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Neonatal Withdrawal Following Pre- and Postnatal Exposure to Methadone in the Rat

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BARR, G. A., A. ZMITROVICH, A. S. HAMOWY, P. Y. R. LIU, S. WANG AND D. E. HUTCHINGS. *Neonatal withdrawal following pre- and postnatal exposure to methadone in the rat.* PHARMACOL BIOCHEM BEHAV **60**(1) 97–104, 1998.— Recent evidence has shown that infant rats undergo precipitated withdrawal following chronic postnatal injection of morphine. In this study we examined whether or not infants exposed to methadone prenatally via the placental blood supply and postnatally via the dam's milk would also experience precipitated withdrawal. Dam's were implanted on gestational day 14 with osmotic minipumps containing one of two concentrations of methadone to supply the opiate throughout gestation and the first postnatal week. Nontreated and pair-fed controls were used. On postnatal day 7, pups were injected with naltrexone and their locomotor activity and ultrasonic vocalizations measured. Methadone exposed pups were more active and vocalized more when injected with naltrexone than with saline. The controls did not show these behavioral changes. The milk of methadone-exposed dams apparently contains sufficient quantities of the opiate for dependence to develop. The results are consistent with other data that demonstrate that very young rat pups can experience an opiate abstinence syndrome that includes increased behavioral activation. © 1998 Elsevier Science Inc.

Abstinence Development Locomotor activity Methadone Opiates Osmotic minipumps Ultrasonic vocalizations Withdrawal

THE neonatal abstinence syndrome that occurs following prenatal exposure to opiates has been well described for the human newborn (9,10), although factors such as multiple drug abuse, nutritional deficits, and poor prenatal care make assessment of the direct effects of opiates quite difficult. One obstacle to developing an adequate animal model of human infant withdrawal from opiates has been the difficulty in demonstrating convincingly that opiate abstinence occurs in the neonatal rat. The earliest age in the rat that adult-like withdrawal symptoms, such as wet dog shakes, diarrhea, and weight loss, occur is around puberty, between 42 and 52 days of age (6,16). The opioid antagonist naloxone administered to neonatal rats following prenatal exposure to methadone consistently failed to induce similar behavioral effects that could be interpreted as representing abstinence (Hutchings, unpublished data). That infants do not show adult-like signs of withdrawal suggests that the physiological systems that mediate the "classical" signs of opioid abstinence and that are readily seen in the adult are immature in the infant rat.

Recently, the existence of an opiate withdrawal syndrome prior to adolescence was shown by measuring behaviors that are appropriate to the age of the developing animal. These responses in the preweaning rat pup include, among others, head and paw movements, stretching, rolling, wall climbing, increased activity, decreased time spent with littermates, and changes in separation induced crying (2,16,20). The nature of the abstinence syndrome changes during development, always including behaviors that are appropriate to the age of the animal, eventually becoming adult like around puberty (6,16).

Methadone is the only drug approved to treat opiate dependence during pregnancy, and does not appear to have any increased long-term neurobehavioral risks for children. Methadone is associated, however, with a neonatal abstinence syndrome characterized by biphasic but transitory CNS arousal.

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The acute phase is characterized by incessant crying, poor state regulation, and hyperactivity that can last several days to weeks. The subacute phase is characterized by restlessness and poor sleep and can persist for 4 to 6 months. Thereafter, the symptoms appear to completely resolve [for review, see (10,12)]. In the present study, we were interested in whether an opiate withdrawal syndrome could be elicited in pups that were exposed to methadone via the placental blood supply and milk. There were four goals. First, we wanted to replicate and extend our morphine observations using methadone, a different opiate. Second, we wanted to expose pups to methadone through the dam's milk, a route of administration more similar to that of the human infant. Human infants exposed in this manner do receive methadone through milk ingestion (18). Third, because our previous work had suggested that there was increased behavioral activity during morphine withdrawal, we measured locomotor activity following precipitated withdrawal in methadone exposed pups. Finally, we wanted to explore further the possible use of ultrasonic vocalizations (USVs) as a metric for withdrawal from methadone in the infant. In part, this entailed determining if pups in precipitated withdrawal would cry more than controls given naltrexone, as they do for morphine given directly to pups (2). The presence of a littermate reduces the vocalizations of the isolated pup. This phenomenon, termed the "companion comfort" effect, is prevented by opiate antagonists, suggesting that the reduction in USV is mediated by the release of endogenous opioids induced by the presence of the companion (5). It was possible that chronic exposure to methadone altered the opioid circuitry such that the quieting effect of the companion is changed. Thus, we examined the companion effect in these studies.

