Gordon A. Barr
Estevan Limon
Richard A. Luthmann
Hunter College and the
Biopsychology Doctoral Program
The Graduate and University Center
City University of New York
New York, NY 10021
E-mail: gbarr@hunter.cuny.edu

Gordon A. Barr
Jianxin Cheng
Shaoning Wang
Division of Developmental Psychobiology
New York State Psychiatric Institute
New York, NY 10032

Analgesia Induced by Local Plantar Injections of Opiates in the Formalin Test in Infant Rats

ABSTRACT: *Morphine injected locally to the paw of an adult or an infant rat is analgesic. Opiates specific to* μ *and* κ *opioid receptors, and less consistently to* δ *opioid receptors, given locally to the site of injury in adult animals are also analgesic in a variety of models of inflammatory pain. To determine which opioid receptor(s) are involved in local analgesia in the immature animal, agonists specific for* μ, κ, *and* δ *opioid receptors were injected into the intraplantar pad in infant rats and the resultant nociceptive behavior and Fos expression assayed in the formalin test. The* κ *opioid receptor agonist U50,488 reduced nociceptive behavior in both phases of the formalin test and reduced Fos expression in the dorsal horn of the lumbar spinal cord, at 3 and 21 days of age. Morphiceptin (*μ *opioid agonist) was analgesic in the 21-day-old pups, but not the 3-day-old pups, measured behaviorally or by Fos expression. DPDPE (*δ *opioid agonist) was not analgesic at either age. We also tested the effects of opioid receptor antagonists on morphine's local analgesic action. Naltrexone, and to a lesser extent the* μ *opioid antagonist CTOP, antagonized morphine's analgesic effect. Kappa and* δ *opioid receptor blockers were inactive. The results demonstrate the ability of the* κ *opioid system to mediate analgesia in the neonate at the site of injury in acute and chronic pain models, that the* μ *opioid agonists are active later in development, but that morphine is analgesic in part through* μ *opioid receptors. © 2003 Wiley Periodicals, Inc.* Dev Psychobiol 42: 111–122, 2003.

Keywords: *analgesia; formalin test; ontogeny; local opiate; intraplantar; U50,488; morphiceptin; opioid antagonists*

Pain is a serious clinical problem for human neonates (Anand & Hickey, 1987) and painful procedures can and do cause localized inflammation (Bellanti, Pung, & Zeligs, 1994). The problem of how to provide safe and effective analgesia for human infants is difficult because opiate drugs act differently in the infant patient than they do in the adult patient (see for reviews Barr, 1995; Fitzgerald, 1995). Furthermore, the relationship of unwanted side effects to therapeutic effects changes with age (Anand, Clancy, & Group, 1994).

Peripherally administered opiates were reported to be analgesic at sites of pain almost 150 years ago (Wood, 1855). More recently, the peripheral antinociceptive properties of opiates in the adult animal have been described more fully. For example, systemically administered opiates, including morphine, codeine, levorphanol, ketocyclazocine, and the κ opioid agonist, U50,488H, are more effective as analgesics against acutely applied pain in inflamed tissue than in normal tissue (Hunskaar & Hole, 1987; Joris, Costello, Dubner, & Hargreaves, 1990; Russell, Jamieson, Callen, & Rance, 1985; Shibata, Ohkubo, Takahashi, Kudo, & Inoki, 1986a; Stein, Millan, Shippenberg, & Herz, 1988a; Stein, Millan, Shippenberg, Peter, & Herz, 1989; Stein & Yassouridis, 1997). Although these data demonstrate increased peripheral sensitivity to opiates due to inflammation, they do not

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rule out a central site of action, especially in light of central changes in opioid function induced by inflammation (Jiang & Gebhart, 1998; Ren, Blass, Zhou, & Dubner, 1997) Injection of opiates that do not readily cross the blood brain barrier or the local injection of small doses of agonists directly into the affected tissue, which are methods used to reduce central actions, also induce analgesia in a hyperalgesic inflamed paw (for detailed reviews see Barber & Gottschlich, 1992; Stein & Yassouridis, 1997). These drugs include the more general opiate agonists morphine, fentanyl, levorphanol, and ethylketocyclazocine (Ferreira & Nakamura, 1979; Joris, Dubner, & Hargreaves, 1987; Levine & Taiwo, 1986; Stein et al., 1989), the μ opioid peptide agonist DAMGO (Ferreira & Nakamura, 1979; Levine & Taiwo, 1989; Stein et al., 1989), δ opioid agonist DPDPE (Stein et al., 1989), κ opioid agonists U50,488H and tifluradam (Haley, Ketchum, & Dickenson, 1990; Stein, Gramsch, & Herz, 1990), and endogenous peptides met-enkephalin (Ferreira & Nakamura, 1979), β-endorphin (Stein et al., 1989), and dynorphin (Shibata Ohkubo, Takahashi, Kudo, & Inoki, 1986b). Lastly, local injection of small doses of the opiate antagonists CTAP, ICI 174,864, methylnaloxone or nor-BNI all reverse the actions of receptor specific agonists in the expected manner (Rios & Jacobs, 1983; Stein, Millan, Yassouridis, & Herz, 1988b; Stein et al., 1989; Takemori, Ikeda, & Portoghese, 1986). Thus, although the mechanisms by which these opiates work remain obscure, there is overwhelming evidence for a peripheral site of action of opiates in damaged tissue.

Whether or not opiates have similar antinociceptive properties in peripheral tissue, inflamed or not, in neonates is not known. Very few studies have examined the ontogeny of inflammation (Barr, 1998; Fitzgerald, 1995; Guy & Abbott, 1992; Jiang & Gebhart, 1998; Lidow, Song, & Ren, 2001; Ruda, Ling, Hohmann, Peng, & Tachibana, 2000; Teng & Abbott, 1998; Williams, Evan, & Hunt, 1990), and we know of only one study that examined the peripheral effects of opiates in young animals (Barr, 1999). There are no reasons to assume that changes in sensory thresholds are the same in neonates and adults nor is there any reason to assume that they have a similar developmental course. There are a number of developmental processes (e.g., development of receptors, perineurial barrier, and so forth) that would suggest that the peripheral analgesic and anti-inflammatory actions of opiates may differ from those of the adult animal (Bellanti et al., 1994; Kar & Quirion, 1995b; Kristensson & Olsson, 1971; O'Grady & Hall, 1992; Panigrahy, Sleeper, Assmann, Rava, White, & Kinney, 1998; Rahman, Dashwood, Fitzgerald, Aynsley-Green, & Dickenson, 1998; Toianen, Uksila, Leino, Lassila, Hirvonen, & Ruuskanen, 1981; van Rees, Dijkstra, & Sminia, 1990; Zhu, Hsu, & Pintar, 1998b). There are two implications of this immaturity. First, clinically, it is important to understand these differences if peripheral administration of opiates is to have broad clinical application. Second, by defining the physiological changes induced by inflammation at different ages, and comparing them to the maturation of peripheral antinociceptive drug action, it should be possible to clarify the peripheral mechanisms by which opiates are analgesic.

