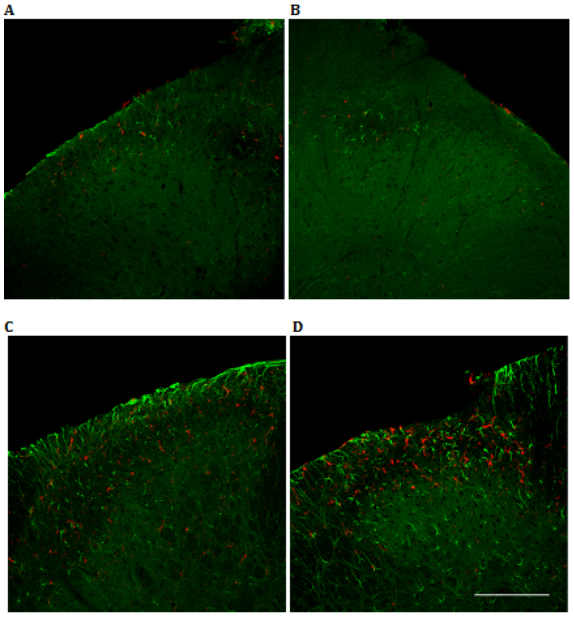

Figure 2: Representative confocal microscopy images of the dorsal horn region from control (A,B) and AS (C,D) animals showing Iba-1 (red) and GFAP (green) expression in L5 (A,C) and L6 (C,D) spinal segments. AS images shown were obtained from hyperalgesia responders in the Day 3 group. Scale bar=200 μm.

Increased spinal cord Iba-1 and GFAP expression, dorsal horn localization and morphological changes

Overall, expression of both Iba-1 and GFAP appeared to be higher in AS animals relative to controls in both L5 and L6 spinal segments (Figure 2). Examination of immuno-staining revealed changes indicative of glial cell activation among AS animals, including increased cell number and localization within the dorsal horn superficial laminae, larger and more densely stained cell bodies, and increased number and length of cell processes.

Hyperalgesia-dependent increase in spinal cord Iba-1 expression

Quantitative analysis of spinal cord Iba-1 expression in cohort 1 animals revealed significant increases in AS animals relative to controls in both L5 and L6 spinal segments (Figure 3A and 3C). Furthermore, analysis of L5 Iba-1 expression in AS animals grouped according to behavioural pain response revealed significant increases in hyperalgesia responders only (Figure 3B). Although increases in L6 Iba-1 expression were observed in both responder and non-responder subgroups versus controls, neither increase reached statistical significance (Figure 3D).