METHOD

Animals and Timing of Pregnancy

Individual nulliparous Wistar females weighing 200–224 g (Hilltop Lab Animals, Inc., Scottdale, PA) were paired with males of the same strain in hanging wire cages. The pans beneath the cages were examined by noon for the presence of sperm plugs. The day a plug was found was designated gestation day 1 (G1). Gravid dams were then randomly assigned to receive one of two doses of methadone, to a nontreated control group, or assigned by weight to a pair-fed control group, with each dam fed an equivalent amount of food as that consumed by a matched subject in the high dose of methadone group (13). All animals were housed in standard Plexiglas cages on wood chips. All dams except those in the pair-feeding condition had continuous access to Purina Lab Chow and water. Lights automatically came on at 0600 h and went off at 1800 h.

Drug Administration

Fourteen-day osmotic minipumps (Alza, Palo Alto, CA) were filled with either saline or one of two concentrations of methadone hydrochloride (Sigma) dissolved in sterile water. A stock concentration of 80 mg/ml was diluted to achieve an average delivery dose of either 9 mg/kg/day (lower dose) or 14 mg/kg/day (higher dose). Pumps were implanted on gestation day (G) 16 and left in place through parturition until testing at postnatal day 7 [for a detailed description of the surgical procedure, see (14)]. The two doses of methadone administered varied over gestation and postnatal days as determined by weight of the dam: the mean lower dose was 8.7 mg/kg/day of G16, 9.0 on day of parturition, and 9.3 on postnatal day 6; the higher mean dose was 13.2 mg/kg/day on G16, 13.8 on day of parturition, and 14.3 on postnatal day 6. Pair feeding and watering were carried out using a yoked design (13). Nontreated controls were left undisturbed. Within 1–5 h after birth, all treated and control offspring were sexed and weighed. Litters were culled when necessary to 12 pups; litters containing fewer than six pups were also sexed and weighed but excluded from further testing.

Assessment of Physical Dependence in Dams

On postnatal day 7, dams were administered 1 mg/kg of naltrexone hydrochloride, and placed in a standard plastic cage for a 30-min observation period. Using a check list previously described (14), the occurrence or frequency of the following withdrawal symptoms were recorded: diarrhea, ptosis, facial tremors (present or absent), wet dog shakes, teeth grinding, and stretching (maximum count of three for each). In addition, an irritability score was assigned based on the animals' reaction (i.e., squealing) in response to handling (ranked from 0 to 3). A composite "abstinence" score was derived from these observational data. In addition, animals were weighed at the beginning and at the end of the 30-min test period. Together, the abstinence score and change in body weight provided a measure of the severity of withdrawal. Immediately after testing, to assess resorptions, all experimental and control dams were sacrificed to determine the number of implantation sites.

