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The Development of Morphine-Induced Antinociception in Neonatal Rats: A Comparison of Forepaw, Hindpaw, and Tail Retraction From a Thermal Stimulus

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BLASS, E. M., C. P. CRAMER AND M. S. FANSELOW. *The development of morphine-induced antinociception in neonatal rats: A comparison of forepaw, hindpaw, and tail retraction from a thermal stimulus.* PHARMACOL BIOCHEM BEHAV **44**(3), 643-649, 1993.—Two parallel experiments in rats 2-21 days of age investigated the onset and characteristics of morphine-induced antinociception. One measure of reactivity to pain, limb retraction from a hotplate, was utilized for three different limbs (forepaw, hindpaw, and tail) to chart the development of opioid sensitivity. Morphine-induced antinociception, even in 2-day-old rats, was obtained for all limbs, in a dose-related fashion, and reached peak sensitivity at 6-7 days of age. Naltrexone did not affect limb retraction latencies in nonmorphine treated rats at any age. These studies demonstrate early antinociception to low doses of an opiate and establish that the pain system, like positive reinforcement systems, is opiate sensitive.

Antinociception Morphine Neonatal rats Thermal stimulus

ALTHOUGH the physiology and pharmacology of developing opioid systems has received considerable attention (for a bibliography see ref. 24), relatively few studies have investigated developmental changes in antinociception known to be mediated by opioids, and these studies have yielded conflicting findings. Kehoe and Blass (11), for example, reported that the low dose of 0.5 mg/kg morphine increased the latency with which 10-day-old pups retract their forepaw from a 46°C hotplate. This antinociception was reversed by the opioid antagonist naltrexone. Similarly, Fanselow and Cramer (4) found that morphine at doses of 2-10 mg/kg lengthened the latency to retract a hindpaw from a 52°C hotplate in 1-day-old rats. Although a 1 mg/kg dose did not produce analgesia in young rats, sensitivity to opiates increased, such that 1 mg/kg was effective at 8 days of age.

In contrast to these findings, Pasternak et al. (15) reported that 2-day-old rats did not exhibit changes in responding to nociceptive stimulation, as measured by the tail-flick response to radiant heat, following a 5-mg/kg dose of morphine. However, 14-day-old pups were analgesic at this dose. This is paradoxical because morphine more readily penetrates the brains of 2-day-old rats (15). Similarly, Giordano and Barr (6) reported that neither morphine nor ketocyclazocine produced analgesia in 3-day-old rats as assessed by tail or hindpaw retraction from a hot water bath.

Studies of opioid physiology and pharmacology indicate that the neonatal period is characterized by substantial changes, such as large increases in met- and leu-enkephalin concentrations in striatum and cortex (16), cerebellar B-endorphin (21). Enkephalin receptors increase markedly between birth and 28 days of age (16). Peak [^{3}H]Met-enkephalin binding in cerebellum is found during the first week, in brainstem during the second, and in forebrain during the third week (21,22). High affinity mu receptors appear in forebrain at birth, decrease during the first week of life, and then rapidly increase (19). Conversely, delta receptor concentration is low at birth but increases monotonically throughout the next 28 days. The concentration of kappa receptors shows less dramatic changes, but the largest increases in low affinity kappa sites are from 7 to 14 days postnatal (18,19). Similarly, high

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affinity ^{3}H-morphine and *D*-Ala2-Met5-enkephalinamide binding sites increased rapidly between Days 2 to 14, while low affinity binding sites showed little change during the same period (15,25). There appear to be at least two populations of opioid binding sites at birth that show differential changes over the first few weeks of life (23). The degree to which these patterns in opioid development are reflected in the ontogeny of behavioral function remains unknown.

Because the question of whether young infants responded to the antinociceptive actions of morphine was unresolved, we independently decided to study limb retraction elicited by a constant thermal stimulus. We contrasted the development of hindpaw, forepaw, and tail retraction for two reasons. First, one source of disparity between the findings of various groups could be that different limbs became sensitive to opiates at different ages. Second, we hoped to identify the behavioral paradigm that yielded the most consistent results, for use in later studies of nociception and tolerance. When the studies were already well underway in each of our labs, we discovered that we were approaching essentially the same problem. Because the results, derived from somewhat different methodologies, were essentially consistent and led us to the same conclusions, we have decided to publish the two studies together.