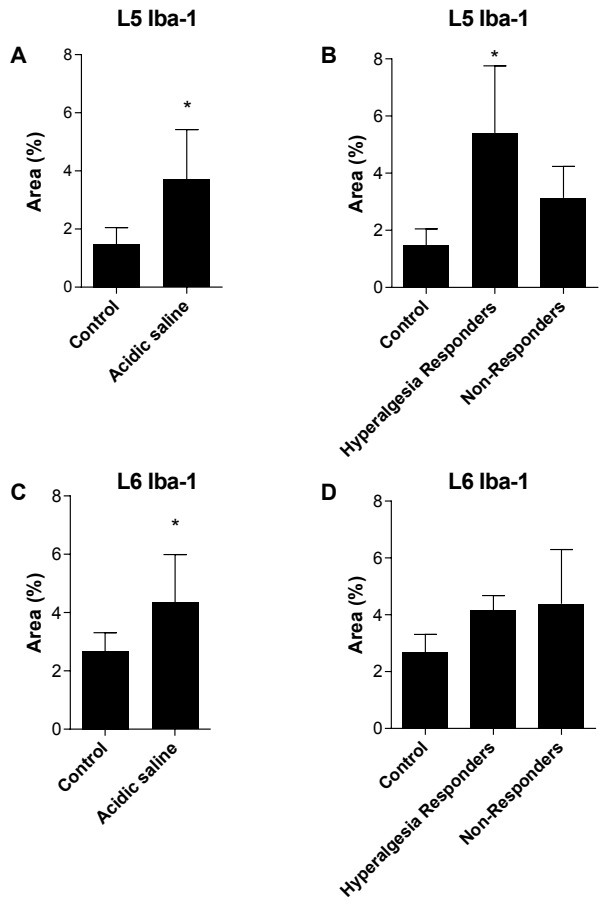

Figure 3: Percent tissue area with Iba-1 immunofluorescence above the intensity threshold in L5 (A,B) and L6 (C,D) spinal cord sections. A and C: Iba-1 area in control (n=4) vs. all cohort 1 AS animals (n=12). B and D: Iba-1 area in control vs. AS hyperalgesia responders (n=3) and non-responders (n=9). Data points represent mean ± SD.*=P<0.05.

Increased GFAP expression in spinal cord is independent of hyperalgesia response

Significant increases in spinal cord GFAP expression were detected in AS animals, relative to controls, in both L5 and L6 segments (Figure 4A and 4C). When analyzed as subgroups, both hyperalgesia responders and non-responder animals showed significantly higher GFAP expression in the L5 and L6 spinal cord versus controls (Figure 4B and 4D). Therefore, increases in GFAP expression occurred independently of hyperalgesia response in AS animals.

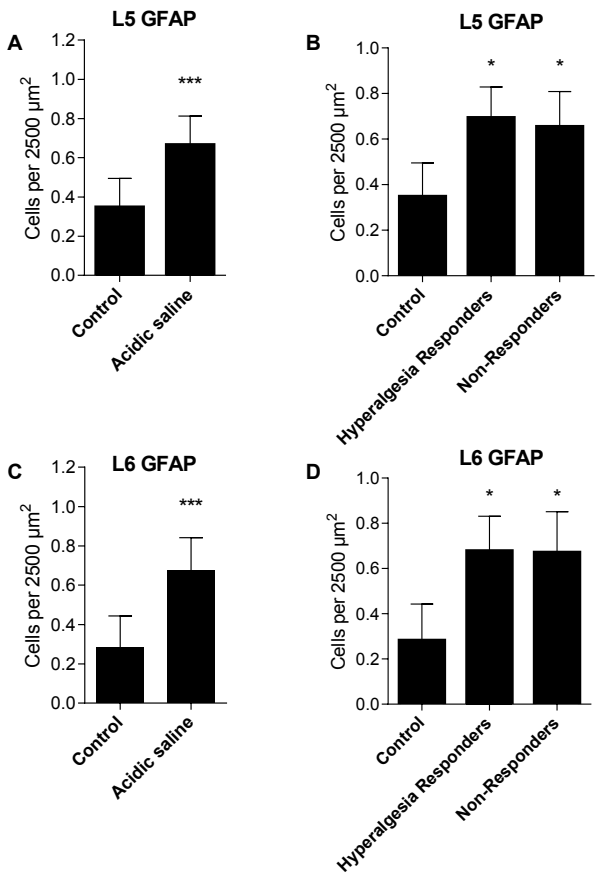

Figure 4: Mean astrocyte cell count per 2500 µm^2 dorsal horn tissue from L5 (A-B) and L6 (C-D) spinal cord sections. A and B: Cell count in control (n=4) vs. all AS animals (n=21). B and D: Cell count in control versus AS hyperalgesia responders (n=5) and non-responders (n=16). Data points represent mean ± SD.*=P<0.05, **=P<0.01,***=P<0.001.