Offspring Testing

USV in the isolate. All behavioral tests of the pups occurred on postnatal day 7. Measurement of ultrasonic vocalization (USV) was carried out according to Hofer and Shair (8). USV was measured using an ultrasonic bat-detector (Ultrasound Advice, QMC Instruments, Model S25, London, UK) set at 40 kHz and a capacitor microphone suspended approximately 10 cm above a 20 × 19 × 21 cm Plexiglas cage. Earphones were used to avoid feedback effects to the pups, and USVs were counted using a soundless electronic counter. An interobserver reliability was 97%, but to control further for individual differences, only one observer was used per litter to allow an accurate comparison between naltrexone- and saline-treated animals within that litter. Twenty minutes prior to challenge, dams were removed and their pups placed on a heating pad maintained at 35–37°C. Pups were sexed, weighed, and marked with an odorless marker. Only six pups per litter were tested, two in the isolation test and two pairs in the companion test. Pups were administered naltrexone (1.0 mg/kg) or an equal volume (10 ml/kg) of saline intraperitoneally, returned to the litter, and tested 15 min later. The dose of naltrexone used was substantial enough to inhibit competitively morphine at the relevant opioid receptor. In studies of precipitated withdrawal in both adults and infants dose–response curves are reasonably flat (3,16).

Companion tests. Companion tests were conducted in which two pups were tested together. Both were administered naltrexone or saline, placed in the test cage, and the summed ultrasounds from both pups counted for 6 min. No attempt was made to separate vocalizations of the individual pups. Within the nontreated, pair-fed, and methadone groups, the order of testing (isolate/companion), sex, and order of drug treatment (naltrexone/saline) were balanced across litters.

Activity testing. Pups were separated from their dam and placed on a heating pad. To dose the entire litter as quickly as possible, each pup was weighed and the mean pup weight used to determine the dosage of naltrexone for each pup in

TABLE 1
MATERNAL AND OFFSPRING EFFECTS

	Nontreated (NT)	Pair-fed (PF)	METH-9	METH-14
Total pregnant (n)	29	21	26	35
Deaths (n)	0	0	0	0
Litters tested (usv/act) (n/n)	15/11	9/9	11/12	12/12
Maternal weight gain (g)	186.7 ± 5.8*	137.8 ± 5.2	178.6 ± 4.8*	176.2 ± 5.9*
% Perinatal mortality	5.3 ± 3.0	10.2 ± 4.2	13.2 ± 4.6†	19.8 ± 4.6*†
% Total offspring mortality	13.9 ± 4.0	20.7 ± 5.6	22.3 ± 4.7	32.1 ± 5.1†
% Born live				
Male	52.7 ± 2.4	54.9 ± 3.4	48.9 ± 2.7	48.8 ± 3.3
Female	47.3 ± 2.4	45.1 ± 3.4	51.1 ± 2.8	51.1 ± 3.3
Litter size	14.9 ± 0.6	13.4 ± 1.0	14.2 ± 0.6	12.6 ± 0.9
Birthweight (g)				
Male	7.2 ± 0.1	6.4 ± 0.1†	6.6 ± 0.1†	6.6 ± 0.1†
Female‡	6.7 ± 0.1	6.2 ± 0.1†	6.2 ± 0.1†	6.3 ± 0.1†
Day 7 weight (g)				
Male	18.7 ± 0.2	15.0 ± 0.5†	15.7 ± 0.5†	15.5 ± 0.4†
Female‡	17.8 ± 0.3	14.6 ± 0.6†	15.2 ± 0.4†	15.3 ± 0.3†

*$p < 0.001$ significantly different from PF.
†$p < 0.013$ significantly different from NT.
‡$p < 0.01$ significantly different from males.
n = number of litters tested.

the litter. Half of the pups were administered 1.0 mg/kg naltrexone and the remainder an equal volume of saline and placed immediately in separate test cages on activity monitors. The two cages were balanced for number of pups and sex [for a detailed description of the activity equipment and its operation, see (11)]. Activity was measured for 30 min, beginning with the first full minute after the test cage was placed on the monitor. Activity testing was performed between 0900 and 1100 h, and ultrasound tests between 1100 and 1600 h. Litters were tested either for activity or ultrasound, never both.

Statistical Analysis

An analysis of variance (ANOVA) was performed on normally distributed data including maternal weight gain, implantation sites, litter size, birth weights, activity, and vocalizations. The Kruskal–Wallis nonparametric test was used to analyze maternal and offspring effects expressed as proportions and Mann–Whitney *U*-tests used for post hoc comparisons. The litter was used as the unit of analysis when multiple pups in each litter were used, and the within-litter factor was treated as a repeated measures variable.

