

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 after reinjury. To further evaluate priming of response by neonatal hindpaw incision, the authors investigated the functional role of spinal microglial p38 mitogen-activated protein kinase after re-incision in adult rodents.

Methods: Plantar hindpaw incision was performed in anesthetized adult rats, with or without previous 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 after 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 after adult incision (55 ± 4 vs. 35 ± 4 cells per section, mean \pm SEM, $n = 6$ to 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 previous neonatal injury and was effective at a lower dose (0.2 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 previous incision.

Conclusions: Neonatal incision primes spinal neuroglial signaling, and re-incision in adult rats unmasks centrally mediated increases in functional microglial reactivity and persistent hyperalgesia. After early life injury, p38 inhibitors may have specific benefit as part of multimodal analgesic regimes to reduce the risk of persistent postsurgical pain. (ANESTHESIOLOGY 2015; 122:1377-90)

PERSISTENT postsurgical 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 after adult and pediatric surgery,⁶⁻⁸ and the intensity of acute postoperative pain is a risk factor for the transition from acute to persistent postsurgical pain in both adults⁴ 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 after 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 previous neonatal surgery increases subsequent perioperative pain and analgesic requirements.¹⁵ Therefore, neonatal pain and injury may represent a specific risk

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 mitogen-activated protein kinase in spinal cord microglia of animals which had received incisional surgery in the neonatal period
- A p38 mitogen-activated protein kinase inhibitor reduced pain behaviors after surgery in adults with previous neonatal surgery, suggesting this enzyme may be a target to reduce exaggerated pain responses after surgery in individuals with a history of neonatal surgery

factor for an increased degree or duration of pain after surgery in later life.

Plantar hindpaw incision is an established model of postoperative pain, producing robust hyperalgesia in adult,

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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 after 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 (morphologic changes identified with ionized calcium-binding adaptor molecule-1, Iba1) both increased and accelerated after incision, and the anti-hyperalgesic effects of the nonspecific microglial inhibitor minocycline are enhanced in adult animals with previous neonatal incision.¹⁹

The mitogen-activated protein kinase (MAPK) p38 is involved in intracellular signaling in spinal microglia. The phosphorylated form (p-p38) is linked to activation of transcription factors that up-regulate synthesis and release of pro-inflammatory mediators.²³ Increased expression of p-p38 is a key component of the microglial–neuronal signaling pathway and provides a functional marker of microglial reactivity that often precedes morphologic changes.^{24,25} In adult rodents, microglial p-p38 MAPK expression peaks 24 h after 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 previous neonatal incision, and to precede previously identified morphologic changes,¹⁹ our primary outcome was group differences in the degree and distribution of p-p38 expression 3 h after adult incision. We also compared dose-dependent antihyperalgesic effects of the p38 inhibitor SB203850. Finally, to determine whether spinal-enhanced responses were driven solely by increased input from the peripheral reinjured tissue, expression of phosphorylated extracellular signal-regulated 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 after neonatal surgical injury and identify microglial p38 inhibition as a specific target to minimize 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 (four to five animals) to control for potential litter variability.

All procedures were performed during anesthesia with 2 to 4% isoflurane (Abbot, AbbVie Ltd., 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 behavioral studies, adult animals were randomly selected from the home cage and numbered by the investigator (F.S.) who measured baseline and all subsequent sensory thresholds. For each cage group, a separate investigator (S.M.W.) 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

After application of chlorhexidine gluconate 0.5% (Vetasept, Animalcare Ltd., United Kingdom), a midline incision was performed on 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, United Kingdom) in adults, which were removed after 5 days. Two experimental groups were compared: (1) animals with neonatal incision at P3 and repeat incision 6 weeks later in early adulthood (neonatal incision plus adult incision; nIN-IN); and (2) age-matched adults undergoing a single incision at 6 weeks of age (adult incision; IN).

Intrathecal Injection

Low lumbar spinous processes were visualized 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, United Kingdom) was dissolved in a solution of 10 or 25% dimethyl sulfoxide (DMSO; Sigma Aldrich) and saline to a final concentration of 1 or 5 mg/ml. The injectate volume (0.2 µl/g) was the same for all groups. Thirty minutes before 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, 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 50 g) 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, USA) 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; after intrathecal injection to evaluate specific drug effects; and at regular intervals after incision (4 h and 1, 2, 3, 7, 10, 14, 21 days).