METHODS

Two parallel experiments, each utilizing similar procedures, were conducted: one at Dartmouth, the other at Johns Hopkins. For the sake of clarity, the procedure used in the Dartmouth portion of the study will be presented first, followed by that utilized in the Hopkins portion. A summary of the two procedures is provided in Table 1.

Subjects (Dartmouth)

Long-Evans hooded rats, bred from stock obtained from Blue Spruce Farms, served as subjects. Pregnant females were housed individually starting 2–8 days prepartum and during the entire nursing period in polypropylene tub cages (25 × 45 × 20 cm) with high protein chow (Agway) and tap water continuously available in the stainless steel lids. Pine shavings were used as bedding. Room temperatures ranged from 22–26°C, with lights on from 0600 to 2000 h EST. Nest areas were checked late each afternoon for newborn pups; pups found at that time were considered born on that day (Day 0). Litters were culled to 10 pups on the day after birth. At each age, eight infant rats were randomly selected from each of four litters for testing.

Design. A three-factor parametric design was used. Withdrawal latencies for forepaw, hindpaw, and tail from a 52°C surface were obtained within-subjects on each test day, with test-limb order counterbalanced. Four doses (0, 1, 2, and 4 mg/kg morphine sulfate in isotonic saline) were administered IP within-litter. That is, two pups from each litter received each dose. Pups were randomly assigned to a drug dose at 2, 7, 14, or 21 days of age (n = 8/cell). The experimenter was blind to the drug dose administered to a particular rat.

Testing procedure. Pups were removed from the home cage, weighed (±0.01 g), and marked with a laundry pen. They were then placed, in groups of eight, in covered plastic tubs (16 cm diameter) with pine shaving bedding, that were set on a heating pad (33°C). Withdrawal latency evaluation began 20 min after drug injection. Each pup was tested three times, once per limb, with tests separated by about 12 min; thus all testing was completed within an hour of injection.

All three tests were conducted using a copper hotplate maintained at 52°C (±0.5°C). To obtain forepaw removal latency, a pup was supported manually just above the hotplate, and one forepaw was extended and allowed to contact the 52°C surface. Latency to lift the paw was recorded when contact was broken. Hindpaw retraction latency was determined by essentially the same procedure. For determining tail sensitivity to heat, the pup was placed on a piece of cardboard so that its tail extended beyond the cardboard's edge. The tail was then lowered onto the hotplate by setting the cardboard support down next to the heated surface. Latency to either lift the tail or to curl it away from the heated surface was recorded. A cut-off of 15 s was used in all cases.

Subjects (Johns Hopkins)

Sprague–Dawley albino rats were studied. They were derived from the Hopkins Psychobiology Colony and treated in essentially the same fashion as above except that Purina laboratory chow was the available food and lights were on from 0700–2100 h. A total of 324 rats from 39 litters were used. Rats were studied at either 2, 6, 9, or 21 days of age; each rat was tested only once.

Design. The study conducted at Hopkins also featured a three-factor parametric design, with age as an independent

TABLE 1

A COMPARISON OF PROTOCOLS UTILIZED AT DARTMOUTH AND JOHNS HOPKINS

	Dartmouth	Johns Hopkins
Subjects	Long–Evans hooded rats	Sprague–Dawley albino rats
Stimulus	Forepaw, hindpaw, or tail contact with 52°C surface	Forepaw, hindpaw or tail contact with 48°C surface
Response measure	Latency to lift stimulated limb	Latency to lift stimulated limb
Drug	0, 1, 2, or 4 mg/kg Morphine sulfate in isotonic saline	Morphine (0.5 mg/kg), naltrexone (0.5 mg/kg), and isotonic saline
Delivery site	IP	IP
Testing	Repeated on 2, 7, 14, and 21 days Dose was assigned randomly on each test day	Days 2, 6, 9, and 21 Each subject was tested once only

factor. Each of nine pups from a litter received either morphine (0.5 mg/kg), naltrexone (0.5 mg/kg) or isotonic saline vehicle and one of its three limbs was tested. Thus, the design was a 4 (age, independent) × 3 (drug, within-litter) × 3 (limb, within-litter) factorial.