RESULTS

Morbidity and Mortality

We have previously reported on these two dose levels of methadone administered on the last two weeks of gestation and their effects on maternal food and water intake and maternal and offspring toxicity (13,14). These data were collected in the present study but are not presented here. Table 1 shows the *n*'s for the various phases of the study and maternal and offspring effects. There were no maternal deaths in any of the treated or control groups. The percent of perinatal mortality (i.e., pups either stillborn or found dead between postnatal days 0–5) was significantly increased for both doses of methadone compared with nontreated controls ($K_3 = 14.09, p < 0.01$, post hoc comparisons, Mann–Whitney *U*-test). At birth males weighed more than females across all groups, $F(1,106) = 75.57$, $p < 0.001$, and for both sexes, the pair-fed, lower dose, and higher methadone-dosed pups weighed less than the nontreated controls [significant one-way ANOVA across males, $F(3,105) = 6.55$, $p < 0.001$, significant post hoc univariate *F*-tests, $p < 0.005$; significant one-way ANOVA across females, $F(3,103) = 3.85$, $p < 0.02$, significant post hoc univariate *F*-tests, $p < 0.02$]. This sex and group difference was still present on postnatal day 7, the day of testing, and was the same for both sexes [significant one-way ANOVA across males, $F(3,86) = 19.09$, $p < 0.001$, with significant post hoc univariate *F*-tests, $p < 0.001$; significant one-way ANOVA across females, $F(3,86) = 14.01$, $p < 0.001$, with significant post hoc univariate *F*-tests, $p < 0.001$].

Maternal Withdrawal Scores

Table 2 shows that in response to naltrexone there were significant differences between treated and control dams in weight loss, $F(3,83) = 52.02$, $p < 0.001$, and abstinence ($K_3 =$

TABLE 2
ABSTINENCE SCORE

Group	*n*	Weight Loss (g)	Mean ± SEM	Median
Nontreated (NT)	19	2.7 ± 0.4	1.6 ± 0.4	1
Pair-fed (PF)	20	2.7 ± 0.4	2.0 ± 0.3	2
METH-9	25	10.4 ± 0.7*	7.7 ± 0.5*	8*
METH-14	24	11.4 ± 0.8*	7.7 ± 0.5*	8*

Weight loss reported occurred during the 30-min test period. Abstinence scores above are derived from the sum of the following observations during the 30 min; diarrhea, ptosis, and facial tremors (present or absent); sensitivity during a hand-held test at min 5 (ranked from 0 to 3); wet dog shakes, teeth grinding, and stretching (max. count of three for each).
*$p < 0.001$ compared to NT and PF.

61.55, $p < 0.001$), Post hoc analyses showed that both the lower and higher dosed dams lost more weight during the 30-min observation period than either control group ($p < 0.001$). The methadone-treated dams had higher scores than the dams in either control group ($p < 0.001$), although the lower dose and higher dose dams did not differ from each other. The incidence of diarrhea, wet-dog shakes, and teeth grinding were particularly elevated. These results are comparable in magnitude to those reported previously (14).

Offspring Activity Measure

Activity counts are shown in Fig. 1. There were no significant differences in activity between any of the treated or controls groups administered saline, although the pair-fed animals, whether administered naltrexone or saline, tended to be less active than the untreated controls. Pups in both methadone groups increased their activity following naltrexone administration compared with saline injected pups of the same group [lower dose: $F(1,11) = 5.47$, $p < 0.05$; higher dose: $F(1,11) = 4.97$, $p < 0.05$].

