

Blockade of Opioid Receptors in the Medullary Reticularis Nucleus Dorsalis, but not the Rostral Ventromedial Medulla, Prevents Analgesia Produced by Diffuse Noxious Inhibitory Control in Rats With Muscle Inflammation

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Abstract: Diffuse Noxious Inhibitory Controls (DNIC) involves application of a noxious stimulus outside the testing site to produce analgesia. In human subjects with a variety of chronic pain conditions, DNIC is less effective; however, in animal studies, DNIC is more effective after tissue injury. While opioids are involved in DNIC analgesia, the pathways involved in this opioid-induced analgesia are not clear. The aim of the present study was to test the effectiveness of DNIC in inflammatory muscle pain, and to study which brainstem sites mediate DNIC- analgesia. Rats were injected with 3% carrageenan into their gastrocnemius muscle and responses to cutaneous and muscle stimuli were assessed before and after inflammation, and before and after DNIC induced by noxious heat applied to the tail (45°C and 47°C). Naloxone was administered systemically, into rostral ventromedial medulla (RVM), or bilaterally into the medullary reticularis nucleus dorsalis (Mdd) prior to the DNIC-conditioning stimuli. DNIC produced a similar analgesic effect in both acute and the chronic phases of inflammation reducing both cutaneous and muscle sensitivity in a dose-dependent manner. Naloxone systemically or microinjected into the Mdd prevented DNIC-analgesia, while naloxone into the RVM had no effect on DNIC analgesia. Thus, DNIC analgesia involves activation of opioid receptors in the Mdd.

Perspective: The current study shows that DNIC activates opioid receptors in the Mdd, but not the RVM, to produce analgesia. These data are important for understanding clinical studies on DNIC as well as for potential treatment of chronic pain patients.

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Diffuse noxious inhibitory control (DNIC) is a mechanism of pain inhibition that occurs when a noxious stimulus is applied outside the test area.^{1,33} Prior studies show that lesioning either the rostral ventromedial medulla (RVM) or periaqueductal grey (PAG) has no effect on DNIC-induced analgesia.^{6,9}

However, DNIC-conditioning stimuli decreases activity of RVM facilitatory ON-cells.²⁴ Neurons in the medullary reticularis nucleus dorsalis (Mdd) respond to high intensity noxious stimuli and may mediate DNIC analgesia.⁸ DNIC reduces activity of convergent dorsal horn neurons through activation of opioids receptors.^{5,6,8,32,44,63} Both the RVM and the Mdd are rich in μ -opioid receptors^{39,40} and thus could play a role in DNIC analgesia.

Although the majority of animal studies characterized DNIC in animals without tissue injury, 3 studies show DNIC is more effective after injury when recording from nociceptive dorsal horn or trigeminal neurons in rats¹⁶⁻¹⁸; this enhanced effect has yet to be confirmed behaviorally. In human subjects, people who do not produce analgesia to DNIC-conditioning stimuli are

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more likely to develop chronic pain.⁶⁴ It is generally thought there is a balance between inhibition and excitation in supraspinal sites so that under normal conditions inhibition is greater than excitation.^{41,42,55} After tissue injury, alterations in this balance result in a loss of inhibition and/or an increase in excitation.^{11,53,57} These data suggest the balance between inhibition and facilitation in chronic pain is altered, and DNIC pathways could be a key component in development of chronic pain.

Previous studies use different nociceptive conditioning stimuli to induce DNIC in animals (electrical stimulation, hot water, acupuncture, or pinch^{30,32,36}) and in humans (heat pain, cold pain, and ischemic pain^{1,29,31,63}). In human subjects, intensities of stimulation (45–47°C) are typically lower when compared to animals (48–50°C); both human and animal studies show intensity is important in producing DNIC analgesia.^{7,33,58} Further, DNIC analgesia is generally tested during the stimulus, and the duration of effect is thought to be short-lasting. Thus, this animal study will investigate: 1) the effectiveness of DNIC using different intensities of conditioning stimuli; 2) the effect of DNIC on both acute and chronic hyperalgesia; 3) if DNIC analgesia outlasts the conditioning stimulus; and 4) participation of endogenous opioids in supraspinal sites in DNIC-induced analgesia.

Materials

Animals

All experiments were approved by the University of Iowa Animal Care and Use Committee and were carried out according to the guidelines of the National Institute of Health. Male Sprague-Dawley rats, weighing between 250 and 350 g (Harlan, St. Louis) were used in this study. The temperature of the behavioral testing room was controlled (23–25°C), and animals were maintained in a 12 hour dark-light cycle with free access to water and food. Behavioral tests were performed between 9 a.m. and 5 p.m.

Induction of Muscle Inflammation

Muscle inflammation was induced in rats by injecting 3% carrageenan (Cg; Type IV carrageenan Lambda; Sigma-Aldrich, St. Louis, MO), dissolved in sterile saline, into the gastrocnemius muscle while the animal was anesthetized with 4% isoflurane.

