Targeting p38 Mitogen-activated Protein Kinase to Reduce the Impact of Neonatal Microglial Priming on Incision-induced Hyperalgesia in the Adult Rat

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Abstract

Background—Neonatal surgical injury triggers developmentally-regulated long-term changes that include enhanced hyperalgesia and spinal microglial reactivity following reinjury. To further evaluate priming of response by neonatal hindpaw incision, we investigated the functional role of spinal microglial p38 mitogen-activated protein kinase following reincision in adult rodents.

Methods—Plantar hindpaw incision was performed in anesthetized adult rats, with or without prior incision on postnatal day 3. Numbers and distribution of phosphorylated-p38 (1, 3, 24 h) and phosphorylated extracellular signal-regulated kinase (15 min, 24 h) immunoreactive cells in the lumbar dorsal horn were compared following adult or neonatal plus adult incision. Withdrawal thresholds evaluated reversal of incision-induced hyperalgesia by p38 inhibition with intrathecal SB203850.

Results—Neonatal injury significantly increased phosphorylated-p38 expression 3 h following adult incision (55 ± 4 *vs.* 35 ± 4 cells per section, mean ± SEM, n = 6-7, P <0.01). Increased expression was restricted to microglia, maintained across lumbar segments, and also apparent at 1 and 24 h. Preincision intrathecal SB203850 prevented the enhanced mechanical hyperalgesia in adults with prior neonatal injury, and was effective at a lower dose (0.2 mg/kg *vs.* 1 mg/kg, n = 8, P <0.05) and for a longer duration (10 *vs.* 3 days). Lumbar neuronal phosphorylated extracellular signal-regulated kinase expression reflected the distribution of hindpaw primary afferents, but was not significantly altered by prior incision.

Conclusions—Neonatal incision primes spinal neuroglial signalling, and reincision in adult rats unmasks centrally-mediated increases in functional microglial reactivity and persistent

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hyperalgesia. Following early life injury, p38 inhibitors may have specific benefit as part of multimodal analgesic regimes to reduce the risk of persistent postsurgical pain.

Introduction

Persistent post-surgical pain occurs in a significant proportion of adults and children.[1,2] There is a need to identify predisposing factors and underlying mechanisms to more specifically target high risk groups with the most effective preventive strategies.[3-5] Severe acute pain continues to be reported following adult and pediatric surgery[6-8] and the intensity of acute postoperative pain is a risk factor for the transition from acute to persistent post-surgical pain in both adults[4] and children.[2] Neonates and infants requiring major surgery or intensive care management are exposed to significant painful stimuli at a time when the developing nervous system is vulnerable to changes in sensory experience.[9,10] Prolonged alterations in sensory function occur in children following neonatal intensive care, with more marked change in those born preterm or who also require surgery.[11-14] Sensitivity to noxious stimuli is increased[11,13] and prior neonatal surgery increases subsequent perioperative pain and analgesic requirements.[15] Therefore, neonatal pain and injury may represent a specific risk factor for an increased degree or duration of pain following surgery in later life.

Plantar hindpaw incision is an established model of postoperative pain, producing robust hyperalgesia in adult, juvenile and neonatal rodents.[16-18] Initial incision during the neonatal period, but not at older ages, increases both the degree and duration of hyperalgesia following subsequent incision.[18,19] In adult rodents, hindpaw incision increases spinal microglial reactivity, and inhibiting microglial function reduces hyperalgesia.[19-22] However, neonatal incision primes the spinal microglial response to subsequent injury, with microglial reactivity (morphological changes identified with ionized calcium-binding adaptor molecule-1, Iba1) both increased and accelerated following incision, and the antihyperalgesic effects of the nonspecific microglial inhibitor minocycline are enhanced in adult animals with prior neonatal incision.[19]

The mitogen-activated protein kinase (MAPK) p38 is involved in intracellular signalling in spinal microglia. The phosphorylated form (p-p38) is linked to activation of transcription factors that upregulate synthesis and release of proinflammatory mediators.[23] Increased expression of p-p38 is a key component of the microglial-neuronal signalling pathway, and provides a functional marker of microglial reactivity that often precedes morphological changes.[24,25] In adult rodents microglial p-p38 MAPK expression peaks 24 h following plantar incision and p38 inhibitors reduce mechanical hyperalgesia.[20,21] We hypothesized that priming of the spinal microglial response by neonatal incision would lead to increased incision-induced p-p38 expression in adulthood. As functional changes were anticipated to occur more rapidly in animals with prior neonatal incision, and to precede previously identified morphological changes,[19] our primary outcome was group differences in the degree and distribution of p-p38 expression 3 h following adult incision. We also compared dose-dependent antihyperalgesic effects of the p38 inhibitor SB203850. Finally, to determine if spinal enhanced responses were driven solely by increased input from the peripheral reinjured tissue, expression of phosphorylated extracellular signal-regulated

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kinase (pERK) was mapped throughout lumbar cord segments that receive afferent input from the plantar hindpaw. These data further demonstrate long-term changes in spinal microglial reactivity following neonatal surgical injury and identify microglial p38 inhibition as a specific target to minimise the enhanced hyperalgesia.

Materials and Methods

Animals

All experiments were performed under personal and project licences approved by the Home Office, London, United Kingdom in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986. Reporting is based on The ARRIVE Guidelines for Reporting Animal Research developed by the National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, United Kingdom.[26] Male Sprague-Dawley rat pups on postnatal day 3 (P3) or adults were obtained from the Biological Services Unit, University College London. All animals were from the same colony, bred and maintained in-house, and exposed to the same caging, diet and handling throughout development. Litters were reduced to a maximum of 12 pups and weaned at P21, with all animals maintained on a 12-h light/dark cycle at constant ambient temperature with free access to food and water. Treatment groups were distributed across multiple litters and/or adult cage groups (4-5 animals) to control for potential litter variability.

All procedures were performed during anesthesia with 2-4% isoflurane (Abbot, AbbVie Ltd., Maidenhead, United Kingdom) in oxygen *via* a nose cone. The handling of rat pups and duration of maternal separation were kept to the minimum possible, and pups were maintained on a warming blanket before return to the dam. For behavioural studies, adult animals were randomly selected from the home cage and numbered by the investigator (FS) who measured baseline and all subsequent sensory thresholds. For each cage group, a separate investigator (SW) created a random, nonsequential sheet for allocation of animals to treatment groups, performed the injections, and retained the blinding sheet until completion of the experiments. Animals tested at all time points and with no missing data were included in the analyses. Tissue slides were also coded by an independent colleague to ensure the experimenter was blinded to treatment group during cell counting.

Plantar incision

Following application of chlorhexidine gluconate 0.5% (Vetasept, Animalcare Ltd., York, United Kingdom), a midline incision was performed on to the plantar aspect of the left hindpaw, and the underlying plantaris muscle was elevated and incised longitudinally as previously described.[16] Incision extended from the distal midpoint of the heel to the level of the first footpad to approximate the same relative length of incision in the hindpaw of pups (postnatal day 3, P3) and young adults (6 weeks of age).[18,19] Skin edges were closed with a single loop suture in pups or two mattress sutures of 5-0 silk (Ethicon, Edinburgh, United Kingdom) in adults which were removed after 5 days. Two experimental groups were compared: (i) animals with neonatal incision at P3 and repeat incision 6 weeks later in early adulthood (neonatal incision plus adult incision; nIN-IN); and (ii) age-matched adults undergoing a single incision at 6 weeks of age (adult incision; IN).

Intrathecal injection

Low lumbar spinous processes were visualised through a small midline skin incision, and a 30-gauge needle was passed in the midline through the lumbar (L) 4/5 or L5/6 intervertebral space to perform intrathecal injection, as previously described.[19] The p38 MAPK inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) (Sigma Aldrich, Gillingham, United Kingdom) was dissolved in a solution of 10% or 25% dimethyl sulfoxide (DMSO; Sigma Aldrich) and saline to a final concentration of 1 mg/ml or 5 mg/ml. The injectate volume (0.2 μl/g) was the same for all groups. Thirty min prior to the adult plantar incision, animals received 0.2 mg/kg SB203580 in 10% DMSO or 1 mg/kg in 25% DMSO, as a higher concentration of DMSO was required to form a solution of 5 mg/ml. Control animals in both IN and nIN-IN groups received equivalent volumes of vehicle (10% DMSO, n = 5) or (25% DMSO, n = 4).

