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The Ontogeny of Opiate Tolerance and Withdrawal in Infant Rats

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FANSELOW, M. S. AND C. P. CRAMER. *The ontogeny of opiate tolerance and withdrawal in infant rats.* PHARMACOL BIOCHEM BEHAV 31(2) 431–438, 1988.—The acquisition of morphine analgesic tolerance was investigated in neonatal rats. Morphine was found to produce a potent analgesia, as measured by latency to retract a hindpaw from a 52°C hotplate, in rat pups as young as 1 day of age. Morphine analgesic tolerance, however, did not develop in rats until the third week of life. Rats given the same daily morphine regimen starting at 15 days of age or older showed rapid tolerance development. The data from four experiments indicate that experience with morphine prior to this age (Day 15) does not impact on the analgesic efficacy of the drug. Similarly, when morphine treatment was discontinued and the rats given a naloxone challenge, withdrawal symptoms were not observed in very young rats. Opiate withdrawal was first detected in rats that started their daily morphine treatment at 30 days of age and were then challenged with naloxone at 52 days of age. Therefore, two correlates of opiate addiction, tolerance and withdrawal, appear to be relatively late-developing phenomena in the rat.

Addiction Analgesia Development Hotplate test Infant Morphine Naloxone Neonate Ontogeny Opiates Perinatal Rat Tolerance Weight loss Withdrawal

A common belief is that the offspring (human or rat) of mothers that received opiates during gestation are born themselves addicted to opiates. That is, they will show withdrawal symptoms if not administered opiates and are tolerant to some of the effects of opiates (4, 5, 19–22, 31, 33). Such effects have been demonstrated when animals are tested much later: rats given well-spaced prenatal administration of opiates show more rapid acquisition of tolerance to morphine despite a prolonged period of opiate abstinence following birth [(2, 14, 15), but see (34)]. Unfortunately, less attention has been paid to the appearance of tolerance *during* the perinatal period. Nor has there been thorough documentation of acutal, adult-like, abstinence-induced withdrawal symptoms following perinatal opiate administration in such animal preparations.

The lack of evidence for pre- or neonatal tolerance is, no doubt, at least partly due to the absence of behavioral measures of the opiate sensitivity of neonates. The neonatal rat is not competent to perform the requisite behaviors used in the standard tests of opiate sensitivity for adult rats (e.g, paw-licking, jumping, locomotion, etc.). Recently a modification of the hotplate analgesiometric technique for neonatal rats has been introduced (7, 9, 10). Rat pups are supported manually just above a thermal stimulus, and either a paw or the tail is allowed to come in contact with the heated surface. Neonatal rats will rapidly retract their appendage, and the animal's competence to respond does not change with age. Morphine, because of its analgesic properties, causes an elevation in the latency to respond. Here, we report a series of studies that employed such a technique to examine changes in responsivity to repeated daily dosages of morphine. In adult rats, it is clear that tolerance develops in response to well-spaced administration of opiates (e.g., once every 24 hours) and that this tolerance is retained over prolonged drug-free periods (25). We also report the neonate's responses when challenged with the opiate antagonist naloxone following prolonged opiate administration. Such procedures lead to pronounced withdrawal symptoms in adult rats (12).

EXPERIMENT 1

In the first experiment, we describe the analgesic response of preweanling rats to repeated daily administration of one of four doses of morphine (or a saline placebo). Morphine exposure continued for 21 days, a length of time chosen to match the length of the rat's gestation period, and was followed on Day 22 by a naloxone challenge. In addition, because tolerance acquisition is facilitated in adults when opiate administration and testing are conducted in a distinctive environment [for review see (3)], an odor cue (peppermint) was presented during each daily exposure.