Diffuse Noxious Inhibitory Control (DNIC)

All rats were acclimated 2× per day for 2 days before the DNIC test to minimize stress during the test. Rats were placed in the restrainer and their tail held in warm (36°C) water for 2 minutes. The DNIC-conditioning stimulus used was immersion of the tail into a hot water bath at 2 different temperatures: 45 and 47°C. The distal 7.0 cm of the tail was immersed into hot water for 2 minutes while the animal was awake. It should be noted that the duration and surface

area of the conditioning stimuli were kept constant in this study since modification of these variables can affect DNIC analgesia. The application of the heat stimulus at these temperatures is similar to that used in awake human subjects, and thus is directly transferable. It was noted that the animal tried to withdraw its tail during this stimulus but the experimenter continued to hold the tail in the temperature. Preliminary studies with higher temperatures (49°C) produced significant distress in awake animals, and thus we were unable to separate stress-induced analgesia from DNIC-induced analgesia. Therefore, we used 2 lower temperatures of 45 and 47°C to induce and test DNIC analgesia.

Behavioral Testing

All animals were tested for cutaneous and muscle mechanical sensitivity. Prior to testing, rats were acclimated to the von Frey filaments and tweezer testing procedures for 2 days and 2 times per day to minimize stress during testing and reduce variability of the outcome measures. For acclimation to von Frey filaments, rats were placed on the elevated table in Lucite cubicles for 10 minutes. For the tweezers, mice were placed in the testing glove for 5 minutes.

Test Stimulus 1: Cutaneous Mechanical Sensitivity

Rats were tested for cutaneous sensitivity with von Frey filaments applied to the paw immediately after the DNIC-conditioning stimulus (within 2 minutes) as previously described.²⁷ A series of filaments with various bending forces (501, 224, 140, 88, 74, 57, 34, and 10 mN) was applied to the plantar surface of the hindpaw. The lowest force at which the withdrawal response was obtained was the cutaneous withdrawal threshold of the paw. A decrease in withdrawal threshold was interpreted as secondary cutaneous hyperalgesia and an increase was interpreted as analgesia.

Test Stimulus 2: Muscle Mechanical Sensitivity

Rats were tested for muscle sensitivity with a pair of tweezers applied to the gastrocnemius muscle as previously described.⁴⁸ To measure the withdrawal threshold, rats were placed in a restraining glove, and the gastrocnemius muscle compressed with a pair of tweezers with calibrated force plates while the hindlimb was extended. Compression was continued until the animal withdrew the leg or vocalized. The maximum force applied at withdrawal was recorded as the compression threshold. A decrease in withdrawal threshold was interpreted as muscle hyperalgesia and an increase was interpreted as analgesia.

Placement of Guide Cannula and Drug Administration

Intracerebral guide cannulae were stereotactically implanted in the RVM or MdD 3 to 5 days before the first intramuscular injection of carrageenan. The rats were anesthetized with an injection of sodium pentobarbital

(Nembutal, 60mg/kg, ip) and secured in a stereotaxic head holder to implant the guide cannula (17.5 mm in length, 26 gauge; Plastics One, Roanoke, VA). After a midline incision, the skull was exposed, and a small hole drilled for placement of the guide cannula. Cannulae were placed 3 mm dorsal to the RVM (interaural: -2.0 mm; mediolateral: $.0$ mm; and dorsoventral: -7.5 mm²⁷) or the MdD bilaterally (interaural: -5.08 mm; mediolateral: ± 1.5 mm; and dorsoventral: -5.8 mm).³⁷ Site specificity was tested by placing the cannulae 3 mm above the MdD into the nucleus cuneatus and either naloxone or vehicle injected. Cannulae were secured to the skull by stainless steel screws and dental acrylic. A dummy cannula (33 gauge, Plastics One) was inserted into the guide cannula to maintain its patency. All rats were allowed 3 to 5 days recovery before testing began.

The rats were microinjected with naloxone hydrochloride, dissolved in sterile saline ($20 \mu\text{g}/.5 \mu\text{l}$; Sigma-Aldrich, St. Louis, MO) or vehicle ($.5 \mu\text{l}/.9\%$ sterile saline) into the RVM or MdD through the guide cannula. For microinjection, a 33-gauge injection cannula was connected to a $10\text{-}\mu\text{l}$ Hamilton syringe through polyethylene-10 tubing backfilled with sterile saline. The microinjection was performed over a 2-minute period and the travel of the air bubble in the tubing was carefully observed to ensure that the drug solution entered the injection cannula. The injection volume of $.5 \mu\text{l}$ was chosen as we previously showed that this volume is sufficient to cover the majority the RVM (nucleus raphe magnus and partial lateral paragigantcellularis) but does not diffuse into the nucleus gigantocellularis 1 mm above the RVM.¹⁹ We also showed the dose of naloxone $20 \mu\text{g}$ blocks μ -opioid receptors in the RVM and prevents TENS-induced antihyperalgesia.²⁷

To examine placement of the cannula into RVM and MdD, an equivalent volume of methylene blue dye was injected at the end of the experiment. Rats were then euthanized and transcardially perfused with 4% paraformaldehyde. After this, the brain was removed and stored in 30% sucrose solution. The brain was cross-sectioned at $40 \mu\text{m}$ on a cryostat and examined under a light microscope for placement of the cannula. Injection sites, mapped from histological sections, are shown in Figs 3 and 4.