The local injection of morphine into the plantar surface of the paw in 3- to 21-day-old pups reduces the nociceptive response in the formalin test (Barr, 1999). In the present experiments, we determined if agonists for specific opioid receptors are effective in producing analgesia in the formalin test, measured both behaviorally and by suppression of c-fos expression in the spinal cord. The goal of the second experiment was to determine through which receptor population morphine acts to induce its local analgesic action.

METHODS

Subjects

Animals were the offspring of Long Evans hooded rats bred and tested at either Hunter College or New York State Psychiatric Institute. Adult animals were allowed free access to food and water. All rats were housed in plastic tubs measuring $40 \times 20 \times 24$ cm and the environmental temperature maintained at a constant $22 \pm 1°C$. Parent animals were fed Purina Lab Chow and water ad libitum. Cages were checked daily at approximately 10 AM and 6 PM. Pups found on that day at either time were termed 0 days of age. Following parturition, litters were culled to ten pups, without regard for the ratio of males to females. All doses of a drug were tested in a single litter. In experiment 1, seven to nine pups were tested at each age and drug dose for the behavioral experiments; four to six pups for each age and drug dose were used for the Fos immunocytochemical studies. In experiment 2, 12 pups were used for each condition. Research protocols were approved by the Institutional Animal Care and Use Committee at both sites and were conducted under the ethical guidelines of the International Association for the Study of Pain, the Society for Neuroscience and the Society for Developmental Psychobiology.

Formalin Test

Pups were injected with 2% formalin (10 μl) into the plantar pad (intraplantar; i.p.l.). Saline-treated controls were not tested because they do not produce any nociceptive responses. The same volume was used for both ages. Although some studies have used increased volumes for older ages because of increase in paw size, there is no clear method to determine the appropriate injection volume at any given age. Furthermore, an increased volume of formalin in older rat pups produces increased Fos expression, suggesting increased noxious input (Yi & Barr, 1995). Furthermore, we were not interested in quantitative

Table 1. Behavioral Rating Scale

Behavior	Score
No response different from untreated animals	0
Favoring: reducing the weight on the paw which remains in contact with the floor	1
Lifting: the paw is raised off the floor	2
Shaking: the paw is lifted and vigorously shaken	3
Licking/biting: the treated paw is licked or nibbled	4

Note. Pups were assigned a number based on the behavior in which they were engaged at the time of observation. The ''paw'' is the injected hindpaw. Each score was mutually exclusive.

comparisons across ages, but rather in the effectiveness of local opiates at each age. Behavior was scan sampled (Martin & Bateson, 1993) every minute for 60 min. Behavior was rated on a five-point scale as adapted from Dubuisson & Dennis (1977) and modified as described previously (Barr, 1999; King, Cheng, Wang, & Barr, 2000). The behaviors recorded are given in Table 1. We have recently described the response of rat pups 7 to 21 days of age to a variety of formalin concentrations and found a highly significant correlations between the formalin concentration and these composite scores ($r^2 = 0.87$ to .99 for 7 to 21-day-old pups; (Watson, Sufka, & Barr, 1999; unpublished data). The formalin test in older pups and adults consists of two phases (Barr, 1998; Dubuisson & Dennis, 1977; Guy & Abbott, 1992; Teng & Abbott, 1998). The first phase is an acute pain response lasting less than 5 min and which is followed by a decline in responding; the second phase occurs about 10–15 min after the formalin injection. In the infant, the interphase and second phase are less apparent before 14 days of age (Barr, 1998; Guy & Abbott, 1992; Teng & Abbott, 1998). This latter phase is thought to represent sensitization of the spinal cord and primary afferents.

Drug Injection

All drugs were injected into the plantar surface of the hindpaw (i.p.l.) or subcutaneously into the back (s.c.) in the same volume as the formalin. The latter control was for systemic effects of the drugs that might occur from diffusion of drug from skin to general circulation. The schedule of injections relative to the formalin treatment is given in the description of the experiments.

EXPERIMENT 1

In the adult animal, drugs that target specific opioid receptors in skin are effective analgesics. Significant maturation of opioid receptors occurs postnatally in the central nervous system (Attali, Saya, & Vogel, 1990; Kar & Quirion, 1995a; Rahman et al., 1998; Zhu, Hsu, & Pintar, 1998a), but the development of these receptors in the periphery has not been described. Thus, it is not known whether or not agonists for μ, δ, or κ receptors would be analgesic following local injection in the infant. In this experiment, we injected agonists to each receptor type and assessed the behavioral and cellular responses to intraplantar injection of formalin at 3 and 21 days of age.

Drugs

The agonists used were U50,488 to target κ opioid receptors, morphiceptin, or DAMGO to target μ opioid receptors and DPDPE to target δ opioid receptors. Each

Table 2. Doses for Local (i.p.l.) and Systemic (s.c) Administration

	3 Day Old		21 Day Old	
Agonist	*Dose i.p.l.*	*Dose s.c.*	*Dose i.p.l.*	*Dose s.c.*
Experiment 1. Behavior				
DPDPE	2.0, 10.0, 50.0 μg (0.2–5.0 mg/kg)	10.0, 50.0 μg (1.0–5.0 mg/kg)	0.4, 2.0, 10.0, 50.0 μg (0.008–1.0 mg/kg)	50.0 μg (1.0 mg/kg)
Morphiceptin	0.024, 0.12, 0.60 μg (2.4–60 μg/kg)	0.12, .60 μg (12.0–60.0 μg/kg)	0.024, 0.12, 0.6, 3.0 μg (0.48–60 μg/kg)	3.0 μg (60 μg/kg)
U50488	0.4, 2.0, 10.0 μg (0.04, 0.20, 1.00 mg/kg)	2.0, 10.0 μg (2.0–10.0 mg/kg)	0.4, 2.0, 10.0 μg (8–200.0 μg/kg)	2.0, 10.0 μg (40–200 μg/kg)
Experiment 1. Fos				
DPDPE	10.0, 50.0 μg (1.0–5.0 mg/kg)	10.0, 50.0 μg (1.0–5.0 mg/kg)	50.0 μg (1.0 mg/kg)	50.0 μg (1.0 mg/kg)
Morphiceptin	0.12, 0.60 μg (12–60 μg/kg)	12.0, 0.60 μg (12–60 μg/kg)	3.0 μg (60 μg/kg)	3.0 μg (60 μg/kg)
U50488	2.0, 10.0 μg (2–10.0 mg/kg)	2.0, 10.0 μg (2–10.0 mg/kg)	10.0 μg (200 μg/kg)	10.0 μg (200 μg/kg)

Note. All doses for i.p.l. and s.c. injection are in μg. In parentheses below, for each drug is the average dose range per kg at both ages. This latter calculation is based on an average weight of 10 g for the 3-day-old pups and 50 g for the 21-day-old pups.

drug was injected into the paw 5 min before the formalin injection. A range of doses was injected locally into the plantar paw to target receptors in skin. Controls for systemic drug effects were typically s.c. injection of the highest one or two i.p.l. doses. Doses were not modified by the body weight of the animal. Thus, the maximum dose expressed in mg/kg was higher for the 3-day-old pups than for the 21-day-old pups. The doses as injected are given and also converted to mg/kg in Table 2. Note that the doses, in mg/kg, overlap between ages and are relevant for the systemic spread of the drug since body weight differences are less importance in the local effects of the drug. The behavioral observations were conducted blind to the dose of the drug.

