Hindpaw incision in early life increases the hyperalgesic response to repeat surgical injury: Critical period and dependence on initial afferent activity

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Pain in early life can enhance the response to subsequent injury, but effects are influenced by both the nature and timing of neonatal injury. Using plantar hindpaw incision, we investigated how postnatal age influences the response to repeat surgical injury two weeks later. The degree and time course of behavioural changes in mechanical withdrawal threshold were measured, and injury-related hyperalgesia was further quantified by flexion reflex electromyographic responses to suprathreshold mechanical stimuli 24 h following incision. Plantar hindpaw incision produces acute mechanical hyperalgesia in neonatal and adult rats, but incision in neonatal pups has an additional effect on the response to subsequent injury. With initial incision at postnatal day (P) 3 or 6, the degree of hyperalgesia following repeat incision 2 weeks later was greater than in animals having a single incision at the same age. At older ages (initial incision at P10, P21 or P40) responses did not differ in repeat and single incision groups. To test the role of primary afferent activity, levobupivacaine sciatic block was performed prior to P6 plantar incision, and controls received saline or subcutaneous levobupivacaine. Repeat peri-operative, but not a single pre-operative sciatic block, prevented the enhanced response to repeat incision two weeks later. Our results show that the first postnatal week represents a critical period when incision increases hyperalgesia following repeat surgery two weeks later, and effects are initiated by peripheral afferent activity. This has potential therapeutic implications for the type and duration of peri-operative analgesia used for neonatal surgery.

1. Introduction

Neonates born prematurely and/or with congenital anomalies often require surgery in early life, and a proportion will need repeated procedures or staged repairs throughout childhood. Pain and injury related to neonatal surgery occurs at a time when the normal development of the nervous system is susceptible to changing levels of neural activity [9]. It is well established that surgery can produce persistent pain in adults [24], but the added complexity of developmentally-regulated responses to pain and injury in early life may result in patterns of response and alterations in sensory processing that differ from those seen at older ages. Using quantitative sensory testing, changes in sensory thresholds have been demonstrated in children many years following neonatal intensive care [15,17] and neonatal thoracotomy [43], and alterations in thermal sensitivity were more marked in those who required surgery in addition to intensive care [51]. Early pain experience has also been shown to increase sensitivity to future experimental and clinical stimuli; such as a prolonged thermal stimulus in children who had required neonatal intensive care [15], immunization following neonatal circumcision [47], and repeat surgery in the same dermatome [32]. Since surgery can be a source of repeated pain experience for patients of all ages, effects of repeated surgical incision at different stages of neural development warrant investigation. Furthermore understanding the mechanisms and developmental periods of susceptibility to injury-induced sensory changes is important for minimising persistent effects of neonatal surgery.

Animal models of neonatal tissue damage indicate that the nature of the injury and the age at which it occurs are both important determinants of subsequent changes in sensory processing. Neonatal inflammation [8,35], skin wounding [27,36] and nerve injury [18,39] have varying persistent effects with different time windows, making it difficult to assess their contribution to the overall impact of early surgery. Furthermore, previous studies have tended to use a different ‘test’ pain stimulus from the original ‘priming’ one [8,16,35,42,44], which may influence investigation of critical periods and underlying mechanisms. In adult rodents, plantar hindpaw incision is an established model of surgical injury [7] and since it also produces acute behavioral hyperalgesia in rat pups from at least one week of age [38,41] we chose it here to further...
investigate effects of neonatal surgical incision. Hindpaw incision has several key advantages. The injury is reproducible and precise spatial coordinates can be identified at all postnatal ages, allowing accurate comparison of the same stimulus at different ages and time intervals. Furthermore, the restricted anatomical location of the injury means that afferent activity at the time of the stimulus can be effectively manipulated by peripheral nerve blockade. We have used behavioral and electromyographic (EMG) measures of hindlimb reflex sensitivity to mechanical stimuli following plantar incision to (i) test the effect of surgical injury at different postnatal ages upon the hyperalgesic response to repeat surgery two weeks later (ii) identify the developmental time window for the increased response to repeat injury and (iii) to evaluate the role of primary afferent activity at the time of initial incision.