METHOD

Subjects

Long-Evans hooded rats, bred from stock obtained from Blue Spruce, were used a subjects. Females were mated to Long-Evans males and were housed individually 2–10 days prepartum and during the entire nursing period in poly-

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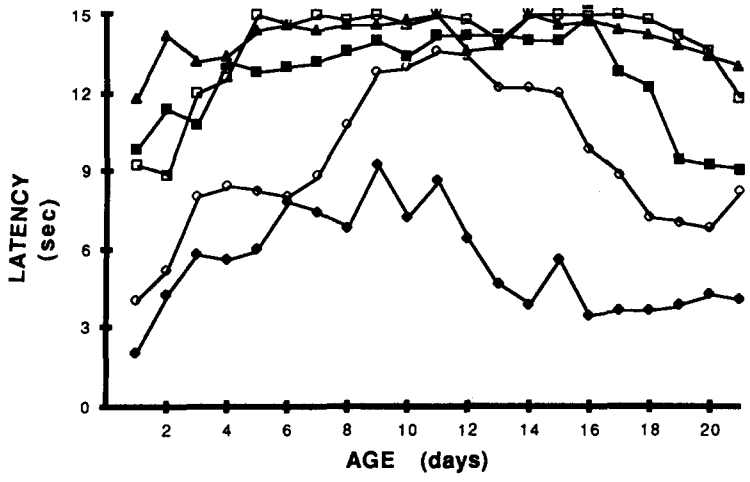

FIG. 1. The data of Experiment 1, indicating the latency of rat pups to retract a paw from the hotplate, are presented in this figure. The rats were given a single daily dose of morphine and tested 20–30 min after this treatment. ◆: 0 mg; ◇: 1mg; ■: 2 mg; □: 5 mg; ▲: 10 mg.

propylene tub cages (25×45×20 cm) with high-protein chow (Agway) and tap water continuously available in the stainless steel lids and pine shavings as bedding. Room temperatures ranged from 22–26°C, with lights on from 0600 to 2000 hr EST. Nest areas were checked late each afternoon for newborn pups; pups found at that time were considered born that day (Day 0). Litters were culled to 10 pups on the day after birth. Dams were primiparous or multiparous; assignment to drug condition was random with regard to parity. The subjects for this experiment were 183 infant rats from 19 litters. Cross-fostering between litters ensured that each drug condition was represented by between 9 and 15 different litters. Eighteen pups died during the course of the experiment. Attrition for the saline, 1, 2, 5, and 10 mg/kg groups was 1, 5, 1, 4, and 7 pups, respectively.

Apparatus

The test apparatus was an IITC, Inc., Model 35-D hotplate. Prior to testing each pup was placed in an 11×9×7-cm glass container that contained a 5×5-cm 8-ply gauze sponge. The gauze was treated with 0.1 ml of Durkee's imitation peppermint extract.

Procedure

Independent groups of rat pups were assigned to five different doses (0, 1, 2, 5, or 10 mg/kg) or morphine sulfate. Starting on the day after birth (Day 1), the pups were weighed and then given a single daily IP injection. Immediately prior to weighing and drug administration, the pups were removed from the nest and individually placed in the peppermint scented containers.

Twenty min after injection the pups were tested for the analgesic effects of morphine. The rats were removed from their containers and supported manually just above the hotplate (52°C), and the bottom of one hindpaw was allowed to come in contact with the heated surface. Pilot work with this procedure indicated that one-day-old rats will lift their paw 2–4 sec, and performance does not change with age. We employed a cut-off latency (15 sec), so that highly analgesic animals did not leave their paw on the heated surface long enough to do any physical damage. Latency to lift the paw was the dependent variable; each hindpaw was tested once, with the tests about 8 min apart. We followed this procedure for 21 days. On Day 22, the procedure was repeated with the exception that all animals instead received the opiate antagonist naloxone hydrochloride (10 mg/kg). In addition to the hotplate test, animals were weighed 4 hours after naloxone injection. The experimenter was always blind to the morphine treatment conditions.

Statistical Analyses

As subjects in the higher dose groups typically went to the cut-off latency, the heterogeneity and normality assumptions of analysis of variance were violated. Additionally, the cells sizes were unequal, limiting analysis of variance's robustness against such violations. Therefore, the hotplate data were analyzed with nonparametric techniques at an alpha level of <0.05. An overall Kruskal-Wallace test was performed on each day and, if warranted, supplemental comparisons were made with the Mann-Whitney U-test.

Weight loss data were analyzed by overall analysis of variance with supplemental comparisons made with Scheffe's test. Alpha was set at $p<0.05$ for these comparisons.