Behavioral Testing

Animals were habituated to the testing environment for 2 days before baseline testing. For measurement of mechanical withdrawal threshold, animals were placed on an elevated mesh platform and a mechanical stimulus (electronic von Frey device; Dynamic Plantar Aesthesiometer, Ugo Basile, Varese, Italy) was applied to the hindpaw plantar surface adjacent to, but not directly over, the incision to avoid disruption of the wound. A linear increase in force was applied (ramp 2.5 g/s to a maximum of 50g) until a withdrawal reflex was evoked. Mechanical threshold was defined as the mean of three responses. Thermal withdrawal latency was determined using a modified Hargreaves Box (PAW thermal stimulator, University Anaesthesia Research and Development Group, University of California San Diego, La Jolla, California) with a glass surface (maintained at 30°C) on which animals were placed in individual Plexiglas cubicles. The thermal nociceptive stimulus from a focused projection bulb positioned below the glass surface was directed to the midplantar hindpaw. Latency was defined as the time required for the paw to show a brisk withdrawal as detected by photodiode motion sensors that stopped the timer and terminated the stimulus. In the absence of a response within 20 s, the stimulus was terminated. Thermal latency was defined as the mean of three responses.

Behavioral testing was performed at the following time points: baseline measures at 6 weeks of age; following intrathecal injection to evaluate specific drug effects; and at regular intervals following incision (4 h and 1, 2, 3, 7, 10, 14, 21 d).

Immunohistochemistry

For spinal cord immunohistochemistry, rats were terminally anesthetised with an intraperitoneal injection of pentobarbitone 100 mg/kg (Euthetal®, Merial, Harlow, United Kingdom) and transcardially perfused with heparinised saline followed by 4% paraformaldehyde with 0.1M phosphate buffer. Tissue was obtained at different time points following adult plantar incision for p-p38 and/or pERK immunohistochemistry. Lumbar spinal cords were dissected and pinned transversely at L4 and longitudinally in the contralateral ventral horn. Tissue was then postfixed overnight at 4°C. Following cryoprotection in 30% sucrose, transverse sections were cut at 20 μm with a cryostat, and

serial lumbar sections were mounted on Superfrost glass slides (Fisher, Superfrost Plus, Fisher Scientific, Houston, TX) and stored at −20°C.

To assess changes in p-p38 immunohistochemistry at time points prior to our previously demonstrated changes in Iba1 immunoreactivity in nIN-IN animals, spinal cords were obtained 1, 3, and 24 h following incision. Sections were pretreated with 70% methanol and 30% hydrogen peroxide solution for 30 min at 4°C, blocked with 3% chicken serum in 0.3% Triton X-100 for 1 h at room temperature, then incubated overnight with rabbit anti-p-p38 antibody (1:400; Cell Signaling, Danvers, CA). Sections were washed twice for 15 min in 0.1M phosphate buffer solution between steps. Sections were incubated with biotinylated secondary antibodies (goat anti-rabbit; 1:400; Vector Stain) for 90 min, placed in ABC complex (1:125; Vector Stain, ABC elite kit, Vector Labs, Burlingame, CA) for 30 min, followed by biotinylated tyramide (1:75; Tyramide Signal Amplification, TSA Staining Kit; Perkin Elmer, Waltham, MA) for 7 min, and then fluorescein isothiocyanate-Avidin (1:600; Vector Stain, Vector Labs) for 2 h at room temperature. For double labelling, spinal sections were incubated with monoclonal antibodies directed at mouse anti-neuronal specific nuclear marker (NeuN, 1:500; Millipore, Hertfordshire, United Kingdom), mouse glial fibrillary acid protein to identify astrocytes (1:200, Cell Signaling) or goat ionized calcium-binding adapter molecule 1 (Iba-1) as a microglial marker (1:100 Abcam, Cambridge, United Kingdom) overnight at room temperature then visualised using anti-mouse/goat Alexa 594 (1:200; Invitrogen, Paisley, United Kingdom) for 2 h at room temperature.

For pERK staining, tissue was obtained 15 min from the commencement of plantar incision to ensure tissue collection was standardized from the onset of injury, and not influenced by the time to perform the incision and/or suturing. Additional comparisons were made 24 h following incision. Spinal cord sections were blocked with 3% goat serum in 0.3% Triton X-100 for 1 h, then incubated overnight at room temperature with rabbit anti-p44/42 (pERK1/2) antibody (1:200; Cell Signaling) and mouse anti-NeuN antibodies (1:500; Millipore). Additional double labelling was performed with goat ionized calcium-binding adapter molecule 1 (Iba-1) as a microglial marker (1:100 Abcam). Slides were then incubated for 2 h with anti-rabbit Alexa 488 (1:200; Invitrogen, Eugene, OR) and anti-mouse or anti-goat Alexa 594 (1:200; Invitrogen, Paisley, United Kingdom). In all protocols, sections were coverslipped with Flouromount (Sigma Aldrich). Negative controls omitting primary antibodies resulted in no immunofluorescence.

Spinal cord sections were visualised at a magnification of x60 on a Leica microscope using the appropriate fluorescent filter. For quantification of p-p38 in the L4-5 spinal cord dorsal horns (laminae I-V) cell counts from 10 randomly chosen sections were averaged, and *n* represents the number of animals. For cephalad-caudal signal tracing, cell counts of p-p38 in serial L4/5 dorsal horn sections were plotted. For pERK, cells in the ipsilateral superficial dorsal horn were counted in serial sections from L2 to L6 as previously described.[27] Images were obtained using an Olympus total internal reflection fluorescence confocal microscope (Olympus, Southend-on-Sea, United Kingdom) with 20x and 40x objectives applied at a z-spacing of 0.6 μm and 0.5 μm respectively.

Statistical Analysis

Our primary outcome was the difference in p-p38 positive cell counts between IN and nIN-IN groups 3 h following adult incision. The number of animals per group was based on previous work following plantar incision in adult rats that used similar methodology and identified significant increases (P <0.01 and P <0.001) in p-p38 counts in the dorsal horn with n = 5-7 animals,[20] and our findings of group differences (IN *vs*. nIN-IN, P <0.01) in the degree, distribution and time course of Iba1 immunohistochemistry with n = 4-6 animals.[19] Phospho-p38 cell counts were obtained from 10 randomly selected L4/5 sections and an average obtained for each animal. Counts in naïve, nIN-IN and IN groups at 1, 3, and 24 h following incision were compared with one-way ANOVA (n = number of animals). In additional control experiments, cell counts were compared in nIN-IN animals 3 h following SB203580 0.2 mg/kg and 1 mg/kg. To quantify and compare the degree and distribution of p38 and pERK staining the area under the curve of cell count in serial sections *versus* spinal cord length was calculated for each animal, and results combined.

Sample sizes for behavioural testing were based on our previously reported group differences between IN and nIN-IN animals with n = 8 (P <0.01-0.001).[19] Values for baseline mechanical withdrawal threshold and thermal withdrawal latency in nIN-IN and IN groups were normally distributed (D'Agostino and Pearson omnibus normality test). In vehicle-treated groups, sensory thresholds were also plotted as the percentage change from baseline by calculating [(baseline preincision threshold − postincision threshold) / baseline threshold] × 100 for each animal at each time point. The degree and the duration of hyperalgesia in both the nIN-IN and IN groups were assessed by within-group comparisons between ipsilateral incised and contralateral paw using two-way repeated measures ANOVA followed by Bonferroni *post-hoc* comparisons. Using within group raw data, mechanical withdrawal thresholds following vehicle, 0.2 mg or 1.0 mg SB203580 were analysed by two-way repeated measures ANOVA (time and drug dose as variables) followed by Bonferroni multiple *post-hoc* comparisons. Within experiment outcomes were not corrected for the number of comparisons.