The expression of Iba-1 and GFAP is not changed in the thalamus ventrobasal complex

No differences in glial marker expression were detected in AS animals compared to controls and very few astrocytes were apparent in the thalamus ventrobasal complex. As seen in Figure 5A, microglia cell counts within the AS group varied considerably and a small, non-significant increase compared to controls was observed. When assessed according to behavioural pain response, neither hyperalgesia responders nor non-responders had significantly different expression of glial cell markers versus controls (Figure 5B). Notably, two animals in the Day 10 group, both non-responders, were found to have markedly increased microglia cell counts, however, accounting for the high variation in the data.

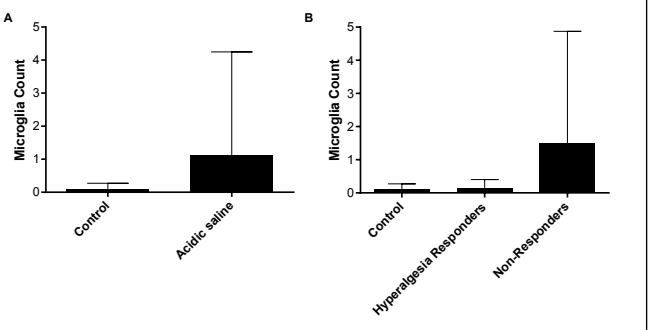

Figure 5: Mean microglia cell count in the thalamus ventrobasal complex. A: Control (n=4) versus all AS model animals (n=21). B: Control versus bilateral hyperalgesia responders (n=5) and non-responders (n=16). Data points represent group mean ± SD.

Discussion

Determining whether gliosis plays a role in chronic widespread musculoskeletal pain disorders is crucial in order to further our understanding of the underlying pathophysiology. In the present study we examined the response of spinothalamic glial cells to repeated insults of low pH to skeletal muscle tissue and describe the relationship between the expression of glial cell markers and the behavioural pain response observed. Although the response of glial cells can vary depending upon the specific injury or disease model, the presence of gliosis is usually characterized by up-regulation of glia-specific activation markers and distinct changes in morphological appearance [34]. Iba-1 and GFAP are well-validated markers of microglia and astrocyte activation, respectively, [35-38]. We found evidence of increased expression of both Iba-1 and GFAP in the lumbar spinal cord dorsal horn, and a correlation between L5 Iba-1 expression and the development of bilateral hyperalgesia. In agreement with published literature in which up-regulation of glial cell markers has been found within the lumbar spinal cord in an experimental pain model [39-41], the increased glial marker expression we observed appeared most apparent within the superficial laminae of the dorsal horn. Furthermore, morphological changes were observed including larger and more densely stained glial cell bodies as well as increased length and number of cell processes. Our findings therefore suggest that gliosis develops within the lumbar enlargement (L5 and L6 segments) of the spinal cord in the acidic saline model of chronic widespread musculoskeletal pain. To our knowledge, this is the first report describing that repeated insult of reduced pH within peripheral skeletal muscle tissue is sufficient to provoke a dorsal horn glial cell response.

The control of synaptic transmission in the CNS is thought to be tripartite, involving pre- and post-synaptic nerve terminals as well as glial cells [42]. The exaggerated transmission of excitatory amino acids in response to the second pH 4.0 injection is critically important to the development of hyperalgesia in this model as demonstrated by the rise in lumbar dorsal horn glutamate and aspartate concentrations following the second, but not the first, acid injection as well as the ability of NMDA receptor blockade during the second, but not the first, injection to prevent the development of hyperalgesia [23,30,31]. Glutamatergic transmission is also involved in the maintenance phase

of hyperalgesia in this model. Increased lumbar dorsal horn glutamate concentrations have been found at 1 week, with mechanical hyperalgesia diminished at this time point upon NMDA and non-NMDA ionotropic glutamate receptor blockade [30,31]. In parallel to these findings, our data demonstrate the presence of astrocyte activation in the lumbar spinal cord dorsal horn 24 hours following the second acidic saline injection and thereafter on days 3,7,10. These findings support the hypothesis of astrocyte involvement in the dysregulation of excitatory amino acid signalling which develops in this model. There may be several mechanisms through which this takes place.