2. Methods

2.1. Experimental animals

All experiments were performed under personal and project licences in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986. Litters of Sprague–Dawley rat pups were obtained from the Biological Services Unit, University College London and equal numbers of male and female pups were randomly divided into treatment groups. Adult male rats were used for experiments commencing after weaning at postnatal day (P) 21. All animals were maintained on a 12-h light/dark cycle at constant ambient temperature with free access to food and water.

2.2. Plantar hindpaw incision

Animals were anesthetised with halothane (2–4%) in oxygen. Alcoholic chlorhexidine gluconate 0.5% (Vetasept, Animalcare Ltd., York, UK) was applied to the left hindpaw. Following a midline incision through the skin and fascia of the plantar aspect of the hindpaw, the plantaris muscle was elevated and incised longitudinally as previously described [6]. To standardise the relative length of the incision at all ages, the incision extended from the midpoint of the heel to the proximal border of the first footpad. Skin edges were closed with two 5–0 sutures (Ethicon, Edinburgh, UK).

2.3. Behavioral testing

The mechanical threshold for hind-limb withdrawal was determined using calibrated von Frey hairs that deliver a logarithmically increasing force (see Table 1). Pups were habituated to handling, lightly restrained on a flat bench surface, and each von Frey hair was applied to the dorsal surface of the hindpaw, as this gave similar but more consistent measures than plantar stimuli in the small paws. Adult rats were habituated on an elevated mesh platform and tested on the plantar surface. Von Frey hairs were applied five times at one-second intervals and the number of evoked flexion reflexes recorded. The maximum force applied was that which evoked five withdrawal responses [52].

2.4. Electromyography recordings

Electromyography (EMG) recordings were performed as previously described [50,53]. Animals were initially anaesthetised with halothane (2–4%) in oxygen. A tracheostomy was performed and the animals ventilated with a T-piece system in conjunction with a Harvard small animal ventilator (Harvard Apparatus Ltd.) in a time-cycled, pressure-limited manner. Oxygen was administered via bobbin-in-glass rotameters and halothane was delivered from a calibrated vaporiser. Heart rate was continuously monitored (Vetronics ERM-8010 ECG and Respiratory Monitor, Vetronics Services, Devon, UK) and body temperature was monitored with a rectal probe and maintained with a thermostatically controlled heat source. Animals were placed in a spinal frame with the left hindpaw secured on a fixed platform with the plantar surface exposed for cutaneous stimulation. Following surgical preparation, the halothane concentration was reduced to an age-appropriate concentration (from 1.1% at P4 to 0.8% in adults) for 30 min to allow equilibration and establish a stable plane of anaesthesia prior to and during EMG recordings.

Bipolar EMG electrodes (Ainsworks, London) comprising stainless steel 30G needles with a central copper wire core were placed through a small skin incision into the belly of the biceps femoris muscle. EMG responses to mechanical stimuli (von Frey hairs) on the plantar surface of the hindpaw were processed (Neurolog, Digimer), displayed on a digital storage oscilloscope (Tektronix TDS 2012), and recorded in 12-s epochs (PowerLab 4S, AD Instruments, Castle Hill, Australia). As mechanical withdrawal threshold increases with age [9,52], more intense mechanical stimuli were required to evoke reflex responses in older animals. Von Frey hairs were sequentially applied to a maximum of hair number 17 (60 g bending force) at P4, hair number 18 (90 g) at P21 and P25, and hair number 20 (180 g) at older ages, resulting in responses being recorded to threshold and 2–3 suprathreshold stimuli at each age. As a result, electromyography recordings in anesthetized animals allow evaluation of changes in threshold as well as in the response to suprathreshold stimuli, and provide a quantitative measure of reflex sensitivity and hyperalgesia at a fixed time point following injury.