Ultrasonic Vocalizations

There were no significant differences between male and female pups in the rate of ultrasound vocalization; therefore, the data were combined within groups for subsequent analysis. Figure 2 shows the mean number of USVs for methadone-treated and control groups over the entire 6-min isolation test session. An overall analysis of variance showed an increased rate of ultrasonic vocalization in all treated and control pups administered naltrexone compared to littermates administered saline, $F(1,36) = 8.37$, $p < 0.01$. Individual ANOVAs showed that this increase was significant only for the higher

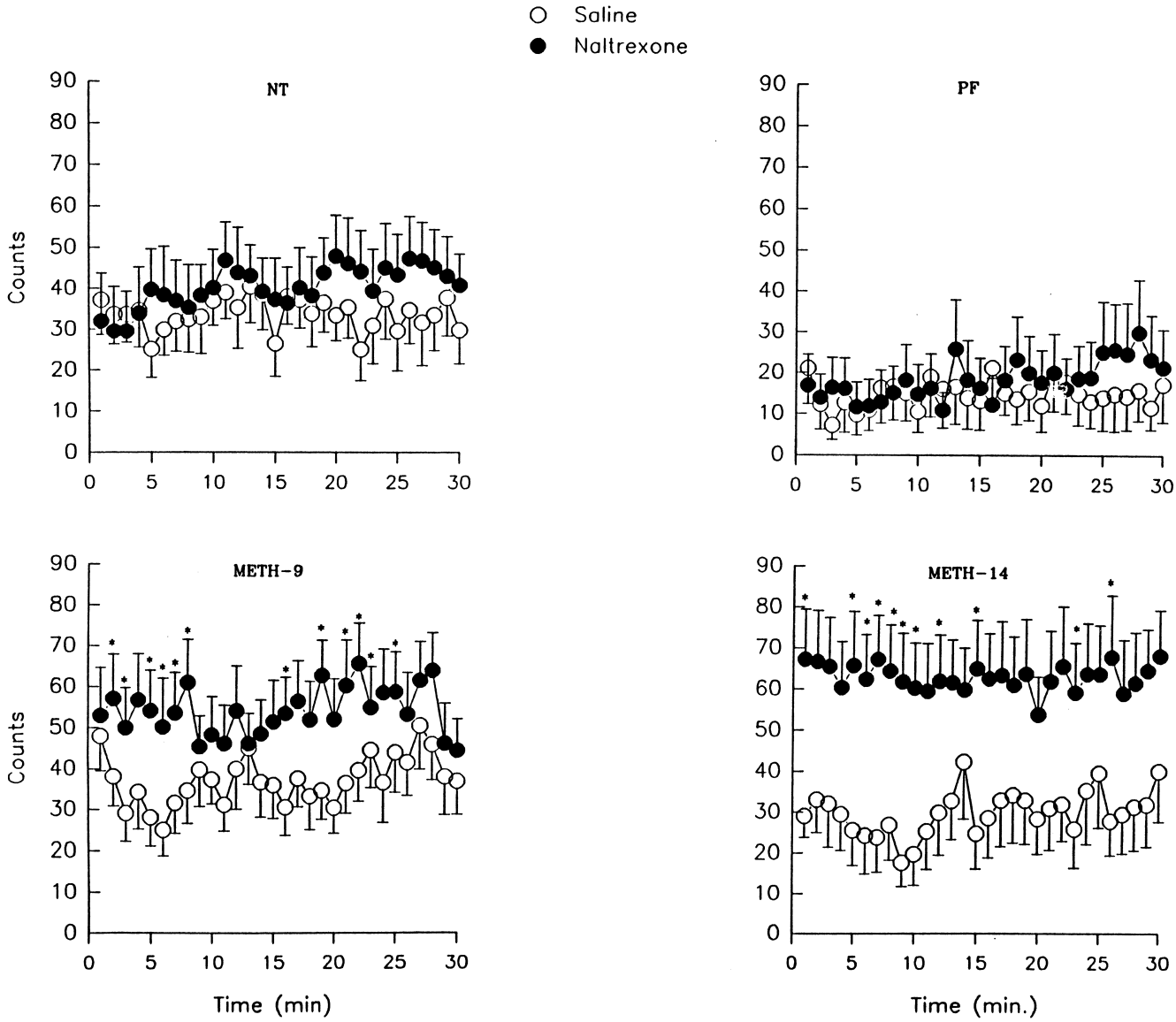

FIG 1. Activity counts (mean ± SEM) for the four experimental conditions. NT: nontreated controls; PF: pair-fed controls; METH-9: lower dose of methadone (9 mg/kg/day); METH-14: higher dose of methadone (14 mg/kg/day). Activity was increased by naltrexone only in pups exposed to methadone.

