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Neonatal complete Freund's adjuvant-induced inflammation does not induce or alter hyperalgesic priming or alter adult distributions of C-fibre dorsal horn innervation

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Abstract

Introduction: Inflammation during the neonatal period can exacerbate pain severity following reinjury in adulthood. This is driven by alterations in the postnatal development of spinal and supraspinal nociceptive circuitry. However, the contribution of alterations in peripheral nociceptor function remains underexplored.

Objectives: We examined whether neonatal complete Freund's adjuvant (CFA)-induced inflammation induced or altered adult development of hyperalgesic priming (inflammation-induced plasticity in nonpeptidergic C fibres) or altered postnatal reorganization of calcitonin gene-related peptide (CGRP)-expressing and isolectin B4 (IB4)-binding C fibres in the spinal dorsal horn (DH).

Methods: After intraplantar injection of CFA at postnatal day (P) 1, we assessed mechanical thresholds in adult (P60) rats before and after intraplantar carrageenan. One week later, intraplantar PGE_2-induced hypersensitivity persisting for 4 hours was deemed indicative of hyperalgesic priming. CGRP expression and IB4 binding were examined in adult rat DH after CFA.

Results: P1 CFA did not alter baseline adult mechanical thresholds, nor did it change the extent or duration of carrageenan-induced hypersensitivity. However, this was slower to resolve in female than in male rats. Rats that previously received carrageenan but not saline were primed, but P1 hind paw CFA did not induce or alter hyperalgesic priming responses to PGE_2. In addition, CFA on P1 or P10 did not alter intensity or patterns of CGRP or IB4 staining in the adult DH.

Conclusion: Complete Freund's adjuvant-induced inflammation during a critical period of vulnerability to injury during early postnatal development does not induce or exacerbate hyperalgesic priming or alter the broad distribution of CGRP-expressing or IB4-binding afferent terminals in the adult dorsal horn.

Keywords: Hyperalgesic priming, Neonatal, Postnatal, Nociceptors, Pain, Inflammation

1. Introduction

Nociceptive pathways undergo substantial structural and functional postnatal maturation.[19] Tissue damage and accompanying pain during the neonatal period has profound effects on the development of somatosensory processing with lasting impact on sensory thresholds and pain perception.[5,21,53] Neonates who undergo surgery or treatment within a neonatal intensive care unit experience significant painful stimuli, and follow-up studies have revealed decreased sensitivity to thermal[23,50] and mechanical[50] stimuli in these patients in later life, as well as increased perception of high intensity or repeat noxious stimuli and increased analgesic requirements during subsequent surgeries.[23,53]

A growing body of preclinical evidence has begun to uncover mechanisms underpinning altered somatosensory processing after neonatal inflammatory injury during a critical period of the first 7 postnatal days in rodents. This has revealed alterations in spinal and supraspinal nociceptive processing that do not occur after similar injuries later in life.[6,30,53,54,57] The consequences of inflammation during the neonatal period on primary afferent fibres (PAFs) in adult life are less clear. Early work claimed that hind paw inflammation in neonatal rodents causes a rostrocaudal expansion of sciatic and saphenous nerve terminal fields in lamina II into adulthood[48]; however, this was found to be transient and followed the timecourse of inflammation.[55] Other studies examining visceral afferents have shown that early-life colonic inflammation increases TRPA1[14] and

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$Na_v1.8$ expression and decreases A-type K^+ currents,[43,44] contributing to visceral hypersensitivity in adult life. It remains to be determined whether alterations in the function or central synaptic connections of PAFs after cutaneous neonatal injury contribute to increased responses to reinjury in adult life.

Hyperalgesic priming is a model of the transition from acute to chronic pain in adult life, and is driven by neuronal plasticity in nonpeptidergic, isolectin B4-positive (IB4+) PAF nociceptors after acute carrageenan or complete Freund's adjuvant (CFA)-induced inflammation or neuropathic injury.[2,25,40,41,45] This results in increased duration and extent of mechanical hypersensitivity upon reinjury, mediated by protein kinase Cε signaling, which occurs in addition to the cAMP-mediated protein kinase A activity within PAFs that contributes to hypersensitivity during the initial injury.[1,2,41,45] We hypothesized that acute inflammation in the neonatal period may induce hyperalgesic priming in adulthood. To test this, we induced inflammation in neonatal rats with CFA and then subsequently examined the development of hyperalgesic priming once rats reached adulthood. We also examined whether neonatal inflammation altered broad distributions of peptidergic and nonpeptidergic PAF termination in the adult superficial dorsal horn (DH).

2. Materials and methods

2.1. Experimental animals

Experimental procedures were conducted in accordance with UK Home Office regulations and followed International Association for the Study of Pain guidelines. Ethical approval was granted by the University of Nottingham Animal Welfare and Ethical Review Board. Time-mated pregnant female Sprague-Dawley rats were purchased from Charles River UK 1 week before parturition. Litters (female and male, litter sizes 9–16 animals) were housed with their dams and animals aged postnatal day (P) 21 or greater were housed in same-sex groups of 2 to 4 animals in individually ventilated cages at constant ambient temperature (21 ± 2°C) and humidity (50%), with access to food and water ad libitum. Animals were maintained on a 12-hour light/dark cycle, and all experimental procedures were performed during their light cycle. Handling and maternal separation of pups was kept to a minimum and litters were weaned at P21.