Experimental Protocol

Experiment 1 tested animals with and without muscle inflammation to compare the effects of the DNIC-conditioning stimuli at 2 different temperatures, 45 and 47°C, on behavioral tests. Behavioral tests were performed before and 24 hours after induction of inflammation (pre-DNIC), considered the acute phase, and 1 week after induction of inflammation, considered the chronic phase. The DNIC-conditioning stimulus was performed at 24 hours and 1 week after induction of inflammation. Behavioral tests were performed immediately after the DNIC-conditioning stimulus and 1 hour after the DNIC-conditioning stimulus. Both 45 and 47°C DNIC-conditioning stimuli were tested and the testing was done in separate groups of animals. A control group was used to compare the DNIC effect in

uninflamed animals that were injected with pH 7.2 saline instead of carrageenan. Each animal was tested individually at a separate time. This was done so that cutaneous and muscle testing could both be done in a short time frame after DNIC (within 5 to 10 minutes) since the analgesia produced by DNIC is thought to be short-lasting.

Experiment 2 tested the effect of naloxone (3 mg/kg, sc, 30 minutes prior to DNIC) administered systemically on the behavioral response to the 47°C conditioning stimulus 24 hours after inflammation. The dose of naloxone was based on previous studies showing effectiveness when administered systemically.⁴⁵ In a separate control group ($n = 4$), the effects of 3 mg/kg naloxone was tested on the mechanical withdrawal thresholds of the paw and muscle at 24 hours after carrageenan and 8 days after carrageenan.

Experiment 3 tested the effect of naloxone microinjected into the RVM ($20 \mu\text{g}/.5 \mu\text{l}$) or the MdD ($20 \mu\text{g}/.5 \mu\text{l}/\text{side}$) on the behavioral response to 47°C conditioning stimulus 24 hours after muscle inflammation. Naloxone was given 10 minutes prior to the DNIC-conditioning stimulus. Testing was done with the experimenter blinded to the injection of vehicle or naloxone. The dose of naloxone were based on previous studies showing effectiveness when microinjected into the RVM.²⁷ The controls groups were injected with the same volume of saline into the RVM or SRD.

Statistical Analysis

The degree of hyperalgesia induced by carrageenan was presented as the mean \pm S.E.M. Percent changes are calculated for the change in DNIC as the withdrawal threshold after DNIC to the withdrawal threshold prior to DNIC but after carrageenan; 0% was no change. For Experiment 1, the effect of DNIC was expressed as a change from the pre-DNIC value either immediately or 1 hour after the DNIC-conditioning stimuli. A repeated measures ANOVA was used for the statistical comparison followed by a Tukey's post hoc test to compare among groups and temperatures. For Experiment 2, a paired t-test compared withdrawal thresholds after naloxone to that prior to naloxone. For Experiment 3, a repeated measures ANOVA compared withdrawal thresholds across time and between groups. A Tukey's test compared differences among groups at individual time periods. To verify the hyperalgesia induced by carrageenan the students t-test compared those injected with saline into the muscle gastrocnemius to those injected with carrageenan. Statistical significance was defined as $P < .05$. Statistical analyses were conducted with SPSS v.17.0 (SPSS Inc, Chicago, IL).

Results

Development of Hyperalgesia After Muscle Inflammation

Carrageenan injected into the gastrocnemius muscle ($n = 14$) reduced the withdrawal threshold of the paw (cutaneous secondary hyperalgesia) and muscle (primary

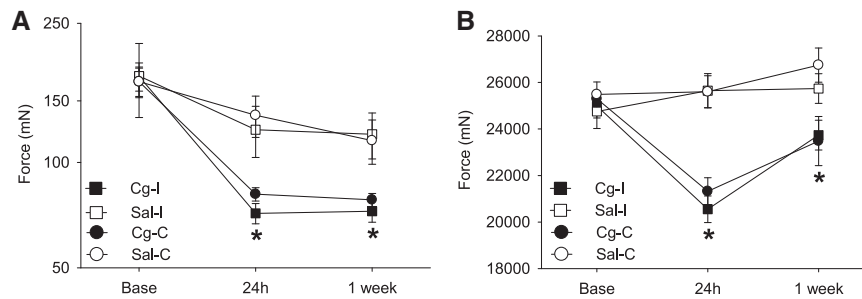


Figure 1. Withdrawal thresholds of the paw (A) and muscle (B) decreased 24 hours after induction of muscle inflammation with 3% carrageenan (Cg) ($n = 16$). Control animals received intramuscular injection of saline (S) ($n = 16$). The ipsilateral (I) side is represented by squares and the contralateral (C) side is represented by circles. Each point represents mean \pm S.E.M. *, significantly different from saline-injected controls.

hyperalgesia) 24 hours after induction of inflammation (acute phase; Fig 1). After 8 days of the inflammation the withdrawal threshold remained decreased for the paw, but not consistently for the muscle (decreases

only observed in the group that received 47°C conditioning stimulus). Moreover, this reduced paw withdrawal threshold occurred for both the ipsilateral and contralateral paws when compared with those that received