dose methadone group, $F(1,10) = 6.56$, $p < 0.05$. There was also an interaction of chronic treatment with test minute, $F(15,180) = 3.16$, $p < 0.001$; the low-dose methadone group and the untreated controls showed a relatively flat pattern of responding, whereas the pair-fed group cried at high levels initially and declined, and the pups treated with the higher dose of methadone increased crying over the course of the test. There were no significant changes in ultrasonic vocalizations for methadone exposed pups given naltrexone for the companion test (Fig. 3). When individual ANOVAs were conducted, there was an effect in the pair-fed controls in which pups administered naltrexone showed a significant increase in number of ultrasounds compared to those administered saline, $F(1,7) = 7.22$, $p < 0.05$.

DISCUSSION

The effects of methadone on maternal food and water intake, maternal weight gain, perinatal mortality, and birth weight were the same as previously reported (13,14,22). Methadone treatment reduced maternal food and water consumption, an effect also seen in previous work (13,14); neither dose

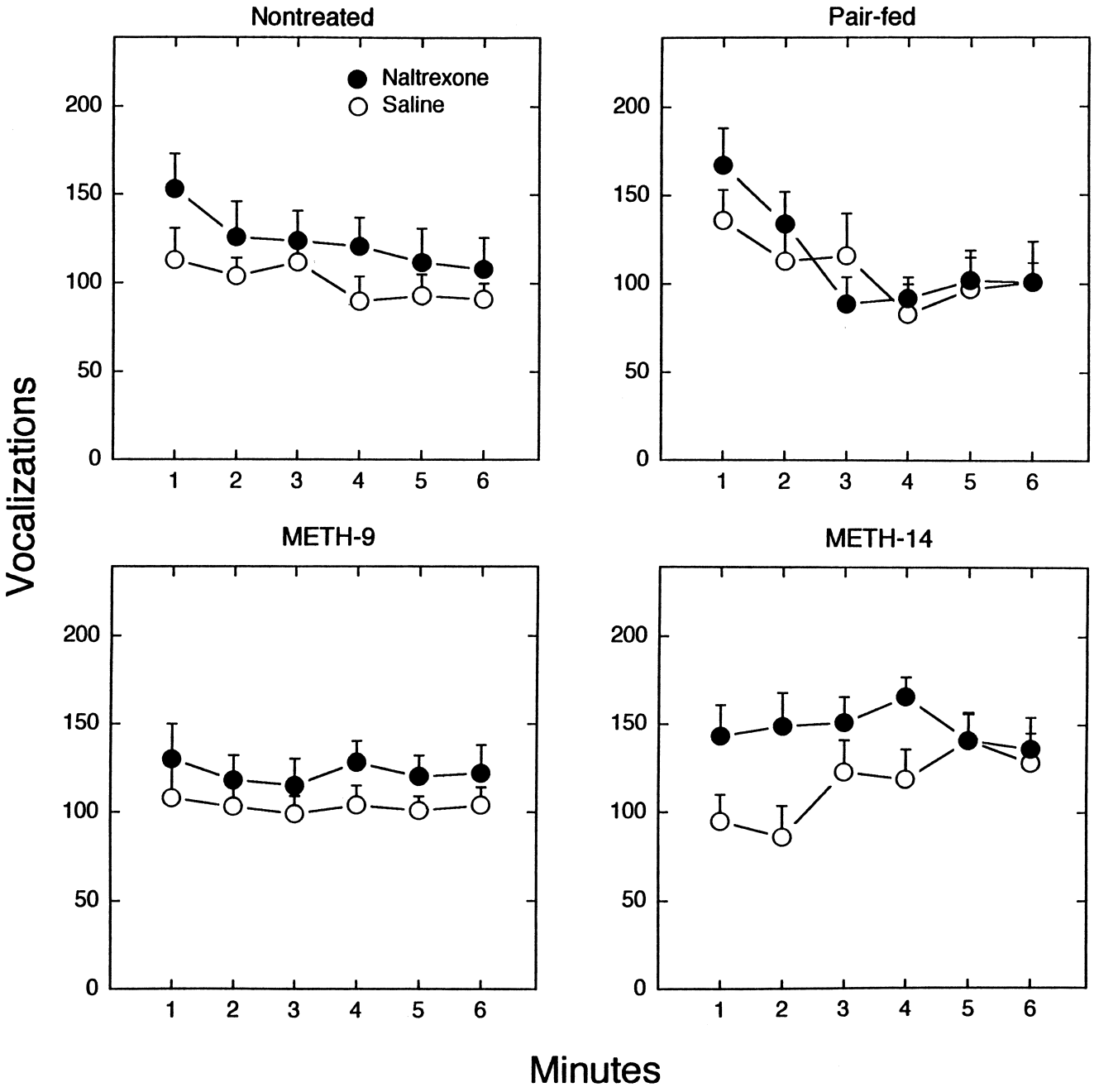

FIG. 2. Ultrasonic vocalizations per minute (mean ± SEM) in a 6-min isolation test for each condition following acute treatment with naltrexone or saline. Abbreviations are as in Fig. 1. Vocalizations were significantly increased by naltrexone only in the METH-14 group.