2.2. Induction of models of inflammatory injury

Inflammation was induced in neonatal rats through an intraplantar injection of complete Freund's adjuvant (CFA; diluted 1:1 with 0.9% saline; Sigma-Aldrich, United Kingdom). A dose of 10 μL was selected as higher doses (25 μL) have been previously shown to cause profound changes in plantar hind paw skin including infiltration by lymphocytes and mast cells for as long as 8 weeks after neonatal inflammation.[55]

Neonatal rats were randomly assigned in either control or treatment groups and then briefly anaesthetised using 4% isoflurane carried by a mixture of 66% N_2O and 33% O_2, whilst placed on a heated mat to preserve body temperature. Using a 27G needle, 10 μL of CFA or saline (control group) was injected subcutaneously into the centre of the plantar surface of the left hind paw.

Once adult (P60), the same cohort of rats were then briefly anaesthetised using 3% isoflurane and received a subcutaneous injection of 5 μL 1% λ-carrageenan (Sigma-Aldrich) in 0.9% saline or 0.9% saline in the centre of the left plantar hind paw between the 2 prominent distal tori. A week later, animals were anaesthetised in the same manner and received an intraplantar injection of 1 μg prostaglandin E_2 (PGE_2) in 5 μL saline or 0.9% saline in the same location. Doses and group sizes were selected based on previous studies examining hyperalgesic priming.[2,9]

2.3. Behavioural testing (von Frey test)

Mechanical paw withdrawal thresholds were assessed by applying von Frey hairs to the plantar surface of the hind paw for 3 seconds, quantified using a modified up-down method.[12] Before baseline measurements, animals were habituated to testing apparatus (elevated Perspex boxes with a fine steel mesh floor; Medical Engineering Unit, University of Nottingham) and hind paw stimulation for 30 minutes on at least 2 occasions. The experimenter was blinded to treatment groups during all testing.

2.4. Immunohistochemistry

After intraplantar injections of CFA or saline at P1 or P10, adult (P67) rats were terminally anaesthetised with sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA; Sigma Aldrich). Lumbar spinal cord segments were collected, postfixed overnight in 4% PFA, and then cryopreserved in 30% sucrose for 24 h. Lumbar spinal cords were sectioned at 30 μm on a freezing microtome (SM201R, Leica, Germany) and immunohistochemistry performed on 8 randomly selected, nonadjacent, L4 or 5 transverse sections.

Free-floating sections were washed, then incubated with blocking solution (3% (vol/vol) goat serum in 0.1 M PBS/0.01% (vol/vol) Triton X-100) for 2 hours at room temperature followed by incubation with rabbit anti-calcitonin gene-related peptide (CGRP; AB15360, Millipore, United Kingdom; 1:1000) and FITC-conjugated isolectin B4 (IB4; Sigma Aldrich L2895; 1:500) overnight at room temperature and then with Alexa Fluor 568-conjugated goat anti-rabbit antibody (A11011, Invitrogen, United Kingdom; 1:500) for 2 hours at room temperature. Sections were mounted on gelatinised slides, allowed to dry overnight, and then coverslipped using Fluoromount-G mounting media (Fisher Scientific, United Kingdom).

2.5. Microscopy and image quantification

Images of the entire DH ipsilateral to early-life intraplantar injections were captured with a Leica DMIRE2 inverted epifluorescence microscope and a 10X objective (0.25 NA) fitted with a Hamamatsu OrcaER camera, controlled using Volocity 6.3 software (PerkinElmer). All images were captured using the same exposure time for each channel (0.5–0.8 seconds). ImageJ/Fiji[49] was used to quantify staining and adjust image brightness/contrast for presentation. Images were processed identically, and the same contrast enhancement was applied to each image.

Images were flat-field corrected (per pixel, image/flat-field) against an image captured of a blank section of slide with the same exposure settings. CGRP and IB4 staining were then quantified by placing a grid 200 × 300 μm, with the 200-μm axis aligned to the dorsal edge of the gray matter, at the centre of the DH, consisting of 9 horizontal lines arranged evenly along the 300-μm axis (ie, every 37.5 μm). A central region was chosen to permit quantification partially within the medial portion of the DH innervated by the tibial nerve (ie, innervating the centre of the plantar surface of the hind paw), and partially within innervation territory of the common peroneal nerve (innervating the centre of the dorsal surface of the hind paw),[15,52] as neonatal injection of CFA induced inflammation of the entire hind paw. The

mean fluorescence intensity of each line was then calculated, providing a profile of fluorescence intensity vs depth from the outer surface of the DH for each target (referred to as the fluorescence intensity profile). Mean fluorescence intensity profiles from 6 to 8 sections per animal were calculated.