2.5. Experimental plan

Table 2 summarises the experimental groups used for single or repeat surgeries performed at different ages. Reflex sensitivity was tested using reflex behavioral thresholds and/or EMG recordings post surgical injury.

Plantar incision was performed in neonatal (postnatal day 3) \( (n = 4) \) and adult rats \( (n = 5) \) and mechanical withdrawal thresholds were determined prior to incision and 4, 24, 48 and 72 h later to assess the degree and duration of behavioral mechanical hyperalgesia. In separate groups of neonatal (P3) and adult (P40) animals, plantar incision was performed, and 24 h later, animals were reanesthetized for EMG recordings. Quantified reflex responses were compared with measures from age-matched non-incised control animals \( (n = 4–6 \text{ all groups}) \).

To determine if surgery during the neonatal period or adulthood had differing effects on the response to repeat incision, rats on
postnatal day (P) 3 or P40 were divided into three treatment groups: (i) initial incision and repeat incision 2 weeks later (ii) initial anesthesia only and single incision 2 weeks later and (iii) non-operated age-matched controls (n = 4–5 all groups). Animal groups were marked and coded to ensure the investigator performing behavioral testing (KT) was blinded to the single or repeat incision grouping. Measurements were performed prior to surgery and at intervals (4, 24, 48 and 72 h, 7 days) post-operatively. In additional experiments (as shown in Table 2), reflex sensitivity was quantified with EMG recordings 24 h following single and repeat incision in neonatal and adult rats (n = 8–10 in all groups). The same investigator (SW) performed all EMG recordings in a standardized manner and was blinded to the single or repeat incision grouping.

To determine if there is a critical developmental period during which surgical injury alters the response to repeat injury, initial incision was also performed at P6, P10, or P21 and a second incision was performed 2 weeks later. Twenty-four hours following incision, EMG recordings quantified the reflex response to mechanical stimuli. Values were compared with age-matched single incision and non-incision controls (n = 8–10 in all groups) (see Table 2).

### 2.6. Sciatic nerve block and response to repeat surgery

The effect of primary afferent blockade on acute behavioral mechanical hyperalgesia and on the subsequent response to repeat incision was evaluated by performing incisions in the presence of local anesthetic blockade of the sciatic nerve. P6 pups were briefly anesthetized with halothane and oxygen via a nose cone and percutaneous sciatic nerve injections of 50 μl of 0.5% levobupivacaine (Chirocaine, Abbott) were performed using the technique previously described in rat pups [19,25]. Following recovery from anesthesia, effective sciatic block was confirmed by ipsilateral motor block and loss of withdrawal reflex response to a suprathreshold mechanical stimulus (vFh 14; 13 g). Pups were then re-anesthetized and within 15 min of the block, plantar incision was performed lateral to the midline to ensure the wound was within the distribution of the sciatic nerve. To compare the effect of duration of primary afferent blockade, separate groups of pups received either a single pre-incision sciatic block or more prolonged perioperative blockade. As the size of the pups precludes the use of implanted catheter techniques, and the duration of sciatic block is relatively brief in pups, a pre-operative block plus three percutaneous injections at 2-h intervals was required to maintain afferent blockade during the early peri-operative period. Prior to subsequent sciatic blocks, resolution of motor block and partial recovery from sensory block was evident. At the same 2-h intervals, littermate control groups received either (i) sciatic injection of 50 μl sterile saline or (ii) subcutaneous interscapular injection of 50 μl of 0.5% levobupivacaine (n = 6 all groups). Mechanical withdrawal thresholds were determined at baseline, post block, and at 4, 24 and 72 h following incision. Two weeks later, a second incision was performed and the EMG response quantified 24 h following incision, and compared with P20 pups that had received a single incision (n = 8).

