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Tonic Nociception in Neonatal Rats

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McLAUGHLIN, C. R., A. H. LICHTMAN, M. S. FANSELOW AND C. P. CRAMER. *Tonic nociception in neonatal rats.* PHARMACOL BIOCHEM BEHAV **36**(4) 859–862, 1990.—The issues of whether infants detect noxious stimuli and whether their nociceptive responses are suppressed by analgesics has been the focus of considerable controversy. Therefore, to more completely assess the nociceptive responses of neonatal rat pups to tonic pain, we tested 3-day-old rat pups using the formalin test. The responses of the young pups to formalin-produced injury were similar to those observed in adult rats, both behaviorally and in terms of their responsivity to morphine-induced antinociception. These results provide the first clear-cut evidence of integrated tonic pain responses in the neonate.

Morphine Tonic nociception Neonate Rat Formalin test

THE question of whether or not neonates experience pain has recently become an important and controversial issue (3, 6, 8, 15, 17, 19–24). Several reports suggest that neonates do not seem to experience pain as intensely as adults; they recover more quickly from pain and display a diminished ability to locate pain (6,20); for review see (3, 8, 17, 23). These reports of diminished pain sensitivity are often based on "casual observations" (21). For example, Merskey (20) reports that circumcision 3–4 days after birth results in "little or no objection from the infant" (p. 118). This reliance upon anecdotal accounts and the paucity of controlled empirical studies has prompted one researcher to state that, "pain in infancy begs more study" [(21), p. 213]. Recent empirical evidence, however, has also lent support to the hypothesis that neonates have diminished nociceptive capabilities. Bronstein and colleagues (6) have postulated, based upon their studies of infant rats presented with viscerally noxious stimuli, that different nociceptive systems develop at different rates. They suggest that these differences in nociception may be the result of maturational differences in sensory, motoric or neural mechanisms.

On the other hand, after reviewing the literature on the neuroanatomical development of pain pathways, Anand *et al.* (3) concluded that, "newborns do have the anatomical and functional components required for the perception of painful stimuli" (p. 1323). Furthermore, recent evidence indicates that infants undergoing painful procedures, such as circumcision, mount a significant physiological stress response that includes changes in heart rate and blood pressure and marked increases in plasma cortisol levels (2,3). In terms of behavior, rats seem to show at least rudimentary responses to nociceptive stimulation during the perinatal period. Tail, frontpaw and hindpaw retraction to a noxious thermal stimulus have all been demonstrated (4). We have recently reported that infant rats as young as 3 days of age show a robust visceral response to intraperitoneal (IP) injections of lithium carbonate that is attenuated by morphine (19). Furthermore, Stickrod *et al.* (25) report that 20-day-old rat fetuses reacted to IP injections of lithium chloride by "wriggling and contracting." These data suggest that the neural and behavioral substrates underlying some forms of nociception *are* intact in very young animals. The purpose of the present experiments was to determine if neonatal rats would show the more integrated responses that adult animals show to a different sort of painful experience, that produced by "tonic pain" (9).

Dennis and Melzack (9) have postulated that the continuous nociception arising from pathology or trauma may be mediated in adults by differing anatomical and biochemical substrates than the nociception elicited in traditional laboratory tests (e.g., tail flick to radiant heat). They have proposed that this "tonic" nociception is subserved by the more slowly conducting unmyelinated pathways, and results in a recuperative response after the damage has been sustained. The formalin test of nociception, as described by Dubuisson and Dennis (11), provides a clinically relevant quantifiable measure of tonic pain in unrestrained animals. It involves a subcutaneous injection of a small amount of formalin into the dorsal surface of the animal's paw. Adult rats and cats respond to the formalin stimulus with the stereotyped recuperative responses of pawlicking and pawlifting (1, 11–13). Therefore, to more completely assess nociception in infant rats, we report a preliminary investigation of the responses of 3-day-old rat pups to formalin treatment. In addition, we have assessed the effects of morphine-induced antinociception on formalin-induced recuperative behavior.

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FIG. 1. A typical example of formalin-induced 'pawlifting.'

