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Characterization of neonatal rat morphine tolerance and dependence

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Abstract

The administration of morphine and fentanyl by continuous intravenous infusion has been shown to produce analgesic tolerance and physical dependence in human neonates. In animals, daily repeated morphine bolus injections is a common method of inducing neonatal rat tolerance and dependence. Yet this method differs from the intravenous route reported to affect human neonates. Alzet osmotic minipumps were implanted in postnatal day 14 rats to provide a continuous morphine infusion more closely mimicking the clinical picture. Rats remained naive or were infused with saline or morphine (0.7 mg/kg/h) for 72 h. Morphine's antinociceptive potency was similar between naive and saline-infused animals, while morphine-infused animals were tolerant. Gender did not contribute to the degree of tolerance observed. Naloxone precipitated withdrawal in the morphine pump-implanted rats was similar to that reported by others. Thus, minipumps provide a useful model for assessing the tolerance and dependence liability of different opioids. © 1997 Elsevier Science B.V.

Keywords: Morphine tolerance, neonatal; Physical dependence, morphine; Alzet osmotic minipump

1. Introduction

Reports indicate that physicians are now more likely to use opioids to manage pain in neonates and infants than in the past (Purcell-Jones et al., 1987; Yaster, 1987; Sukhani, 1989; McLaughlin et al., 1993). Concern has developed over the possible overzealous management of pain and its consequences with the increased use of opioids. Opioids are routinely administered intravenously to provide continuous analgesia and sedation during extracorporeal membrane oxygenation and mechanical ventilation, for the treatment of life-threatening pulmonary diseases in neonates and infants (Arnold et al., 1990, 1991; Roth et al., 1991; Leuschen et al., 1993). Considerable evidence indicates that iatrogenic tolerance and dependence can develop in this population receiving morphine or fentanyl by continuous i.v. administration (Maguire and Maloney, 1988; Norton, 1988; Arnold et al., 1990, 1991; Noerr, 1991; French and Nocera, 1994; Katz et al., 1994; Franck and Vilardi, 1995). Iatrogenic tolerance was indicated when a given dose of morphine or fentanyl became ineffective and the patient required increasingly larger doses to provide the same level of analgesia observed with smaller doses. Physical dependence to morphine was characterized by the presence of withdrawal signs in 48% of infants within a 24 h period, while 50% to 84% of neonates removed from fentanyl exhibited abstinence (Norton, 1988; Arnold et al., 1990; French and Nocera, 1994).

Although the ontogeny of opioid antinociception has been examined in neonatal animals, surprisingly little is known about neonatal tolerance and physical dependence. Morphine tolerance and dependence have been observed in neonatal rats, but reports disagree about the age at which this can occur. Some researchers report tolerance in 9-day-old rats (Van Praag and Frenk, 1991; Barr and Wang, 1992), whereas others did not observe tolerance until the rats were 15-days-old (Fanselow and Cramer, 1988; Windh et al., 1995). In each of these studies, morphine was repeatedly administered by bolus injection using a variety of dosing and injection schedules. Such differences could account for the divergent results among these studies. In addition, it is likely that repeated administration leads to fluctuations in central nervous system opioid concentrations that could affect the development of tolerance. Furthermore, bolus injections require repeated handling and stress of neonatal rats and dams that could affect the development of tolerance. These concerns led us to begin using subcutaneously implanted Alzet osmotic minipumps

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to render neonatal rats tolerant and physically dependent to opioids. We have successfully used osmotic minipumps to render postnatal day 9 (P9) rats tolerant and dependent to fentanyl (Thornton and Smith, 1997). Minipumps have the advantage of delivering drug at a constant rate to provide stable plasma and tissue opioid concentrations for long periods of time. In addition, minipump drug delivery more closely mimics the intravenous route reported to induce tolerance and dependence in human neonates. Therefore, morphine-filled osmotic minipumps were used to test the hypothesis that continuous morphine administration would render neonatal rats tolerant and physically dependent to morphine.

2. Methods and materials

2.1. Source of neonatal rats

Nulliparous female Sprague Dawley dams and appropriately aged litters of 10 pups (5 females and 5 males) were purchased from Zivic–Miller (Zedianople, PA). The animals were housed in the animal care facilities at the Medical College of Virginia with a 12 h light–dark cycle, and allowed food and water ad libitum. The animals arrived at the facility at age postnatal day 8 (P8) and remained there until P14. At P14, the neonates were surgically implanted with osmotic minipumps, and remained with the dam before conducting the experiments 72 h later. Experiments were conducted with approval of the Institutional Care and Use Committee at the Medical College of Virginia.