To compare the overall behavioral response in the two weeks following incision, changes in sensory threshold were plotted against time and the hyperalgesic index for each animal was calculated as the area over the curve from baseline (0) to 14 days,[28] such that a larger area over the curve represents a greater change from baseline and greater degree and/or duration of hyperalgesia[19] and comparisons were made with unpaired two-tailed Student's t-test.

Data were analysed using Prism Version 6.0 (GraphPad, San Diego, CA), and P <0.05 was considered statistically significant.

Results

Incision-induced phospho-p38 expression in spinal microglia is primed by prior neonatal incision

As we have previously shown that microglial morphological changes following adult incision occur earlier (24 h *vs.* 3 days) in animals with prior neonatal incision,[19] and p-p38 expression in spinal microglia peaks 24 h following single adult incision,[20] we used p-p38

expression to compare functional microglial reactivity up to this time point (1, 3, and 24 h, fig. 1). Representative spinal cord sections show p-p38 immunoreactive cells in the medial dorsal horn from an IN (fig. 1Ai) and nIN-IN (fig. 1Aii) animal. Three hours following adult incision p-p38 immunoreactive cell counts were increased in both IN (n = 8) and nIN-IN (n = 7) groups, but to a greater degree in animals with prior neonatal incision (P <0.01, one way ANOVA followed by Bonferroni *post-hoc* comparisons; fig. 1B). To compare both the degree and distribution of p-p38 expression, cell counts in serial sections of L4/5 cord 3 h following incision were plotted from cephalad to caudal (fig. 1C). Cell counts were consistently higher in the nIN-IN group, and significant group differences were found when the area under the cell count *versus* spinal length curve was calculated for each animal (P <0.05 unpaired two-tailed Students t-test; fig. 1D). Double labelling with the microglial marker Iba-1 confirmed expression of p-p38 in microglia 3 h following incision, but there was no overlap with astrocyte (glial fibrillary acid protein) or neuronal (NeuN) markers (fig. 2).

To further evaluate the time course of microglial reactivity, p-p38 cell counts were also compared at 1 and 24 h. One hour following incision, p-p38 expression in the nIN-IN (n = 5) group was significantly higher than in nonincised controls (n = 4) (nIN *vs.* naive, P <0.001, one way ANOVA followed by Bonferroni *post-hoc* comparisons), whereas values in the IN group (n = 5) were lower (IN *vs.* nIN-IN, P <0.05) and did not differ significantly from the naïve group (fig. 1A). At 24 h, increased pp38 expression persisted in both IN (n =7) and nIN-IN (n = 5) groups. Overall, p-p38 expression was enhanced by prior neonatal incision, with a main effect of incision group ($F_{1,31}$ = 28.9, P <0.001) as well as time ($F_{2,31}$ = 6.71, P = 0.0038) (two-way ANOVA with time and incision group as variables followed by Bonferroni *post-hoc* comparisons).

Antihyperalgesic efficacy of p38 inhibition is enhanced following initial neonatal incision

SB203850 was prepared in a solution of 10% DMSO (0.2 mg/kg) or 25% DMSO (1 mg/kg). Mechanical withdrawal thresholds measured 15 min following intrathecal injection (but prior to incision) confirmed that drug and/or vehicle alone did not alter reflex thresholds (table 1). Within either the IN or nIN-IN groups, sensory thresholds did not differ at any time point between vehicle control animals receiving 10% DMSO (n = 5) or 25% DMSO (n = 4)(IN P = 0.94; nIN-IN P = 0.14; repeated measures two-way ANOVA with time and DMSO concentration as variables followed by Bonferroni *post-hoc* comparison). Therefore, data are combined as a single vehicle control group in subsequent analyses.

Baseline sensory thresholds of both the previously injured hindpaw and the contralateral uninjured paw were increased compared to age-matched adult control animals (table 1) as previously reported following neonatal incision[19] and neonatal hindpaw inflammation.[29] Mechanical withdrawal threshold and thermal latency from vehicle controls are expressed as percentage change from baseline for each animal to compare IN (n = 9) and nIN-IN (n = 9) groups in figure 3, with the raw data also shown in figure 4. The enhanced hyperalgesia in animals with prior neonatal incision replicates our previous findings,[19] and group differences are not altered by intrathecal DMSO. The increased degree (nIN-IN > IN from 4 h to 10 days postincision, P <0.05 to P <0.001, two-way repeated measures ANOVA with

time and incision group as variables followed by Bonferroni *post-hoc* comparisons; fig. 3A), and duration of mechanical hyperalgesia (ipsilateral *vs.* contralateral paw; two-way repeated measures with time and paw as variables; P <0.001 all time points to 10 days in nIN-IN group; P <0.05 to 3 days in IN group) was also reflected by a significant increase in the mechanical hyperalgesic index (nIN-IN > IN, P <0.01 Student's *t*-test; fig 3B). Similarly, the degree of change in thermal latency following adult incision was greater (nIN-IN > IN from 4 h to 10 days, P <0.05 to P <0.001, two-way repeated measures ANOVA with time and incision group as variables followed by Bonferroni *post-hoc* comparisons; fig. 3C), and the duration was longer in animals with prior neonatal incision (ipsilateral *vs.* contralateral paw; P <0.001 all time points to 10 days in nIN-IN; P <0.01 to 3 days in IN group; two-way repeated measures with time and paw as variables), and a significant increase in the thermal hyperalgesic index (nIN-IN > IN, P <0.01 Student's *t*-test; fig. 3D).

To determine if increased p-p38 expression in the spinal cord was linked to the enhanced behavioural response, dose-dependent effects of an intrathecal p38 inhibitor were compared. Intrathecal SB203580 reduced adult incision-induced mechanical hyperalgesia in both IN and nIN-IN groups, but dose requirements differed (fig. 4). A single pre-incision dose of 1mg/kg SB203580 (n = 6) reduced mechanical hyperalgesia for 3 days following adult incision when compared to both vehicle (n = 9) and 0.2 mg/kg (n = 8) groups (P <0.01, two-way repeated measures ANOVA followed by Bonferroni *post-hoc* comparisons; fig. 4A). The mechanical hyperalgesic index (0-14 days) was also reduced by 1 mg/kg but not 0.2 mg/kg SB203580 (P <0.001 one way ANOVA followed by Bonferroni *post-hoc* comparisons; fig. 4B). By contrast, in nIN-IN animals a lower dose of 0.2 mg/kg SB203580 (n = 8) significantly reduced mechanical hyperalgesia for a longer time period (P <0.01 to 7 days, two-way repeated measures ANOVA followed by Bonferroni *post-hoc* comparisons; fig. 4C). Intrathecal SB203580 1 mg/kg (n = 8) produced anti-hyperalgesic effects for 10 days following incision, and produced a greater dose-dependent reduction in the mechanical hyperalgesic index (fig. 4D). Plotting the degree of reversal of hyperalgesia (with vehicle treated animals normalized to zero) allowed comparison of dose-dependent effects, and demonstrated increased efficacy in the nIN-IN *versus* IN group (fig. 4F). Control experiments in nIN-IN animals confirmed that intrathecal SB203580 0.2 mg/kg (n = 3) and 1 mg/kg (n = 4) reduced p-p38 expression (P <0.05 one way ANOVA; fig. 4E).

As previously reported after adult plantar incision,[20] p38 inhibition had no effect on thermal hyperalgesia (fig. 4G and H). Consistent with the group differences expressed as percentage change in figure 3C, analysis of the thermal latency raw data demonstrates significant reductions at all time points to 3 days in the IN vehicle control group (n = 9; P <0.01-0.001, two-way repeated ANOVA with time and treatment as variables followed by Bonferroni *post-hoc* comparisons; fig. 4G) and to 10 days following nIN-IN (n = 9, P <0.01-0.001; fig. 4H). Thermal latency was not altered by SB203580 with no main effect of treatment in either the IN ($F_{2,20}$ = 0.173, P = 0.17) or nIN-IN ($F_{2,22}$ = 0.68, P = 0.52; two-way repeated ANOVA with time and treatment as variables followed by Bonferroni *post-hoc* comparisons) groups.