2.6. Statistical analysis

Data are presented as mean ± SEM, with n = 1 defined as a single animal. Data were tested for normality (Shapiro–Wilk tests) and analysed with parametric or nonparametric statistical tests where appropriate, with $P < 0.05$ considered statistically significant. Data were analysed using Mann–Whitney *U* test, 2-way, or repeated-measures (RMs) 2- or 3-way analysis of variance (ANOVA), with Bonferroni *post hoc* tests where appropriate. Hyperalgesic priming was considered to have occurred after statistically significant decreases in mechanical threshold from baseline 4 hours post-PGE_2 injection. All statistical analyses were performed using GraphPad Prism 8.1.

3. Results

3.1. Neonatal complete Freund's adjuvant-induced inflammation has no effect on adult inflammation-induced mechanical hypersensitivity in male or female rats

Inflammation was induced in male and female neonatal rats (aged P1) through intraplantar injection of CFA (10 μL) or sham injection (saline) under anaesthesia. Complete Freund's adjuvant induced substantial swelling and erythema of the entire hind paw, extending to the ankle, which resolved within 7 days and was not present in sham groups. At P60, there was no significant difference in mechanical hind paw withdrawal thresholds between rats receiving CFA or sham injection (**Fig. 1A**; Mann–Whitney test, $U = 176$, $P = 0.09$; n = 24 and 20 for CFA and sham, respectively).

To investigate the potential effects of neonatal inflammation on adult responses to reinjury, rats received an injection of 1% λ-carrageenan (5 μL; carra) into the centre of the plantar surface of the hind paw. Carrageenan induced a significant decrease in mechanical threshold when examined 1, 4, and 24 hours after injection in both neonatal CFA- and saline-treated groups, and thresholds returned to baseline within 1 week (**Fig. 1B**; 3-way RM ANOVA with Bonferroni posttests; Time x Adult treatment interaction, $F_{4,160} = 9.174$; $P < 0.001$; n = 10–12). Neonatal CFA had no impact on adult responses to carrageenan at any time point (Time × Neonatal treatment × Adult treatment interaction, $F_{4,160} = 0.477$; $P = 0.752$).

We then tested for potential sex differences in the effects of neonatal CFA-induced inflammation on adult responses to carrageenan-induced inflammation (**Fig. 2**). Carrageenan-induced decreases in mechanical thresholds from baseline lasted significantly longer in female vs male rats (**Fig. 2A**; 3-way RM ANOVA with Bonferroni posttests vs baseline; Time × Sex interaction, $F_{4,72} = 2.56$; $P = 0.046$; n = 4–6). Female rats also demonstrated a significantly lower area-under-curve of mechanical threshold over time (**Fig. 2B**; 2-way ANOVA; Sex $F_{1,18} = 5.005$, $P = 0.038$). Neonatal treatment with CFA had no effect on carrageenan-induced changes in mechanical threshold in either male or female rats (**Fig. 2A**: 3-way RM ANOVA; Time × Neonatal treatment × Sex interaction $F_{4,72} = 0.399$; $P = 0.808$; **Fig. 2B**: 2-way ANOVA; Neonatal treatment × Sex interaction $F_{1,18} = 0.840$, $P = 0.372$).

3.2. Neonatal complete Freund's adjuvant-induced inflammation does not alter the development of hyperalgesic priming

One week after intraplantar injection of carrageenan or saline (animals now aged P67), the development of hyperalgesic priming was examined through an intraplantar injection of PGE_2 (1 μg in 5 μL saline) or saline; decreased mechanical thresholds that were maintained for at least 4 hours postinjection were deemed to be indicative of hyperalgesic priming.[2] PGE_2 injection resulted in a significant decrease in mechanical thresholds over time (**Fig. 3**; 2-way RM ANOVA; Time × Treatment interaction $F_{8,76} = 3.68$, $P = 0.0011$; n = 5–6). Pre-PGE_2 injection, there were no significant differences in mechanical threshold between groups (**Fig. 3**; mean mechanical threshold = 23.5 ± 0.77 g; Bonferroni posttests between groups, $P > 0.85$ for all comparisons). One hour post-PGE_2 or saline injection, rats in all 4 treatment groups that received PGE_2 displayed a significant decrease in mechanical threshold from baseline (neonatal/P60/P67 treatments: CFA/carra/PGE_2; sham/