2.2. Surgical implantation of alzet osmotic minipumps

Morphine sulfate (Research Biochemicals International, Natick, MA) was dissolved in sterile pyrogen-free isotonic saline (Baxter Healthcare, Deerfield, Il) and cold sterilized by filtration through a Millex-HV, 25 mm, 0.45 μm syringe filter (Millipore, Bedford, MA). Alzet 1003D osmotic minipumps were loaded with morphine or saline in a laminar flow hood using sterile procedures as described in detail in the *Alzet Osmotic Minipumps: Technical Information Manual* from Alza, Palo Alto, CA. The Alzet 1003D pump is recommended for animals weighing at least 10 g and infuses solution at 1 μl/h for 72 h (Alza, Palo Alto, CA). The loaded pumps were then primed by placing them in sterile isotonic saline at 37°C for 3 h before implanting them in the rats. Pump delivery beginning at 4 h (Alza) allowed the neonatal rats one hour to recover from anesthesia. Therefore, time zero of the study began one hour after implantation of the pump.

Neonatal rats at P14 were briefly anesthetized with methoxyflurane (Metofane®, Pitman–Moore, Mundelein, Il). Following induction of anesthesia (as noted by the absence of the righting reflex and foot pinch response), the pups were placed on a 37°C heating pad. The skin on the back near the base of the tail was depilated before swabbing the skin with 70% ethanol. Sterile scissors were used to make a 1 cm incision approximately 1.5 cm from the base of the tail. Alza recommends that the incision be made at the base of the neck of adult animals. However, in early implantation trials of neonates we observed that the rostral position of the incision and the pump interfered with feeding posture. By making the incision near the base of the tail the incision and caudal position of the pump did not interfere with movement and the feeding posture of the animal. After the incision, a sterile preloaded Alzet 1003D osmotic minipump was inserted under the skin and the incision was closed with Vetbond Tissue Adhesive (3M Animal Care Products, St. Paul, MN). The pump was inserted so that the delivery port was 180° from the incision to prevent drug leakage from the incision. The area was swabbed with 10% povidine–iodine, and the animal was allowed to recover. The animals were injected i.p. with 60,000 U of potassium penicillin G to prevent infection and 0.5 ml of isotonic saline s.c. to prevent hypovolemia according to IACUC (Institute on Animal Care and Use Committee) guidelines at the Medical College of Virginia. Within each litter of 5 females and 5 males, two rats were anesthetized but remained naive, while 8 were randomly assigned to receive saline- or morphine-filled pumps. The pups were returned to the dam and were challenged 72 h later with morphine s.c. for generation of dose–response curves.

2.3. Tail-flick test

For tests of antinociception, P17 rats after 72 h of drug infusion were removed from the dam and kept warm (37°C) on a heating pad. Heating pads were used because they are unable to thermoregulate on their own and hypothermia can contribute to a diminished pain sensitivity (Phifer and Terry, 1986). The tail-flick test used to assess antinociception was developed by D'Amour and Smith (1941) and modified by Dewey et al. (1970). Before injection of drug, the base-line (control) tail-flick latencies were measured for each animal. As in previous studies of neonatal rats in this laboratory, the intensity of the heat stimulus was adjusted to yield a base-line latency of 3–4 s, and a 10 s cut-off was used to prevent tissue damage (Enters et al., 1991; McLaughlin and Dewey, 1994; Thornton and Smith, 1997). Previous experiments in this laboratory have established that peak morphine antinociception in P17 rats occurs at 30 min, with antinociception lasting 180 min. Thirty min after drug administration, the test latency was measured and the data were transformed to the percentage of maximum possible effect (%MPE) according to the method of Harris and Pierson (1964). This was calculated as: $\%MPE = [(\text{test latency} - \text{control latency})/(10 - \text{control latency})] \times 100$. The ED_{50} value and 95% confidence limits for dose–response curves were