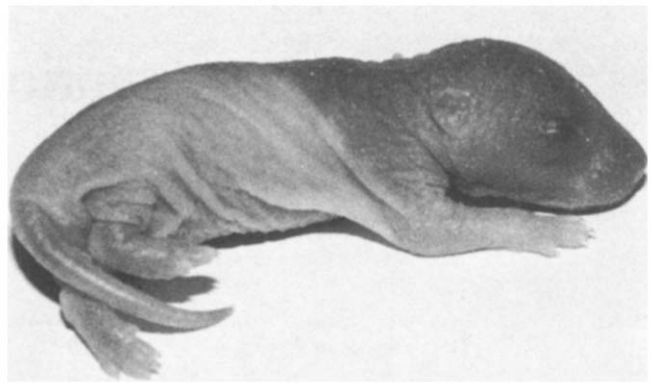

FIG. 2. Another example of formalin-induced recuperative behavior in a neonatal rat pup. [Note that this 'pawlift' is very similar to the adult 'lying' behavior described in Fanselow (12).]

GENERAL METHOD

Subjects

Long-Evans-derived rat pups, 3 days of age, were employed in all experiments. Females were mated in our colony with Long-Evans males (Blue Spruce, Altmont, NY). Approximately one week prior to parturition, the dams were housed individually in plastic tub cages (24 × 43 × 28 cm) until the conclusion of the study. The dams were checked daily in the late afternoon for pups, with the day of birth designated as Day 0. The colony was maintained on a 14:10 light-dark cycle at approximately 26°C with Prolab 3000 chow and tap water available ad lib. The litters employed in this study were not culled, however, litters containing less than 8 pups were not used. Seventy pups from 10 litters yielded an N of 10/cell in each experiment.

EXPERIMENT 1

Procedure

Two 3-day-old rat pups were randomly selected from each litter. They were removed from their dam, weighed and numbered with permanent ink. Each animal had 5.0 μl of a dilute formalin solution (15%) or isotonic saline injected into the dorsal surface of the right hindpaw. This concentration and injection site are commonly used in the adult formalin test, e.g., (12), however the volume was decreased ten-fold to compensate for the pups' smaller paws. The testing apparatus consisted of a cardboard box (29.5 × 15.5 × 10.0 cm) with one side folded down to facilitate observation. A heating pad set to approximately 31°C was placed under the box to keep the pups warm during testing.

Immediately after the formalin injection and continuing for 60 min, the pups' behavior was time-sampled and recorded at 30-sec intervals. As is the case with adult versions of the formalin test (12), the pups' behaviors were categorized as: 'pawlift,' 'pawlick,' or 'other.' A score of 'pawlift' was given when the injected paw was completely lifted from the testing surface (Fig. 1). This often consisted of the pup rolling its hips to the side, away from the injected paw, with the front paws remaining on the floor (Fig. 2). A score of 'pawlick' was given if the injected paw was brought to the mouth and licked (Fig. 3). Finally, a score of 'other' was given for any other behavior. The behavior most common in this category was sleeping. For purposes of analysis, the 'pawlift' and 'pawlick' categories were collapsed and analyzed as 'recuperative behavior' unless otherwise indicated. The data in both experiments were analyzed using a repeated measures ANOVA. All animals in both experiments were euthanized immediately after testing with 0.2 ml sodium pentobarbital (50 mg/ml, IP).

Results

As can be observed in Figs. 1 and 2, the behavioral responses elicited by the formalin injection were similar to the stereotyped recuperative responses to formalin observed in adult animals [see (1) and (12) for photographs of adults and (11) for drawings]. As illustrated in Fig. 4A, there was a significant formalin by time interaction, $F(11,198) = 1.91$, $p < 0.05$. This pattern of gradual onset and plateau is similar to that observed in adults (11). Also similar to adult animals (13), only a few of the saline-injected animals displayed any recuperation. As can be seen in Fig. 4B, this resulted in a reliable difference between formalin- and saline-injected rats for formalin-induced pawlifting, $F(1,18) = 63.35$, $p < 0.0001$, and total recuperative behavior, $F(1,18) = 63.54$, $p < 0.0001$. Finally, as can be seen in Fig. 4C, several pups engaged in pawlicking. Although pawlicking was a relatively low frequency behavior, it was only observed in the pups injected with formalin [proportion of pups exhibiting at least 1 pawlick, $F(1,18) = 3.86$, $p = 0.065$].[2] Many more formalin-injected pups were noted as pulling their paws toward their mouths, as in pawlicking, however, the paw was not brought close enough to be licked. These findings are especially surprising in light of the pups'

FIG. 3. A typical example of formalin-induced pawlicking in a neonatal rat pup. (As in the first two figures, this behavior is very similar to that elicited by formalin in adult rats.)