Immunohistochemistry

For spinal cord immunohistochemistry, rats were terminally anesthetized with an intraperitoneal injection of pentobarbitone 100 mg/kg (Euthatal®; Merial, United Kingdom) and transcardially perfused with heparinized saline followed by 4% paraformaldehyde with 0.1 M phosphate buffer. Tissue was obtained at different time points after 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. After cryoprotection in 30% sucrose, transverse sections were cut at 20 μ m with a cryostat, and serial lumbar sections were mounted on Superfrost glass slides (Fisher, Superfrost Plus, Fisher Scientific, USA) and stored at -20°C.

To assess changes in p-p38 immunohistochemistry at time points before our previously demonstrated changes in Iba1 immunoreactivity in nIN-IN animals, spinal cords were obtained 1, 3, and 24 h after 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, USA). Sections were washed twice for 15 min in 0.1 M phosphate buffer solution between steps. Sections were incubated with biotinylated secondary antibodies (goat anti-rabbit; 1:400; Vector Stain, ABC elite kit; Vector Labs, USA) for 90 min, placed in ABC complex (1:125; Vector Stain) for 30 min, followed by biotinylated tyramide (1:75; Tyramide Signal Amplification, TSA Staining Kit; Perkin Elmer, USA) for 7 min, and then fluorescein isothiocyanate-Avidin (1:600; Vector Stain; Vector Labs) for 2 h at room temperature. For double labeling, spinal sections were incubated with monoclonal antibodies directed at mouse antineuronal specific nuclear marker (NeuN, 1:500; Millipore, 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, United Kingdom) overnight at room temperature and then visualized using anti-mouse/goat Alexa 594 (1:200; Invitrogen, 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 after 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 labeling 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, USA) and anti-mouse or anti-goat Alexa 594 (1:200; Invitrogen). In all protocols, sections were coverslipped with Fluoromount (Sigma Aldrich). Negative controls omitting primary antibodies resulted in no immunofluorescence.

Spinal cord sections were visualized at a magnification of $\times 60$ on a Leica microscope using the appropriate fluorescent filter. For quantification of p-p38 in the L4-5 spinal cord dorsal horn (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, United Kingdom) with $\times 20$ and $\times 40$ objectives applied at a z-spacing of 0.6 and 0.5 μ m, respectively.

Statistical Analysis

Our primary outcome was the difference in p-p38-positive cell counts between IN and nIN-IN groups 3 h after adult

incision. The number of animals per group was based on previous work after 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 $n = 5$ to 7 animals²⁰ and our findings of group differences (IN *vs.* nIN-IN, $P < 0.01$) in the degree, distribution, and time course of Iba1 immunohistochemistry with $n = 4$ to 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 naive, nIN-IN, and IN groups at 1, 3, and 24 h after 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 after SB203580 0.2 and 1 mg/kg. To quantify and compare the degree and distribution of p-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 behavioral testing were based on our previously reported group differences between IN and nIN-IN animals with $n = 8$ ($P < 0.01$ to 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] \times 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 after vehicle, 0.2 or 1.0 mg SB203580, were analyzed 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 2 weeks after 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 *t* test.

Data were analyzed using Prism Version 6.0 (GraphPad, USA), and P value less than 0.05 was considered statistically significant.

Results

Incision-induced Phospho-p38 Expression in Spinal Microglia Is Primed by Previous Neonatal Incision

As we have previously shown that microglial morphologic changes after adult incision occur earlier (24 h *vs.* 3 days) in animals with previous neonatal incision,¹⁹ and p-p38

expression in spinal microglia peaks 24 h after 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 after 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 previous 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 after 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 Student *t* test; fig. 1D). Double labeling with the microglial marker Iba-1 confirmed expression of p-p38 in microglia 3 h after 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 after 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 naive group (fig. 1A). At 24 h, increased p-p38 expression persisted in both IN ($n = 7$) and nIN-IN ($n = 5$) groups. Overall, p-p38 expression was enhanced by previous 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 after Initial Neonatal Incision