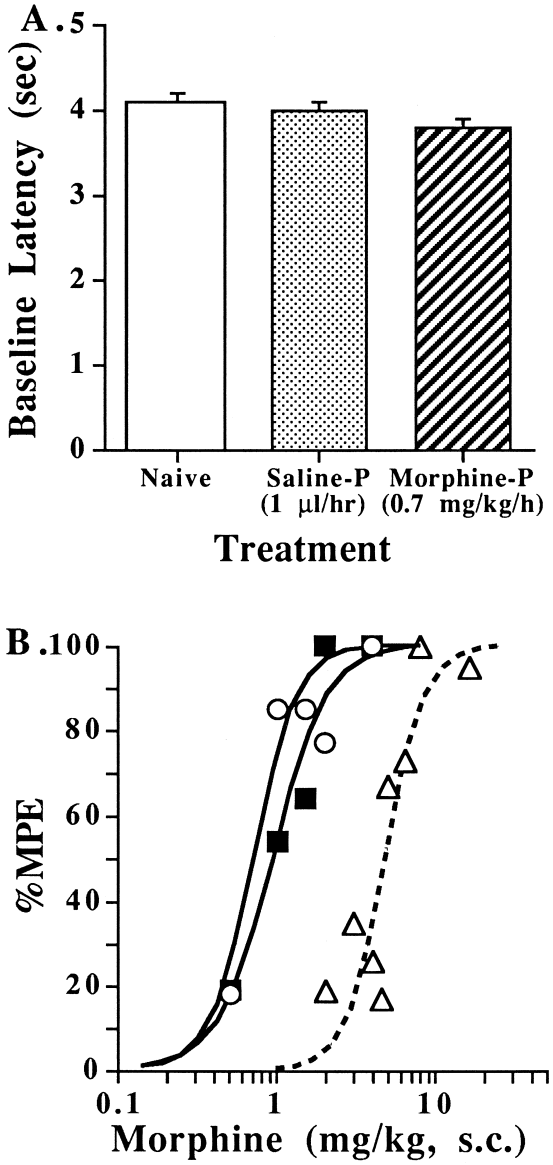

Fig. 1. (A) Base-line tail-flick latencies in naive, saline- and morphine-infused rats. P14 rats remained naive or were surgically implanted with 1003D Alzet osmotic minipumps. After 72 h of infusion of saline (1 μl/h) or morphine (0.7 mg/kg/h), base-line tail-flick latencies were obtained from P17 rats before administration of morphine, as described in panel B. (B) Tolerance to the antinociceptive effects of morphine. After obtaining base-line tail-flick latencies, morphine was administered to naive (○, ————), saline (■, ————) or morphine (▲, ---) infused P17 rats. Thirty minutes later, test latencies were obtained for calculation of %MPE.

calculated using the method of Tallarida and Murray (1987). Potency ratios and 95% C.L. were calculated using the method of Colquhoun (1971).

2.4. Withdrawal testing

Naloxone (5 mg/kg, s.c.) was administered to naive P17 neonates, or P17 neonates chronically infused with saline or morphine for 72 h. After naloxone administration, the animal was moved to a cage for a 15 min observation period. A cage measuring 50×31 cm was marked with a grid of 30 squares (8×7 cm) for measurement of spontaneous activity. The average number of lines crossed in 25 min was expressed as lines/animal. Other behaviors were quantified as the number of animals exhibiting the sign/total number of animals observed. Spontaneous activity and hypothermia were analyzed using analysis of variance (ANOVA) followed by post hoc analysis with the Tukey's test. Non-parametric data were analyzed using the χ^2 test.

3. Results

Experiments were conducted to test the hypothesis that continuous morphine administration renders P17 neonatal rats tolerant to morphine. For these experiments, P14 rats remained naive or were surgically implanted with Alzet 1003D osmotic minipumps as detailed in Section 2. Rats at P14 were infused for 72 h with saline (1 μl/h) or morphine (0.7 mg/kg/h), and at P17 were tested for tolerance. Baseline tail-flick latencies following 72 h of infusion were not different among the groups (Fig. 1A). However, the potency of acutely administered morphine was significantly reduced in rats infused at 0.7 mg/kg/h (Fig. 1B). Not only was the ED_{50} value increased (Table 1), but morphine's potency was decreased 4.9-fold. It is noteworthy that saline-pump implanted rats exhibited similar ED_{50} values as naive animals, which indicated the absence of a pump effect on antinociception. The role of gender in the degree of morphine tolerance was also examined. As seen in Table 2, naive male and female rats were equally sensitive to the acute antinociceptive effects of morphine. In addition, both male and female morphine-infused rats were equally tolerant to the acute antinociceptive effects of morphine 72 h later. The ED_{50} values between tolerant male and female rats were not different, which indicated the absence of an effect of gender in tolerance development.