To check that the sciatic nerve block had not affected the normal development of the reflex response, the effect of 4 × 2-h sciatic nerve blocks with levobupivacaine, sciatic injection of saline, or general anesthesia alone were determined at baseline (P6), 24 h (P7) and 2 weeks later (P20). At P21, EMG reflex responses were quantified as previously described (n = 4–6 all groups). To check the effects of the nerve block upon the morphological integrity of the nerve, repeated P6 injections of saline or levobupivacaine were performed as described above (n = 3 both groups) and three days later animals were terminally anesthetized with pentobarbitone, transcardially perfused with 4% paraformaldehyde and glutaraldehyde, and the ipsilateral and contralateral sciatic nerves removed. Ultra-thin sections of the nerves were stained with toluidine blue and examined for structural abnormalities, disruption of the peri-neurum, and infiltration by inflammatory cells.

### 2.7. Statistical analysis

For each test condition in the behavioral studies, the number of withdrawal responses was plotted against the mechanical force in grams applied by sequential von Frey hairs. As each increase in von
Frey hair number corresponds to a log₁₀ increase in applied force, a sigmoidal stimulus response curve with variable slope was constructed using non-linear regression. The mid point of the curve (50% effective force, EF₅₀) was determined and designated the threshold [31,52]. Repeated behavioral measures were analysed by two-way ANOVA with time and treatment as variables and Bonferroni post-tests (GraphPad Prism Version 4).

The duration of the EMG response was outlined from the display of the raw data and the integral of the root mean square (RMS) of the signal was calculated (EMG response) (Chart, Powerlab AD Instruments). The EMG response was plotted against the von Frey hair number (mechanical stimulus) and the area under the stimulus–response curve (AUC) calculated to quantify the overall “reflex response” (Prism 4, GraphPad, San Diego, USA) [53]. Results were compared with Student’s t-test or one-way ANOVA and post hoc comparisons depending on the number of experimental groups. P < 0.05 was considered statistically significant.

3. Results

3.1. Acute mechanical hyperalgesia following incision in neonatal and adult rats

To confirm that plantar incision in the first postnatal week produces changes in reflex sensitivity, we compared effects in P3 and adult animals. Plantar hindpaw incision produces behavioral mechanical hyperalgesia in both neonatal (Fig. 1A) and adult rats (Fig. 1B) as demonstrated by a leftward shift in the mechanical stimulus and withdrawal response relationship. In P3 pups, the mechanical withdrawal threshold calculated from the midpoint of the stimulus–response curve (i.e. EF₅₀) is 1.04 ± 0.05 g at baseline, but is reduced to 0.41 ± 0.03 g at 4 h (P < 0.05; one-way ANOVA with Dunnett’s post hoc multiple comparison to baseline) and 0.80 ± 0.04 g at 24 h following plantar incision. Changes at 24 h are influenced by the normal pattern of increasing mechanical threshold with age as demonstrated by the marked right-shift in the stimulus–response curve 7 days after incision. Withdrawal thresholds at baseline are higher in adult animals (91.1 ± 2.3 g at baseline) but are significantly reduced to 4.3 ± 1.5 g at 4 h and remain low for 7 days following incision (P < 0.05 all time points; one-way ANOVA with Dunnett’s multiple comparison to baseline). As previously reported, the duration of mechanical hyperalgesia is shorter in younger animals [41].

To further evaluate reflex sensitivity and hyperalgesia, flexor reflex electromyography (EMG) recordings were also performed to quantify changes in both threshold and in the response to suprathreshold stimuli (Fig. 1C). Compared to age-matched non-incised animals, the EMG reflex response was significantly greater 24 h following plantar incision in both neonatal and adult animals (Fig. 1D; P < 0.05 Student’s unpaired two-tailed t-test). Effects did not differ between male and female pups. Due to developmental differences in reflex responses (increased duration and amplitude and lower threshold in younger pups) [11,53], the raw value for the area under the stimulus–response curve is larger in neonatal pups, but incision produces a similar relative increase at both ages (mean 126% increase following P3 incision and 114% increase following P40 incision).