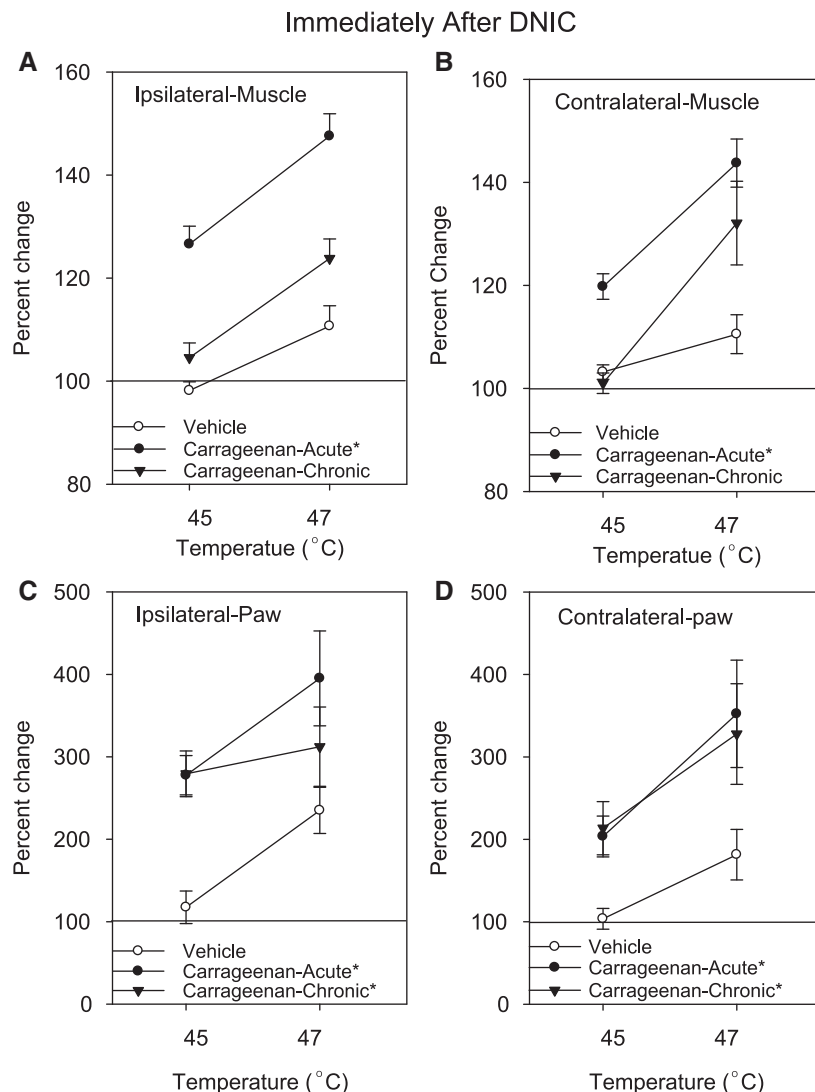


Figure 2. Graphs representing DNIC-induced analgesia, immediately after the conditioning stimulus, on the muscle (A, B) and paw (C, D) withdrawal thresholds in saline-injected controls ($n = 10$), acute inflammation ($n = 6$), and chronic ($n = 10$) muscle inflammation. The DNIC-conditioning stimulus was given at 2 intensities, 45°C and 47°C. DNIC = diffuse noxious inhibitory controls. Each point represents mean \pm S.E.M. *, significantly different from controls.

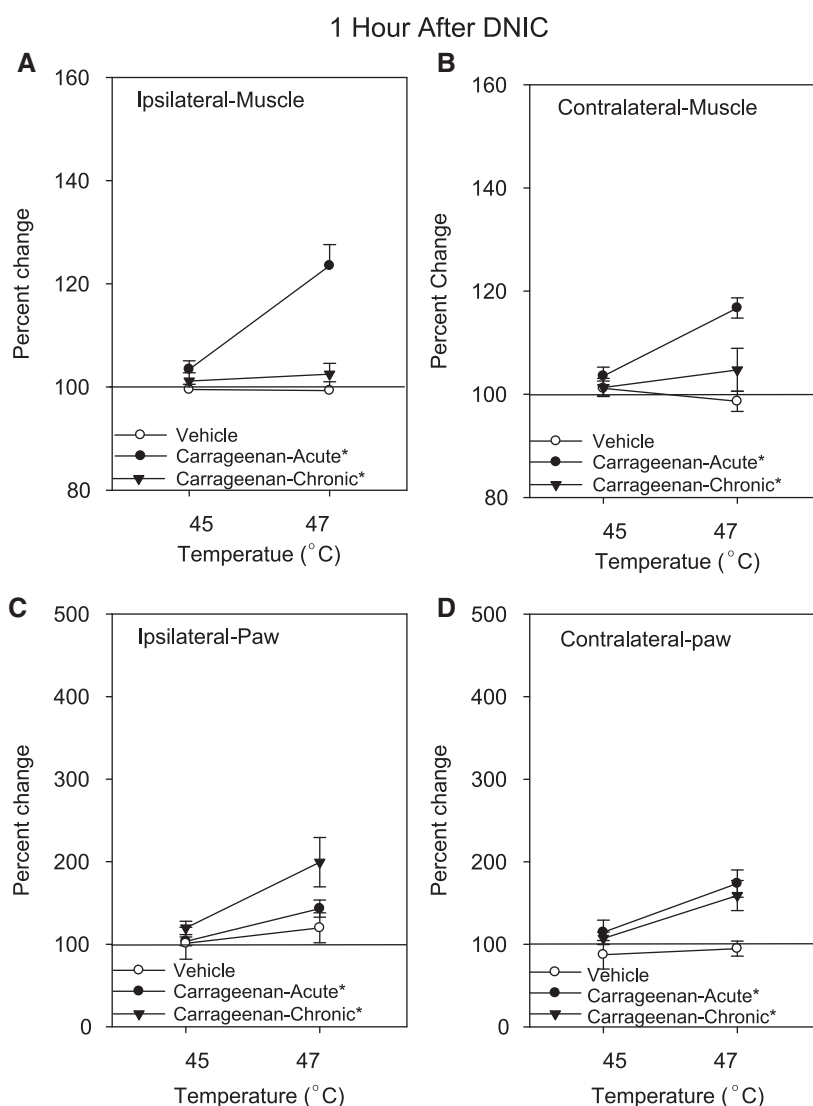


Figure 3. Graphs representing DNIC-induced analgesia, 1 hour after the conditioning stimulus, on the muscle (**A, B**) and paw (**C, D**) withdrawal thresholds in saline-injected controls, acute inflammation, and chronic muscle inflammation. The DNIC-conditioning stimulus was given at 2 intensities, 45°C ($n = 6$) and 47°C ($n = 10$). DNIC = diffuse noxious inhibitory controls. Each point represents mean \pm S.E.M. *, significantly different from control.