Immunohistochemistry

After testing, a subset of pups were deeply anesthetized with sodium pentobarbital and perfused transcardially with 0.1 M PBS, pH 7.2, followed by 4% buffered paraformaldehyde, pH 7.2. Tissue was cryoprotected with sucrose (30%), and frozen sections of the lumbar enlargement (L4, L5) of the spinal cord were cut at 30 μm. Immunocytochemistry was conducted simultaneously on tissue from rats of different ages and experimental conditions in order to control for subtle differences that may have occurred between assays, although in previous work we have analyzed assay to assay variability and found little (Yi & Barr, 1995). The primary antibody was rabbit-anti-Fos (Ab-5; Oncogene Sciences, Manhasset, NY) diluted 1:2,000 in PBS with 2% BSA. We used the ABC method (Hsu, Raine, & Fanger, 1981) as described in the Vectastain kit except that the tissue was incubated in the primary antibody overnight at room temperature on a shaker. Diaminobenzadine was the chromagen. For each animal, 5–10 sections were selected and counted as described previously (Yi & Barr, 1995). The counts were conducted blind to the drug, dose, and route of administration.

Statistics

Data from the minute by minute observations were averaged into 3 min bins. These data were analyzed by factorial ANOVAs. Each age was analyzed separately. Two main effects, dose and bins, and the interaction of those two main effects were analyzed. The doses were all tested within a single litter at any given age, and therefore drug dose, bins, and the interaction were analyzed by a within subjects design. For the Fos data, factorial ANOVAs were run at each age. The factors were the dose or the drug and the region of the spinal cord assayed; however for simplicity sake, only changes in total numbers of labeled cells are discussed.

Results and Discussion

κ Opioid receptor agonist

The specific κ opioid agonist, U50,488H, was the most effective treatment in reducing the behavioral response to formalin (Figure 1). At 3 days of age, both the medium and high dose administered to the paw were analgesic. The high dose of the subcutaneous control was also analgesic, reducing the response to a degree similar to that of the intraplantar treatment. The high potency of opiates in the infant has been reported before in the formalin test and is likely due to both pharmacodynamic factors and pharmacokinetic differences (Abbott, Franklin, & Westbrook, 1995). Although the highest s.c. dose was also analgesic at

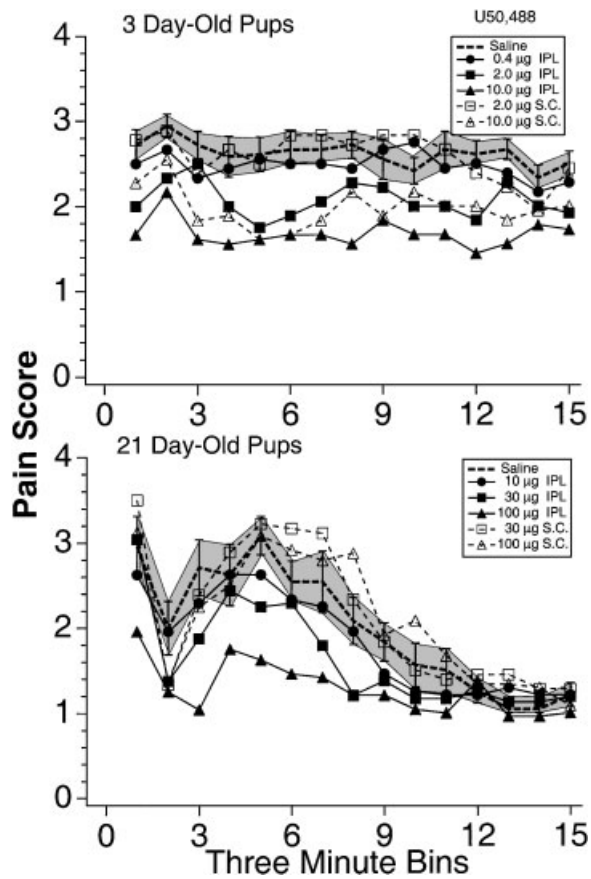

FIGURE 1 The effect of U50,488 on the behavioral response to formalin over the 45 min test period. U50,488 was given 15 min before formalin (2%; 10 μl). Presented are means and standard errors of 3-min bins for the saline controls (shaded gray) and means without error bars for the drug conditions. The two higher doses were analgesic across the entire test session, and the 100-μg dose reduced responding in both the early and late phases at 21 days of age. Note the lack of a biphasic response pattern at 3 days of age.

3 days of age, the middle intraplantar dose was selectively more effective than the comparable s.c. dose. A similar result was found using morphine at 3 days of age (Barr, 1999). Note that the κ opioid drug was analgesic at all time points. Thus, the κ opioid system is functional in the periphery as early as 3 days of age.

At 21 days of age, U50,488H was highly potent and site specific. All three intraplantar doses significantly reduced nociceptive responding, whereas no s.c. dose did. At 21 days of age, the major effects were in the second phase, although the highest dose given i.p.l. also reduced responding in the first bin. Note that the potency of U50,488 at both ages was approximately the same when equated on a mg/kg basis.

Fos expression in the spinal cord was also reduced in pups treated locally with U50,488 (Figures 2 and 3). At 3 days of age, the total number of Fos labeled cells was decreased at both doses given locally. There was a slight reduction in the number of labeled cells at the higher s.c. dose, but the reduction was significantly less than that of the comparable dose given locally. As has been reported before, the majority of Fos-labeled cells induced by formalin were in the medial superficial dorsal horn (Yi & Barr, 1995). At 21 days of age, there was again