It could be argued that neonatal maturation during the 72 h infusion from P14 to P17, and not tolerance, con-

Table 1
Tolerance to morphine in P17 neonatal rats after a 72 h infusion of morphine (0.7 mg/kg/h) from osmotic minipumps. P14 rats remained naive or were implanted with an osmotic minipump containing saline or morphine. Seventy-two hours later, antinociception in P17 rats was measured in the tail-flick test 30 min after s.c. administration of morphine. ED_{50} values and potency ratio values with accompanying 95% confidence limits were calculated using least squares linear regression analysis of graded dose–response data

Treatment	ED_{50} (mg/kg)	Potency ratio
Naive	0.6 (0.4 to 0.9)	
Saline pump (1 μl/h)	0.9 (0.7 to 1.2)	1.3 (0.9 to 1.9)
Morphine pump (0.7 mg/kg/h)	4.7 (4.0 to 5.5)[a]	4.9 (4.0 to 6.3)[a]

[a] Significantly different from saline-pump implanted rats.

Table 2
Role of gender in morphine tolerance in P17 neonatal rats after a 72 h infusion of morphine (0.7 mg/kg/h) from osmotic minipumps. P14 rats remained naive or were implanted with an osmotic minipump containing saline or morphine. Seventy-two hours later, antinociception in P17 rats was measured in the tail-flick test 30 min after s.c. administration of morphine. ED_{50} values and potency ratio values with accompanying 95% confidence limits were calculated using least squares linear regression analysis of graded dose–response data

Treatment	Male ED_{50}	Female ED_{50}
Naive	0.4 (0.1 to 1.5)	0.5 (0.3 to 0.9)
Saline pump (1 μl/h)	1.0 (0.6 to 1.5)	1.0 (0.8 to 1.3)
Morphine pump (0.7 mg/kg/h)	6.2 (3.4 to 11.3)[a]	5.0 (4.3 to 5.9)[a]

[a]Significantly different from saline pump-implanted rats.

tributed to the apparent reduction in the potency of morphine on P17. However, morphine ED_{50} values in naive P14 and P17 rats were not significantly different (P14 = 1.4 mg/kg versus P17 = 1.9 mg/kg). It could also be argued that neonatal maturation may have affected the distribution of morphine into the brain. Animals injected with antinociceptive doses of morphine also received [^{3}H]morphine (0.5 μCi) for estimation of tissue levels as described by Thornton and Smith (1997). Our results indicate that the brain effective concentration-50 values (i.e., nanograms of morphine equivalents/gram brain tissue that elicit a 50% MPE) were not statistically different between P14 and P17 rats (P14 = 183 ng/g versus. P17 = 259 ng/g). Therefore, neither maturational effects on antinociception nor morphine tissue distribution played a role in the development of morphine tolerance.

Table 3
Behavioral profile of morphine withdrawal precipitated by 5 mg/kg naloxone in P17 rats. Rats at P14 remained naive or were infused for 72 h with saline (1 μl/h) or morphine (0.7 mg/kg/h) from osmotic minipumps. At P17 the rats injected with naloxone were observed for 15 min for signs of dependence. These behaviors are indicated as the number of animals exhibiting the sign to the total number of animals observed

Withdrawal signs	Naive	Saline pump	Morphine pump
Spontaneous activity (lines/animal)	39	32	338[a]
Micturition	1/6	0/6	3/6
Defecation	5/6	5/6	6/6[c]
Face washing	6/6	4/6	3/6
Wall climbing	0/6	0/6	6/6[b]
Abnormal posture	0/6	0/6	6/6[b]
Forepaw treading tremors	0/6	0/6	2/6
Scream on touch	0/6	0/6	1/6
Wet-dog shakes	0/6	0/6	4/6
Spontaneous jumping	0/6	0/6	5/6[b]
Mastication	4/6	3/6	6/6
Ptosis	0/6	0/6	6/6[b]
Rhinorrhea	0/6	0/6	4/6
ΔT_b (°C)	0.0	−0.4	−1.9[a]

[a]Significantly different from respective saline pump-implanted rats, ANOVA with post hoc Tukey's test.
[b]Significantly different from respective saline pump-implanted animals, χ^2
[c]Diarrhea.