Incision-induced pERK expression is similar in animals with and without prior neonatal injury

To test whether prior neonatal injury alters the pattern of neuronal activation in the spinal dorsal horn, pERK expression was quantified and compared in IN and nIN-IN groups 15 min following the commencement of adult incision (n = 10 both groups) and also at 24 h (n = 4 both groups, fig. 5). A representative lumbar section 15 min following commencement of plantar incision demonstrates pERK immunoreactivity in the ipsilateral superficial dorsal horn (fig. 5Ai) with higher power images showing colocalization with the neuronal marker NeuN (fig. 5Aii-iv). At both 15 min (fig. 5Bi, ii) and 24 h (fig. 5Biii,iv), pERK staining colocalized with NeuN but not with the microglial marker Iba1 (fig. 5Bii, iv). Counts of pERK from L4/5 segments (10 sections per animal) were significantly higher at 15 min *versus* 24 h (P <0.001) but did not differ between IN and nIN-IN groups at either time point (two way ANOVA followed by Bonferroni *post-hoc* comparisons; fig. 5C). The segmental expression of pERK in serial lumbar cord sections (L2 to L5) followed a similar pattern in both groups (fig. 5D) and correlated with the primary afferent terminal fields of the plantar nerves innervating the hindpaw (fig. 5E). Quantification of the area under the cell count *versus* cord length graph found no significant differences between the IN and nIN-IN animals in L2/3 or L4/5 (fig. 5F).

Discussion

Neonatal hindpaw incision produces long-term alterations in injury response, with enhanced neuroglial signalling in the spinal cord and an increased degree and duration of hyperalgesia following adult incision. In animals with prior neonatal incision, functional microglial reactivity was enhanced as indicated by a greater degree and more rapid onset of p-p38 expression in dorsal horn microglia, and associated hyperalgesia was more effectively targeted by the p38 inhibitor SB203580. Whereas increased microglial p-p38 expression in the prior neonatal incision group was evident throughout lumbar segments, the pattern of neuronal pERK expression following adult incision was not significantly different. These findings support and extend our previous work identifying microglial priming by neonatal injury as key to centrally-mediated enhanced injury responses in later life. In addition, p38 inhibitors may have a specific preventive role as part of multimodal analgesic regimes for those with an increased risk of persistent postsurgical pain following early life injury.

Neuroglial interactions in the spinal cord modulate pain sensitivity, sustain central sensitization, and contribute to the transition from acute to persistent pain.[30-33] Microglia have significant functional plasticity, and can be primed by prior experience.[34] Adult animals with prior neonatal incision were indistinguishable from age-matched naïves in terms of baseline microglial Iba1 immunoreactivity.[19] However, repeat incision unmasked an increased degree, distribution and duration of microglial reactivity. Morphological changes are indicative of reactive phenotypes but may not always predict function,[30,35] and so here we evaluated activation of the intracellular signalling MAPK p38. Phosphorylation of p38 leads to upregulated release of pro-inflammatory mediators from microglia, and amplification of pronociceptive signals in the dorsal horn.[36,37] Skin and muscle incision in adult rodents increases p-p38 cell counts in the spinal cord from 1 h to 3 days,[20,21,38] and p-

p38 protein at 1-5 days.[39] Although neuronal p-p38 expression has been identified at much later time points in some injury models,[21] p-p38 is expressed exclusively in microglia during the first 1-3 days following plantar incision[20,40] or nerve injury.[23] Here we show that prior neonatal incision significantly enhanced the response following adult incision, with a more rapid onset and greater degree of microglial p-p38 expression that extended throughout the L4/5 cord. These data support our hypothesis that neonatal injury primes spinal microglia and that reinjury in later life unmasks enhanced microglial reactivity, in turn driving an increased degree and duration of hyperalgesia.

The enhanced spinal response could simply be a consequence of increased primary afferent input from the reinjured tissue. We previously demonstrated that the same standardized primary afferent input (electrical stimulation of the tibial nerve) produced a greater degree of hyperalgesia and microglial reactivity in adult animals with prior neonatal incision, suggesting that the long-term changes are spinally-mediated and not solely driven by peripheral reinjury.[19] As peripheral noxious stimuli induce ERK phosphorylation in spinal dorsal horn neurons in an intensity-dependent and somatotopically-appropriate manner,[25] we now mapped incision-induced pERK expression to identify potential alterations in primary afferent input from the reinjured tissue. Following adult plantar incision increased neuronal pERK expression in the ipsilateral L4-5 spinal cord has been reported at 30 min[41] or at 1 to 5 min “after the establishment of incisional pain”[42] (rather than timing from the beginning of incision as here), with more prolonged increases in pERK protein (maximal at 4 h).[43] Neuronal pERK expression was increased throughout the lumbar cord in a distribution consistent with the primary afferent terminal field of the medial and lateral plantar nerves from the hindpaw.[44] By 24 h, pERK expression had decreased in both groups but remained restricted to neurons, whereas *de novo* microglial expression has been reported at later time points following spinal nerve ligation.[45,46] The lack of significant differences in the degree or distribution of neuronal pERK expression suggest similar primary afferent input in both groups, supporting our hypothesis that centrally-mediated mechanisms are important mediators of long-term changes in pain response. As neonatal incision also produces developmentally regulated and long-term increases in excitatory[47,48] and reductions in inhibitory[49] synaptic signalling in the dorsal horn, changes in neuronal and/or microglial reactivity may contribute to the enhanced central hyperalgesic response.

Strategies to minimise the long-term impact of neonatal surgery may be directed at improving analgesia at the time of initial injury and/or more specifically targeting mechanisms underlying enhanced hyperalgesia following subsequent surgery. Neuroimmune interactions represent a major potential therapeutic target for management of persistent pain.[30,50] However as microglia have multifaceted responses with a spectrum of activity from proinflammatory through to antiinflammatory roles in tissue repair, capturing beneficial effects alone is problematic.[51] Microglia also have specific roles during postnatal development and inhibiting synaptic pruning and remodelling or the phagocytosis of apoptotic debris associated with programmed cell death may produce adverse effects on neural circuitry.[50-52] As local anaesthetic blockade with slow-release bupivacaine prevented nerve-injury induced increases in p-p38[53,54], blocking primary afferent input may prevent priming, while avoiding more direct microglial inhibition. We have previously shown that perioperative sciatic nerve blockade at the time of neonatal incision prevented the enhanced

hyperalgesic response to future surgery.[18] In addition, we have recently evaluated the ability of sciatic blockade to prevent changes in descending modulation following neonatal incision (Walker et al. Surgical injury in the neonatal rat alters the adult pattern of descending modulation for the rostroventral medulla. Anesthesiology 2015;122:xx-xx). Descending pathways that modulate spinal reflex sensitivity undergo significant postnatal maturation,[10,55-57] and injury-induced changes may underlie the delayed emergence and generalized distribution of elevated sensory thresholds following neonatal hindpaw inflammation[29] or incision.[19] Enhanced inhibitory modulation from the rostroventral medulla has been demonstrated following neonatal inflammation,[58] and we are currently assessing the pattern of inhibition or facilitation of spinal reflex sensitivity by rostroventral medulla stimulation[55] in adults with prior neonatal incision.

Reducing microglial reactivity at the time of adult incision may minimise the impact of prior neonatal injury. We previously demonstrated selective antihyperalgesic effects with low dose intrathecal minocycline in adult rats undergoing repeat incision.[19] Although intrathecal minocycline inhibits both morphological changes and reduces p-p38,[28] there are additional nonspecific effects on spinal synaptic signalling[59] and peripheral antiinflammatory effects following systemic administration.[19,60] Inhibition of p38 represents a more specific target.[25,61] Pretreatment with intrathecal SB203580 reduced hyperalgesia following anterior thigh skin/muscle incision and retraction[21] and spinal nerve ligation,[62] and in some studies was more effective in the induction than maintenance phase.[23] When administered 24 or 72 h following adult plantar incision intrathecal SB203850 had no effect on mechanical thresholds,[40] but here administration prior to incision had dose-dependent antihyperalgesic effects that outlasted the duration of action of the drug (*i.e.*, preventive analgesic effects).[63] Inhibition of p38 specifically targeted the enhanced mechanical hyperalgesia in animals with prior neonatal injury, as dose requirements were lower and effects prolonged. However, MAPK inhibitors directed at p38[20] or ERK[43] do not alter thermal hyperalgesia following hindpaw incision. Modality-specific mechanisms have not been elucidated but the focus on mechanical hyerpalgesia[39] may be clinically appropriate as movement-evoked pain significantly effects post-operative mobilization and morbidity.[20,43,64]

Clinical implications

Repeat incision represents a clinically relevant model as multiple surgeries are not uncommon during childhood,[65,66] particularly in those with complications of preterm birth or complex congenital anomalies. Neonatal surgery has been associated with increased pain and perioperative analgesic requirements following subsequent surgery in infancy.[15] As microglial precursors colonize the human central nervous system during early development, with a major influx in human fetal spinal cord between 14 and 16 postgestational weeks,[67] the developmental trajectory and phenotypic diversity of microglia are suited to roles in long-term changes in spinal connectivity and sensitivity. In addition to biological factors associated with increased central sensitization and persistent pain, early life experience influences psychological factors, such as pain catastrophizing and parental responses,[13] associated with persistent postsurgical pain in children[2,68] and adults.[4] This further emphasizes the need for improved understanding and management of neonatal pain to minimize long-term consequences.