SB203580 was prepared in a solution of 10% DMSO (0.2 mg/kg) or 25% DMSO (1 mg/kg). Mechanical withdrawal thresholds measured 15 min after intrathecal injection (but before 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 with age-matched adult control animals (table 1)

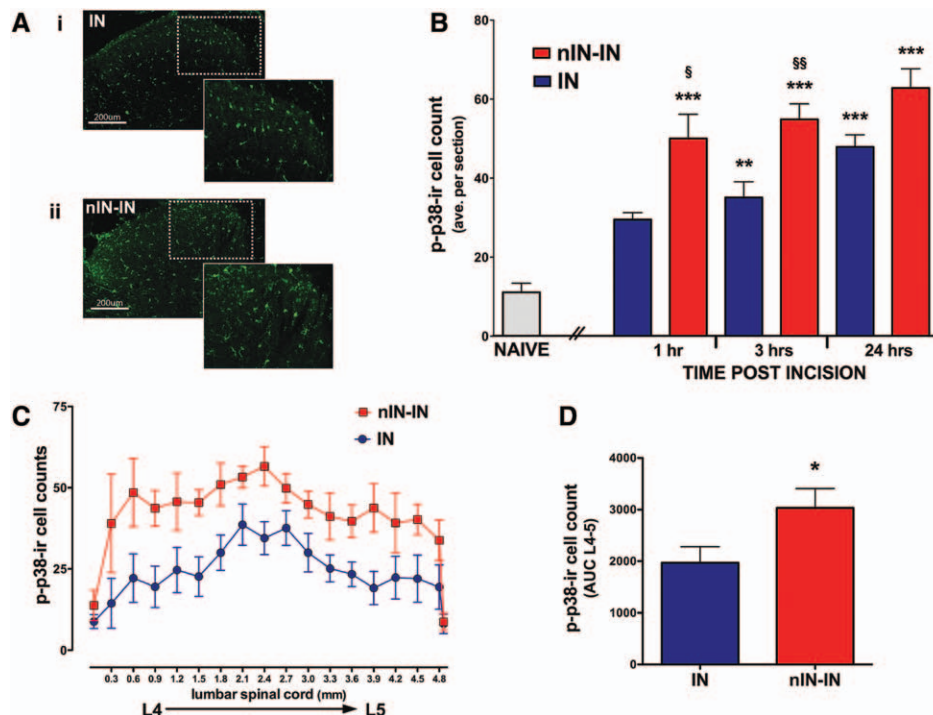


Fig. 1. Expression of phosphorylated p38 (p-p38) in the ipsilateral lumbar dorsal horn after plantar incision. (A) Examples of p-p38 immunoreactivity in the ipsilateral superficial dorsal horn with the inset at higher magnification are shown 3 h after adult incision in an IN (A*i*) and nIN-IN (A*ii*) animal. Scale bar = 200 μ m. (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 naive animals and at time points (1, 3, and 24 h) after adult incision (IN) and in animals with previous neonatal and adult incision (nIN-IN). Counts were significantly increased compared with naive ($n = 4$) 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 \pm SEM, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA followed by Bonferroni *post hoc* comparisons. Counts were significantly higher in the nIN-IN versus IN group at 1 h ($\$P < 0.05$) and 3 h ($\$P < 0.01$). (C) Graphical display of cell counts versus spinal length from serial sections of the 4th and 5th lumbar cord segments (L4 to L5) 3 h after incision. The average cell count per five 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 to L5) versus spinal length was calculated for each animal. Data points = mean \pm SEM; nIN-IN ($n = 7$) > IN ($n = 8$), * $P < 0.05$ unpaired two-tailed Student *t* test.