Astrocytes in the dorsal horn surround glutamatergic synapses and normally maintain tight control of extracellular glutamate concentrations through glutamate re-uptake by Na^+-dependent glutamate transporters GLAST and GLT-1, thereby regulating the ability of glutamate to excite spinothalamic neurons. Handling of glutamate by astrocytes is, however, sensitive to a variety of extracellular signals, including mediators secreted by microglia and neurons. In response to pro-inflammatory stimuli, glutamate transporters along with glutamate uptake capacity has been shown to decrease in astrocytes *in vitro* [43-45], while the expression and functional capacity of GLT-1 has been found to decrease significantly in chronic pain *in vivo* [46]. Furthermore, excessive spinal cord glutamate accumulation along with pain-related behaviours that are dependent upon ionotropic glutamate receptor activation have been invoked spontaneously *in vivo* upon blockade of high affinity glutamate transporters in the spinal cord [47]. Therefore, the ability of the second injection in the AS model to provoke rises in dorsal horn excitatory amino acid concentrations along with bilateral hyperalgesia could reflect a decrease in astrocyte-mediated glutamate clearance. Future studies could test this hypothesis by examining the cellular localization and magnitude of expression of glutamate transporters at different time points over the course of this model, specifically assessing differences before and after each acid injection.

Impaired glutamate re-uptake by astrocytes is not the only possible explanation for the exaggerated release of excitatory amino acids observed following the second injection. As previously demonstrated, cobalt chloride, a calcium channel antagonist capable of blocking synaptic voltage-gated calcium channels as well as NMDA and non-NMDA glutamate receptors prevented the rise in dorsal horn excitatory amino acids following the second injection [30]. Indeed, the release of glutamate has been shown to occur through calcium-dependent vesicular release from both neurons and astrocytes [48-50]. Therefore, up-regulation of GFAP may point to an enhanced capacity for calcium-induced glutamate release from astrocytes in the lumbar dorsal horn following the second injection. Additionally, astrocytes express NMDA, AMPA and kainate ionotropic glutamate receptors as well as metabotropic glutamate receptors (*i.e.*, mGluR) [51,52]. Activation of these receptors has been shown to trigger further glutamate release by astrocytes, with a rise in intracellular calcium found to be the common underlying intracellular mechanism [49,53-55]. Indeed, increased intracellular calcium concentrations in stimulated astrocytes have been directly associated with NMDA-mediated excitatory transmission in neurons [55-57]. Therefore, in cases of increased extracellular glutamate concentrations in the dorsal horn, for example, due to pro-inflammatory dysregulation of glutamate clearance or metabolism, excitatory synaptic transmission can be amplified further through glutamate-induced glutamate release from glia via AMPA, kainite, and/or mGlu receptor activation. The role of astrocytes in mediating excitatory synaptic communication is thus of critical importance. Taken together with previous findings, our data support the hypothesis that glial cells, in particular astrocytes, are involved in the pro-nociceptive changes in excitatory transmission that develop in the lumbar spinal cord in the AS model.

Enhancement of pain-like behaviours has been directly correlated with glial cell activation in previous studies [39-41,58,59]. Consistent with these observations, we found increased lumbar spinal cord expression of Iba-1 in bilateral hyperalgesia responder animals whereas no difference in Iba-1 expression was detected in non-responder animals versus controls. In contrast, however, increased lumbar GFAP expression was observed in both hyperalgesia responders and non-responders compared to controls. Therefore, spinal cord glial cell activation is likely not the only pathophysiologic change required for a bilateral hyperalgesia response to develop in this animal model. Supporting this notion, previous studies have indicated that the second acidic saline injection in the AS model produces changes in the RVM (*i.e.*, increased release of excitatory neurotransmitters and reduced release of inhibitory neurotransmitters), and, that blockade of NMDA receptors within either the RVM or the nucleus reticularis gigantocellularis (NGC) is sufficient to abolish mechanical hyperalgesia [27,29]. Therefore, interplay between ascending nociceptive input from the spinal cord dorsal horn and descending excitatory facilitation from the RVM and NGC likely mediates the development of bilateral hyperalgesia in this model of musculoskeletal pain. Based on the results described here, it is expected that non-responder animals did not develop the same RVM changes in response to acidic saline injections, and therefore, despite the increased spinal cord GFAP expression detected, the absence of RVM hyper-excitation may have precluded the development of mechanical hyperalgesia in the non-responder subgroup.