RESULTS AND DISCUSSION

Group means for latency to pawlift on Days 1–21 are presented in Fig. 1. On Day 1, the higher doses of morphine produced analgesia. Statistical analyses of these data confirmed this description; the 2, 5, and 10 mg/kg groups differed reliably from saline. However, the 1 mg/kg morphine dose did not produce an elevation of pawlift latencies relative to saline controls.

The 10 mg/kg animals maintained their high level of analgesia over the 21 days of morphine administration (i.e., they always differed reliably from controls). The 2 and 5 mg/kg animals showed enhanced analgesia over days so that by Day 4 they were indistinguishable from the 10 mg/kg animals. While animals receiving 1 mg/kg of morphine did

TABLE 1

MEAN (±SEM) WEIGHT LOSS 4 HR AFTER A NALOXONE CHALLENGE OF RATS GIVEN VARIOUS DOSES MORPHINE SULFATE (OR PLACEBO) BEGINNING AT VARIOUS AGES

Age Start	Dose (mg/kg)	% Body Wt. Lost
1	0	5 (0.5)
1	2	3 (0.4)
1	10	4 (0.5)
10	0	2 (0.3)
10	2	2 (0.4)
10	10	1 (0.1)
15	0	7 (0.7)
15	2	8 (1.0)
15	10	6 (0.4)
30	0	2 (0.3)
30	2	3 (0.3)
30	10	5 (0.3)
45	0	2 (0.3)
45	2	3 (0.3)
45	10	5 (0.05)
70	0	2 (0.02)
70	2	3 (0.03)
70	10	5 (0.02)

not appear to be analgesic initially, by Day 8 they showed reliably longer pawlift latencies than saline controls. On day 21, all four doses of morphine were producing statistically reliable analgesia relative to saline controls. Thus, we obtained no evidence for tolerance to the analgesic effects of morphine in neonatal rats. In fact, we observed the opposite pattern, the neonatal animals appeared to become increasingly sensitive to morphine over the drug administration period.

The animals with a history of morphine exposure were not more reactive on the hotplate following the naloxone challenge on Day 22. The mean response latencies of the 0, 1, 2, 5, and 10 mg/kg groups were 5.3, 5.8, 5.1, 4.3, and 8.5 sec, respectively; these group differences were not statistically reliable. Furthermore, the animals with a history of morphine treatment did not show the weight loss that is characteristic of withdrawing adults (see Table 1). Thus, with two measures of withdrawal used in adults (weight loss and pain sensitivity), we found no evidence of naloxone-precipitated withdrawal in neonates exposed to morphine for 21 days. We also did not see any obvious drug-related changes in the incidence of "wet dog shakes," although this behavior was not quantified. Thus we were unable to find either opiate tolerance or abstinence syndrome in neonates. It should be noted that if normal adults are subjected to these procedures both tolerance and withdrawal symptoms are easily demonstrated (see data provided in Experiment 2).

EXPERIMENT 2

The obvious question raised by Experiment 1 is, at what age does adult-like tolerance to daily morphine administration first appear? Experiment 2 sought to answer that question by using procedures similar to those of the first experiment but starting opiate administration at older ages.

METHOD

Subjects

Long-Evans rats bred and housed as described in Experiment 1, were used in this experiment. Rats that began testing at 5, 10, and 15 days of age remained with their dam until the end of testing. Those tested beginning at 30, 45, and 70 days of age were separated from their dam at 25 days of age and were housed in same-sex groups. The experiment used a total of 187 rats divided so that each drug group was represented by between 9 and 14 subjects.