Testing procedure. The procedure used in this portion of the study was similar to that described above. Rats were separated from the dam as a group, weighed and injected with their respective substance by an experimenter blind to the contents of the syringe. The stainless-steel surface of the hotplate was maintained at 48°C (±0.2) by circulating hot water from a temperature-controlled bath through copper tubing that covered the underside of the plate. Forepaw and hindpaw latencies were obtained with a timer that recorded when contact was made and broken with the plate; tail-lift latencies were determined by holding the rat and lowering it gently onto the heated surface, so that the tail completely contacted the plate; tail lift was recorded.

Data Analyses

For that portion of the experiment conducted at Dartmouth, a two-factor analysis of variance (ANOVA) was performed on data from each age group, with test limb as a repeated measure and drug dose as an independent measure. For the data collected at Johns Hopkins, age, drug, and limb effects were assessed with three-factor ANOVA.

RESULTS

The essential findings of these studies are: (a) Morphine, in doses as low as 0.5 mg/kg, caused increased latencies in limb retraction among rats 2–14 days of age. (b) These increases occurred in a dose-related manner. (c) Rats 6–7 days of age were most responsive to morphine, as judged by increased limb withdrawal latency. (d) Naltrexone (0.5 mg/kg) administration did not influence the latency with which a limb was removed from a 48°C surface in drug-free rats. (e) Assessing opioid influences on nociception in rats 21 days of age requires methods that differ from the ones used in these studies.

Figure 1 presents limb retraction latencies for saline-, morphine-, and naltrexone-treated rats 2, 6, 9, and 21 days of age (Johns Hopkins study). Analysis revealed statistically significant effects of age [$F(3, 291) = 21.90$, $p < .01$] and drug [$F(2, 291) = 43.849$, $p < .01$], as well as interactions between age and drug [$F(95, 291) = 6.99$, $p < .01$], age and limb [$F(6, 291) = 4.60$, $p < .01$], and drug and limb [$F(4, 291) = 2.55$, $p < .05$]. The main effect of limb and the three-way interaction were insignificant.

The age by drug interaction was largely due to elevated responsivity to morphine in 6-day-old pups and no responsivity to this dose of morphine in 21-day-old pups. Naltrexone did not systematically cause a change in limb withdrawal at the 0.5 mg/kg dose utilized in these experiments. None of the post-hoc Newman-Keuls analyses showed a difference between naltrexone and saline. The limb by age interaction reflected heightened nociceptive responsivity of the tail in comparison to the other two limbs at 2 and 6 days of age, and not difference between the limbs at 9 and 21 days of age. The drug by limb interaction was due to a modest reduction in morphine-induced analgesia in the tail.

Figure 2 demonstrates dose-dependent responsivity for all three limbs in 2-, 7-, 14- and 21-day-old rats (Dartmouth study). Among 2-day-old rats, effects of drug [$F(3, 28) = 14.26$, $p < .01$], limb tested [$F(2, 56) = 8.86$, $p < .01$] and their interaction [$F(6, 56) = 2.28$, $p < .05$] were all significant. At Day 7, the drug [$F(3, 40) = 7.13$, $p < .01$] and limb effects [$F(2, 80) = 25.8$, $p < .01$] remained significant, although the interaction was not. Among 14-day-old rats, effects of drug, limb tested, and their interaction were all significant [$F(3, 32) = 25.82$, $p < .01$; $F(2, 64) = 26.47$, $p < .01$; and $F(6, 64) = 9.20$, $p > .01$, respectively]. Finally, at Day 21, there were significant drug, limb, and interaction effects [$F(3, 32) = 7.00$, $p < .01$; $F(2, 64) = 141.30$, $p < .01$; and $F(6, 64) = 2.67$, $p < .05$, respectively]. In addition, among all limbs, at all ages, except the tail at Days 14 and 21, trend analysis revealed significant linear dose-related effects.