The hypothesis was also tested that continuous morphine administration renders P17 rats physically dependent to morphine. For these experiments, P14 rats remained naive or were surgically implanted with osmotic minipumps. Rats at P14 were infused for 72 h with saline (1 μl/h) or morphine (0.7 mg/kg/h), and at P17 were tested for dependence. Morphine pump-implanted P17 rats administered naloxone (5 mg/kg, s.c.) displayed a precipitated withdrawal syndrome (Table 3). Morphine-infused rats displayed the highest level of spontaneous activity, which often resulted in wall climbing behavior. Although many naive and saline-rats defecated, only the morphine pump-implanted rats exhibited diarrhea. All of the morphine-infused rats exhibited abdominal stretching similar to the visceral nociception elicited by *p*-phenylquinone. Other signs of dependence included forepaw treading/tremors, spontaneous jumping, ptosis, rhinorrhea and hypothermia.

4. Discussion

4.1. Acute antinociceptive effects of morphine in neonatal rats

Naive P17 rats injected with morphine exhibited dose-dependent antinociception (Fig. 1B, Table 1). These results indicate that μ-opioid systems are sufficiently developed for neonatal rats to respond to morphine. Based on comparisons between human age and the rat neonatal period, P14 to P17 rats are comparable to full-term human infants (Dobbing, 1974). In neonatal rats as young as P3 — which are comparable to preterm human infants — dose-dependent morphine antinociception in the tail-flick test and formalin test have been demonstrated (McLaughlin and Dewey, 1994). We have also shown that morphine is active in P3 rats, with potency increasing and reaching a plateau from P9 to P21 (unpublished findings). Thus, from P9 to P21 morphine's potency did not vary significantly. Others have similarly demonstrated an age-related increase in the potency of morphine (Auguy-Valette et al., 1978; Zhang and Pasternak, 1981). Surprisingly, these findings parallel another report indicating that brainstem μ-opioid receptor density progressively increases in neonatal rats from P1 to P21 (Xia and Haddad, 1991). Thus, throughout the neonatal period opiates such as morphine are effective antinociceptive agents.

4.2. Morphine tolerance in neonatal rats

Our results support the hypothesis that continuous morphine administration renders neonatal rats tolerant to morphine. The first evidence of tolerance was the absence of

antinociception to the morphine infused from the pump, as indicated by comparable baseline tail-flick latencies among the groups (Fig. 1A). The second evidence of tolerance was the finding that the potency of acutely administered morphine in morphine-infused rats was reduced relative naive and saline-infused rats (Fig. 1B). To our knowledge, this is the first reported attempt at using osmotic minipumps to render neonatal rats tolerant to morphine, although fentanyl tolerance has been demonstrated (Thornton and Smith, 1997). Thus, osmotic minipumps may provide a reliable method of examining the tolerance and dependence liability of many opioids in neonatal rats.

Other groups have utilized repeated bolus injections to render neonatal rats tolerant and dependent to morphine (Fanselow and Cramer, 1988; Kitchen and Boswell, 1990; Van Praag and Frenk, 1991; Barr and Wang, 1992; Jones and Barr, 1995; Windh et al., 1995). However, controversy exists about the earliest neonatal age at which tolerance and dependence can occur. Morphine tolerance has been reported in rats as young as P7 (Barr and Wang, 1992), although investigators in two studies did not observe tolerance until P15 (Fanselow and Cramer, 1988; Windh et al., 1995). Others have observed tolerance in P9, P10, P15, P20 and P27 rats (Kitchen and Boswell, 1990; Van Praag and Frenk, 1991). Furthermore, physical dependence has been observed in neonatal rats as young as P7 (Jones and Barr, 1995). In each of these studies, morphine was repeatedly administered by bolus injection using a variety of dosing and injection schedules. Such differences could account for the divergent results among these studies. Thus, with the exception of two studies, tolerance and dependence has been observed throughout the neonatal period. More support for even earlier effects have been noted with prenatal opioid exposure. Dams exposed to morphine or methadone give birth to physically dependent neonatal rats (Lapointe and Nosal, 1982; Enters et al., 1991). Thus, the majority of data, including ours, indicates that neonates are susceptible to opioid tolerance and dependence throughout the neonatal period.