Inhibition of p38 represents a potential analgesic target for persistent pain,[61] and p38 inhibitors are currently in clinical trials.[61,69-71] Efficacy will depend on formulations with adequate potency and selectivity[72] being administered to an appropriate patient group at the right time. In adults with established neuropathic pain[70,71] or active rheumatoid arthritis[73] p38 inhibitors have shown small or no analgesic benefit. Although laboratory studies suggest reduced efficacy during the maintenance phase of incisional pain,[21,40] our data indicate benefit with preincision administration of p38 inhibitors. Preinjury dosing is specifically applicable to clinical anesthetic practice and an oral p38 inhibitor (SCIO-469) prior to adult dental extractions reduced pain to a similar degree as ibuprofen and prolonged time to first analgesia compared to placebo.[69] The partial reversal of mechanical hyperalgesia by p38 inhibitors shown here suggests use in multimodal therapy to reduce movement-evoked pain. Benefits of perioperative multimodal analgesic therapy are well-established in both adult and pediatric practice.[74-76] The lower dose requirements and preventive analgesic effects of p38 inhibition following repeat surgery suggest a specific benefit in patients with prior surgery in early life. Current evidence for pharmacological prevention of persistent postsurgical pain is limited[77] and studies including children are required.[78] Identifying specific mechanisms underlying persistent pain will ensure the most appropriate treatments are evaluated in at-risk groups, to ultimately inform improvements in perioperative management and reduce both the degree and duration of postoperative pain.

Acknowledgments

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Final Boxed Summary Statement

What we already know about this topic:

* In rodents, neonatal surgery augments pain behaviors after surgery in adults, although the mechanisms are unclear

What this article tells us that is new:

* In rats, incisional surgery in adulthood resulted in greater phosphorylation of the signaling enzyme, p38 MAP kinase in spinal cord microglia of animals which had received incisional surgery in the neonatal period

* A p38 MAP kinase inhibitor reduced pain behaviors after surgery in adults with prior neonatal surgery, suggesting this enzyme may be a target to reduce exaggerated pain responses after surgery in individuals with a history of neonatal surgery

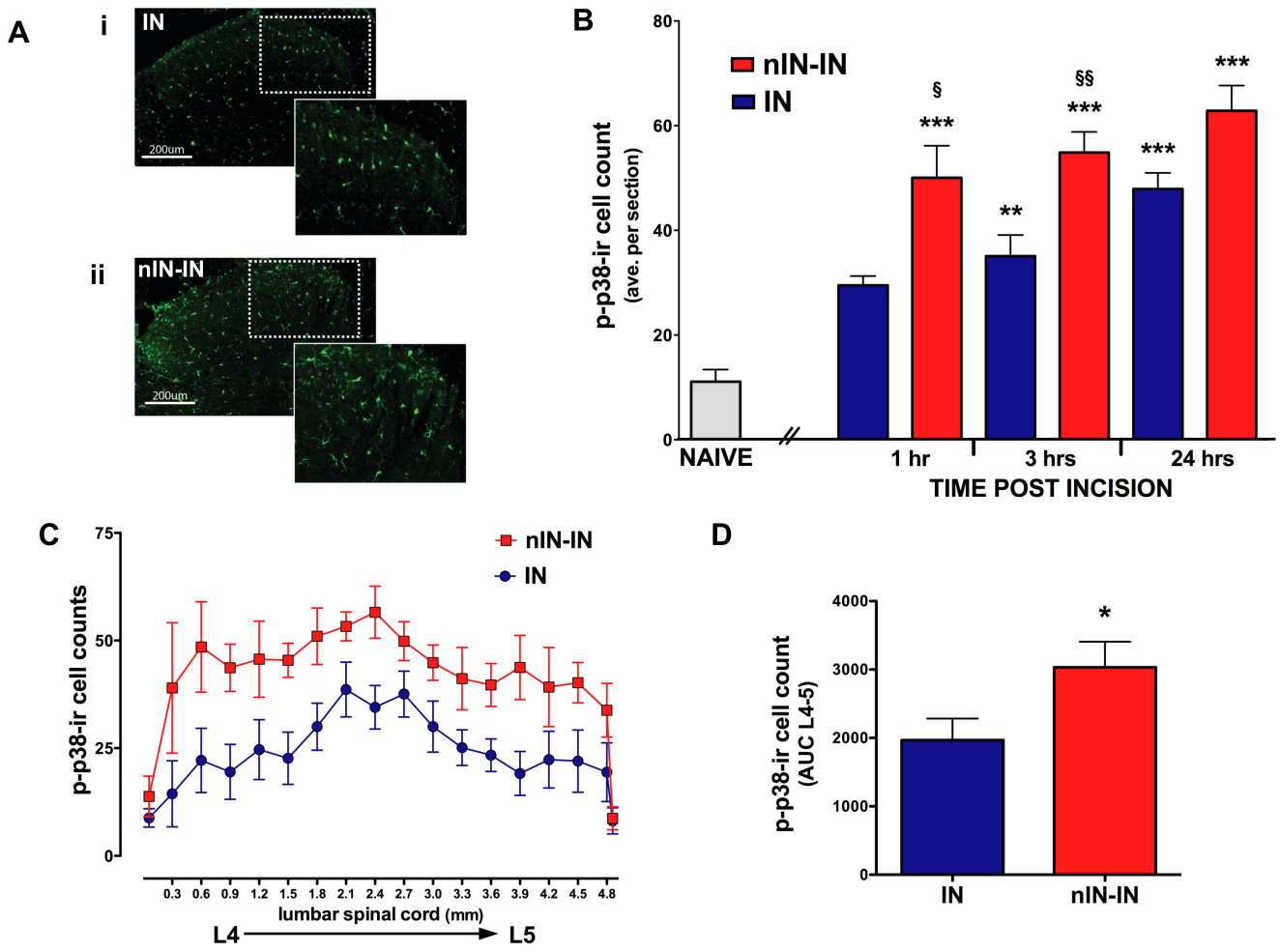

Figure 1.

Expression of phosphorylated p38 (p-p38) in the ipsilateral lumbar dorsal horn following plantar incision. *(A)* Examples of p-p38 immunoreactivity in the ipsilateral superficial dorsal horn with the inset at higher magnification are shown 3 h following adult incision in an IN (Ai) and nIN-IN (Aii) animal. Scale bar = 200 μm. *(B)* Counts of p-p38 immunoreactive positive cells (p-p38-ir; average from 10 lumbar sections per animal) in the superficial dorsal horn are shown for naïve animals and at time points (1, 3, and 24 h) following adult incision (IN) and in animals with prior neonatal and adult incision (nIN-IN). Counts were significantly increased compared with naïve (n = 4) at 1 h (n = 5), 3 h (n = 7), and 24 h (n = 8) in the nIN-IN group, but at 3 h (n = 8) and 24 h (n = 5) in the IN group. Bars = mean ± SEM, ** P <0.01, *** P <0.001, one-way ANOVA followed by Bonferroni *post-hoc* comparisons. Counts were significantly higher in the nIN-IN *vs.* IN group at 1 (§ P <0.05) and 3 h (§§ P <0.01). *(C)* Graphical display of cell counts *vs.* spinal length from serial sections of the 4th and 5th lumbar cord segments (L4-L5) 3 h following incision. The average cell count per 5 serial sections was calculated for each animal. Group values are plotted from cephalad to caudal. *(D)* The area under the p-p38-ir cell count (area under the curve [AUC] L4-5) *vs.* spinal length was calculated for each animal. Data points = mean ± SEM; nIN-IN (n = 7) > IN (n = 8), * P <0.05 unpaired two-tailed Student's t-test.