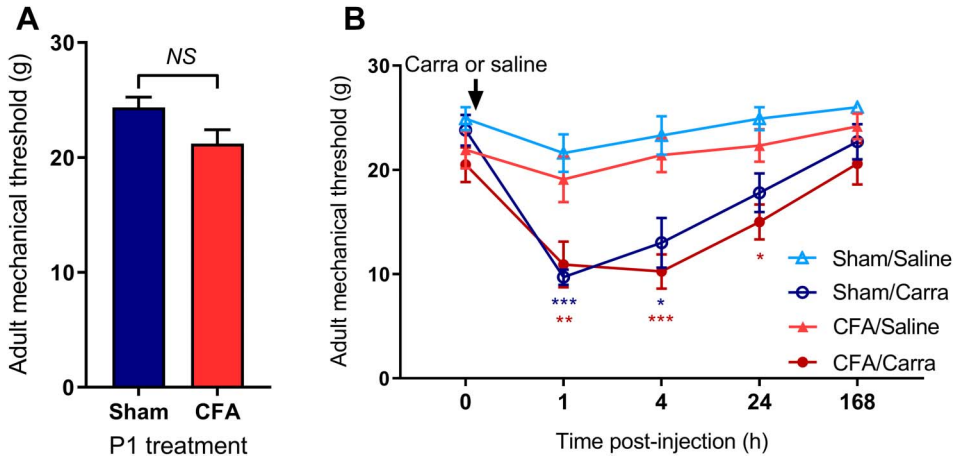

Figure 1. Neonatal CFA-induced inflammation does not alter adult mechanical thresholds or responses to repeat inflammation. (A) Intraplantar CFA (10 μL) at P1 did not alter mechanical paw withdrawal thresholds in adult (P60) rats (Mann–Whitney test; $P = 0.09$; n = 20 and 24 for sham and CFA, respectively). (B) Carrageenan (5 μL; carra) in adult rats induced a significant decrease in PWT lasting at least 24 hours (3-way RM ANOVA; Time x Adult treatment interaction $P < 0.001$; Bonferroni posttests vs sham/saline, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n = 10–12); however, neonatal CFA had no impact on adult responses to carrageenan at any time point (Time × Neonatal treatment × Adult treatment interaction $P = 0.752$). CFA, complete Freund's adjuvant.

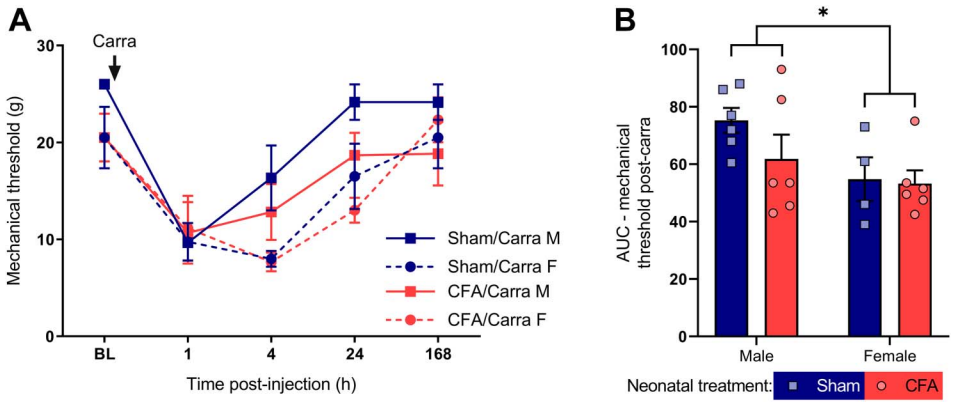

Figure 2. Sex differences in mechanical hypersensitivity after intraplantar carrageenan injection in adulthood after neonatal CFA-induced inflammation. (A) Carrageenan (carra; 1%, 5 μL) induced a significant decrease in mechanical thresholds from baseline in all groups, and this was different between male and female rats (3-way ANOVA; significant Time and Time × Sex interactions; $P = < 0.001$ and 0.046, respectively; n = 4–6). However, neonatal treatment (CFA or sham injection) had no effect on adult responses to carrageenan (Time × Neonatal treatment × Sex interaction, $P = 0.808$) (B) Area-under-curve analysis of alterations in mechanical threshold also showed a significant difference between male and female rats, but no effect of neonatal treatment (2-way ANOVA; Sex $P = 0.038$ (*); Sex × Neonatal treatment $P = 0.371$). ANOVA, analysis of variance; CFA, complete Freund's adjuvant.

carra/PGE_2; CFA/saline/PGE_2; sham/saline/PGE_2), whereas those injected with saline did not. At 4 hours, only those groups that previously received carrageenan still exhibited reduced mechanical thresholds (**Fig. 3**; Bonferroni posttests compared to baseline; $P = 0.0011$; n = 5–6). Saline injection induced no alteration in mechanical threshold from baseline.

Neonatal CFA did not alter adult primed responses to PGE_2 at any time point (Bonferroni posttests between groups at 1 and 4 hours postinjection; CFA/carra/PGE_2 vs sham/carra/PGE_2, $P > 0.99$; n = 5–6) nor did neonatal CFA induce hyperalgesic priming (CFA/saline/PGE_2 vs sham/saline/PGE_2, $P > 0.99$; n = 5–6).