Procedure

To determine the age at which opiate analgesic tolerance first appears, we started tests with naive rats at 5, 10, 15, 30, 45 or 70 days of age. Rats started at 15 days of age or younger were tested with the paw lift procedure described in the first experiment. It is difficult to test subjects older than 30 days of age with this analgesic test (7). Therefore, analgesia testing was halted on Day 29 for the subjects started at 10 and 15 days of age. Subjects in the three older age groups were given the standard hotplate paw-lick test (6). This consists of placing a rat on the hotplate and taking the latency to lick a paw. Rats were removed from the plate followng a lick or a jumping response. A 90 sec cutoff latency was used for this procedure. The 5-, 10- and 15-day-old pups were placed in the glass containers described in Experiment 1 prior to testing. The 30- and 45-day-olds were placed in plastic tub cages rather than the glass containers. The 70-day-old rats were placed in standard wire mesh home cages. All animals were exposed to the peppermint scented gauze prior to testing. All age groups were tested with morphine dosages of 10 mg/kg, 2 mg/kg or saline placebo treatment, except for the animals started at 5 days, which were only tested with 5 mg/kg of morphine or a placebo. As before, treatment consisted of single daily IP injections of the assigned morphine (or saline) dose. The last day of the experiment was a naloxone test day. All unspecified details were as described for the first experiment. Statistical analyses were conducted in a manner similar to that described in Experiment 1. Each age group was analyzed separately.

RESULTS AND DISCUSSION

The animals that were started at 30, 45 and 70 days of age all showed the same pattern of results (see Fig. 2). Initially, the 10 mg/kg dose produced a potent analgesia, as evidenced by a statistically reliable elevation in response latencies. However, with repeated administrations, 10 mg/kg morphine progressively lost its ability to raise response latencies. The 2 mg/kg dose never produced a change in response latencies, relative to placebo controls, in these older animals.

In contrast, among the 5-day-old rats morphine produced a potent analgesia, elevating pawlift latencies to near the 15 sec cut-off latency, and the analgesic efficacy of the drug never waned over repeated daily administrations (see Fig. 3).

The data of the rats started at 10 and 15 days of age are depicted in Fig. 4. Potent analgesia was produced by both drug dosages on the first day of testing. The animals that were started at 15 days of age showed rapid tolerance to both

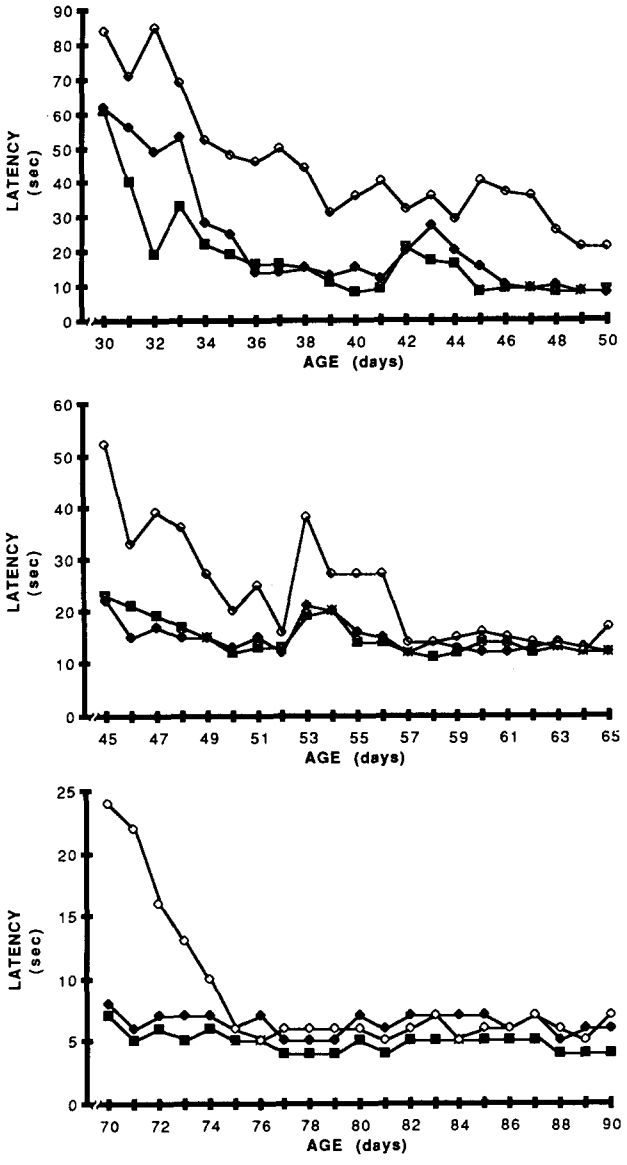

FIG. 2. This figure presents the latency of rats to either lick a paw or jump from the hotplate following a single daily injection of the indicated dose of morphine. The upper graph is for the rats that started drug treatment at 30 days of age, the middle graph is for the rats that started at 45 days of age, and the bottom graph is for rats that started at 70 days of age. ■: 0 mg; ◆: 2 mg; ◇: 10 mg.