3.2. Effect of repeat injury on the degree of mechanical hyperalgesia

To test if the response to repeated surgical injury differs in neonatal and adult animals, the degree of behavioral mechanical hyperalgesia following a second incision (prior incision two weeks earlier) was compared with age-matched animals undergoing a single incision (Fig. 2). The mechanical threshold is reduced in P17 single and repeat incision groups at 4 and 24 h and in adult animals at all time points to 7 days (P < 0.05 single or repeat incision versus saline, two-way ANOVA with time and treatment as

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Fig. 1. Acute effects of plantar incision in neonatal and adult animals. The mechanical force applied by sequential von Frey hairs is plotted on a log₁₀ scale against the response (number of withdrawal responses with five applications) at baseline and time points following plantar incision in postnatal day 3 (P3; n = 5) pups (A) and adult rats (B; n = 4). The mechanical withdrawal threshold is calculated from the midpoint of the curve (i.e. EF₅₀) is 1.04 ± 0.05 g at baseline, but is reduced to 0.41 ± 0.03 g at 4 h (P < 0.05; one-way ANOVA with Dunnett’s post hoc multiple comparison to baseline). Data points mean ± SEM. (C) Examples of EMG recordings to graded mechanical stimuli (von Frey hairs, vFh 14 to 17) and the method for calculating the area under the mechanical stimulus versus EMG response curve (AUC) are shown diagrammatically. (D) Twenty-four hours following plantar incision in neonatal (postnatal day 3, P3) or adult (P40) rats, the flexor reflex EMG response (area under mechanical stimulus versus EMG response curve; AUC EMG) was significantly increased when compared to age-matched non-incised controls. Bars = mean ± SEM; n = 4–6 all groups. P < 0.05 Student’s unpaired two-tailed t-test.
variables and Bonferroni post hoc comparison). There is a main effect of treatment (neonatal group $F_{(2,66)} = 33.5, P < 0.001$; adult group $F_{(2,54)} = 1029, P < 0.0001$), and a significant interaction with time (neonatal group $F_{(10,66)} = 31.4, P < 0.001$; adult group $F_{(10,54)} = 49.8, P < 0.001$). The increase in reflex thresholds in neonatal animals at 72 h (now aged P6) and 7 days (now aged P10) is consistent with normal developmental changes in the reflex response. Due to these changes in baseline with age, time accounts for more variance in the neonatal group (82% versus 25%) and further analysis compared the response to single versus repeat incision within the separate age groups. When the first incision was performed as a neonate (P3), the decrease in mechanical threshold 24 and 48 h following a second incision two weeks later was significantly greater than in animals having a single incision at this age (Fig. 2A; $P < 0.05$ single versus repeat incision, two-way ANOVA with time and treatment as variables and Bonferroni post hoc comparison). When the first incision was performed as an adult (P40), the degree of behavioral mechanical hyperalgesia following the second injury two weeks later was the same as that following a single injury at this age (Fig. 2B).

In neonatal and adult animals, the hyperalgesic response to repeat injury was also measured by flexion reflex EMG responses to graded von Frey hair mechanical stimuli (Fig. 3). As the baseline reflex response changes with age (as seen in Fig. 1D), the value for non-incised controls was normalized to 100% at all ages, and the effect of single or repeat incision expressed as a percentage change from control (% change reflex response = [(incision AUC - control AUC)/control AUC] * 100). Consistent with the behavioral measures, age-related differences in the response to single and repeat incision were noted. Prior incision in neonatal but not in adult animals was associated with increased hyperalgesia following a second incision two weeks later, as the reflex response in the repeat incision group was significantly greater than the single incision group ($P < 0.01$ single versus repeat, two-way ANOVA with Bonferroni post hoc comparison) (Fig. 3).