saline into the gastrocnemius muscle. This bilateral hyperalgesia has been found more frequently in musculoskeletal pain models.^{14,15,43,49,50,60,61}

Effect of DNIC on Muscle Withdrawal Threshold

Overall Effect

For the muscle withdrawal thresholds there was: 1) a temperature-dependent effect with 47°C producing greater analgesia than 45°C ($F_{1,42} = 14.2$; $P = .0001$); 2) greater analgesia on the inflamed side when compared to the contralateral side ($F_{1,42} = 15.3$, $P = .0001$); and 3) a significant difference between groups ($F_{2,42} = 3.1$, $P = .05$) with the control group significantly different from the acute inflammation group ($P = .015$), but not from the chronic inflammation group ($P = .09$ [Fig 2]). There was also a significant effect for changes in the muscle withdrawal threshold 1 hour after DNIC that

was dependent on temperature of stimulation and group (inflammation \times group: $F_{2,42} = 14.2$, $P = .0001$). In the animals with acute muscle inflammation there was a significant increase in withdrawal thresholds 1 hour after the 47°C conditioning stimulus when compared to controls ($P = .0001$) and those with chronic inflammation ($P = .0001$ [Fig 3]).

Normal Animals

In animals without inflammation, the 47°C ($n = 8$), but not the 45°C ($n = 6$), conditioning stimulus significantly increased ($10.0 \pm 4\%$) the muscle withdrawal threshold (Fig 2). All values returned to baseline 1 hour after the DNIC-conditioning stimuli.

Acute Inflammation

In animals with acute inflammation, the 45°C ($n = 6$) and 47°C ($n = 8$) conditioning stimulus increased the muscle withdrawal threshold (Fig 2). The 45°C conditioning

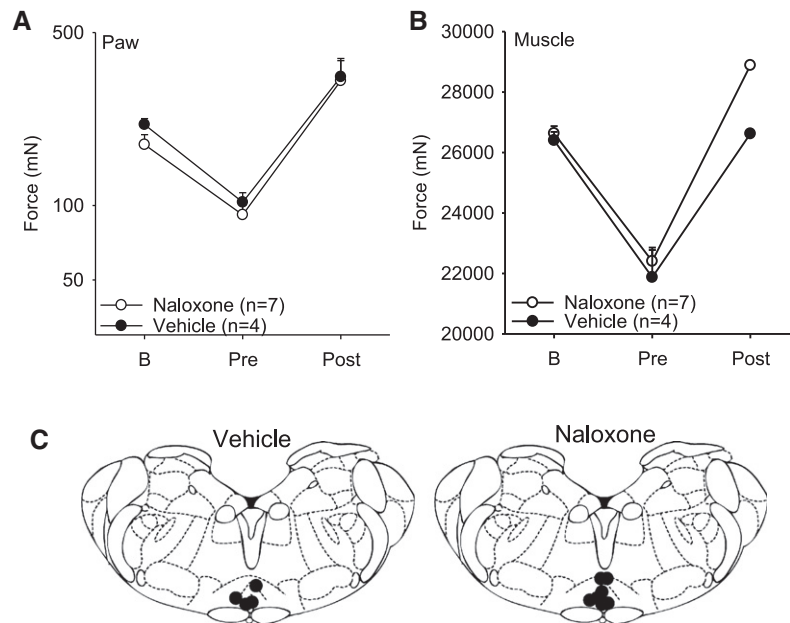


Figure 4. Paw (A) and muscle (B) withdrawal thresholds before and 24 hours after muscle inflammation, and after the 47°C conditioning stimulus. Naloxone or vehicle was microinjected into the RVM prior to the conditioning stimuli. Each point represents mean \pm S.E.M. B = baseline testing before inflammation; RVM = rostral-ventromedial medulla; DNIC = diffuse noxious inhibitory controls. (C) Coronal sections of the medulla showing the microinjection sites for the group injected with naloxone or vehicle.

stimulus increased the ipsilateral paw withdrawal threshold (IPL) by $26 \pm 3\%$ and the contralateral paw withdrawal threshold (CTL) by $20 \pm 2\%$. Moreover, DNIC delivered at an intensity of 47°C was more robust than 45°C and increased the withdrawal thresholds ipsilaterally by $48 \pm 4\%$ and contralaterally by $44 \pm 5\%$. One hour post-DNIC hyperalgesia returned similar to that observed pre-DNIC for the 45°C conditioning stimulus but there was still a significant increase for the group treated with the 47°C conditioning stimulus (Fig 3).

Chronic Inflammation

In animals with chronic inflammation, the 45°C ($n = 6$) conditioning stimulus was ineffective while the 47°C ($n = 8$) conditioning stimulus increased the muscle withdrawal thresholds both ipsilaterally ($24 \pm 3.8\%$) and contralaterally ($32 \pm 8.1\%$) [Fig 2]. The hyperalgesia returned to baseline 1 hour after DNIC (Fig 3).