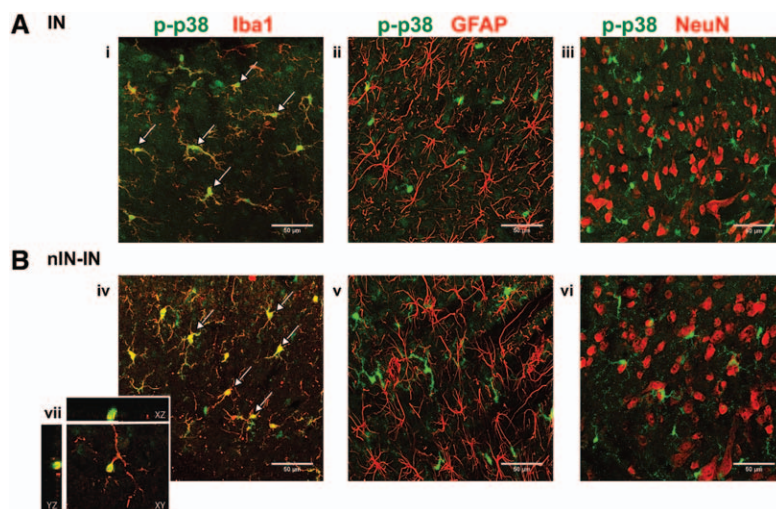


Fig. 2. Phospho-p38 is expressed by microglia in the spinal cord after plantar incision. High-power images from the ipsilateral spinal dorsal horn 3 h after adult incision (IN) (A*i*–A*iii*) and neonatal plus adult incision (nIN-IN) (B*iv*–B*vii*). Double labeling demonstrates colocalization of p-p38 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 μ m.

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		
Left	Right	Left	Right	Pre	Post	Postdrug	Pre	Postdrug	Postdrug
IN (n = 23)	8.1 ± 0.4 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)	26.1 ± 1.0 (n = 6)	27.8 ± 1.9	27.3 ± 1.4
nIN-IN (n = 25)	10.2 ± 0.1 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)	31.8 ± 0.8 (n = 8)	31.6 ± 1.7	31.9 ± 1.5

* $P < 0.001$ IN (left hindpaw) versus nIN-IN (left, previously incised hindpaw) Student unpaired two-tailed t test.
 IN = adult incision group; nIN-IN = neonatal incision plus adult incision group; post = measure 20–25 min after injection but before incision; pre = baseline measure before intrathecal injection.

as previously reported after neonatal incision¹⁹ 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 previous 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 t test; fig. 3B). Similarly, the degree of change in thermal latency after 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 previous 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 t test; fig. 3D).

To determine whether increased p-p38 expression in the spinal cord was linked to the enhanced behavioral 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 preincision dose of 1 mg/kg SB203580 (n = 6) reduced mechanical hyperalgesia for 3 days after adult incision when compared with 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 to 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 antihyperalgesic effects for 10 days after 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