Rats appear to be more responsive to the exogenous opiate on Day 7 than at either 2 or 14 days of age. This is clear when comparing forepaw, hindpaw, and tail retraction latencies at 2 and 7 days of age and in forepaw and hindpaw latencies between 7- and 14-day-old rats. Support is lent, therefore, to the idea of heightened sensitivity to morphine in Day 6–7 rats. The dose-response functions presented in Fig. 2 also support the availability, in a graded manner, of endogenous analgesic systems to different amounts of exogenous opiates even in 2-day-old rats. This held for retraction latencies in all three limbs. By Day 7, 1 mg/kg produced a response latency that exceeded the imposed 15-s cut-off limit.

In short, the mechanisms needed to detect changes in circulating opiate levels and to make these changes available to antinociceptive systems are functional by Day 2, and the response properties of the three limbs to increased morphine levels are not differentially affected developmentally. As shown in both Figs. 1 and 2, rats 6–7 days of age are markedly responsive to morphine, but this increased sensitivity does not extend to rats that are 9–14 days of age.

Figure 2 also illustrates the behavior of Day 21 rats, in which forepaw and hindpaw withdrawal is insensitive to the effects of morphine injections at 1 and 2 mg/kg and markedly diminished at 4 mg/kg. In light of the short latencies of forepaw and hindpaw removal, failure to remove the tail from the hotplate is difficult to understand. It may reflect a change in tail morphology, such as increased insulation from hair growth, which decreased sensitivity to heat.

DISCUSSION

Three classes of findings concerning the development of opioid-mediation of nociception emerge from these studies: first, the time course of development of responsiveness to an exogenous opiate; second, the similarities in response characteristics among the three limbs; and third, the failure of naltrexone to affect baseline antinociceptive responses.

Most importantly, the present findings demonstrate that rats 2 days of age and older can utilize morphine as an antinociceptive agent. This implies that the endogenous opiate system is functional, at least in terms of the availability of receptors and their potential for binding an exogenous opiate and transducing the signal arising from that change into behavioral modulation (i.e., increased pawlift latency). The present studies further suggest that antinociception caused by morphine injections peaks at 6–7 days of age, at least within the age range used here. The agreement between findings from the two laboratories that conducted this study independently, using different parameters of stimulation, rats of different strains, and different schedules of testing, provides validity to the three main ontogenetic points concerning age of onset, maximum sensitivity, and decline in responsivity as weaning approaches. The effectiveness of opioid agonists in suppressing nociceptive responses in rats as young as 2 days of age is not limited to morphine, which is a predominantly mu opioid