dosages. In contrast, tolerance development in the animals started at 10 days of age was weak and retarded. The analgesic efficacy of the drug did not begin to diminish until Day 16 for the low dose animals and not until Day 23 for the high dose animals. Thus Day 15 seems to be the first age at which an adult-like pattern of analgesic tolerance appears. Consistent with this hypothesis is the fact that the animals of Experiment 1 given low doses (1 and 2 mg/kg) starting at Day 1 postpartum did not show any diminution in analgesic efficacy until after age 15 (see Fig. 1). Indeed, the data obtained from the animals that first received morphine at 1, 5, and 10 days of age suggest that high doses of morphine (5 or 10 mg/kg) may actually delay the onset of tolerance.

Comparison of the Figs. 1, 2, 3 and 4 suggests that morphine's analgesic potency decreases with age. This is especially striking with the 2 mg/kg dose, which is a potent analgesic in animals tested at ages younger than 30 days but is ineffective in animals older than 30 days. This observation is consistent with other investigations of the development of the analgesic potency of morphine (7).

When given the naloxone challenge, animals that started 10 mg/kg morphine treatment at ages 30, 45 and 70 all showed a rapid and large loss of body weight, suggesting opiate withdrawal (see Table 1). Animals that began opiate treatment at earlier ages did not. Indeed, as can be seen in Table 1, animals whose morphine regimen (10 mg/kg) started on Day 1 actually showed reliably less weight loss following naloxone challenge than did the morphine naive (placebo) controls. The earliest age at which we have reliably demonstrated withdrawal is 52 days postpartum (i.e., rats that began opiate treatment at Day 30). While it is possible that withdrawal could be demonstrated in juvenile rats between 35 and 50 days, ages which we did not test because they would have received different types of analgesiometric tests, it appears that opiate withdrawal symptoms develop relatively late.

EXPERIMENT 3

In Experiment 1, the analgesic response to morphine appeared to increase during the first 10 days (see Fig. 1). Because all pups received morphine (or the saline placebo) continuously throughout this period, it was not possible to determine if this trend resulted from previous exposure to the opiate or from maturation of opiate analgesia systems. Thus, Experiment 3 was conducted to examine the role of previous morphine exposure on this increasing sensitivity to morphine during the first 10 days. Pups receiving morphine daily were compared to littermates receiving morphine only a single time. Since this sensitization effect was most apparent at low doses, the 1 mg/kg dose was employed.

METHOD

Except where specified below, the procedures were similar to those of Experiment 1. Two pups from each of 4 litters were given morphine sulfate (1 mg/kg) daily from postnatal Days 1–9. On Days 3, 6 and 9, two previously unexposed pups from each litter were also given morphine.

Hotplate tests of analgesia were conducted, as described in Experiment 1, at Days 3, 6, and 9 for both the pups receiving daily morphine injections and those receiving morphine for the first time. Forty-eight pups from six litters served as subjects. Data analyses were conducted using matched-pair *t*-tests among littermates at each age (1).

RESULTS AND DISCUSSION

Pups receiving morphine daily showed the expected rise in morphine-elicited analgesia over days (see Fig. 5). Their littermates receiving a single morphine injection also became increasingly analgesic at older ages, indicating that the rise in sensitivity to morphine was due to maturation rather than opiate exposure. At no age did pups injected daily differ significantly from their littermates injected for the first time. Thus, our previous findings were not the result of either experience-induced sensitization or a build-up of unmetabolized morphine.