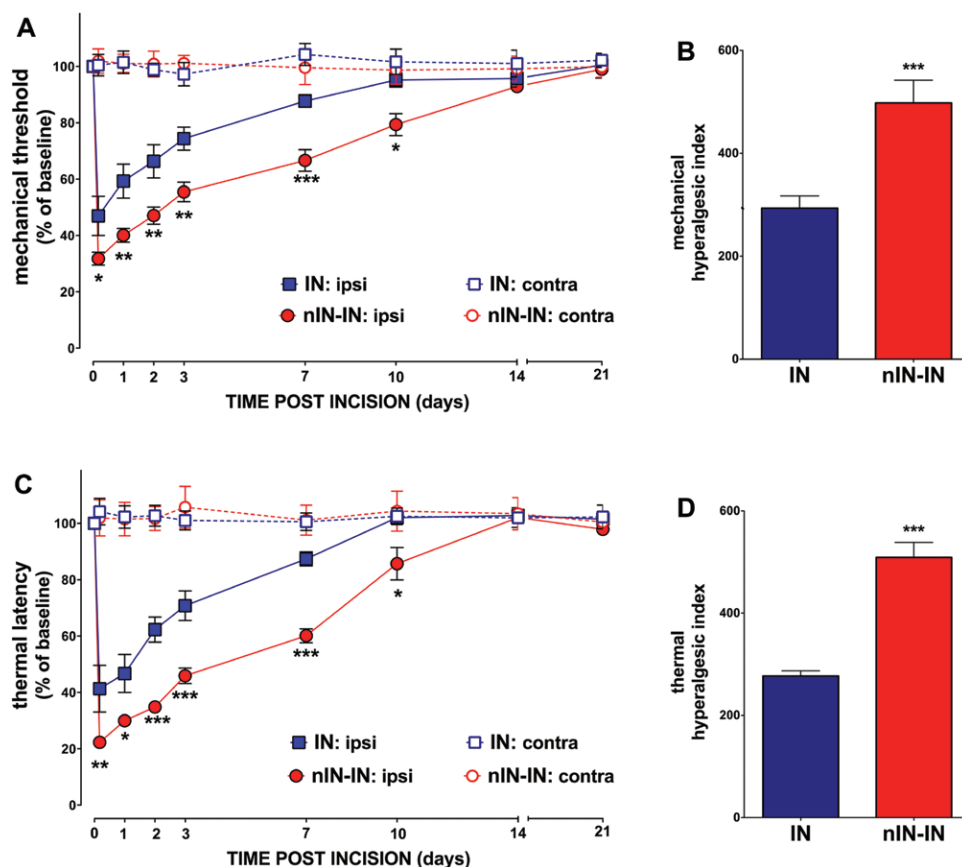


Fig. 3. Previous neonatal incision increases the degree and duration of incision-related mechanical and thermal hyperalgesia in adulthood. (A) Mechanical withdrawal threshold of the incised (ipsi) and contralateral (contra) paws of vehicle control animals are expressed as percentage change from baseline ($[(\text{baseline preincision threshold} - \text{postincision threshold}) / \text{baseline threshold}] \times 100$) and plotted against time for 3 weeks after incision in previously naive adults (IN) and adults with previous 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 \pm SEM, $n = 9$ per group, IN ipsi versus 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 \pm SEM, $n = 9$ per group, $***P < 0.001$ unpaired two-tailed Student *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 postincision. Data points = mean \pm 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 \pm SEM, $n = 9$ per group, $***P < 0.001$ unpaired two-tailed Student *t* test.

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. 4, G 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 ($n = 9$; $P < 0.01$ to 0.001 , two-way repeated-measures ANOVA with time and treatment as variables followed by Bonferroni *post hoc* comparisons; fig. 4G) and to 10 days after nIN-IN ($n = 9$, $P < 0.01$ to 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-measures 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 Previous Neonatal Injury

To test whether previous 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 after 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 after commencement of plantar incision demonstrates pERK immunoreactivity in the ipsilateral superficial dorsal horn (fig. 5Ai) with higher power images showing colocalization with the