To test the critical age period for producing this enhanced response to repeat injury, initial plantar hindpaw incision was also performed at P6, P10, or P21 and the reflex hyperalgesia following a second incision 2 weeks later was tested. At all ages, plantar incision produced hyperalgesia that was quantifiable with EMG recordings. There was a main effect of treatment ($F_{(2,121)} = 217.6, P < 0.0001$) which accounted for 66% of the total variance, and an interaction with age ($F_{(8,121)} = 10.3, P < 0.01$) which accounted for 11% of the total variance. Twenty-four hours following incision, the reflex response was significantly greater in both the single and repeat incision groups at all ages ($P < 0.01$ single or repeat incision versus non-incised control, two-way ANOVA with Bonferroni post hoc comparison). As seen at P3, prior incision at P6 was associated with an enhanced hyperalgesic response to a second incision two weeks later (Fig. 3; $P < 0.01$ single versus repeat incision, two-way ANOVA with Bonferroni post hoc comparison), but when the first incision was performed at older ages (i.e. P10, P21 or P40) the response to repeat incision did not differ from age-matched single incision groups.

### 3.3. Effect of primary afferent blockade at the time of initial surgery

To determine if blocking primary afferent activity modifies acute mechanical hyperalgesia following neonatal incision, the effects of pre-operative or more prolonged peri-operative sciatic blockade on mechanical hindpaw withdrawal thresholds before and after P6 incision were measured (Fig. 4A). As expected, mechanical hyperalgesia was apparent in the saline group as the mechanical withdrawal threshold of the incised paw was significantly decreased from baseline 4 and 24 h following incision ($P < 0.01$ one way repeated measures ANOVA with Bonferroni post hoc comparison). In addition, the threshold in the saline group was lower when compared to both sciatic block groups at 4 and 24 h ($P < 0.05$ saline versus pre- or peri-operative sciatic block, two way repeated measures ANOVA and Bonferroni post hoc comparison).
Fig. 4. Effect of sciatic nerve block on the acute behavioral response to plantar incision at P6 and the response to a second incision. (A) Behavioral mechanical withdrawal thresholds of the ipsilateral hindpaw in P6 pups at baseline and 4, 24 and 72 h following plantar incision are plotted. Treatment groups include: a single pre-operative injection of levobupivacaine (pre-op sciatic block 1×) or a pre-operative block plus 3 × 2-h post-operative blocks ( peri-operative sciatic blocks 4×). Control animals received 4 × 2-h injections of saline (saline). Bars = mean ± SEM; n = 7–8. * P < 0.05 saline versus pre-operative sciatic (1 ×) and versus peri-operative sciatic (4×); two-way ANOVA with time and treatment as variables and Bonferroni post hoc comparison. (B) The reflex response following single incision at P20 (single incision) is compared with P6 + P20 incision (repeat incision) in animals treated at the time of P6 incision with: 4 × 2-h injections of saline (saline); 4 × 2-h subcutaneous injections of the local anesthetic levobupivacaine (s.c. LA); a single pre-operative levobupivacaine sciatic block (pre-operative sciatic 1 ×); or 4×2-h levobupivacaine sciatic blocks (peri-operative sciatic 4×). The reflex response to repeat incision was significantly greater compared with the single incision group in animals treated with saline, subcutaneous local anesthetic or a single pre-operative block, but the repeated peri-operative sciatic blocks did not differ from the single incision group. AUC EMG = area under mechanical stimulus versus EMG–response curve; Bars = mean ± SEM; n = 6–8 all groups; P < 0.05 one-way ANOVA with Bonferroni’s multiple comparison to single incision group.

4. Discussion

Our results show that the hyperalgesic response to repeat surgery after a two-week interval is enhanced by prior neonatal experience, and this effect is dependent on the age at which the initial injury occurs. Planter hindpaw incision produces acute hyperalgesia in neonatal and adult rats, but incision in neonatal pups has an additional effect on the response to subsequent injury, that is not seen following initial incision at older ages. The first postnatal week represents a critical period during which incision produces a segmental hyperalgesia that is unmasked by repeat injury two weeks later. This effect is prevented by peri-operative sciatic nerve blockade suggesting a mechanistic role for primary afferent activity at the time of initial incision.