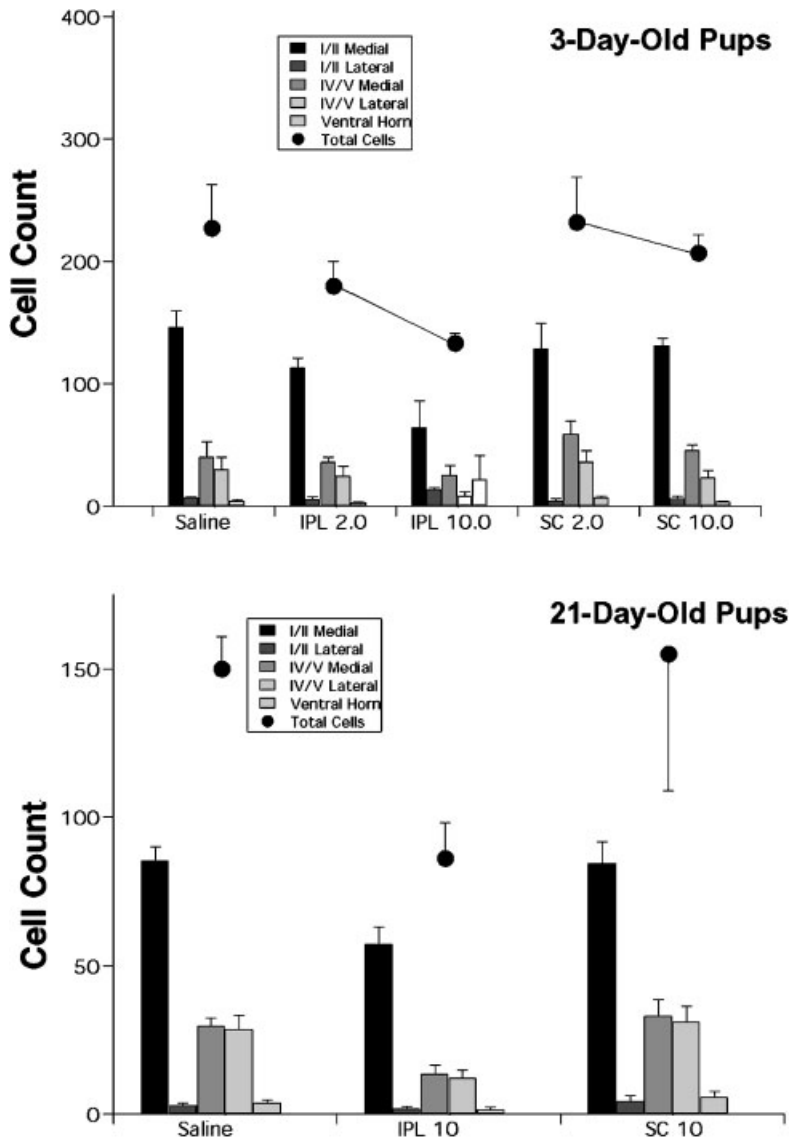

FIGURE 2 The effects of U50,488 on Fos-like immunoreactivity in the lumbar enlargement unilateral to the injection site of formalin. The bars correspond roughly to Rexed laminar as labeled. The circles represent the total number of cells in the section on the side unilateral to treatment. At 3 days of age, Fos expression was significantly reduced at both doses given locally and at the higher dose given s.c. At 21 days of age, only the i.p.l. dose significantly reduced FOS expression.

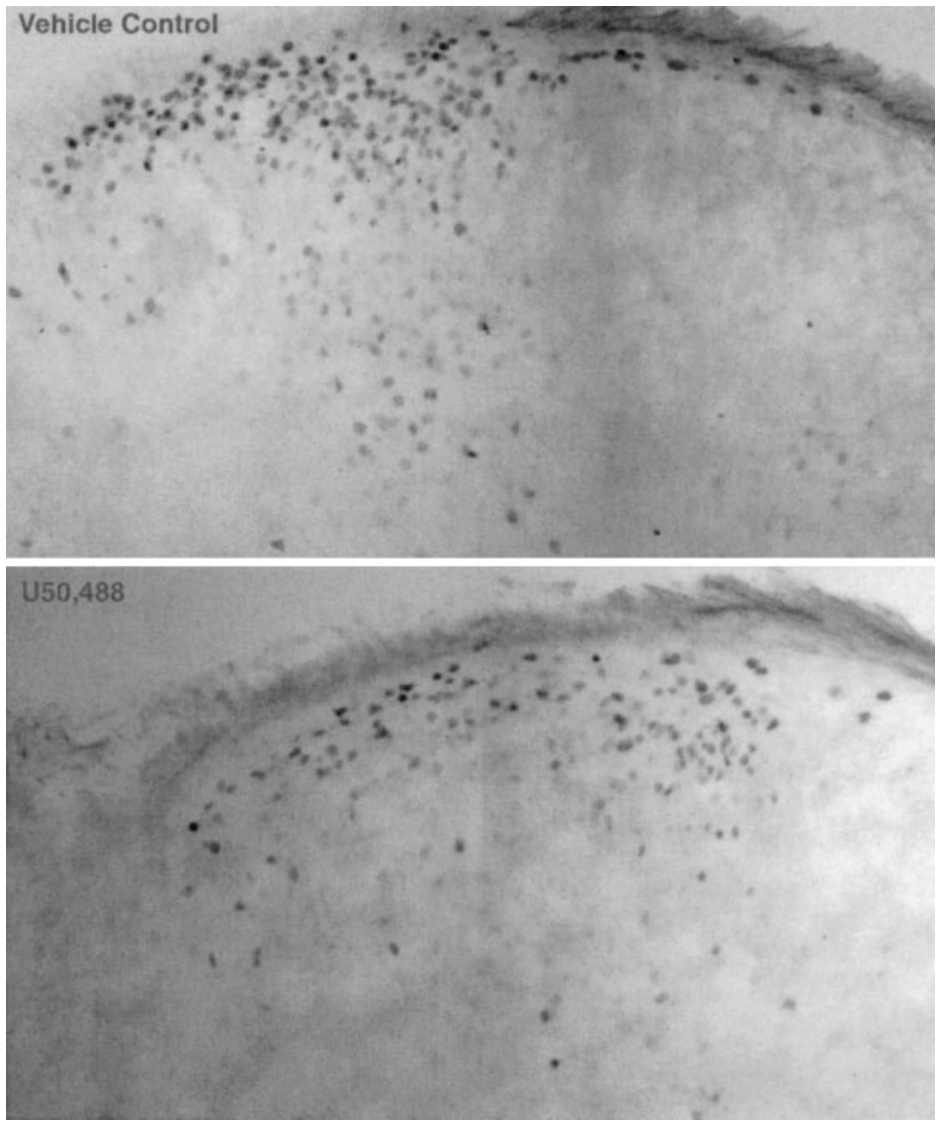

FIGURE 3 Photomontage of Fos expression in Control (**top**) and U50,488 (**bottom**) treated 3-day-old pups. Both are from the right dorsal horn and the pups were given formalin as described. Note the decreased number of labeled cells in the U50,488-treated animals.

a significant decrease in the number of Fos labeled cells following i.p.l. injection compared to the s.c. or to saline controls.

μ Opioid Receptor Agonists

At 3 days of age, the μ opioid receptor agonist morphiceptin was ineffective. Morphiceptin given i.p.l. was not different from saline or drug given subcutaneously (Figure 4). (DAMGO similarly was not analgesic at this age; data not shown.) However, at 21 days of age, the two highest doses of morphiceptin produced significant analgesia compared to saline or to a comparable high dose given s.c. The major effect was a reduction of responding in the second phase, an effect parallel to that of local morphine (Barr, 1999). Subcutaneous injections of morphiceptin were ineffective at either age.

There were no significant effects of morphiceptin on Fos expression injected either s.c. or i.p.l. in 3-day-old pups, in accordance with the behavioral data (Figure 5). At 21 days of age, there was a significant reduction of total Fos positive cells with the local injection (3.0 μg), corroborating the behavioral data.