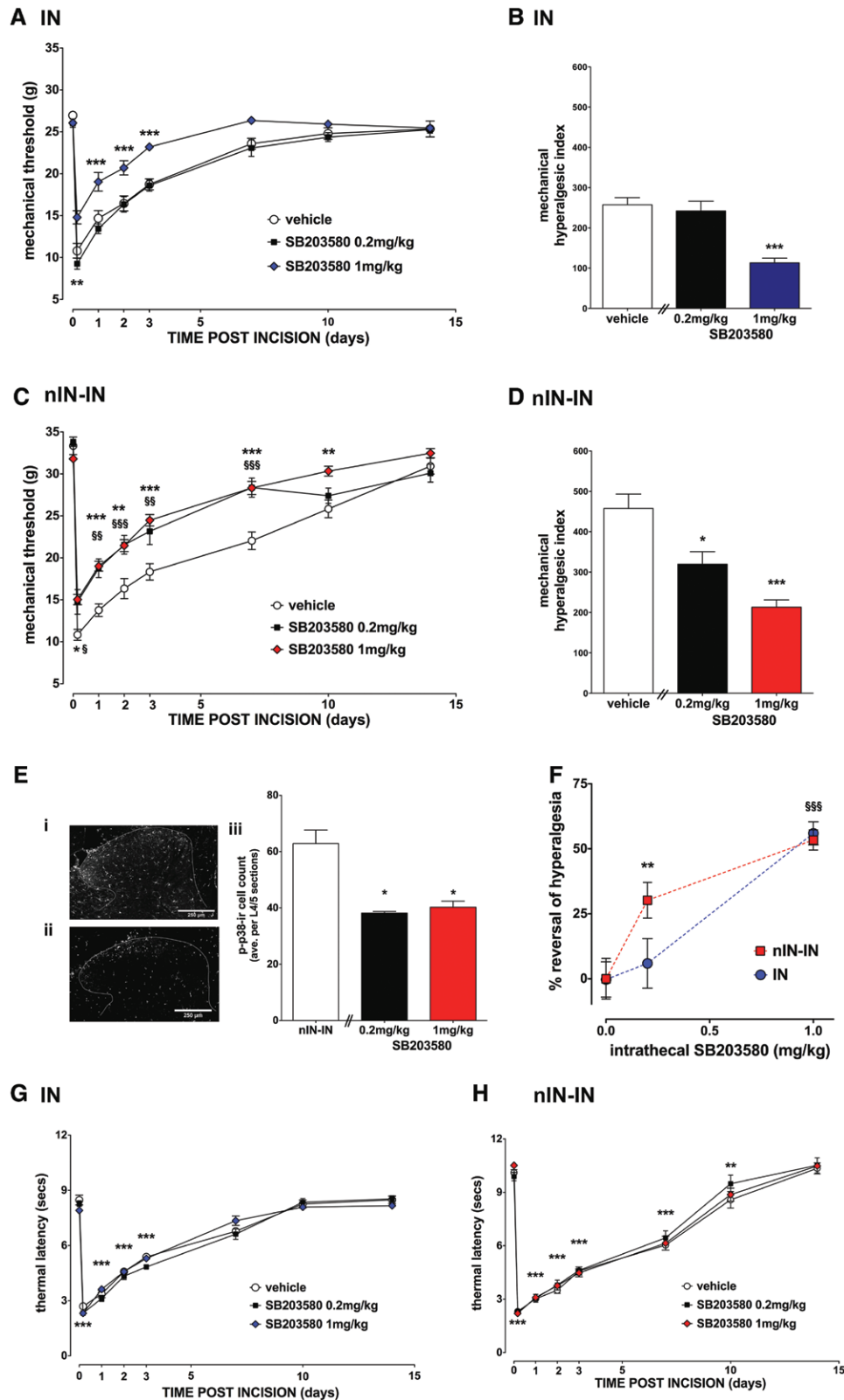


Fig. 4. Preincision administration of the p38 inhibitor SB203580 dose-dependently reduces mechanical hyperalgesia. (A) Mechanical withdrawal threshold is plotted against time after adult incision (IN). Preincision administration of 1 mg/kg SB203580 ($n = 6$) significantly reduces mechanical hyperalgesia for 3 days when compared with both vehicle control ($n = 9$) and 0.2 mg/kg SB203580 ($n = 8$) groups. Data points = mean \pm 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

neuronal marker NeuN (fig. 5, Aii–Aiv). At both 15 min (fig. 5, Bi and Bii) and 24 h (fig. 5, Biii and Biv), pERK staining colocalized with NeuN but not with the microglial marker Iba1 (fig. 5, Biii and Biv). 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 signaling in the spinal cord and an increased degree and duration of hyperalgesia after adult incision. In animals with previous 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. Although increased microglial p-p38 expression in the previous neonatal incision group was evident throughout lumbar segments, the pattern of neuronal pERK expression after 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 after 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 experience.³⁴ Adult animals with previous neonatal incision were indistinguishable from age-matched naives in terms of baseline microglial Iba1 immunoreactivity.¹⁹ However, repeat incision unmasked an increased degree, distribution, and duration of microglial reactivity. Morphologic changes are indicative of reactive phenotypes but may not always predict function,^{30,35} and so here, we evaluated activation of the intracellular signaling MAPK p38. Phosphorylation of p38 leads to up-regulated release of proinflammatory 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 to 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 to 3 days after plantar incision^{20,40} or nerve injury.²³ In this study, we show that previous neonatal incision significantly enhanced the response after 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