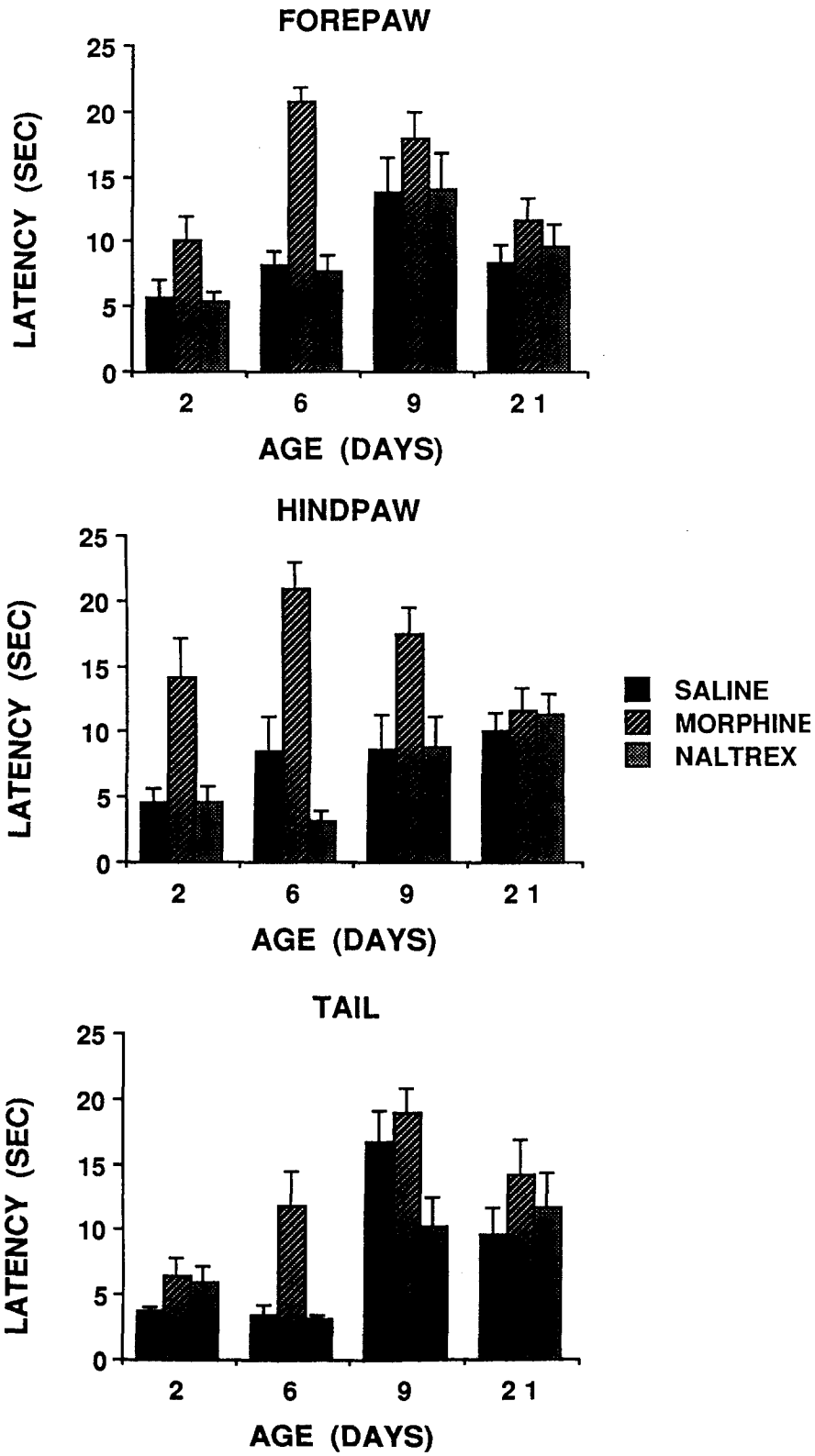

FIG. 1. Mean latency (±SEM) in which forepaw, hindpaw or tail was removed from a 48°C surface after injections of isotonic saline, 0.5 mg/kg morphine, or 0.5 mg/kg naltrexone in rats 2, 6, 9 and 21 days of age.