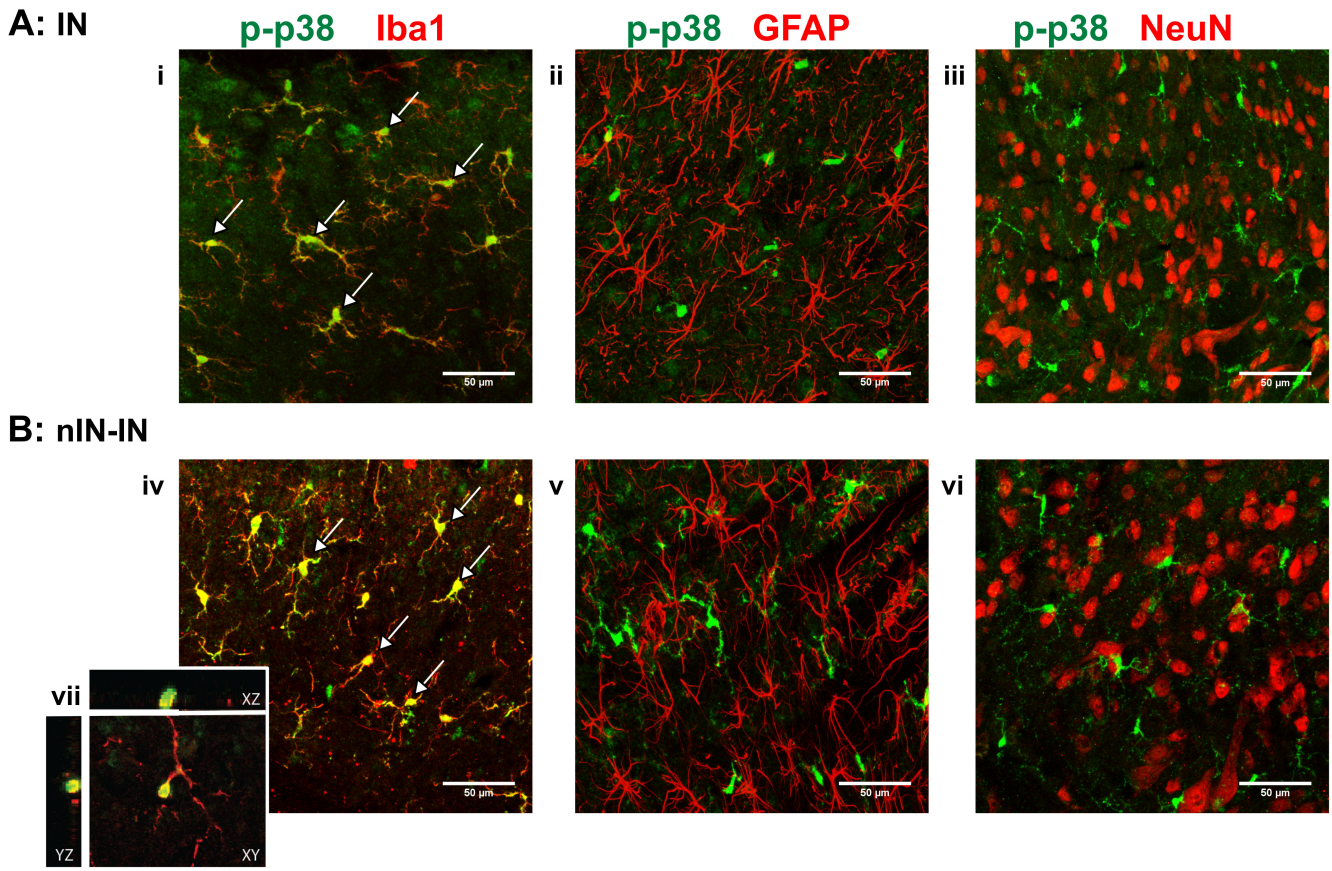

Figure 2.

Phospho-p38 is expressed by microglia in the spinal cord following plantar incision. High power images from the ipsilateral spinal dorsal horn 3 h following adult incision (IN) (A i-iii) and neonatal plus adult incision (nIN-IN) (B iv-vii). Double- labelling demonstrates co-localisation of p-p38 with the microglial marker ionized calcium-binding adaptor molecule (Iba1) (i, iv), confirmed in xz, xy and yz planes (vii). Phospho-p38 is not coexpressed with glial (glial fibrillary acid protein [GFAP]) or neuronal (NeuN) markers at this time point. Scale bar = 50 µm.

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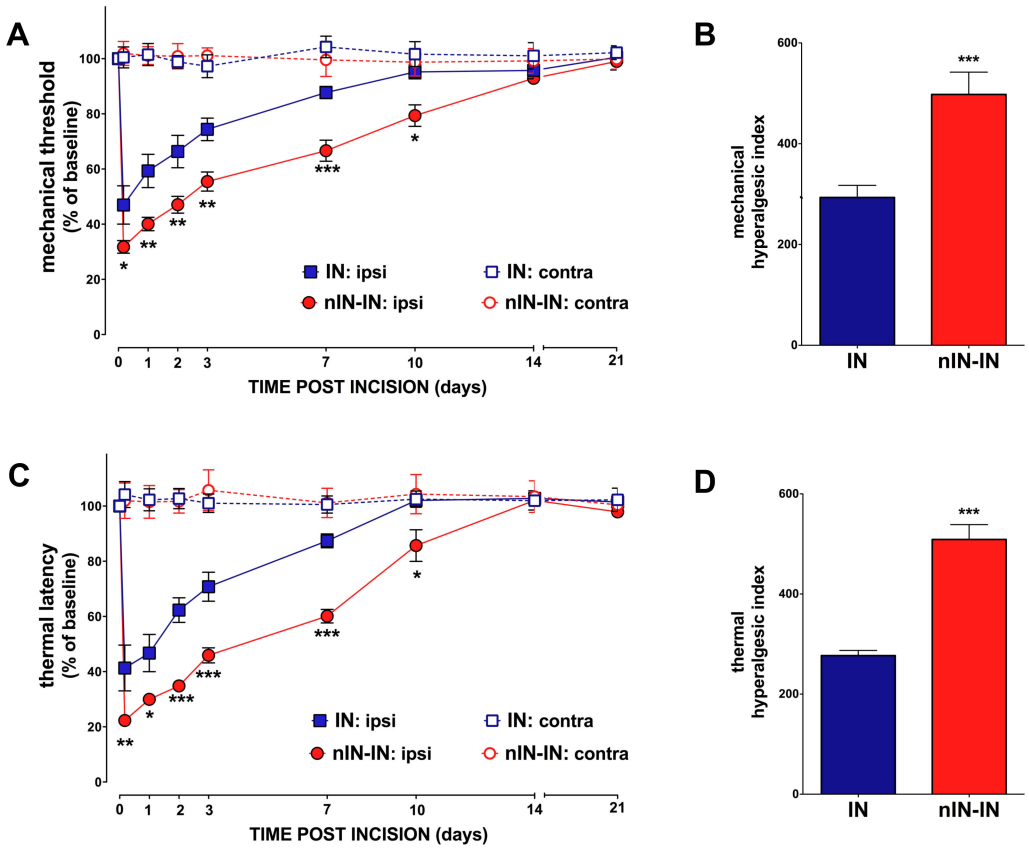

Figure 3.