Fig. 4. (Continued) threshold vs. time graph from 0 to 14 days) also demonstrated a significant reduction after 1 mg/kg SB203580, but no effect with 0.2 mg/kg in the adult incision (IN) group. Bars = mean \pm SEM; *** $P < 0.001$ one-way ANOVA followed by Bonferroni *post hoc* comparisons. (C) Incision-related mechanical hyperalgesia in animals with previous neonatal incision (nIN-IN) is reduced by 0.2 or 1 mg/kg SB203580 ($n = 8$ both groups). Data points = mean \pm SEM; vehicle ($n = 9$) *versus* 0.2 mg/kg $SP < 0.05$, $SSP < 0.01$, $SSSP < 0.001$; vehicle *versus* 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 after both 0.2 and 1 mg/kg SB203580 in nIN-IN animals. Bars = mean \pm 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 after 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 after SB203580 (iii). Bars = mean \pm 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 after 0.2 mg/kg only in the nIN-IN group. Data points = mean \pm SEM; nIN-IN vehicle *versus* drug, $**P < 0.01$ 0.2 mg/kg *versus* vehicle in nIN-IN group $SSSP < 0.001$, 1 mg/kg *versus* 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 after 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 \pm 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 after 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 \pm 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).

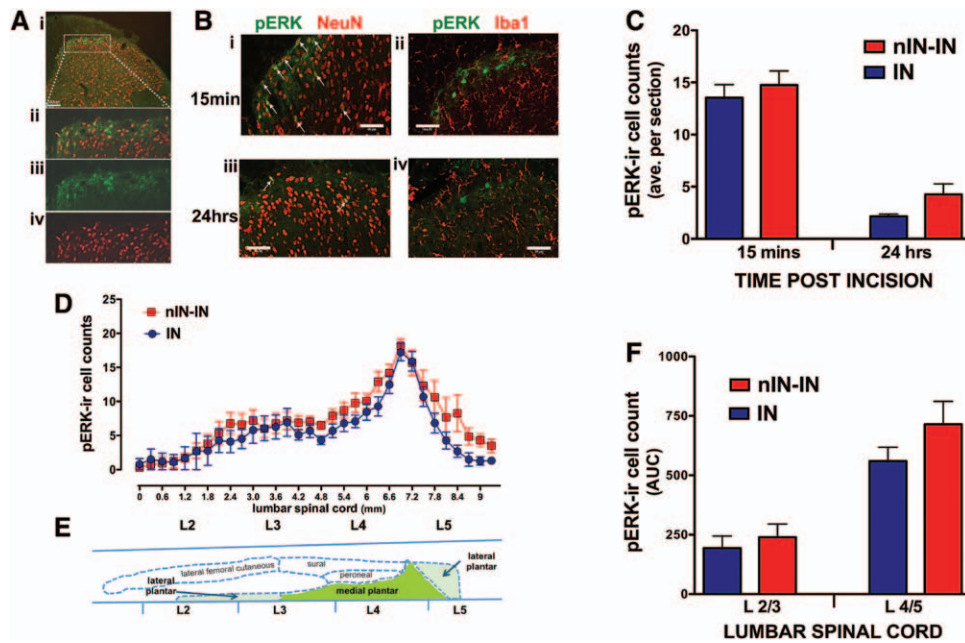


Fig. 5. Degree and distribution of phosphorylated extracellular signal-regulated kinase (pERK) expression in the lumbar spinal cord after plantar incision. (A) Representative pERK staining with double labeling of ipsilateral dorsal horn (i, scale bar = 100 μ m) and high-power view (ii) confirming expression of p-ERK (green, iii) in neurons (NeuN, red, iv) 15 min after commencement of plantar incision. (B) Double-labeling of sections at 15 min (i, ii) or 24 h (iii, iv) demonstrated coexpression of pERK with NeuN but not the microglial marker ionized calcium-binding adaptor molecule-1 (Iba1) at both time points (scale bar = 50 μ m). (C) Counts of pERK-immunoreactive positive cells (pERK-ir) in adult-only (IN) and previous neonatal plus adult incision (nIN-IN) groups are shown 15 min after 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 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). (D) The degree and distribution of pERK-IR cells 15 min after 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 \pm SEM, $n = 10$ animals per group. (E) The distribution of pERK staining correlates with the segmental distribution of afferent fibers from the medial and lateral plantar nerves (redrawn from Molander C, Grant G: Laminar distribution and somatotopic organization of primary afferent fibers from hindlimb nerves in the dorsal horn. A study by transganglionic transport of horseradish peroxidase in the rat. *Neuroscience* 1986; 19:297–312.⁴⁴ Reproduced with permission from Elsevier. Adaptations are themselves works protected by copyright. So, 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 versus 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 \pm SEM, $n = 10$ per group.