Planter hindpaw incision is an established post-operative pain model in adult rats [46], and the mechanisms underlying hyperalgesia and the efficacy of analgesics differ from inflammation and nerve injury [7,28]. Age-related effects of surgical injury warrant specific evaluation, and using anatomical hindpaw landmarks to injure the same relative area ensures a consistent and reproducible stimulus despite changes in size with age. Consistent with data from P14 pups [41], a single plantar incision at P3 or P17 produced behavioral mechanical hyperalgesia, but the duration was shorter than in adults. We also quantified sensitivity by flexion reflex EMG recordings to assess changes in threshold and in the response to suprathreshold stimuli, both of which contribute to injury-related hyperalgesia. We have previously used this methodology to evaluate C-fiber induced mechanical hyperalgesia in rat pups [53] and have now confirmed that plantar incision produces injury-related hyperalgesia at all postnatal ages.

Different forms of neonatal injury have been associated with alterations in the response to future noxious stimuli. Severe CFA-induced neonatal inflammation increases the response to inflammation, formalin, and capsaicin injection of the previously injured paw in adulthood [16,42,46]. Mild neonatal inflammation from hindpaw carrageenan is associated with increased hyperalgesia when the ipsilateral, but not contralateral paw is re-inflamed [35] or surgically incised [8] in adulthood. As surgery produces skin wounding and nerve injury in addition to inflammation [24,55], but the developmental profile of these different forms of injury varies [18,27,36,39], we specifically evaluated the impact of hind-
paw incision in early life upon the pain evoked by repeat surgical injury. Initial incision at P3 was associated with increased mechanical hyperalgesia (as assessed by behavioral withdrawal thresholds and EMG measures of reflex sensitivity) following a repeat incision two weeks later, whereas prior experience had no impact when the initial incision occurred in adulthood.

A developmental window or critical period during which injury triggers persistent sensory changes in rodents has been reported following inflammation. Severe inflammation at P0 or P3 but not at P14 produces long-term expansion of sciatic terminal fields in the spinal cord [42]; mild inflammation at P3 but not P14 is associated with alterations in visceral sensitivity in the adult [54]; and an enhanced response to repeat inflammation is seen if mild inflammation is induced between P0 and P5, but not at P8 or older [35]. Surgical laparotomy in P0 mice produces long-term sensory changes with reduced sensitivity to visceral stimuli in adulthood [44], but segmental effects of repeat incision have not been evaluated in this model. By comparing effects of surgical injury across a range of ages, we have now shown that the first postnatal week is a critical period for altered responses to surgery, as hindpaw incision at P3 or P6, but not at older ages, was associated with increased hyperalgesia following repeat incision two weeks later. An enhanced response to re-inflammation of a neonatally-inflamed paw is apparent at time points from 4 days until several weeks after the initial injury [35]. Repeat incision was performed after 2 weeks to allow complete wound healing and resolution of primary hyperalgesia at all ages, so the exact time of onset of enhancement is not clear. We are currently evaluating effects of neonatal incision on sensory processing in adulthood [48].

As the enhanced response to repeat surgery occurs only after neonatal incision, the injury must trigger plastic changes in pain pathways through developmentally regulated mechanisms that differ from those following adult plantar incision. The efficacy of peri-operative sciatic block indicates a crucial initiating role for primary afferent activity arising from the skin incision. Such activity may cause local changes in sensory terminals, such as hyperinnervation [27] or alterations in receptor transport and distribution [21], or central effects such as sensitization of nociceptive circuits in the spinal cord. Plantar incision in P7 and P28 pups increases excitability in large DRG neurons, but ongoing activity persists beyond the period of behavioral hyperalgesia in the younger pups and may contribute to longer-term changes [40]. In the adult, plantar incision produces spontaneous activity in A-beta as well as A-delta and C primary afferent fibers [13,28,33], in a pattern that differs from inflammation and nerve injury [26,29]. As the central distribution and functional response to C- and A-fiber afferent inputs change during the postnatal period [2,20,30,53], and A-beta fiber activation produces sensitization in the superficial dorsal horn in young but not adult animals [20,30], activation of A-beta fibres by incision may contribute to differing effects in early development.