Prior neonatal incision increases the degree and duration of incision-related mechanical and thermal hyperalgesia in adulthood. *(A)* Mechanical withdrawal threshold of the incised (ipsi) and contralateral (contra) paws of vehicle control animals are expressed as percentage change from baseline ([(baseline preincision threshold − postincision threshold) / baseline threshold] × 100) and plotted against time for 3 weeks following incision in previously naïve adults (IN) and adults with prior hindpaw incision (nIN-IN). The degree of change in threshold in the incised paws was significantly greater in the nIN-IN group from 4 h to 10 days postincision. Measures in contralateral paws did not differ from baseline at any time point. Data points = mean ± SEM, n = 9 per group, IN ipsi *vs.* nIN-IN ipsi * P <0.05, ** P <0.01, *** P <0.001 two way repeated measures ANOVA followed by Bonferroni *post-hoc* comparisons. *(B)* The mechanical hyperalgesic index (area over the mechanical threshold curve for each animal from baseline to 14 days) was significantly greater in the nIN-IN group. Bars = mean ± SEM, n = 9 per group, *** P <0.001 unpaired two tailed Students t-test.

(C) Changes in thermal withdrawal latency of the incised and contralateral paws are plotted against time. Significant differences between nIN-IN and IN ipsilateral paws were seen from 4 h to 7 days post incision. Data points = mean ± SEM, n = 9 per group, * P <0.05, ** P <0.01, *** P <0.001 two-way repeated measures ANOVA followed by Bonferroni *post-hoc* comparisons. *(D)* The thermal hyperalgesic index was also significantly greater in the nIN-

IN group. Bars = mean ± SEM, n = 9 per group, *** P <0.001 unpaired two tailed Students t-test.

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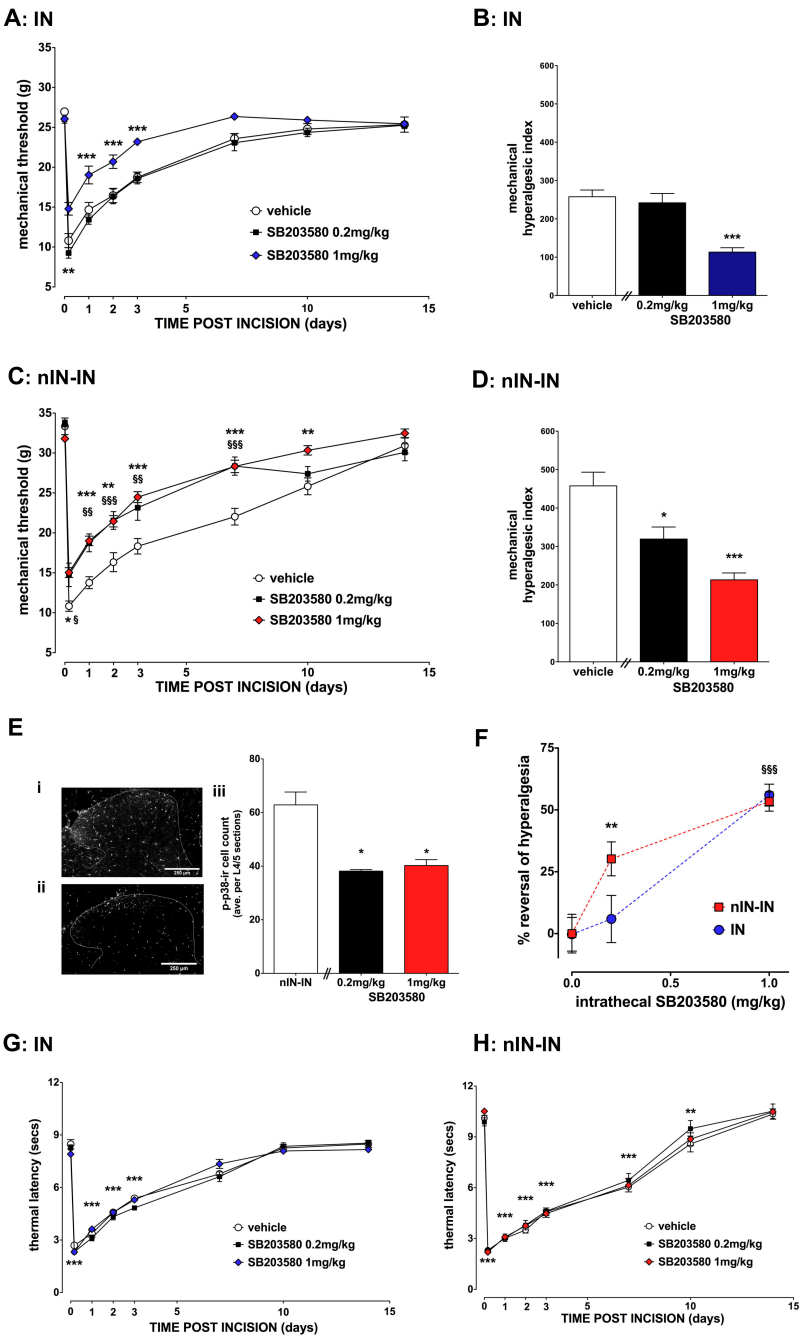

Figure 4.

Preincision administration of the p38 inhibitor SB203580 dose-dependently reduces mechanical hyperalgesia. *(A)* Mechanical withdrawal threshold is plotted against time following adult incision (IN). Preincision administration of 1 mg/kg SB203580 (n = 6) significantly reduces mechanical hyperalgesia for 3 days when compared to both vehicle control (n = 9) and 0.2 mg/kg SB203580 (n = 8) groups. Data points = mean ± SEM; ** P <0.01, *** P <0.001; two-way repeated measures ANOVA with time and treatment as variables followed by Bonferroni *post-hoc* comparisons. *(B)* The mechanical hyperalgesic

index (area over the threshold versus time graph from 0-14 days) also demonstrated a significant reduction following 1 mg/kg SB203580, but no effect with 0.2 mg/kg in the adult incision (IN) group. Bars = mean ± SEM; *** P <0.001 one-way ANOVA followed by Bonferroni *post-hoc* comparisons.

(C) Incision-related mechanical hyperalgesia in animals with prior neonatal incision (nIN-IN) is reduced by 0.2 mg/kg or 1 mg/kg SB203580 (n = 8 both groups). Data points = mean ± SEM; vehicle (n = 9) *vs.* 0.2 mg/kg § P <0.05, §§ P <0.01 §§§ P <0.001; vehicle *vs.* 1 mg/kg * P <0.05 ** P <0.01 *** P <0.001; two-way repeated measures ANOVA with time and treatment as variables followed by Bonferroni *post-hoc* comparisons. *(D)* The mechanical hyperalgesic index is significantly reduced following both 0.2 mg/kg and 1 mg/kg SB203580 in nIN-IN animals. Bars = mean ± SEM; * P <0.05 *** P <0.001; one-way ANOVA followed by Bonferroni *post-hoc* comparisons. *(E)* Representative spinal dorsal horn sections from nIN-IN animals following vehicle (i) or 0.2 mg/kg SB203580 (ii). Cell counts (average of 10 L4/5 sections per animal, n = 3-5 animals per group) confirm reduction in p-p38 cell counts following SB203580 (iii). Bars = mean ± SEM; * P <0.05 one way ANOVA. *(F)* To compare efficacy between IN and nIN-IN groups, the hyperalgesic index in the vehicle group was normalized to zero and the percentage reversal shown for each drug dose. Efficacy is demonstrated following 0.2 mg/kg only in the nIN-IN group. Data points = mean ± SEM; nIN-IN vehicle *vs.* drug, ** P <0.01 0.2 mg/kg *vs.* vehicle in nIN-IN group §§§ P <0.001 1 mg/kg *vs.* vehicle in IN and nIN-IN group; two-way ANOVA with incision group and drug dose as variables followed by Bonferroni *post-hoc* comparisons. *(G)* Thermal withdrawal latency is significantly reduced from baseline for 3 days following adult incision (IN) in vehicle (n = 9) 0.2 mg/kg SB203580 (n = 8) and 1 mg/kg SB203580 (n = 6) groups. Data points = mean ± SEM, *** P <0.001 one-way repeated measures ANOVA followed by Bonferroni *post-hoc* comparisons. Values are not altered by SB203850 at any time point (two-way ANOVA with time and treatment as variables followed by Bonferroni *post-hoc* comparisons). *(H)* Thermal withdrawal latency is significantly reduced from baseline for 10 days following neonatal plus adult incision (nIN-IN) in vehicle (n = 9) 0.2 mg/kg SB203580 (n = 8) and 1 mg/kg SB203580 (n = 8) groups. Data points = mean ± SEM, ** P <0.01, *** P <0.001 one-way repeated measures ANOVA followed by Bonferroni *post-hoc* comparisons. Values are not altered by SB203850 at any time point (two-way ANOVA with time and treatment as variables followed by Bonferroni *post-hoc* comparisons).