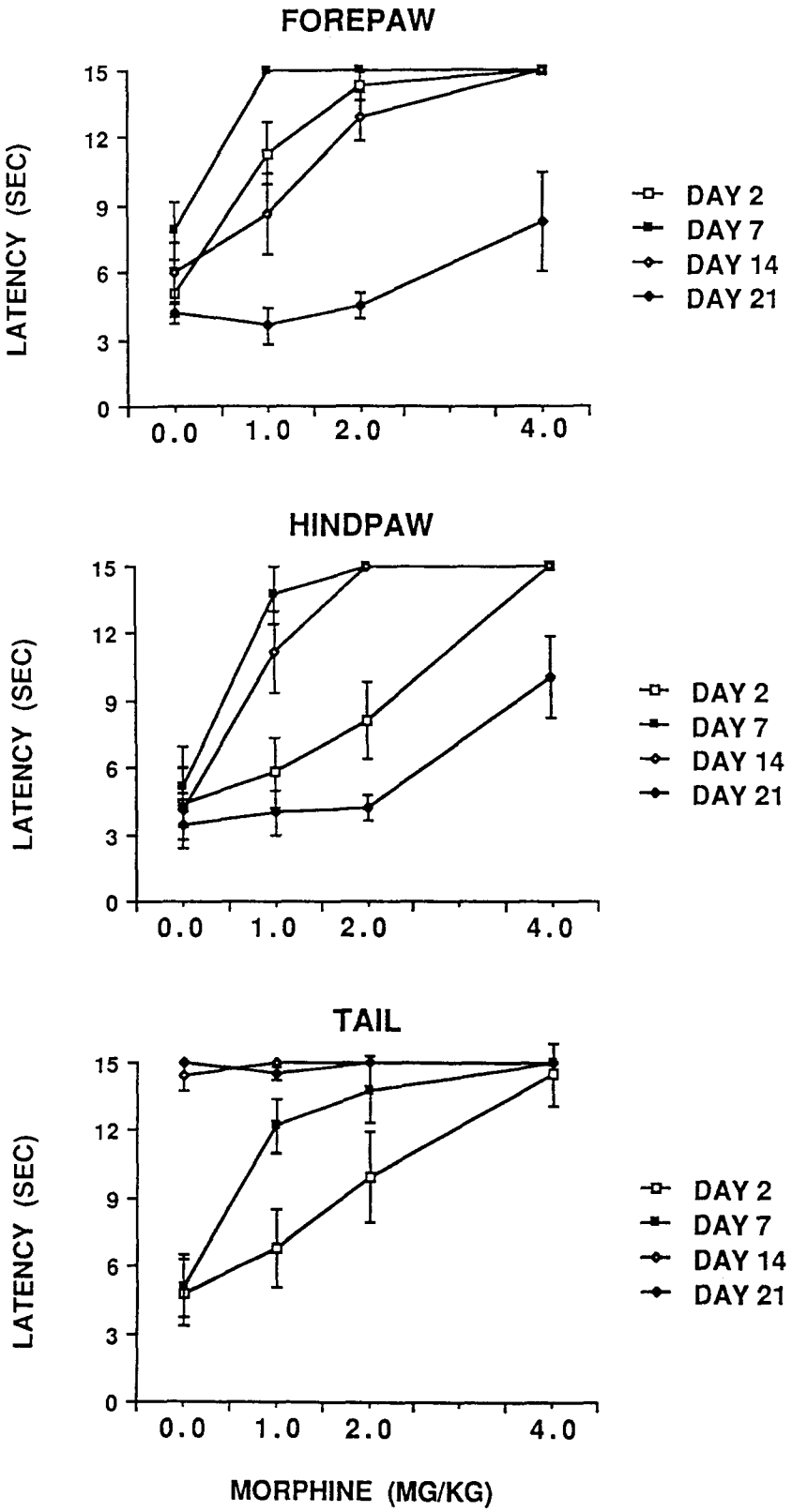

FIG. 2. Mean latency (±SEM) of limb removal from a 52°C surface in rats 2, 7, 14 and 21 days of age, injected with either 0, 1, 2, or 4 mg/kg morphine.

agonist. Helmstetter, Calcagnetti, Cramer, and Fanselow (7) found that two kappa opioid agonists, ethylketocyclazocine and bremazocine, also produced analgesia in 2-day-old rats, and Blass, Jackson, & Smotherman (3) reported that a 20-ml μl pulse of milk delivered to the mouth of newborn rats elevated heat escape latencies.

Our findings contrast with those of Giordano and Barr (6), who reported that neither morphine nor ketocyclazocine produced analgesia in 3-day-old rats as assessed by tail or hindpaw retraction from a hot water bath. Similarly, Pasternak et al. (15) found that the tail-flick response was not inhibited by morphine in 2-day-old rats. Procedural differences may account for these discrepant sets of findings. The test protocols used by Giordano and Barr (6) yielded mean response latencies in control rats of less than 1 s. Comparable latencies (range 1.6–2.9 s) were reported by Pasternak et al. (15). In contrast, mean tail-withdrawal latencies of saline-injected rats in the present studies were about 4 s, as were baseline latencies in Helmstetter et al. (7). Thus, neonatal rats appear not to be opioid-sensitive to intense heat stimulation that caused rapid limb withdrawal.

There appears to be a similar pattern in adult rats. Jensen and Yaksh (8) reported that 5 μg of morphine injected into either medullary raphe or pararaphe sites caused analgesia only when the test was conducted with a low-intensity heat stimulus that produced a long-latency tail-flick (about 4 s baseline). No analgesic effect of this treatment was detected when a high-intensity stimulus that produced a short baseline latency (about 2 s) was used. Jensen and Yaksh (8) suggest that the response to the high-intensity stimulus was spinally mediated, because spinally transected rats did not respond to the low-intensity stimulus. In general, when the heat escape response is supraspinally mediated, morphine produced a more potent analgesia at more CNS sites than when a spinally mediated response (short latency tail-flick) was studied (8). In support of this idea, neonatal rats continued to respond briskly to the high-intensity stimulation following spinal transection (25).