Effect of DNIC on Paw Withdrawal Threshold

Overall effect

For paw withdrawal thresholds there was: 1) a temperature-dependent effect with 47°C producing greater analgesia than 45°C ($F_{1,42} = 7.4$; $P = .01$); 2) greater analgesia on the inflamed side when compared to the contralateral side ($F_{1,42} = 8.6$, $P = .005$); and 3) a significant difference between groups ($F_{2,42} = 6.0$, $P = .005$) with the control group significantly different from the acute inflammation group ($P = .004$) and from the chronic inflammation group ($P = .025$ [Fig 2]). There was also a significant effect for changes in the paw withdrawal threshold 1 hour after DNIC that was dependent on:

1) temperature of stimulation ($F_{1,42} = 16.9$, $P = .0001$); and 2) differences between groups ($F_{2,42} = 6.1$, $P = .005$) with the control group significantly lower than the acute inflammation ($P = .02$) and the chronic inflammation ($P = .001$) group (Fig 3).

Normal Animals

DNIC at 45°C ($n = 6$) had no effect on the paw withdrawal thresholds in animals without inflammation. DNIC at 47°C ($n = 8$), however, significantly increased with paw withdrawal thresholds in animals without inflammation by $135 \pm 29\%$ ipsilaterally, and $181 \pm 31\%$ contralaterally (Fig 2).

Acute Inflammation

In animals with acute inflammation, however, the DNIC-conditioning stimulus at either 45°C ($n = 6$) or 47°C ($n = 8$) significantly increased the paw withdrawal threshold bilaterally. The 45°C conditioning stimulus increased the paw withdrawal threshold ipsilaterally by $178 \pm 24\%$ and contralaterally by $103 \pm 25\%$ (Fig 2). One hour after the 45°C DNIC-conditioning stimulus, the withdrawal thresholds returned to baseline values. There was a larger increase in the paw withdrawal thresholds (ipsilateral $295 \pm 57\%$; contralateral $152 \pm 65\%$) after the 47°C conditioning stimulus when compared to 45°C (Fig 3). This increase persisted 1 hour after application of the 47°C DNIC conditioning stimulus (ipsilateral $43 \pm 10\%$; contralateral $74 \pm 16\%$).

Chronic Inflammation

In animals with chronic inflammation both the 45°C ($n = 6$) and the 47°C ($n = 8$) conditioning stimulus significantly increased the paw withdrawal threshold bilaterally (45°C: ipsilateral $178 \pm 24\%$; contralateral

103 \pm 25%; 47°C: ipsilateral 295 \pm 58%; contralateral 252 \pm 65% [Fig 2]). There was a significant increase in withdrawal thresholds of the paw 1 hour after the 47°C conditioning stimulus (ipsilateral 99 \pm 29%; contralateral 59 \pm 18%) when compared to baseline (Fig 3), but not for the 45°C conditioning stimulus.

Effect of Naloxone on DNIC Analgesia

Systemic Naloxone

To verify the participation of endogenous opioids in the mechanism of DNIC as previously described,^{4,32} naloxone, an opioid antagonist, was injected subcutaneously 30 minutes before the 47°C conditioning stimulus ($n = 6$). The muscle withdrawal threshold did not increase (ipsilateral 11 \pm 6%; contralateral 9 \pm 5%) when compared to values in those that did not receive naloxone (ipsilateral 48 \pm 4%; contralateral 44 \pm 5%), confirming a role for opioids in DNIC-induced analgesia. However, the paw withdrawal threshold still increased significantly after systemic naloxone (ipsilateral 246 \pm 45%; contralateral 247 \pm 66%). As a control, we tested if systemic naloxone (3 mg/kg, sc) alone had any effect on the muscle or paw withdrawal thresholds ($n = 4$); there was no significant change 30 minutes after naloxone (Table 1).

RVM Naloxone

Since the RVM is implicated in opioid induced analgesia,⁸ we tested if naloxone ($n = 7$) microinjected into the RVM prevents the analgesic effect of DNIC delivered with the 47°C conditioning stimulus. As seen in Fig 4A and 4B, naloxone had no effect on the analgesia produced by DNIC on either muscle or paw withdrawal thresholds when compared to microinjection of vehicle as a control ($n = 4$). Thus, opioid receptors in the RVM do not mediate the effects of DNIC.

MdD Naloxone

Since prior literature suggests the MdD mediates the analgesic effects of DNIC,⁶ we tested if microinjection of naloxone administered into the MdD before the 47°C conditioning stimulus prevented DNIC analgesia. Microinjection of naloxone ($n = 7$) into the MdD bilaterally prevented DNIC-induced analgesia for both the muscle ($n = 6$) and paw ($n = 7$) withdrawal thresholds when compared to vehicle controls ($P < .05$; $n = 5$ muscle; $n = 6$ paw [Fig 5]). In animals with misplaced cannula

there was still an increase of 221 \pm 39% for the paw withdrawal threshold in those injected with naloxone ($n = 5$) that was significantly greater than those injected with naloxone in the MdD (100 \pm 54%). In 4 animals, the cannulae were placed in the cuneate nucleus; in 1 animal, the cannulae was placed lateral reticular nucleus. Thus, opioid receptors in the MdD mediate the analgesic effects of DNIC.