However, what are the advantages of osmotic minipumps if others have induced morphine tolerance and dependence by repeated bolus injection? A major strength of the model is that it closely mimics the continuous i.v. infusion regimens reported to induce tolerance and dependence in human neonates (Maguire and Maloney, 1988; Franck and Vilardi, 1995; Mainous, 1995). With bolus administration morphine is usually injected once or twice daily, during which time tissue morphine levels likely fluctuate between injections. Some spontaneous withdrawal may occur between doses which could affect the development of tolerance and dependence. Minipumps can deliver drug at a constant rate for extended periods of time. In addition, the infusion pump dose can be adjusted to alter the observed degree of tolerance and dependence. We are currently examining the influence of morphine infusion dose in neonatal rats. Furthermore, by infusing drug continuously minipumps may reduce the toxicity often associated with bolus injection of drug.

Another potential confound to the development of tolerance and dependence with bolus drug administration is the required repeated handling and stress of the neonatal rats and dams. Several studies indicate that handling stress in neonatal rats alters beta-endorphin levels, serotonin turnover and receptor binding, and GABA receptor binding (Iny et al., 1987; Bolden et al., 1990; Smythe et al., 1994). Injection stress has been implicated in the inability of repeated cocaine administration to induce behavioral sensitization in neonatal rats (Meyer and Yacht, 1993). Regarding opioid antinociception in adult rats, restraint stress presumably potentiates opioid antinociception through endogenous opioid systems (Adams et al., 1987; Levesque and Holtzman, 1993). Thus, the stress of repeatedly handling and injecting neonatal rats may ultimately affect the degree of opioid tolerance and dependence. Osmotic minipump implantation limits the handling of neonates and the dam to a single period, thus reducing the potential confound of stress.

4.3. Role of gender in morphine tolerance

It was also important to assess whether gender contributed to the expression of neonatal rat morphine tolerance. Several reports indicate that sexually mature male and female rats are differentially sensitive to opioid antinociception (Islam et al., 1993; Kepler et al., 1991), as well as non-opioid antinociception (Kiefel and Bodnar, 1992). Specifically, centrally administered morphine elicited a greater magnitude of antinociception in male than in female rats (Kepler et al., 1991). However, our results clearly demonstrate two findings: (1) naive male and female rats were equally sensitive to the acute antinociceptive effects of morphine, and (2) morphine-infusion produced an equal degree of tolerance between male and female rats (Table 2). Thus, in the early postnatal period gender does not play a role in morphine antinociception and tolerance. To our knowledge, the earliest ages at which gender begins to affect opioid activity remains to be determined.

4.4. Morphine-induced physical dependence

Our results support the hypothesis that continuous morphine administration renders neonatal rats physically dependent to morphine. Following naloxone administration, the morphine-infused rats exhibited a robust withdrawal syndrome. The primary signs were spontaneous activity — which often resulted in wall climbing behavior — diarrhea, abdominal stretching, forepaw treading/tremors, spontaneous jumping, ptosis, rhinorrhea and hypothermia. These signs were very similar to those observed in P14 rats chronically treated with morphine by repeated injection (Jones and Barr, 1995). Several lines of evidence

indicate that these behaviors constitute a true withdrawal syndrome. First, the similarity of signs with Jones and Barr (1995) suggests that physical dependence was the result of chronic μ-opioid receptor activity. Second, these signs occur in neonates undergoing either passive or precipitated withdrawal. And third, these signs were triggered by naloxone only in morphine-infused rats. The onset of signs began almost immediately, peaked by 10 min, and were reduced in severity by 25 min. Other signs such as weight loss and ultrasonic vocalization have been reported in neonatal rats chronically treated with morphine (Fanselow and Cramer, 1988; Barr and Wang, 1992). Therefore, chronic administration of morphine by osmotic minipump resulted in neonatal rats becoming dependent on morphine.

4.5. Summary

In conclusion, chronic morphine administration via osmotic minipumps appears to render neonatal rats tolerant and dependent to morphine. Future studies will be conducted to examine the long-term consequences of chronic postnatal opioid exposure, so that the potential effects of iatrogenic tolerance and dependence in human infants can be examined.

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