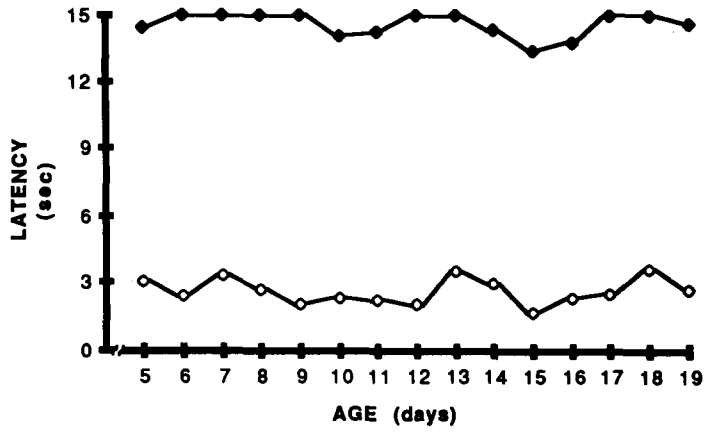

FIG. 3. This figure depicts the latency of rats to pawlift as a function of morphine or saline treatment. The drug treatment was started at 5 days of age. ◆: 5 mg; ◇: 0 mg.

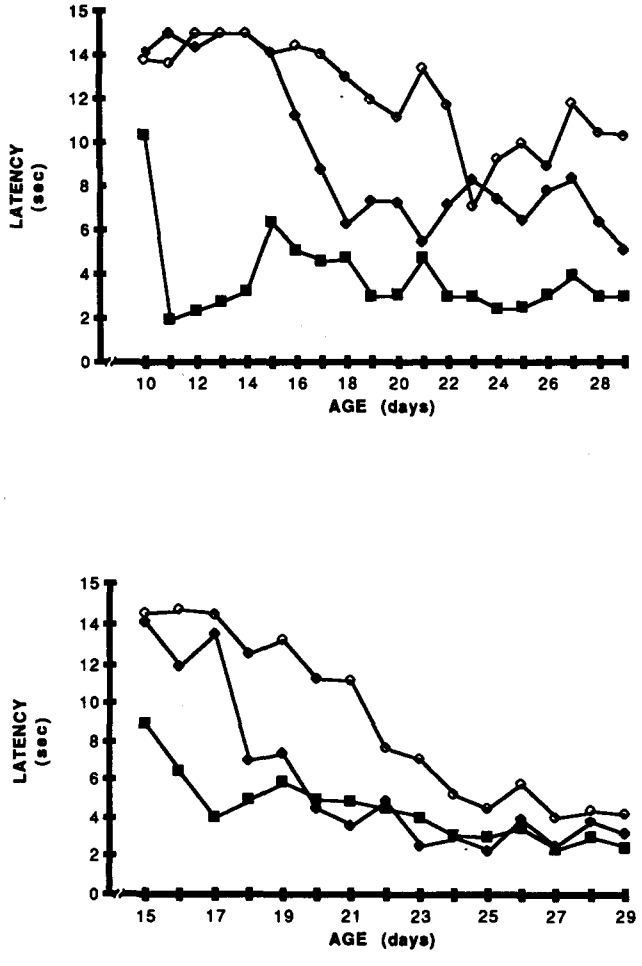

FIG. 4. This figure shows that latency of rats to pawlift as a function of the indicated dose of morphine. Animals started treatment at either 10 (upper graph) or 15 (lower graph) days of age. ■: 0 mg; ◆: 2 mg; ◇: 10 mg.

EXPERIMENT 4

Inspection of the data of the first two experiments suggests that receipt of morphine prior to 15 days of age offered no savings in the rate of tolerance development. Indeed, the trend was for early opiate exposure to retard tolerance acquisition. To determine the effect of morphine exposure prior to age 15 on the initial appearance of tolerance and withdrawal, a split-litter design was used in which one group of pups received morphine from a young age and their littermates from Day 15, the age at which our procedures first detect tolerance.

METHOD

Five pups from each litter began receiving daily injection of morphine sulfate (0, 2, or 10 mg/kg) on Day 8. They were tested daily for analgesic response. The other half of each litter began the same regimen on Day 15. On Day 30, all pups were challenged with naloxone, tested for analgesia, and weighed hourly for 4 hours. Unspecificed details were as described for Experiment 1. Seventy-four pups from 9 litters served as subjects that contributed to the data analysis. An additional 16 pups died during the course of the experiment (4 from the saline group, 6 from the 2 mg group, and 6 from the 10 mg group).