3.3. Complete Freund's adjuvant on P1 or P10 does not alter broad distributions of CGRP and IB4+ primary afferent terminals in the adult spinal dorsal horn

The influence of intraplantar injection of CFA at P1 (during the "critical period" of long-term consequences of neonatal injury) and P10 (after this critical period) on adult distributions of nonpeptidergic (IB4+) nociceptor innervation within the DH was examined by measuring isolectin B4 (IB4) binding-associated fluorescence intensity along a path through the DH, perpendicular to and originating at the exterior of the SC (fluorescence intensity profile). IB4 binding was concentrated to a band within the superficial laminae of the DH, approximately 30 to 120 μm from the exterior (**Figs. 4A and D**). There were no differences in fluorescence intensity profile of IB4 binding within the DH ipsilateral to neonatal injury between groups (**Fig. 4E**; intraplantar CFA or sham injection at P1 or P10; 3-way RM ANOVA; Depth × Age at injury × Injury interaction: $F_{8,72} = 0.51$; $P = 0.84$; n = 3–4). Peptidergic (CGRP+) nociceptor innervation was also examined; CGRP immunostaining was restricted to a narrow band of high-intensity staining in the superficial DH, approximately corresponding with laminae I and II_o (**Figs. 5A and D**). As with IB4 staining, no differences were found in the fluorescence intensity profiles of DH CGRP staining between groups (**Fig. 5E**; 3-way RM ANOVA; $F_{8,\ 72} = 1.23$, $P = 0.30$, n = 3–4).

4. Discussion

Pain during the neonatal period has a substantial impact on the postnatal development of nociceptive circuitry, leading to persistent global increases in acute mechanical and thermal thresholds (hypoalgesia) and increased responses to reinjury at the site of initial inflammation (hyperalgesia).[53] Previous studies investigating mechanisms driving these changes have identified alterations in spinal and supraspinal nociceptive circuitry. Within the spinal cord, increased activation of microglia,[6] facilitated long-term potentiation of DH interneurons,[32] and increased PAF input, together with decreased inhibitory input, onto lamina I projection neurons,[34] are among the mechanisms that are proposed to lead to increased hyperalgesia in adulthood. Global hypoalgesia is thought to arise from decreased rostral ventromedial medulla (RVM)-mediated descending facilitation and a predominance of inhibition of DH nociceptive processing developing after neonatal injury,[54] increased opioid tone within the RVM and periaqueductal gray,[31] and a decrease in the intrinsic excitability of lamina II interneurons.[33]

Although studies have shown that early-life colonic inflammation increases visceral afferent expression TRPA1[14] and $Na_v1.8$ and decreases A-type K^+ currents,[43,44] few studies have investigated changes in primary afferent function after cutaneous inflammation in the neonate. Here, we investigated whether hyperalgesic priming, PKCε-mediated neuroplasticity of IB4+ afferent responses to repeat inflammation,[2] may contribute to exacerbated responses to reinjury in adulthood. We have shown that neonatal CFA-induced inflammation does not induce hyperalgesic priming of primary afferent nociceptors or alter its development in adult life. In parallel, distributions of peptidergic and nonpeptidergic C-fibre terminals in the adult DH were unaltered after neonatal CFA-induced inflammation.

4.1. Neonatal complete Freund's adjuvant-induced inflammation had no effect on adult mechanical thresholds before or after adult inflammatory injury

Complete Freund's adjuvant-induced inflammation at P1 had no effect on baseline mechanical thresholds in adult life. Our findings correlate with several previous studies showing that hind paw CFA or carrageenan injection during the neonatal period did not result in changes in mechanical or thermal thresholds in adult life.[36,48,55] These collective results are in contrast to the long-term effects of other models of neonatal injury, including hind paw surgical incision,[6,54,56] which result in global mechanical and thermal hypoalgesia in adult life. We also found that neonatal CFA had no effect on inflammation-induced changes in mechanical

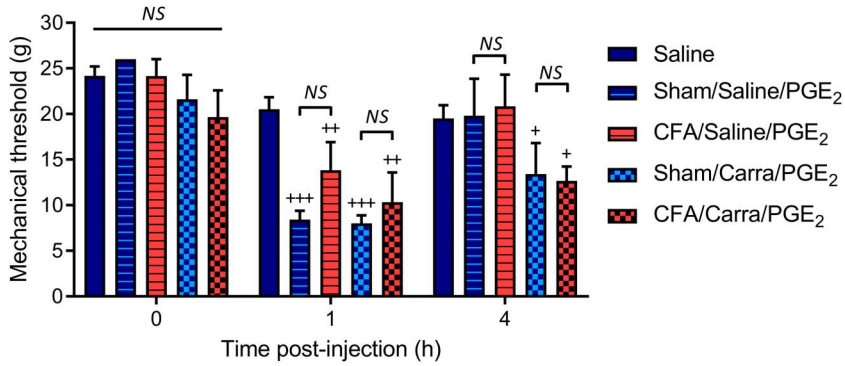

Figure 3. Neonatal CFA-induced inflammation has no effect on the development of hyperalgesic priming in adult rats. Mechanical PWT after intraplantar injection of PGE_2 (1 μg in 5 μL saline) or saline, 7 days after carrageenan or saline injection and after neonatal CFA or saline administration. One hour after injection, all groups receiving PGE_2 experienced a significant decrease in PWT, whilst those receiving saline did not; at 4 hours, only those groups previously receiving carrageenan maintained decreased PWTs (2-way RM ANOVA with Bonferroni posttests vs baselines; $+P < 0.05$; $++P < 0.01$; $+++P < 0.001$; n = 5–6). Neonatal CFA had no effect on adult responses to PGE_2 (Bonferroni posttests, $P > 0.05$). ANOVA, analysis of variance; CFA, complete Freund's adjuvant.

threshold in adulthood. This correlates with Walker et al.[55] who found no alterations in adult thresholds after neonatal CFA or carrageenan, but is in contrast to several previous studies, in which neonatal hind paw CFA,[36,48] carrageenan,[46] surgical incision,[6,56] or repetitive pin-prick[11] caused a greater magnitude of decrease in mechanical threshold and thermal withdrawal latencies upon reinjury compared to control animals that did not experience neonatal injury.