δ Opioid Receptor Agonist

DPDPE was ineffective at either age (Figure 6). No dose was more effective than saline or the s.c. controls. However, at 21 days of age, the formalin scores following

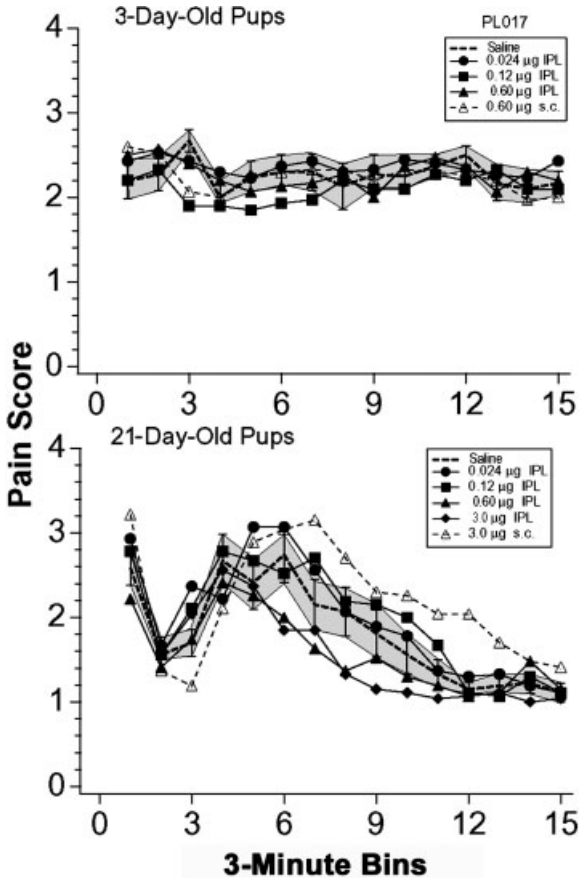

FIGURE 4 The effect of morphiceptin on the behavioral response to formalin over the 45 min test period. Details are as in Figure 1. Morphiceptin was ineffective at 3 days of age but reduces responding at 21 days of age at the two higher doses.

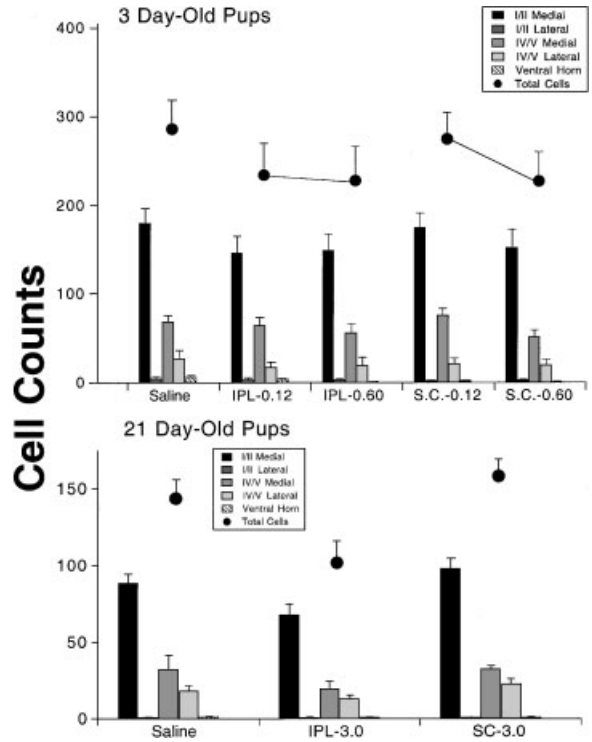

FIGURE 5 Effects of morphiceptin on Fos-like immunoreactivity in the lumbar enlargement ipsilateral to the injection site of formalin. The bars correspond roughly to Rexed laminar as labeled. Details are as in Figure 2. At 3 days of age, fos expression was not significantly reduced at any doses or route of administration. At 21 days of age, only the i.p.l. dose significantly reduced fos expression.

the s.c. dose of DPDPE were significantly higher than were those of the comparable i.p.l. dose or the saline control. There were no differences in Fos expression in any i.p.l. treatment condition at either age (Figure 7).

EXPERIMENT 2

Morphine is an effective analgesic drug given i.p.l in the formalin test (Barr, 1999). In the adult morphine acts largely if not exclusively through μ opioid receptors but because there are no data on the development of opioid receptors in peripheral tissue, there is no reason to assume that morphine acts similarly in the infant. This study therefore used antagonists to μ, δ, or κ opioid receptors to determine the receptor type targeted by local morphine injection in the formalin test of 10-day-old pups.

Subjects

Ten-day-old pups were used in this experiment because they show a robust analgesic response to morphine given i.p.l. without any corresponding analgesia induced by the same dose given s.c. (Barr, 1999). Thus testing 3-day-old pups would be complicated by the systemic effects. Three-day-old pups show equivalent analgesia to 10-day-old pups when morphine is given locally but are more sensitive to the s.c. route. Because it is difficult to demonstrate a specific local action of morphine in younger pups, these experiments used older animals. Pups were bred and treated as described for Experiment 1.

Drug Treatment

Pups were injected with saline (all volumes were 10 μl) or one of four opioid receptor antagonists directly into the plantar pad of the hindpaw before the administration of morphine. The antagonists were: naltrindole (δ blocker; 6.25 μg/injection; equal to 250 μg/kg), nor-BNI (κ blocker; 6.25 μg/injection; 250 μg/kg), CTOP (μ blockers; 0.5 μg; 20 μg/kg), or naltrexone (non-specific antagonist; 6.25 μg; 250 μg/kg). Morphine (3.0 μg/injection; 120 μg/kg) or saline was injected 15 min later.

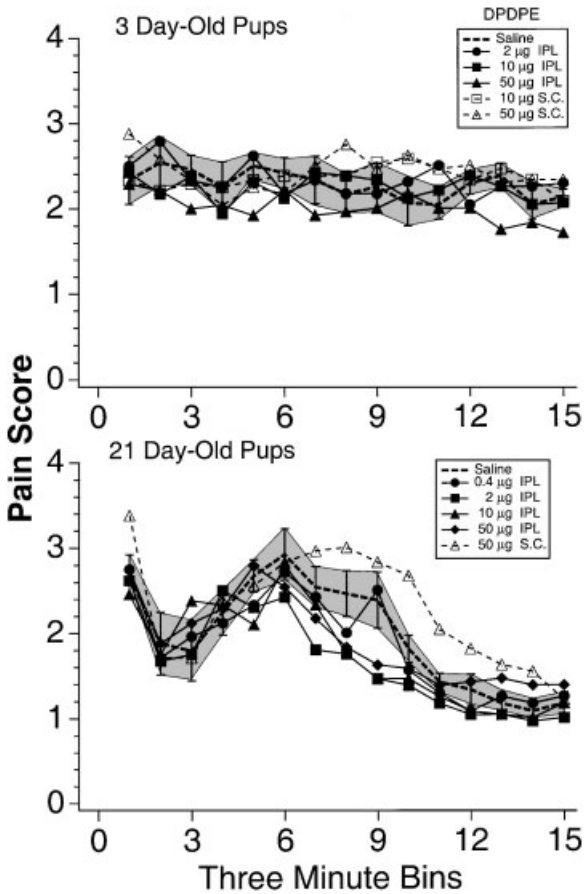

FIGURE 6 The effect of DPDPE on the behavioral response to formalin over the 45-min test period. Details are as in Figure 1. DPDPE was ineffective at either age for both routes of administration.