RESULTS AND DISCUSSION

As shown in Fig. 6, pups receiving morphine from Day 8 did not differ from their littermates receiving morphine from Day 15 in their acqusition of tolerance. When challenged with naloxone on Day 30, pups previously injected with morphine, regardless of the duration of that exposure, were no more reactive to the hotplate than saline controls nor did they lose significantly more weight (see Fig. 7). Thus, an additional week of morphine exposure prior to 15 days of age had no effect on the development of tolerance and withdrawal.

GENERAL DISCUSSION

These experiments assessed the analgesic effects of a daily morphine injection in rats that began receiving drug

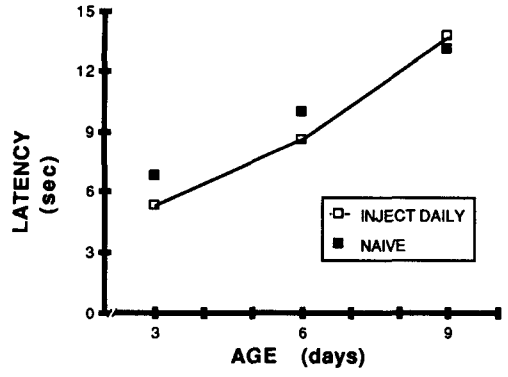

FIG. 5. This figure compares rats that received a single daily 1 mg/kg dose of morphine from birth (inject daily) to animals that received the same drug for the first time (naive) at various ages. The dependent measure is the latency to retract a paw from the hotplate. □: Inject daily; ■: naive.

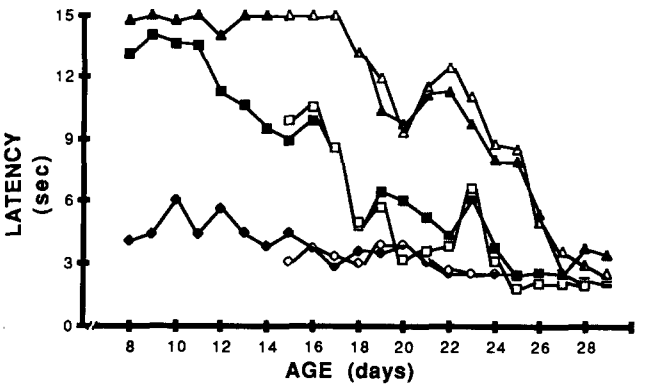

FIG. 6. Rats either received daily injections starting at Day 8 (closed points) or Day 15 (open points). The morphine doses were either 10 (triangles), 2 (squares), or 0 mg/kg (circles). The response measure was latency to withdraw a hindpaw from the hotplate.

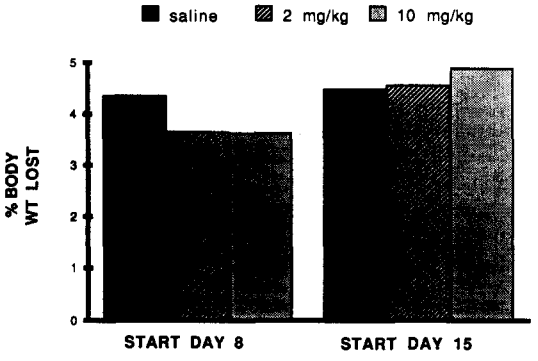

FIG. 7. This figure presents the percentage of body weight lost following a naloxone challenge (10 mg/kg) as a function of previous morphine history. Prior to this test of opiate withdrawal, the rats received either 10, 2, or 0 mg/kg of morphine sulfate daily starting from either 8 or 15 days of age.

treatment at various ages. The data of all 4 experiments indicate that injection of morphine prior to 15 days of age does not alter sensitivity to the analgesic effects of the drug. Experiment 3 indicated that the increase in morphine-induced analgesia observed during the first week postpartum appears to be a maturational rather than an experiential phenomenon. Table 2 presents a summary of these data, along with some additional data we have collected. The table indicates the number of drug treatments necessary before a reduction in morphine's effectiveness is first observed and also the point when the drug was no more effective than placebo. From these data it is very clear that with these procedures opiate tolerance does not develop during the first two postnatal weeks. An interesting trend is that rats exposed to morphine at the youngest ages (e.g., from Day 1) seem relatively delayed in manifesting tolerance. As can be seen in Table 2, pups receiving morphine beginning on Day 15 show reductions in their latencies to pawlift almost immediately, while those beginning on Day 1 remain highly analgesic for several days beyond Day 15. Therefore these data suggest that mechanisms necessary for the expression of tolerance do not develop until the third week postpartum, and drug exposure prior to this time does not lead to earlier acquisition of tolerance and may, in fact, delay tolerance. Withdrawal responses typical of adults apparently do not develop until somewhat beyond the fifth week (see Table 1).