These inconsistencies between this study and previous work may be explained by the extent of painful experience occurring during the neonatal period. Several studies have shown that administration of CFA or carrageenan at P1-3 does not induce

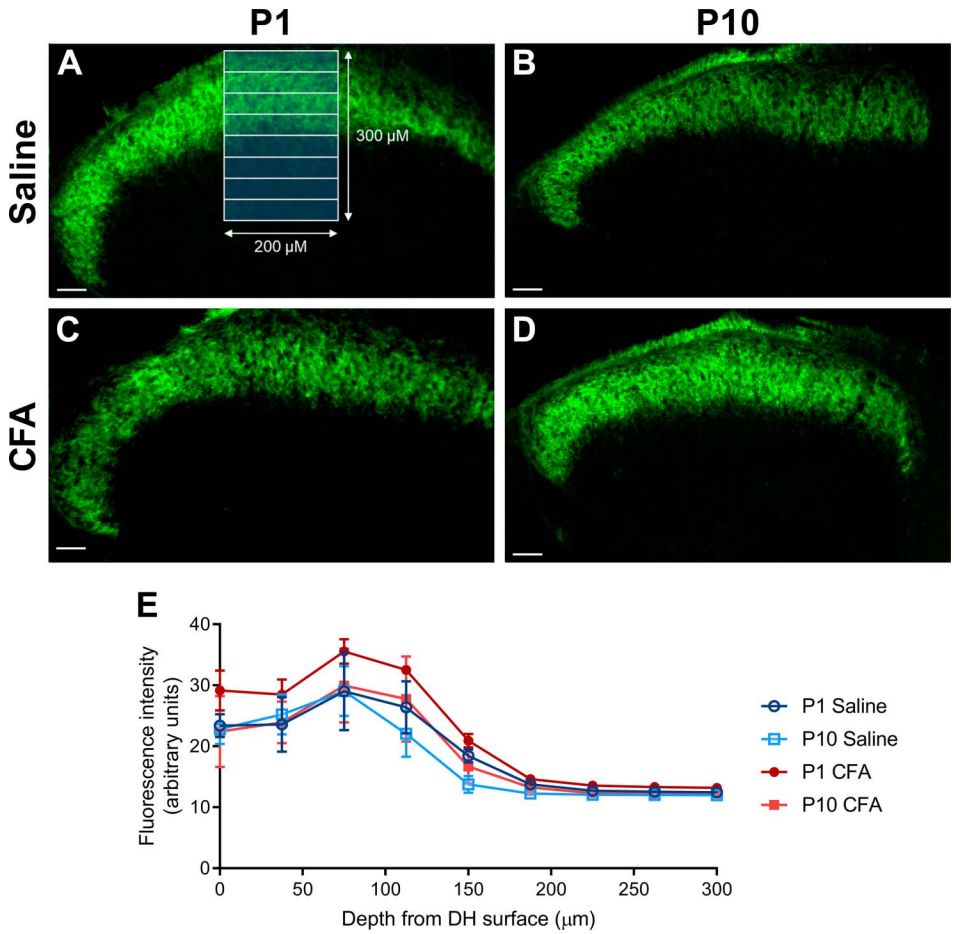

Figure 4. Intraplantar CFA at P1 or P10 has no effect on adult nonpeptidergic primary afferent fibre terminal staining in the dorsal horn (DH). (A–D) Representative images of IB4 binding in the ipsilateral DH at P67 after intraplantar CFA or saline at P1 or 10. Scale bars = 50 μm. Quantification method is shown in (A): a 200 × 300-μm grid was placed as shown across the dorsal horn. The mean fluorescence intensity across each of the 9 horizontal lines was then calculated to provide a profile of fluorescence intensity vs depth from dorsal surface of the dorsal horn gray matter. (E) Fluorescence intensity profiles of IB4 binding in the ipsilateral DH. No significant differences were observed between groups (3-way RM ANOVA; $P = 0.84$; n = 3–4). ANOVA, analysis of variance; CFA, complete Freund's adjuvant; RM, repeated-measures.