[2]Pups exhibiting at least one pawlick received a score of 1, pups not exhibiting these behaviors were given scores of 0. An ANOVA was performed on these proportions. A discussion of this binary scoring technique for the formalin test can be found in Fanselow [p. 82, (12)].

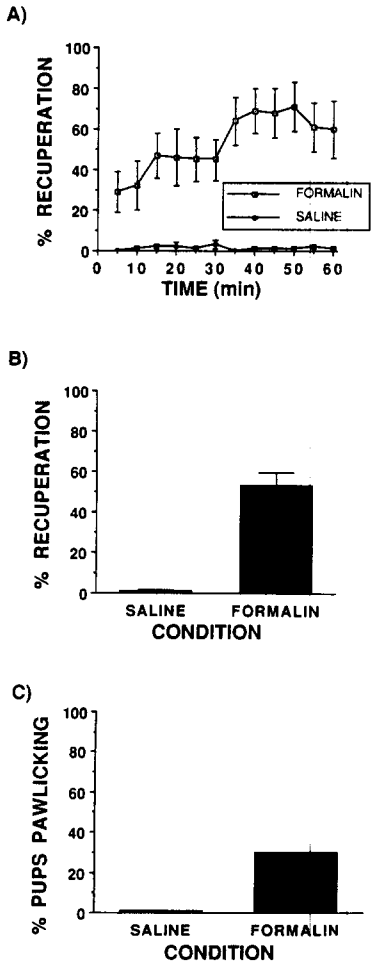

FIG. 4. (A) The percentage of samples (±SEM) scored as recuperative behavior (pawlifting and pawlicking) as a function of 5-min blocks during a 60-min test. (B) Mean (±SEM) percentage of time spent engaged in recuperative behavior during a 60-min test. (C) The proportion of pups pawlicking during a 60-min test.

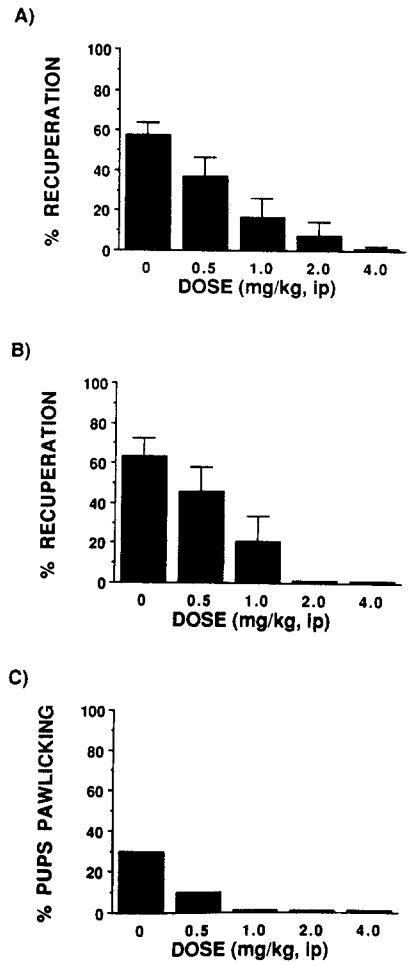

FIG. 5. (A) The effect of morphine on mean (±SEM) percentage of time spent engaged in recuperative behavior during a 60-min test. (B) The effect of morphine on mean (±SEM) percentage of time spent engaged in recuperative behavior during the 30–40-min peak-recuperation response. (C) The effect of morphine on the proportion of pups pawlicking during a 60-min test.

rather limited behavioral repertoire. Therefore, using the behavioral categories employed in the adult formalin test, pawlifting and pawlicking, formalin produced reliable effects.

EXPERIMENT 2

Reports of adult nociception indicate that the formalin test is highly sensitive to the antinociceptive properties of morphine (1). Therefore, in the second experiment, we assessed the effect of morphine on formalin-induced nociception in neonatal rats.