Pups were tested in the formalin test 5 min later as described above.

Results

None of the antagonists had any effect on the pain responses when they were injected with saline instead of morphine (data not shown). CTOP and naltrexone altered morphine-induced analgesia (Figure 8). Naltrexone fully reversed morphine's analgesic actions, resulting in responses identical to that of pups not given morphine (saline-saline group) and responses significantly different from pups given saline and morphine. CTOP was less effective. Indeed pups given CTOP-morphine were not statistically different from pups given naltrexone-morphine, or saline-saline, but also did not differ from pups given saline before the morphine injection. Thus, there is reversal of morphine's effects by CTOP, but unlike naltrexone, the analgesia was not fully blocked.

Discussion

In the adult animal, local injections of opiates are analgesic in a variety of pain paradigms. Intraplantar injection of morphine is likewise analgesic as early as 3 days of age. Here we found that drugs that target either the μ or κ opioid receptor are analgesic, although the μ preferring drugs were not effective in the 3-day-old pup. Indeed, U50,488 was as effective as any centrally administered drug in the infant. The δ opioid specific peptide, DPDPE, was ineffective at either age. Morphine's analgesic actions are likely mediated through the μ opioid receptor since only CTOP and naltrexone treatment reduced morphine's analgesic actions.

There are several caveats that qualify the above results. First, we did not test antagonists that target different subtypes of the opioid receptor. Thus, it is possible that morphine's effects are mediated by actions at a subtype of the κ or δ opioid receptor. For example, it is possible that an agonist that targeted the δ_2 receptor subtype (e.g., deltorphin II) would have been analgesic despite the ineffectiveness of DPDPE. We think this unlikely however since available data suggest that morphine produces its analgesic action through a μ opioid site in the adult. That CTOP did not fully antagonize morphine's analgesic action at 10 days of age is possibly due to an inadequate dose to fully block the μ opioid receptor, or to a half life shorter than that required to be fully active throughout the entire test session, or to actions of morphine not mediated by the μ opioid receptor.

U50,488 was the most effective analgesic. This is consistent with the adult data that show that κ opioids are effective locally in the formalin test measured electrophysiologically or behaviorally (Haley et al., 1990). Of note, U50,488, unlike other opioids given locally, reduced both the acute pain response (first phase) and the later second phase thought to represent peripheral and central sensitization (Hong & Abbott, 1995; Nozaki-Taguchi & Yamamoto, 1998). Most opioid receptor agonists reduce the second phase preferentially. We do not know in this case whether the reduction in the second phase is an independent effect on neural processes that mediate the late phase, or is secondary to the reduced magnitude of the early phase.

The relative lack of effectiveness at 3 days of age of both morphiceptin and DAMGO was surprising. In particular the ineffectiveness of these drugs at 3 days of age was unexpected in light of the prior data showing that morphine was an effective analgesic at that age and that it likely acts through a μ opioid receptor. These drugs were effective analgesics at 21 days of age. In fact, we would have predicted that these drugs would have been more potent in the neonate. Locally administered opiates are most effective in inflamed tissue. One of the

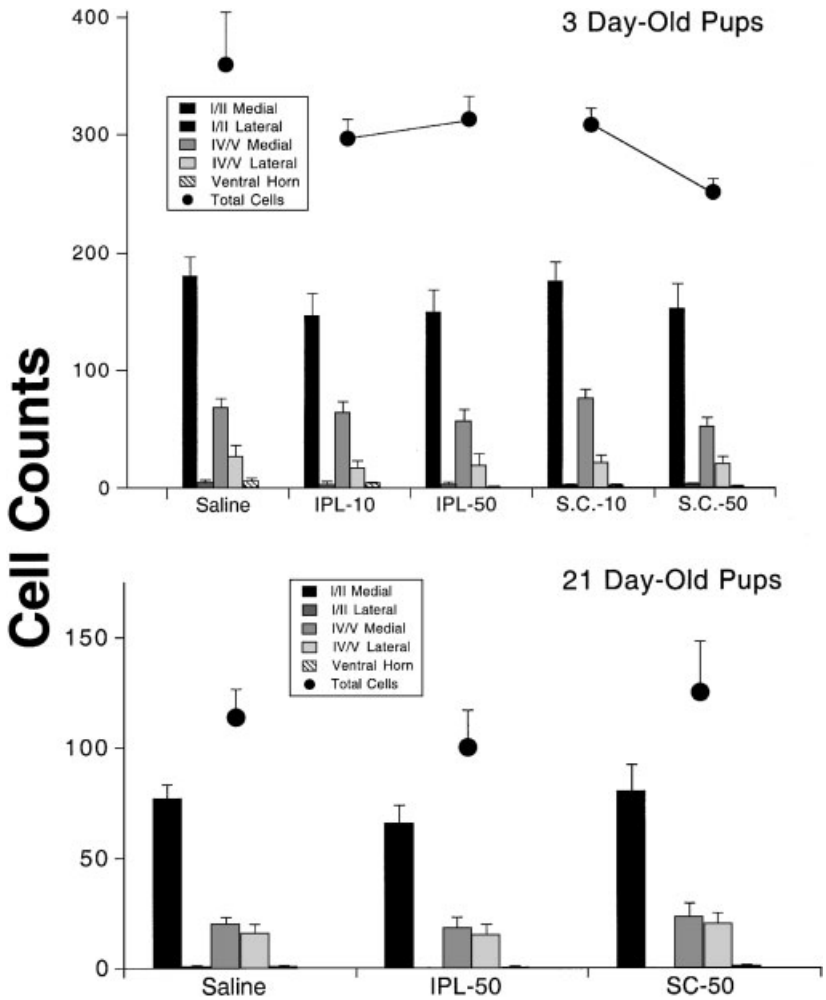

FIGURE 7 Effects of DPDPE on Fos-like immunoreactivity in the lumbar enlargement ipsilateral to the injection site of formalin. The bars correspond roughly to Rexed laminar as labeled. Details are as in Figure 2. DPDPE had no significant effect on Fos expression.

consequences of inflammation is disruption of the perineurial barrier that blocks access of opiates to peripheral nerves (e.g., Stein & Yassouridis, 1997). In these experiments, inflammation was not induced prior to treatment with opiates, but we have argued that because the perineurial barrier matures slowly postnatally, opiates should be effective in the neonate in the absence of inflammation (Barr, 1999). This seems to be the case at least for morphine and U50,488.