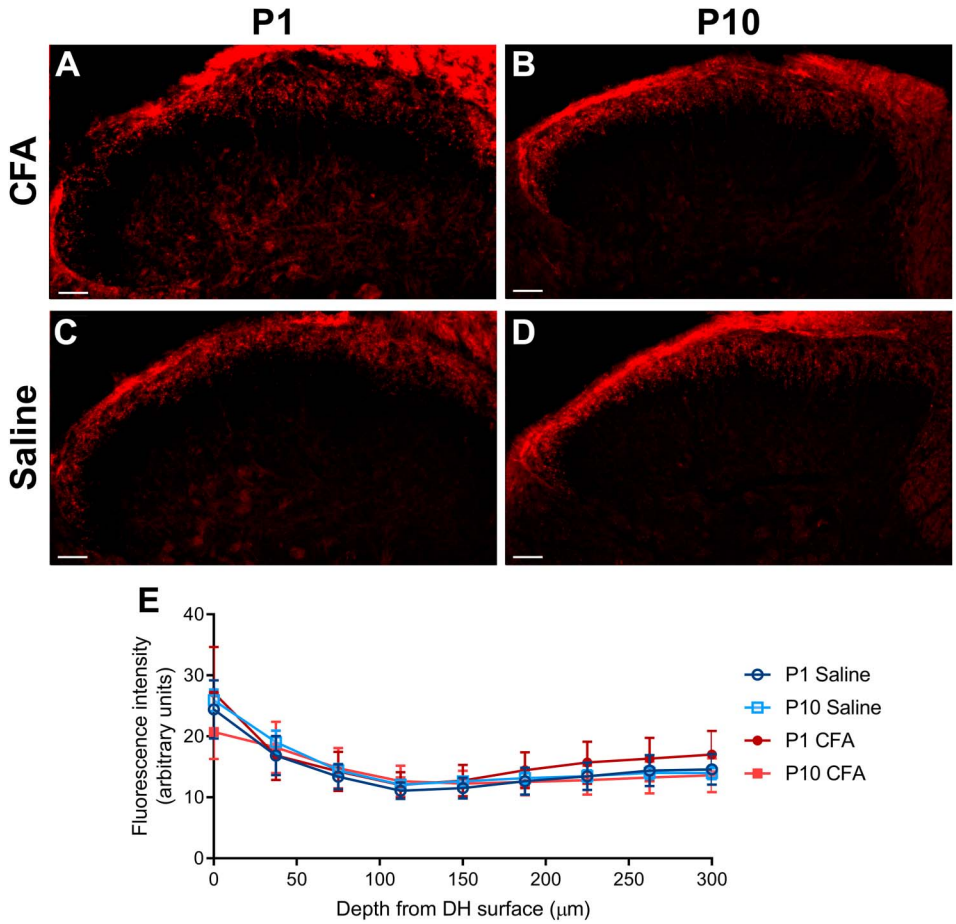

Figure 5. Intraplantar CFA at P1 or P10 has no effect on adult peptidergic primary afferent fibre terminal staining in the dorsal horn (DH). (A–D) Representative images of CGRP immunostaining in the ipsilateral DH at P67 after intraplantar CFA or saline at P1 or 10. Scale bars = 50 μm. (E) Fluorescence intensity profiles of CGRP staining in the ipsilateral DH. No significant differences were observed between groups (3-way RM ANOVA; $P = 0.30$; n = 3–4). ANOVA, analysis of variance; CFA, complete Freund's adjuvant; RM, repeated-measures.

acute decreases in mechanical threshold despite the presence of inflammation.[3,48,55] By contrast, others have shown that surgical hind paw incision[54,56] and injection of carrageenan[46] induces short-lived mechanical hypersensitivity. Perioperative sciatic nerve block during the neonatal incision injury prevents the development of long-term somatosensory alterations after neonatal hind paw incision,[54,56] suggesting that nociceptive drive is required to develop long-term alterations in somatosensation, and the presence of peripheral inflammation alone may not be sufficient to generate this effect.

Previous studies showing neonatal CFA-induced vulnerability to reinjury in adulthood[24,36,48] used a larger dose (25 μL dose of CFA diluted 1:1 in saline) than used in this study (10 μL). 25 μL of CFA administered to neonatal rats generates chronic inflammatory changes persisting well into adulthood,[36,55] accounting for long-term vulnerability to inflammation despite a lack of acute hypersensitivity during the initial injury.

4.2. Sex differences in inflammation-induced mechanical hypersensitivity

In this study, carrageenan-induced decreases in mechanical withdrawal thresholds were slower to resolve in females than in males. This correlates with other reports of increased severity or duration of mechanical or heat hypersensitivity after intraplantar carrageenan in female rodents,[13,35] although this effect was not shown by Joseph et al.[26] who found that duration and severity was increased in male compared to female rats. Females have been shown to produce a larger proinflammatory immune response to tissue damage than males, potentially resulting in the accumulation of increased levels of proinflammatory cytokines that result in peripheral sensitization.[47]

Reports of sex differences in the effects of neonatal injury on somatosensation in later life are mixed. Neonatal carrageenan injection was reported to induce a greater degree of global hypoalgesia and enhanced hyperalgesia upon reinjury in adult female rats than in males.[31] By contrast, neonatal hind paw incision enhanced hyperalgesia upon reinjury to a similar extent in male and female rats, although this was dependent on microglial function in males but not in females.[39] Another study reported that neonatal hind paw incision induced mechanical and thermal hypersensitivity in male, but not female, adolescent (P28–40) mice.[10] Here, neonatal treatment had no impact on the extent or resolution of adult inflammation-induced mechanical hypersensitivity in either sex.