nerve) produced a greater degree of hyperalgesia and microglial reactivity in adult animals with previous 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. After adult plantar incision, increased neuronal pERK expression in the ipsilateral L4-5 spinal cord has been reported at 30 min⁴¹ 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 after spinal nerve ligation.^{45,46} The lack of significant differences in the degree or distribution of neuronal pERK expression suggests 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 inhibitory⁴⁹ synaptic signaling in the dorsal horn, changes in neuronal and/or microglial reactivity may contribute to the enhanced central hyperalgesic response.

Strategies to minimize 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 after 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 remodeling or the phagocytosis of apoptotic debris associated with programmed cell death may produce adverse effects on neural circuitry.^{50–52} As local anesthetic 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 after neonatal incision.⁵⁵ Descending pathways that modulate spinal reflex sensitivity undergo significant postnatal maturation,^{10,56–58} and injury-induced changes may underlie the delayed emergence and generalized distribution of increased sensory thresholds after neonatal hindpaw inflammation²⁹ or incision.¹⁹ Enhanced inhibitory modulation from the rostroventral medulla has been demonstrated after neonatal inflammation,⁵⁹ and we are currently assessing the pattern of inhibition or facilitation of spinal reflex sensitivity by rostroventral medulla stimulation⁵⁶ in adults with previous neonatal incision.

Reducing microglial reactivity at the time of adult incision may minimize the impact of previous 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 signaling⁶⁰ and peripheral antiinflammatory effects after systemic administration.^{19,61} Inhibition of p38 represents a more specific target.^{25,62} Pretreatment with intrathecal SB203580 reduced hyperalgesia after anterior thigh skin/muscle incision and retraction²¹ and spinal nerve ligation,⁶³ and in some studies was more effective in the induction than maintenance phase.²³ When administered 24 or 72 h after adult plantar incision, intrathecal SB203850 had no effect on mechanical thresholds,⁴⁰ but here administration before incision had dose-dependent antihyperalgesic effects that outlasted the duration of action of the drug (*i.e.*, preventive analgesic effects).⁶⁴ Inhibition of p38 specifically targeted the enhanced mechanical hyperalgesia in animals with previous neonatal injury, as dose requirements were lower and effects prolonged. However, MAPK inhibitors directed at p38²⁰ or ERK⁴³ do not alter thermal hyperalgesia after hindpaw incision. Modality-specific mechanisms have not been elucidated but the focus on mechanical hyperalgesia³⁹ may be

clinically appropriate as movement-evoked pain significantly effects postoperative mobilization and morbidity.^{20,43,65}

Clinical Implications

Repeat incision represents a clinically relevant model as multiple surgeries are not uncommon during childhood,^{66,67} particularly in those with complications of preterm birth or complex congenital anomalies. Neonatal surgery has been associated with increased pain and perioperative analgesic requirements after subsequent surgery in infancy.¹⁵ Because 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,⁶⁸ 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^{2,69} 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,⁶² and p38 inhibitors are currently in clinical trials.^{70–72} Efficacy will depend on formulations with adequate potency and selectivity⁷³ being administered to an appropriate patient group at the right time. In adults with established neuropathic pain^{71,72} or active rheumatoid arthritis,⁷⁴ 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) before adult dental extractions reduced pain to a similar degree as ibuprofen and prolonged time to first analgesia compared with placebo.⁷⁰ 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.^{75–77} The lower dose requirements and preventive analgesic effects of p38 inhibition after repeat surgery suggest a specific benefit in patients with previous surgery in early life. Current evidence for pharmacological prevention of persistent postsurgical pain is limited⁷⁸ and studies including children are required.⁷⁹ 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.

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Competing Interests

The authors declare no competing interests.

Correspondence

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