There are several possibilities for why the μ opioids were ineffective at the younger age. First, because we tested agonists in pups as young as 3 days and the antagonist studies were done at 10 days of age, it is possible that μ opioid receptors in the periphery mature between 3 and 10 days of age. If true, this would be a different developmental trajectory from μ opioid receptor development in the central nervous system (Kar & Quirion, 1995b; Rahman et al., 1998; Zhu et al., 1998b). We know of no data that address this issue. Second, higher doses of the μ opioid agonists might have been effective by local injection but then they likely would also have been analgesic following s.c. injection, since the infant appears very sensitive to systemic opiates. Third, it is possible that DAMGO and morphiceptin were ineffective because they were peptides and less stable in skin than morphine or because their effectiveness was reduced by the subsequent injection of formalin. This would not, however, explain the effectiveness of morphiceptin in the 21-day-old pups.

The data on δ opioid agonists in adults are not consistent. In the formalin test, the δ opioid agonist DSTBUTLET was ineffective (Haley et al., 1990) but DPDPE was analgesic (Nozaki-Taguchi & Yamamoto, 1998). In other pain models, the results with δ opioid agonists are equally unclear. In the prostaglandin test, DPDPE and DSLET were inactive (Levine & Taiwo,

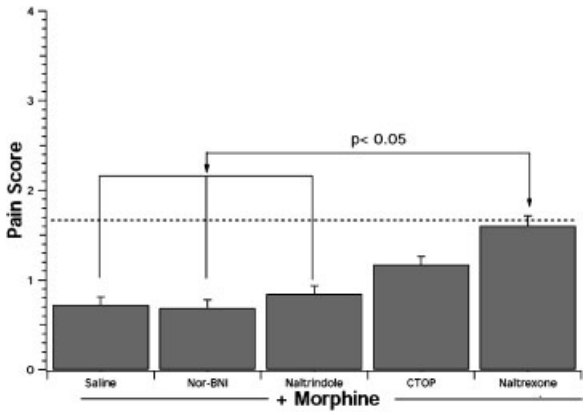

FIGURE 8 Effects of various opioid receptor antagonists on morphine induced analgesia at 10 days of age. Morphine and the antagonists were given i.p.l. followed by formalin. The mean scores summed over 45 min are provided. The dashed line indicates basal response levels to formalin. The degree of morphine induced analgesia is depicted by the saline pretreatment group. CTOP partially but incompletely reversed morphine's analgesic effects. Note that neither nor-BNI nor naltrindole had any effect on morphine analgesia. The antagonists by themselves, without morphine, had no effect on the response to formalin (data not shown).

1989); however DPDPE was effective in CFA-induced hyperalgesia (Stein et al., 1989). δ Opioid receptors develop late in the central nervous system (McDowell & Kitchen, 1986; Rahman et al., 1998; Zhu, et al., 1998b) and their development in skin is not known. The possible late development of the δ opioid receptor could account for the lack of analgesic effect of DPDPE.

In summary, the κ opioid receptor agonist U50,488 was a very effective agonist at local sites of nociception. It had the unique property of reducing pain in the first and second phase in the formalin test. Thus, drugs acting similarly might be clinically useful where concern in infants is for both acute painful procedures and long-term changes due to pain and inflammation. In contrast, the μ and δ opioid agonists were ineffective at the youngest ages.

NOTES

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REFERENCES

Abbott, F. V., Franklin, K. B. J., & Westbrook, R. F. (1995). The formalin test: scoring properties of the first and second phases of the pain response in rats. Pain, 60, 91–102.

Anand, K. J. S., Clancy, G. T., & Group, S. S. (1994). Creating a prospective database for neonatal analgesic practices. Pediatric Research, 35, 212A.

Anand, K. J. S., & Hickey, P. R. (1987). Pain and its effects in the human neonate and fetus. New England Journal of Medicine, 317, 1321–1329.

Attali, B., Saya, D., & Vogel, Z. (1990). Pre- and postnatal development of opiate receptor subtypes in rat spinal cord. Developmental Brain Research, 53, 97–102.

Barber, A., & Gottschlich, R. (1992). Opioid agonists and antagonists: an evaluation of their peripheral actions in inflammation. Medical Research Review, 12, 525–562.

Barr, G. A. (1995). Ontogeny of nociception and antinociception. In P. V. Thadani (Ed.), Biological mechanisms and perinatal exposure to drugs. National Institute of Drug Abuse 158, 172–201.

Barr, G. A. (1998). Maturation of the biphasic behavioral and heart rate response in the formalin test. Pharmacological Biochemical Behavior, 60, 329–335.

Barr, G. A. (1999). Antinociceptive effects of locally administered morphine in infant rats. Pain, 81, 155–161.

Bellanti, J. A., Pung, Y.-H., & Zeligs, B. J. (1994). Immunology. In G. B. Avery M. A. Fletcher, & M. G. MacDonald (Eds.), Neonatology: pathophysiology and management of the newborn (pp. 1000–1027) New York: J.B. Lippincott Co.

Dubuisson, D., & Dennis, S. G. (1977). The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain, 4, 161–174.

Ferreira, S., & Nakamura, M. (1979). Prostaglandin hyperalgesia. ii. The peripheral analgesic activity of morphine, enkephalins and opioid antagonists. Prostaglanden, 18, 191–201.

Fitzgerald, M. (1995). Developmental biology of inflammatory pain. British Journal of Anaesthesia, 75, 177–185.

Guy, E. R., & Abbott, F. V. (1992). The behavioral response to formalin in preweanling rats. Pain, 51, 81–90.

Haley, J. E., Ketchum, S., & Dickenson, A. H. (1990). Peripheral k-opioid modulation of the formalin response: an electrophysiological study in the rat. European Journal of Pharmacology, 191, 437–446.

Hong, Y. G., & Abbott, F. V. (1995). Peripheral opioid modulation of pain and inflammation in the formalin test. European Journal of Pharmacology, 277, 21–28.

Hsu, S., Raine, L., & Fanger, H. (1981). Use of avidin-biotin-peroxidase complex (abc) in immunoperoxidase techniques: a comparison between abc and unlabeled antibody (pap) procedures. Journal of Histochemistry Cytochemistry, 29, 577–580.

Hunskaar, S., & Hole, K. (1987). The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain, 30, 103–114.

Jiang, M. C., & Gebhart, G. F. (1998). Development of mustard oil-induced hyperalgesia in rats. Pain, 77, 305–313.

Joris, J., Costello, A., Dubner, R., & Hargreaves, K. (1990). Opiates suppress carrageenan-induced edema and hyper-

thermia at doses that inhibit hyperalgesia. Pain, 43, 95–101.

Joris, J., Dubner, R., & Hargreaves, K. (1987). Opioid analgesia at peripheral sites: a target for opioids released during stress and inflammation? Anesthesia Analgesia, 66, 1277–1281.

Kar, S., & Quirion, R. (1995a). Neuropeptide receptors in developing and adult rat spinal cord: an in vitro quantitative autoradiography study of calcitonin gene-related peptide, neurokinins, mu-opioid, galanin, somatostatin, neurotensin and vasoactive intestinal polypeptide receptors. Journal of Comparative Neurology, 354, 253–281.