Since the thalamus ventrobasal complex (VBC) is immediately afferent to the spinal cord dorsal horn along the ascending spinothalamic tract, examination of this region was included in the present investigation. Although no evidence of increased Iba-1 or GFAP expression in the VBC was found, the possibility of glial cell involvement here cannot be entirely discounted. Microglia activity within the VBC could potentially develop during a later time course within this model, given that a non-significant increase in Iba-1 expression was observed at day 10, the final time point of our study. Further data from an increased number of hyperalgesia responder animals captured from a broader range of time points is necessary to draw conclusions around thalamic gliosis in this animal model.

Despite the variable rates of bilateral hyperalgesia response reported in the AS model [23,24], the rate of response observed in our study was lower than expected. Interestingly, animals which developed hyperalgesia in our study were restricted to two of the four time point groups. In consideration of this observation, we note that the experimental protocol for each time point group was initiated on a different calendar day (*i.e.*, the experimental schedules for each group were stratified over the calendar). Therefore, environmental factors and/or stressors experienced (*ex.*, cage cleaning by animal care staff on the day of an injection) could have differed between groups. It has been previously demonstrated that environmental stress from confined restraint increases serum cortisol levels and increases pain-like behavioral responses (*i.e.*, reduced von Frey paw withdrawal thresholds), and, that sound stress enhances bradykinin-induced hyperalgesia in rats [41,60]. Therefore, variation in environmental factors across experimental groups may be responsible for the

between-group differences in hyperalgesia response observed in our study.

Taken together with previous studies in this research model along with clinical evidence of elevated inflammatory CSF markers [18,19], our experimental findings support the need for further investigation into the role of gliosis in chronic widespread musculoskeletal pain. The present pilot study involved only immunohistological detection of glial cell activation. Further investigations in this model are therefore recommended to test for additional indicators of gliosis and CNS neuro-inflammation. These may include measurement of Iba-1 and GFAP mRNA levels, quantification of NF-κB expression and phosphorylation to the active state (p-NF-κB), expression and phosphorylation of the NMDA receptor subunit NR2B, as well as concentrations of cytokines such as IL-1β, IL-6 and TNFα. This area of investigation may also be further advanced through the use of imaging modalities to assess the location and magnitude of glial cell activation both in research models and human subjects. Such methods may include positron emission tomography (PET) to image tracers such as ^{18}F-PBR111 or ^{11}C-PBR28 which bind to translocator protein (TPSO) expressed by activated glial cells. For example, PET imaging studies with ^{11}C-PBR28 have detected increased thalamic glial cell activity in chronic low back pain as well as T11-T12 spinal cord neuro-inflammation in chronic lumbar radiculopathy [61,62]. Alternatively, the use of non-radioactive fluorinated TPSO ligands may allow for glial cell imaging through the use of ^{19}F-MRI [63,64]. Candidate ^{19}F-MRI tracers could include the high affinity TPSO ligand FEDAA1106 or the fluorinated benzodiazepines SAS 643 and SAS 646 [65,66].

In conclusion, the evidence of glial cell involvement presented here supports the need for further investigation into the contribution of gliosis to the pathophysiology underlying chronic widespread musculoskeletal pain disorders. Additional investigations both in research models and human subjects are recommended to further advance the research findings of this pilot study.

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