Discussion

DNIC Increases Pain Thresholds in Uninjured Animals and Humans

Numerous studies have shown that application of a noxious conditioning stimulus produces analgesia in both animals and humans, and a concomitant reduction in dorsal horn activity.^{34,63} Different studies describe recruitment of DNIC in uninjured animals^{34,59} and humans^{20,47} using different conditioning stimuli applied to the skin with the majority of these studies showing analgesia during the test stimulus. We extend these previous findings by showing DNIC analgesia: 1) immediately after the conditioning stimulation; 2) for secondary hyperalgesia; 3) for muscle hyperalgesia; and 4) can last up to 1 hour in animals with muscle inflammation. Our data are in agreement with prior studies recording effects of DNIC on inhibiting dorsal horn neuron activity in rats which show persistent inhibition after removal of the conditioning stimulus.³³ For example, DNIC induced by intraperitoneal injection of acetic acid results in analgesia to the tail flick test immediately, to the hot plate test for 15 minutes, and to the vocalization threshold to electrical stimulation for up to 30 minutes.¹² On the other hand, in lightly anesthetized rats, 49 to 51°C conditioning stimuli (but not 45 to 48°C) increased the tail flick latency to heat but had no effect on the paw withdrawal latency to heat. Thus, effectiveness and length of action of DNIC differs depending on the test stimulus, intensity of stimulus, and outcome measured.

The current study also shows that DNIC analgesia is dependent on the intensity of the conditioning stimulus, with a greater intensity producing a greater analgesia and is in agreement with prior work in animals.^{7,33,59} A recent study in human subjects agrees with this concept, showing greater pain intensity and greater DNIC analgesia with the cold-pressor test when compared

Table 1. Muscle and Paw Withdrawal Thresholds Before and After Treatment With 3 mg/kg Naloxone. Data Are Mean \pm S.E.M. and Given in mN of Force

	MUSCLE WITHDRAWAL THRESHOLD (mN)		PAW WITHDRAWAL THRESHOLD (mN)	
	IPISILATERAL	CONTRALATERAL	IPISILATERAL	CONTRALATERAL
Baseline	2890 \pm 261	2516 \pm 325	253 \pm 121	120 \pm 33
24 h post-Cg	1365 \pm 141	2591 \pm 187	33 \pm 13	68 \pm 32
30 min post-naloxone	1432 \pm 441	2108 \pm 390	38 \pm 22	68 \pm 32
8 days post-Cg	2362 \pm 482	2765 \pm 121	86 \pm 51	116 \pm 36
30 minute post-naloxone	2000 \pm 553	2464 \pm 362	85 \pm 49	207 \pm 103

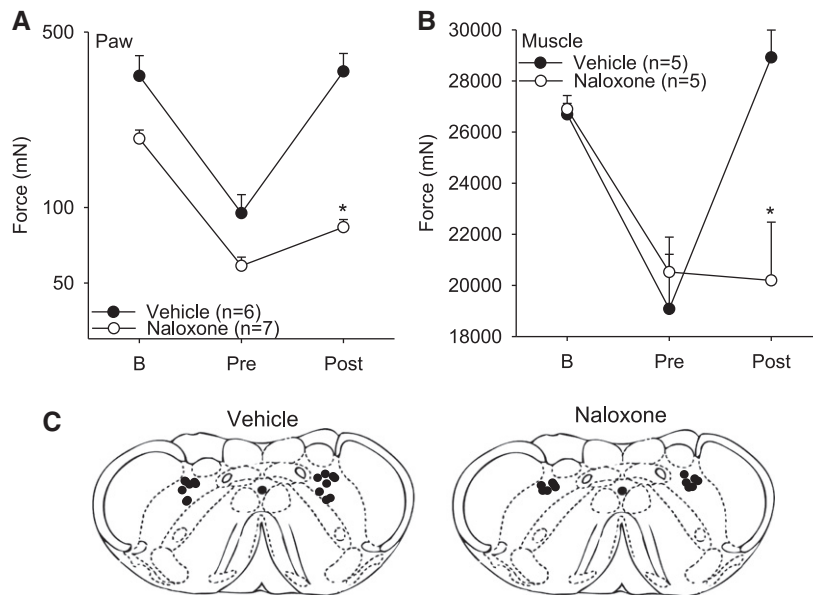


Figure 5. Paw (A) and muscle (B) withdrawal thresholds before and 24 hours after muscle inflammation, and before and after the 47°C conditioning stimulus. Naloxone or vehicle was microinjected into the MdD prior to the conditioning stimuli. Each point represents mean \pm S.E.M. B = baseline testing before inflammation. (C) Coronal sections of the medulla showing the microinjection sites for the group injected with naloxone and vehicle. MdD = medullary reticularis nucleus dorsalis; DNIC = diffuse noxious inhibitory controls.

to intramuscular infusion of hypertonic saline.¹ On the other hand, Granot et al²³ show no correlation between pain scores and endogenous analgesia in human subjects; however, DNIC-conditioning stimuli resulting in pain below 20/100 do not produce analgesia. Thus, intensity of the DNIC conditioning stimulation appears critical to produce analgesia in both animals and healthy subjects.