Kar, S., & Quirion, R. (1995b). Neuropeptide receptors in developing and adult rat spinal cord: an in vitro quantitative autoradiography study of calcitonin gene-related peptide, neurokinins, mu-opioid, galanin, somatostatin, neurotensin and vasoactive intestinal polypeptide receptors. Journal of Comparative Neurology, 354, 253–281.

King, T. E., Cheng, J., Wang, S., & Barr, G. A. (2000). Maturation of NK1 receptor involvement in the nociceptive response to formalin. Synapse, 36, 254–266.

Kristensson, K., & Olsson, Y. (1971). The perineurium as a diffusion barrier to protein tracers. Differences between mature and immature animals. Acta Neuropathology, 17, 127–138.

Levine, J. D., & Taiwo, Y. O. (1986). Hyperalgesic properties of 15-lipoxygenase products of arachidonic acid. PNAS, 83, 5331–5334.

Levine, J. D., & Taiwo, Y. O. (1989). Involvement of the mu-opiate receptor in peripheral analgesia. Neuroscience, 32, 571–575.

Lidow, M. S., Song, Z. M., & Ren, K. (2001). Long-term effects of short-lasting early local inflammatory insult. Neuroreport, 12, 399–403.

Martin, P., & Bateson, P. (1993). Measuring behaviour. Cambridge, UK: Cambridge University Press.

McDowell, J., & Kitchen, I. (1986). Ontogenesis of d-opioid receptors in rat brain using [3h]-[d-pen2, d-pen5]enkephalin as a binding ligand. European Journal of Pharmacology, 128, 287–289.

Nozaki-Taguchi, N., & Yamamoto, T. (1998). Involvement of nitric oxide in peripheral antinociception mediated by kappa- and delta-opioid receptors. Anesthesia Analgesia, 87, 388–393.

O'Grady, M. P., & Hall, N. R. S. (1992). Interactions between the developing immune and neuroendocrine systems. In H. N. Shair, G. A. Barr, & M. A. Hofer (Eds.), Development psychobiology: new methods and changing concepts, (pp. 223–238), New York: Oxford.

Panigrahy, A., Sleeper, L. A., Assmann, S., Rava, L. A., White, W. F., & Kinney, H. C. (1998). Developmental changes in heterogeneous patterns of neurotransmitter receptor binding in the human interpeduncular nucleus. Journal of Comparative Neurology, 390, 322–332.

Rahman, W., Dashwood, M. R., Fitzgerald, M., Aynsley-Green, A., & Dickenson, A. H. (1998). Postnatal development of multiple opioid receptors in the spinal cord and development of spinal morphine analgesia. Brain Res Dev Brain Research, 108, 239–254.

Ren, K., Blass, E. M., Zhou, Q. Q., & Dubner, R. (1997). Suckling and sucrose ingestion suppress persistent hyperalgesia and spinal fos expression after forepaw inflammation in infant rats. Proceedings of the National Academy of Sciences of the United States of America, 94, 1471–1475.

Rios, L., & Jacobs, J. (1983). Local inhibition of inflammatory pain by naloxone and its n-methyl quaternary analog. European Journal of Pharmacology, 96, 277–283.

Ruda, M. A., Ling, Q. D., Hohmann, A. G., Peng, Y. B., & Tachibana, T. (2000). Altered nociceptive neuronal circuits after neonatal peripheral inflammation. Science, 289, 628–631.

Russell, N. J. W., Jamieson, A., Callen, T. S., & Rance, M. J. (1985). Peripheral opioid effects on neurogenic plasma extravasation. British Journal of Pharmacology, 84, 45.

Shibata, M., Ohkubo, T., Takahashi, H., Kudo, T., & Inoki, R. (1986a). Peripheral analgesic actions of opioid peptides and morphine analogues [Japanese]. Folia Pharmacology Japonica, 88, 101–107.

Shibata, M., Ohkubo, T., Takahashi, H., Kudo, T., & Inoki, R. (1986b). [peripheral analgesic actions of opioid peptides and morphine analogues]. Nippon Yakurigaku Zasshi, 88, 101–107.

Stein, C., Gramsch, C., & Herz, A. (1990). Intrinsic mechanisms of antinociception in inflammation: Local opioid receptors and β-endorphin. Journal of Neuroscience, 10, 1292–1298.

Stein, C., Millan, M., Shippenberg, T., & Herz, A. (1988a). Peripheral effects of fentanyl upon nociception in inflamed tissue of the rat. NL, 84, 225–228.

Stein, C., Millan, M., Yassouridiss, A., & Herz, A. (1988b). Antinociceptive effects of mu and kappa agonists in inflammation are enhanced by a peripheral opioid receptor-specific mechanism. European Journal of Pharmacology, 155, 255–264.

Stein, C., Millan, M. J., Shippenberg, T. S., Peter, K., & Herz, A. (1989). Peripheral opioid receptors mediating antinociception in inflammation: evidence for involvement of mu, delta and kappa receptors. Journal of Pharmacology and Experimental Therapeutics, 248, 1269–1275.

Stein, C., & Yassouridis, A. (1997). Peripheral morphine analgesia. Pain, 71, 119–121.

Takemori, A. E., Ikeda, M., & Portoghese, P. S. (1986). The u, k and d properties of various opioid agonists. European Journal of Pharmacology, 123, 357–361.

Teng, C. J., & Abbott, F. V. (1998). The formalin test: a dose-response analysis at three developmental stages. Pain, 76, 337–347.

Toianen, P., Uksila, J., Leino, A., Lassila, L., Hirvonen, T., & Ruuskanen, O. f. (1981). Development of themitogen responding tc ells and natural killer cells in the human fetus. Immunological Reviews, 57, 89–105.

van Rees, E. P., Dijkstra, C. D., & Sminia, T. (1990). Ontogeny of the rat immune system: an immunohistochemical approach. Develop. Comparative Immunology, 14, 9–18.

Watson, G. S., Sufka, K. J., & Barr, G. A. (1999). The behavioral response of the preweaning rat in the formalin test. Society of Neuroscience Abstracts, 25, 1681.

Williams, S., Evan, G. I.. & Hunt, S. P. (1990). Spinal c-fos induction by sensory stimulation in neonatal rats. Neuroscience Letters, 109, 309–314.

Wood, A. (1855). New method of treating neuralgia by the direct application of opioids to the painful points. Edinburgh Medical Surgery Journal, 82, 265–281.

Yi, D. K., & Barr, G. A. (1995). The induction of fos-like immunoreactivity by noxious thermal, mechanical and chemical stimuli in the lumbar spinal cord of infant rats. Pain, 60, 257–265.

Zhu, Y., Hsu, M. S., & Pintar, J. E. (1998a). Developmental expression of the mu, kappa, and delta opioid receptor mrnas in mouse. Journal of Neuroscience, 18, 2538–2549.

Zhu, Y. X., Hsu, M. S., & Pintar, J. E. (1998b). Developmental expression of the mu, kappa, and delta opioid receptor mrnas in mouse. Journal of Neuroscience, 18, 2538–2549.