Recent approaches to opiate tolerance have made clear that the parameters with which morphine is administered may lead to two distinct types of tolerance, long-term and short-term (3, 11, 16). By long-term tolerance we mean that reduced drug reactions are noted even over long periods of abstinence (25). This form of tolerance appears to be mediated by a Pavlovian conditioning process (8, 24, 26) and is facilitated when low to moderate dosages are given in a distinctive context at widely spaced intervals (3). With short-term tolerance, relatively short abstinence periods appear to result in elimination of the tolerance. The short-term process is facilitated by closely spaced or chronic (e.g., pellet implant) administrations of relatively high doses of the drug (32). This short-term tolerance is not Pavlovian in that it is observed even if there is a relatively large change between opiate administration and testing environment (3,27). The administration procedures that we have employed in the studies reported here were those that should favor the long-term process. Drug administrations were widely spaced (i.e., 24 hr), the dosages were low to moderate ($\leq$10 mg/kg), and they were given in a distinctive environment (individually in peppermint scented containers away from the nest). Therefore, our finding that tolerance only emerges for drug administration after 15 days postpartum may be specific to the long-term process, and it is quite possible that younger animals will show short-term tolerance. In this regard, one possible explanation of our failure to find tolerance is that infant rats are unable to form associations, as would be necessary for the Pavlovian form of tolerance. We find this an unlikely explanation, because neonatal rats have been shown to be capable of learning a variety of associations involving odor (13,23).

It is interesting to note that the period surrounding 15 days of age is characterized by marked changes in endogenous opioid receptor/ligand systems (17, 18, 28, 29, 36). This correlation between the development of endogenous opioid physiology/pharmacology and the emergence of tolerance

TABLE 2

NUMBER OF DAYS OF MORPHINE EXPOSURE UNTIL THE FIRST INDICATION OF TOLERANCE (SIGNIFICANT REDUCTION IN LATENCY TO PAWLIFT) AND UNTIL FULLY TOLERANT (INSIGNIFICANTLY DIFFERENT FROM SALINE CONTROLS) AMONG RATS BEGINNING MORPHINE ADMINISTRATION AT VARIOUS AGES

Age Start	Number of Days to First Reduction		Number of Days Until Fully Tolerant	
	2 mg	10 mg	2 mg	10 mg
1	18	20	>21	>21
8	6	11	16	19
10	7	10	11	>21
12	3	8	6	20
15	2	4	5	12
17	1	5	2	15

may provide a key to the mechanisms underlying opiate tolerance. Further research will be necessary to explore this possibility.

In conclusion, these data clearly indicate that neonatal rats do not acquire tolerance and that they do not show abstinence-related behaviors when challenged with opiate antagonists in the same way that adults do. To be sure, our opiate-experienced neonates had problems, in many cases similar to those reported for pups exposed to opiates prenatally (35). They were retarded in their growth, gaining weight at a slower than normal rate. At Day 22 of Experiment 1, the animals that started receiving 10 mg/kg of morphine on Day 1 weighed only 82% of their placebo controls' weight. These animals' eyes opened 1–3 days later than saline controls. However, these are problems quite separate from a tolerance/abstinence syndrome. Rather, they may reflect a teratological effect of drug exposure. In fact, malnutrition can produce many of the same later effects as early opiate exposure (30). Our failure to find tolerance and abstinence in neonates calls for a reexamination of the concept of the neonatal abstinence syndrome in rats. Additionally, these data suggest that the search for mechanisms of opiate tolerance should concentrate on systems that functionally mature at about 15 days after birth.

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