4.3. Neonatal complete Freund's adjuvant did not alter the development of, or induce, hyperalgesic priming

This study showed that prior application of carrageenan in adults generated a prolonged PGE_2-mediated hyperalgesic response, in agreement with previous demonstrations of type I (injury-

induced) hyperalgesic priming after hind paw carrageenan or CFA injections in adulthood.[2,9,26] However, neonatal CFA did not alter the response to carrageenan, nor the development of hyperalgesic priming (revealed after intraplantar injection of PGE_2), compared with rats that received saline in the neonatal period. This finding is of interest because in adults, hyperalgesic priming persists for several months after the initial injury.[2,17,42] In addition, development of priming in adults is not dependent on the hyperalgesia accompanying the initial noxious insult,[2] meaning that a lack of hyperalgesia accompanying neonatal inflammation would not be expected to be sufficient to explain the failure to induce hyperalgesic priming in adulthood. This may suggest that PKCε-mediated alterations in nociceptive processing differ between neonates and adults. This could be due to a lack of functional IB4+ nociceptor innervation of the DH during the first postnatal week,[7] or differences in the acute inflammatory process, or PKCε-mediated signalling pathways. Regardless of mechanism, we speculate that previously observed long-term effects of painful neonatal experience[6,53,56] are unlikely to be related to mechanisms involved in type I hyperalgesic priming. However, PGE_2 can induce hypersensitivity for at least 24 hours in primed animals[17,26]; given that we did not examine the time-course of resolution of PGE_2-medated hypersensitivity, our data cannot exclude the possibility that neonatal CFA may alter the duration of primed responses to PGE_2. Furthermore, our findings do not preclude the possibility that other models of neonatal injury that induce long-term vulnerability to reinjury (such as hind paw incision[6]) may induce hyperalgesic priming or exacerbate the development of priming in adulthood.

4.4. Early life complete Freund's adjuvant does not alter peptidergic and nonpeptidergic afferent termination in the adult dorsal horn

Primary afferent termination patterns in the DH undergo substantial postnatal maturation; peptidergic C fibres are functional from the late gestational stages and sparsely project to the DH during this period, whereas IB4-binding C fibres are do not terminate in the DH until P5 and noxious stimulus-mediated activity is not present until P10.[4,20] In addition, neonatal inflammation increases CGRP expression and IB4 binding in dorsal root ganglia neurons during this period of postnatal maturation.[8] Here, we have shown that hind paw CFA-induced inflammation at P1 (during the "critical period" of long-term consequences of neonatal injury) or P10 (after the critical period)[46] does not alter the intensity or broad distribution of CGRP-associated immunofluorescence or IB4 binding in the adult DH. Regardless of treatment, CGRP+, peptidergic immunostaining was visible in the superficial DH, approximately in laminae I and II, whilst IB4 staining was present deeper in lamina II to III, reflecting normal adult patterns.[51]

Previous studies demonstrating alterations in PAF innervation of the DH after neonatal injury identified rostrocaudal expansion of nerve terminals[48] and increases in lamina III to V CGRP immunoreactivity[37]; however, these studies used a dose of CFA that induces chronic inflammation lasting well into adulthood,[55] which does not reflect neurodevelopmental plasticity or resemble human neonatal experience. Our findings do not exclude the possibility of PAF synaptic reorganization from contributing to long-term alterations in somatosensation; despite bulk patterns of C-fibre termination not changing after neonatal CFA-induced inflammation, it is possible that the integration of PAF connectivity with specific populations of second order neurons may be altered, as has been demonstrated for NK1-expressing projection neurons.[34]

4.5. Conclusions and implications for future directions

We have shown that CFA-induced inflammation during the neonatal period does not induce or alter the development of hyperalgesic priming. Furthermore, we have shown that early-life CFA-induced inflammation did not alter postnatal reorganization of C-fibre input into the DH. These findings point towards other potential mechanisms, which may include a contribution of the descending control pathways. The RVM undergoes significant postnatal development,[22,28,29] and neonatal injury significantly alters adult descending pathways from the RVM.[54] Although selective ablation of serotonergic input to the DH (ie, arising from the RVM[16,18]) attenuates the initial, acute phase of PGE_2-mediated hyperalgesia in adult rodents, it does not influence the chronic phase, demonstrating that this pathway does not contribute to the development of hyperalgesic priming.[27] By contrast, ablation of dopaminergic projection neurons arising from the hypothalamic A11 nucleus resulted in a failure of the development of hyperalgesic priming in adulthood.[38] To date, the postnatal development of hypothalamic descending control of nociception has yet to be characterised, and therefore whether the lack of hyperalgesic priming after neonatal CFA-induced inflammation herein arises due to functional different hypothalamic descending control pathways in neonates, compared to adults, remains to be explored. Future studies examining synaptic integration of PAFs into DH circuitry and supraspinal control of spinal nociceptive processing may further elucidate the mechanisms underpinning life-long alterations in somatosensation after painful neonatal experience.

Disclosures

The authors have no conflicts of interest to declare.

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