DNIC Is Altered in Pain Conditions

The current study shows that DNIC is more effective in animals with muscle inflammation when compared to those without inflammation; the 45°C conditioning stimulus produces analgesia in animals after muscle injury but not in those without muscle injury. These data agree with prior animal studies showing that DNIC is more effective in animals with hind paw inflammation or neuropathic pain when recording central nociceptive neurons in the dorsal horn or trigeminal nucleus.^{17,18} Interestingly, in humans, cold-pressor-induced DNIC responses applied concomitantly with a hypertonic saline-induced muscle pain resulted in less analgesia.¹ This may be comparable to that observed in the current study when the DNIC-conditioning stimulus was given with the muscle withdrawal threshold stimulus which resulted in no analgesia in animals without tissue injury.

We propose that the injury produces changes in spinal and supraspinal sites that alter the effectiveness of DNIC. Our results show that DNIC analgesia is greater on the inflamed side when compared to the contralateral side. This finding was surprising since the analgesia produced by DNIC is systemic and affects all limbs. It may be that in areas of neuron sensitization after tissue injury, effects of DNIC are greater. Indeed opioid agonists delivered spinally or supraspinally are more effective in reducing dorsal horn neuron activity or hyperalgesia after

inflammation,^{26,51,52} suggesting enhanced activity in the opioid system.

The current study showed that DNIC analgesia was stronger in animals with inflammation when compared to uninjured animals. In contrast, human subjects with chronic pain show reduced analgesia to DNIC-conditioning stimuli.^{10,28,29,31,35,38,46,62} One possibility for the differences between human subjects with chronic pain and animals with tissue injury is the time from initial injury. In human subjects with rheumatoid arthritis the loss of DNIC analgesia occurs in those with long-standing disease (>5 years) but not those with newly diagnosed disease (<1 year).³⁵ In animals with cutaneous inflammation, there is enhanced descending facilitation at early time periods that is then masked at later time periods by enhanced descending inhibition.⁵⁴ In agreement, in animals with inflammation, DNIC analgesia was greater in the acute stage at a time when there is enhanced inhibition, but not in a more chronic stage.^{17,18} Thus, these data support the conclusion that there is a shift in the balance between uninjured and injured animals that depends on the time after injury.

Effect of Naloxone on DNIC

The current study shows that the effects of DNIC are not mediated by opioid receptors located in the RVM. This is in agreement with prior studies that show lesions of the RVM do not prevent the effects of DNIC.^{5,6} On the other hand, the current study shows, for the first time, that blockade of opioid receptors in the MdD prevents DNIC analgesia. Our data agree with prior studies that systemic activation of opioid receptors reduces C-fiber-evoked activity induced in MdD neurons,⁴ and lesions of the MdD prevent analgesia produced by low doses of naloxone.⁵⁶ Recent work shows expression of mu-opioid receptors in the MdD in

equivalent levels to that in the RVM, and nearly 100% of spinally projecting neurons express the mu-opioid receptors.^{39,40} Thus, we conclude nociceptive stimuli activate opioid receptors on MdD neurons that project to the spinal cord to reduce dorsal horn neuron activity and result in analgesia.

The current study shows that blockade of opioid receptors systemically reduces DNIC analgesia after inflammation. Surprisingly, however, the current study shows that only the DNIC-induced reduction in muscle hyperalgesia, but not cutaneous hyperalgesia, was prevented by systemic blockade of opioid receptors. One explanation is that different outcome measures have different sensitivity to systemic naloxone. Prior animal studies show a naloxone-reversible blockade of DNIC analgesia when recording activity of dorsal horn neurons in response to noxious electrical stimulation in uninjured animals.³² In healthy human subjects, naloxone blocked the increase in the spinal nociceptive reflex threshold produced by DNIC,⁶³ but had no effect on the increase in cutaneous heat pain produced by DNIC.²¹

Alternatively, it is possible that systemic naloxone enhanced the response to cutaneous stimuli, thus masking the effects of naloxone on DNIC. Early studies show a bidirectional effect of naloxone on nociception such that low doses produce hyperalgesia and higher doses produce analgesia.^{2,3,13} The dose of systemic naloxone used in the current study was the same dose that increased cutaneous hyperalgesia in arthritic rats.¹³ However, in our hands, this dose of naloxone had no effect on either muscle or cutaneous hyperalgesia. Opposite excitatory and inhibitory signals sent from different pools of neurons located in the spinal cord and the MdD could result in a lack of effect of naloxone on DNIC analgesia.

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Limitations

One limitation is the application of the DNIC-conditioning stimuli in the awake animal which could produce stress in the animal and thus result in stress-induced analgesia. To minimize this as a concern, we: 1) acclimated animals to the test stimuli; 2) used heat intensities lower than that previously used in animals; and 3) used heat intensities similar to that used in human subjects. Furthermore, stress-induced analgesia is regulated by opioids and involves the RVM.^{22,25} Our results show that naloxone microinjected into the RVM does not block the DNIC analgesia; thus, we do not believe that there is a stress-induced analgesia in the current study.

Another limitation of the behavioral study was our inability to test during the DNIC-conditioning stimuli. This was not possible as we had to restrain the animals during the DNIC in a separate holder to that used for the behavioral tests. However, we did test immediately after completion of the conditioning stimuli and were able to show changes at this time that likely reflect DNIC-analgesia.

Conclusion

In conclusion, this study showed that a greater intensity of noxious conditioning stimuli produces greater analgesia, and DNIC reduces both primary muscle and secondary cutaneous hyperalgesia during muscle inflammation. We further show that DNIC utilizes opioid receptors in the MdD, but not the RVM, to produce analgesia.

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