The analgesic effects of morphine in the present study increased with age to a maximum at 6–7 days but then began to decrease. A similar pattern was observed by Fanselow and Cramer (4). It is difficult to relate this maximum morphine sensitivity to any particular aspect of endogenous opioid receptor development, which is still dynamic in 6-day-old rats. Spain et al. (19) found that ^{3}H-DAGO binding reached its lowest point between 4 and 7 days of age, with binding of this ligand higher at 1 and 10 days of age. In brain stem, ^{3}H-naloxone binding shows its most dramatic increase between ages 2 and 5 (23). Presumably, the nonmonotonic pattern in morphine's effects over this period (Days 2–21) reflects an interaction between the unfolding of opioid receptor development described in the introduction of this paper, in combination with alterations in morphine pharmacokinetics, such as changes in enzymatic and metabolic drug disposition, the decrease in the drug's ability to penetrate the brain with age (9,15), and sensorimotor development.

The early availability of an antinociceptive opioid receptor system compliments that of an opioid-mediated system of positive affect or reinforcement in rats. In an important study, Stickrod, Kimble, and Smotherman (20) demonstrated that rats at 20-day fetal age could be classically conditioned to prefer the taste of apple juice when it was paired with morphine injections. Several studies have demonstrated a relationship between the taste of sugars, milk, fats and carbohydrates, and antinociception that is opioid-mediated (1,2,3,17). These substances are also rewarding via opioid pathways in infant rats, as judged by their abilities to serve as unconditioned reinforcers in classical conditioning and by their failure to support classical conditioning in rats treated by naltrexone (10,12,13). Positive and negative (present study) affective systems therefore appear to be behaviorally functional at or before birth and interact reciprocally despite their remarkable immaturity in terms of adult levels of receptor availability and circulating peptides.

There are apparent similarities in the antinociceptive response properties of the three limbs: the same age of onset of morphine sensitivity (at least within the limits of this study) and the ability to obtain dose-related functions from the three limbs, at least at 2 and 7 days of age. These findings again contrast with those of Giordano and Barr (6), who report a rostro-caudal developmental pattern of sensitivity. Another important similarity stems from the failure of naltrexone to systematically affect the retraction latency of any limb. Furthermore, each of the limbs tested gave results with about the same degree of individual variability. Taken together, these results lead us to the conclusion that any of the three limbs studied would be suitable for tests of antinociception in young pups; however, the tail would prove less useful in studies that included rats beyond the first week or so.

Figure 1 demonstrates that naltrexone at 0.5 mg/kg did not reduce limb withdrawal latency in any systematic fashion in rats tested immediately after removal from the nest. This may be contrasted with the effectiveness of naltrexone, at this dose, in reversing the antinociceptive effects caused by morphine injections (11), intraoral infusions of various substances (1,2,3,17), and forced isolation from the dam (12). Moreover, naltrexone counters the calming effects of morphine injections and intraoral infusions of various substances on isolation-induced ultrasonic vocalizations (11). Failure of naltrexone to affect withdrawal latencies in animals tested immediately after removal from nest or group housing implies that systems mediating antinociception do not have a tonic opioid component in the absence of either stressful antecedents or certain classes of ingestive experiences. The endogenous opioid contribution appears to be nascent, ready to be engaged by classes of events that differ qualitatively, and to respond quickly to the onset of these events. Although one cannot rule out naltrexone-induced hyperalgesia without a full dose-response study, this conclusion is entirely consistent with the literature on stress-induced analgesia in adult animals. Specifically, endogenous opioids produce analgesia only when the animal is first subjected to a stressful event (14). Opioid antagonists do not affect baseline pain sensitivity but do cause relative hyperalgesia in adults when administered in conjunction with an environmental event that precipitates analgesia (e.g.,see ref. 5).

In summary, these studies have revealed the early availability of opioid systems as evaluated by changes in antinociceptive behavior following morphine injection. Because the endogenous system seems to be functional in rats at an early age, questions now arise as to their behavioral and physiological contributions to normal development within the nest setting.

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