Procedure

Five 3-day-old rat pups were removed from each of 10 litters, weighed and numbered, following the procedure described above. The pups were injected with either morphine sulphate (0.5, 1.0, 2.0, 4.0 mg/kg, IP) or saline. Immediately after the morphine injection, all of the pups received a 5.0 μl injection of dilute formalin (15%) into the dorsal surface of the right hindpaw. The behavioral scoring and testing apparatus are described in Experiment 1. For the purposes of calculating the Analgesic Dose 50 (AD_{50}), an animal was classified as analgesic if it recuperated less than 10% of the 30–40-min peak-recuperation test period. Using regression analysis, the AD_{50} was defined as the dose at which 50% of the pups were analgesic. This criterion was previously employed by Abbott *et al.* (1) for adult rats.

Results

As can be seen in Fig. 5A, morphine attenuated recuperative behavior in a dose-dependent manner, $F(4,45)=9.67$, $p<0.001$. A linear trend analysis indicates a highly significant linear trend, $F(4,45)=36.41$, $p<0.001$, and Scheffe's test confirms that the 4.0, 2.0 and 1.0 mg/kg doses were significantly different from saline at the 0.05 level. Analysis of the 30–40-min peak-recuperation time period typically reported in adults (1) confirms these results (Fig. 5B), $F(4,45)=9.95$, $p<0.001$. Finally, morphine also attenuated the proportion of pups exhibiting at least 1 pawlick (Fig. 5C) [pawlick, $F(4,45)=2.55$, $p=0.052$].[2] There was also a significant dose by time interaction, but the onset of morphine-induced antinociception parallels the time course of responses to

formalin, such that it is difficult to tease apart the two effects (11,18). Finally, the AD_{50} calculated for pups was similar to that reported for adults. Our AD_{50} for the 30–40-min peak effect portion of the test was 0.88 mg/kg, only slightly different than the dose of 1.06 mg/kg previously reported in adults (1), suggesting that the antinociceptive properties of morphine in the formalin test of tonic nociception are comparable for adults and neonates. This is surprising in light of recent reports indicating that neonatal rats are especially sensitive to morphine-induced antinociception in the hotplate test (14).

GENERAL DISCUSSION

Our results indicate that in the formalin test of tonic nociception neonatal rat pups exhibit behavioral responses similar to those observed in adults. The presence of this stereotypic recuperative behavior in such immature animals suggests that it may depend entirely upon neurosensory and neuromotor maturation and not upon experiential factors. Thus, infant rats respond as if a variety of nociceptive stimuli are experienced as painful (4, 5, 14, 16, 18, 19, 25). The results from Experiment 2 indicate that morphine-induced antinociception is present in neonatal rat pups with a dose-response relationship similar to that previously reported in adults (1). These data complement those of others showing that morphine is a potent analgesic agent in neonatal rats (5, 14, 16, 18). Thus, while neonates may be motorically immature and experiential factors may play a role in the elaboration of pain (20), it is clear that neonatal rats do respond to a tonic pain stimulus.

Several reports in the literature suggest that neonates do not experience pain as intensely as adults [(6,20); for review see (3, 8, 17, 23)]. For example, in a recent survey of pediatric anaesthetists in the U.K. and Ireland, 13% responded that newborn infants less than one month old did not feel pain and an additional 7% were not sure (23). One result of this uncertainty is a reluctance to utilize anesthetic and antinociceptive agents in the very young (3, 21–24). Recent data, however, indicate that the stress associated with poor pain management can be detrimental, and may even increase mortality and morbidity (3). We suggest, therefore, that our adaptation of the tonic test of nociception not only demonstrates that infant rats do display the "more complex and purposive" (7) tonic nociception, but our adaptation of the formalin test may also yield a clinically relevant model of nociception in neonates. In addition, the pups' responses observed in our adaptation of the formalin test are similar to those observed in adult preparations, both behaviorally and in terms of their responsivity to morphine-induced antinociception. This will allow direct ontogenic comparison of nociception between neonates and adults not possible with the hotplate test in which both the behavioral and antinociceptive responses are somewhat different (14).

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