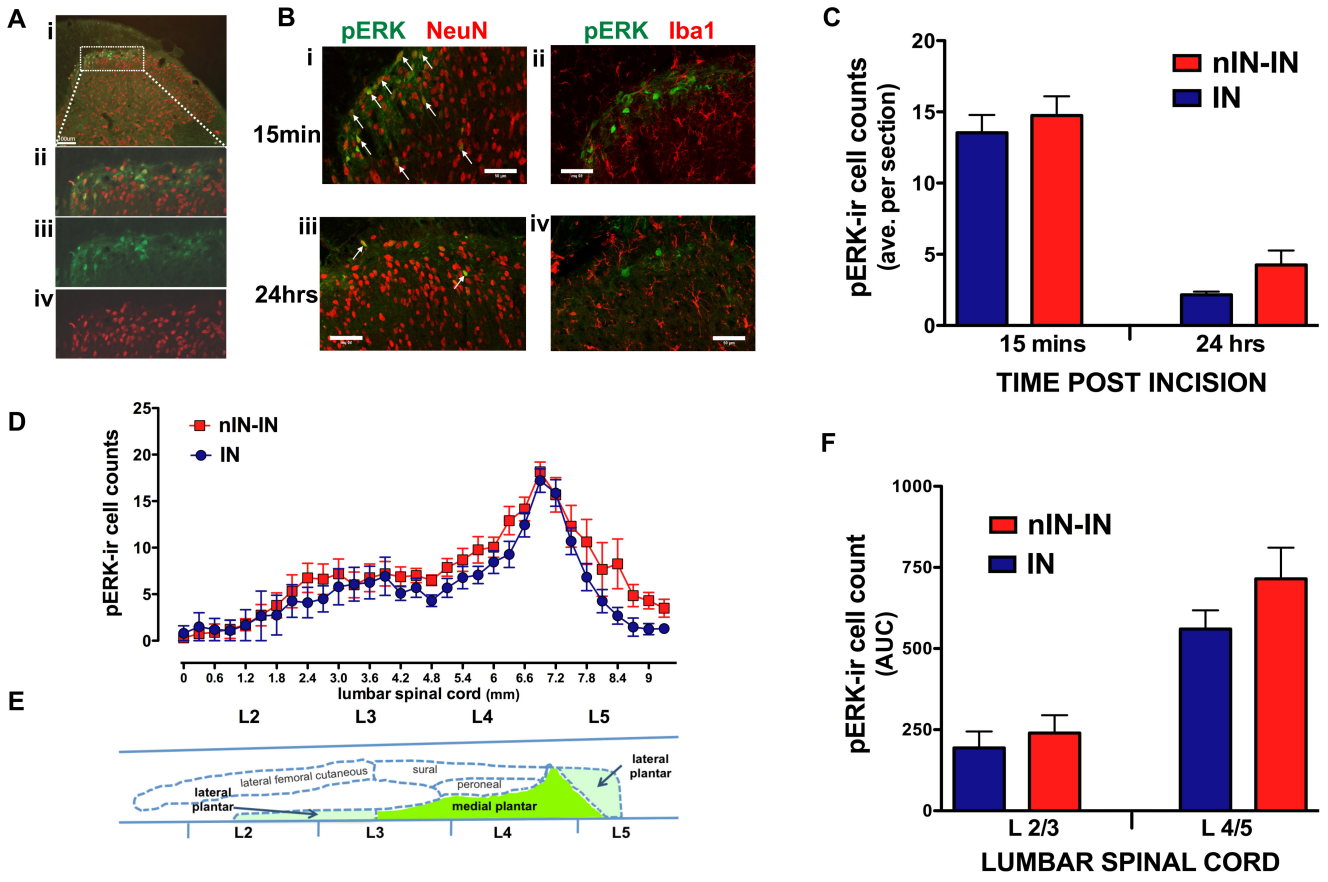

Figure 5.

Degree and distribution of phosphorylated extracellular signal-regulated kinase (pERK) expression in the lumbar spinal cord following plantar incision. *(A)* Representative pERK staining with double labelling of ipsilateral dorsal horn (i, scale bar = 100 μm) and high power view (ii) confirming expression of p-ERK (green, iii) in neurons (NeuN, red, iv) 15 min following commencement of plantar incision. *(B)* Double-labelling of sections at 15 min (i, ii) or 24 h (iii, iv) demonstrated coexpression of pERK with NeuN but not the microglial marker Iba1 at both time points (scale bar = 50 μm). *(C)* Counts of pERK-immunoreactive positive cells (pERK-ir) in adult-only (IN) and prior neonatal plus adult incision (nIN-IN) groups are shown 15 min following the commencement of incision (n = 10 per group) and at 24 h (n = 4 per group). For each animal, the cell count was averaged from 10 randomly selected lumbar cord (L4/5) sections. Cell counts were significantly higher at 15 min *vs.* 24 h ($P < 0.001$) but did not differ between IN and nIN-IN groups at either time point (two way ANOVA followed by Bonferroni *post-hoc* comparisons). *(D)* The degree and distribution of pERK-IR cells 15 min following incision was quantified from serial lumbar sections and mapped against spinal length. The average cell count per five serial sections from L2-5 cord was calculated for each animal. Group values are plotted from cephalad to caudal and showed a similar pattern in both IN and nIN-IN groups. Data points = mean ± SEM, n = 10 animals per group. *(E)* The distribution of pERK staining correlates with the segmental distribution of afferent fibres from the medial and lateral plantar nerves (redrawn from Molander C, Grant G: Laminar distribution and somatotopic organization of primary

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afferent fibers from hindlimb nerves in the dorsal horn. A study by transganglionic transport of horseradish peroxidase in the rat. Neuroscience 1986; 19: 297-312; reproduced with permission from Elsevier. Adaptations are themselves works protected by copyright. So in order to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation). *(F)* The area under the curve (AUC) for cell counts *vs.* spinal length was calculated for L2/L3 and L4/L5, and did not differ between treatment groups (nIN-IN *vs.* IN $P > 0.05$, one-way ANOVA). Bars = mean ± SEM, $n = 10$ per group.

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Table 1

Preincision Sensory Thresholds in Adult Animals

	SENSORY THRESHOLDS: Adult Baseline				INTRATHECAL DRUG TREATMENT GROUPS: Mechanical Threshold (g)					
	thermal latency		mechanical threshold		vehicle		SB203580 0.2 mg/kg		SB203580 1 mg/kg	
	left	right	left	right	pre	post	pre	post-drug	pre	post-drug
IN (n=23)	8.1 ± 0.4 s	8.3 ± 0.6 s	26.3 ± 1.2 g	26.6 ± 1.1 g	27.0 ± 1.1 (n = 9)	28.0 ± 1.3	26.0 ± 1.4 (n = 8)	27.8 ± 1.9	26.1 ± 1.0 (n = 6)	27.3 ± 1.4
nIN-IN (n=25)	10.2 ± 0.1 s*	10.2 ± 0.5 s	32.7 ± 2.1 g*	32.2 ± 1.1 g	33.3 ± 3.1 (n = 9)	29.2 ± 7.6	33.7 ± 1.2 (n = 8)	31.6 ± 1.7	31.8 ± 0.8 (n = 8)	31.9 ± 1.5

g = grams; IN = adult incision group; nIN-IN = neonatal incision plus adult incision group; post = measure 20-25 minutes following injection but prior to incision; pre = baseline measure prior to intrathecal injection; s = seconds.

* *P* <0.001 IN (left hindpaw) *versus* nIN-IN (left, previously incised hindpaw) Student's unpaired two-tailed *t*-test.