Plantar incision produces alterations in the stimulus–response characteristics of dorsal horn neurons and central sensitization in adult rodents [23,34,49]. In P7 pups, greater evoked spike activity and a rapid expansion of receptive field size are seen in the dorsal horn [38]. As there are significant postnatal changes in excitatory and inhibitory circuits in the spinal cord and in their modulation by descending pathways [1,9,14], activity-dependent alterations in nociceptive circuitry during this period may underlie persistent functional changes. c-Fos expression in the dorsal horn is increased ipsilaterally to repeat inflammation [46], capsaicin injection [16] or plantar incision [8] when a neonatally-inflamed paw is re-injured in adulthood. However, differences in intracellular signalling [8] suggest that it is not just the degree, but also the mechanisms of central sensitivity that are altered by early injury.

The duration of primary afferent blockade was critical for modulating the response to neonatal plantar incision. Two different patterns of response were identified: (i) a reduction in behavioral mechanical hyperalgesia that persisted beyond the duration of sciatic blockade (i.e. preventive analgesic effect [22]), and (ii) modulation of the enhanced response to repeat incision two weeks later. In adult rats, single pre-incision administration of local anesthetic (either peripheral infiltration [34], ankle blocks plus plantar infiltration [45], or intrathecal injection [5]) reduced behavioral mechanical hyperalgesia at 4 h, but not 24 h. This lack of preventive effects in adults may be influenced by the longer duration of hyperalgesia relative to the local anesthetic block. By contrast, in younger pups a single pre-incision sciatic block (seen here in P6 pups and previously reported at P14 [37]) does reduce mechanical hyperalgesia 24 h following hindpaw incision. More prolonged peri-operative sciatic blockade at P6 had an additional impact on the response to a second incision two weeks later that was not seen following a single pre-operative block, suggesting that the duration of an analgesic intervention may be critical for modulating more persistent effects following neonatal surgery.

As systemic administration of local anesthetic can produce analgesic effects following hindpaw incision [23], effects of peripheral nerve blockade need to be differentiated from effects of systemic absorption [3]. In the current experiments, sciatic but not subcutaneous administration of levobupivacaine prevented the enhanced response to repeat incision, suggesting a specific role for primary afferent blockade. The reduction in reflex response following repeat incision was not due to impaired development of the reflex, as repeated sciatic blocks (in the absence of surgical injury) had no effect on behavioral thresholds or EMG responses, and produced no histological signs of sciatic nerve injury.

Our findings are clinically relevant from two different perspectives. Firstly, increased peri-operative analgesic requirements at the time of repeat surgery has been shown in infants, but similar studies have not been performed following initial surgery at older ages [32]. The current data suggests that the enhanced hyperalgesic response to repeat incision after two weeks specifically relates to an initial injury in the neonatal period. While direct parallels between rat and human postnatal development are difficult, spinal cord development during the first postnatal week in rodents correlates with the human preterm and neonatal period [10,12], suggesting that initial surgery during this time may be associated with age-related changes in sensory processing. Secondly, our findings have implications for the ability of analgesia at the time of the initial injury to modulate longer-term alterations in pain. Topical local anaesthesia at the time of neonatal circumcision reduced but did not completely prevent the enhanced behavioral response to immunization several months later [47]. Here we find that the ability to prevent subsequent enhanced hyperalgesia was dependent on the duration of the initial peripheral nerve blockade. This data provides important insights into the plastic changes in pain processing triggered by early pain experience and has potential therapeutic implications for the type and duration of peri-operative analgesia used for neonatal surgery.

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