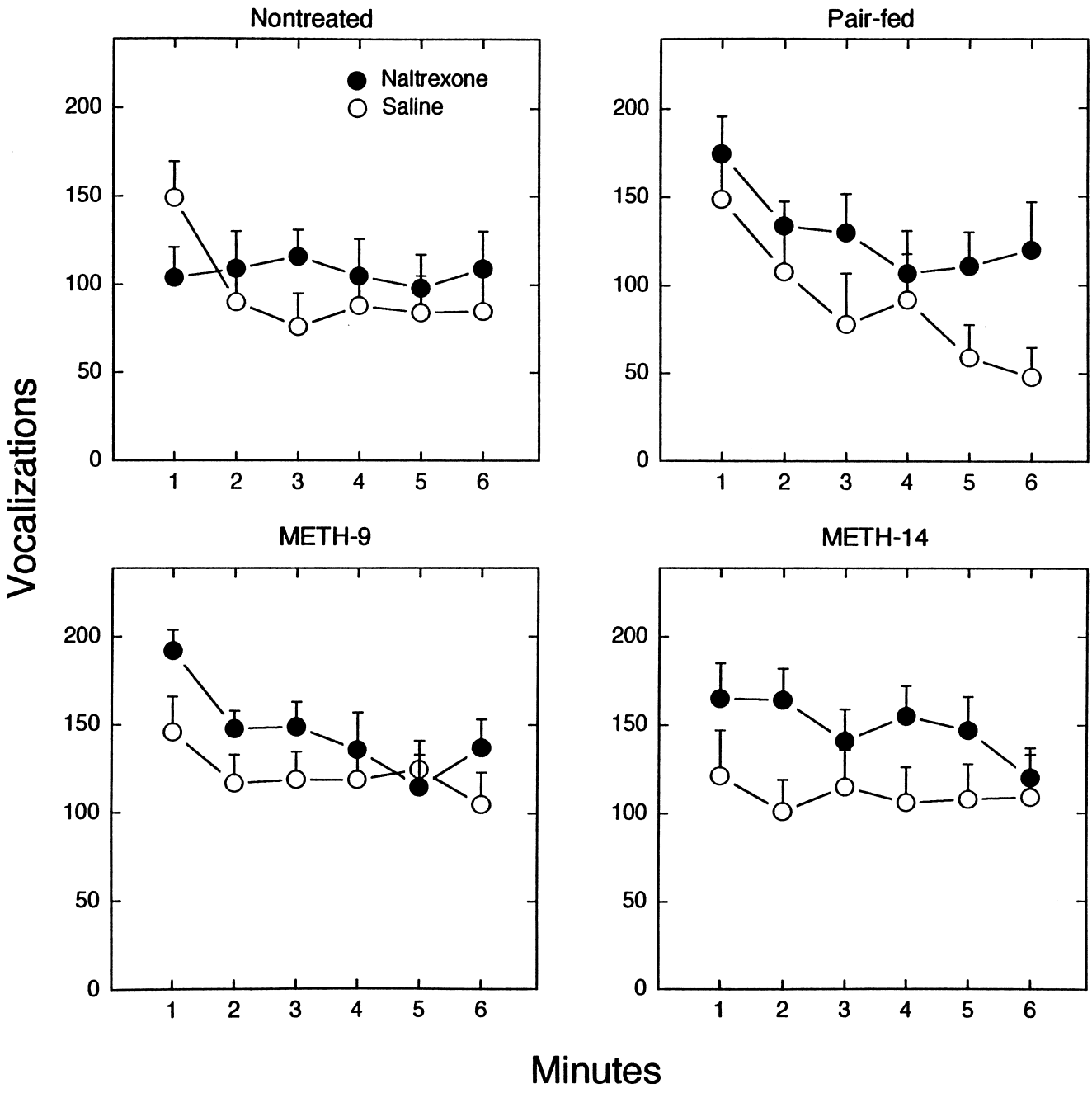

FIG. 3. Ultrasonic vocalizations per minute (mean ± SEM) in a 6-min companion test for each condition following acute treatment with naltrexone or saline. The number of vocalizations is combined for both pups. Abbreviations are as in Fig. 1. Vocalizations were significantly increased by naltrexone only in the pair-fed control group.

affected resorptions, number of pups born live, or litter size. The maternal abstinence scores obtained here were also similar to our previous observations. All of the signs of "classical" opioid abstinence were observed among the methadone-treated dams administered naltrexone, including diarrhea, ptosis, facial tremors, "wet dog" shakes, teeth grinding, stretching, sensitivity to touch, and weight loss. Thus, the administration of methadone by the osmotic minipump produced physical dependence in the dams.

Compared to the untreated control pups, birthweights were reduced in the pair-fed and methadone-treated offspring. The relationship between pup bodyweight and maternal weight gain is not clear. During the first postnatal week when the dams continued to receive methadone, the decrement in maternal food and water intake persisted. Similarly, the lower body weight of the pair-fed and drug-treated offspring persisted until postnatal day 7. Although we cannot explain why these animals weighed less, it does stress the importance of in-

cluding a nontreated control group so that effects associated with the vehicle controls can be detected. Such controls are important so that effects due primarily to methadone can be distinguished from those that are secondary to nutritional and effects associated with surgery.

Prenatal exposure to the high dose of methadone increased the mortality that occurred either at birth or before postnatal day 5. We do not know why these pups died, but possibly the continued exposure to methadone compromised their ability to nipple attach and suckle. The dams' maternal behavior may also have been disrupted by drug administration. It should be noted that in previous studies (13,14) in which pups were fostered to surrogate dams that were not receiving methadone, mortality of the offspring was not increased by prenatal methadone exposure.

Naltrexone administered at postnatal day 7 increased locomotor activity across all chronic treatment groups. The activity of pups given either the lower dose or higher dose of methadone were significantly increased compared to controls. This locomotor activation likely represents precipitated opiate abstinence, an observation consistent with the increased motor activity reported in the adult (15). That naltrexone increased activity following chronic methadone administration is also consistent with previous findings for morphine, and suggest that it is a behavioral component of opiate abstinence in the infant (2,16,20). We did not do a detailed analysis of the behaviors that constituted the increased activity here. For precipitated withdrawal using morphine as the opiate, the increased activation is comprised of very specific behaviors at 7 days of age. These behavioral changes include increased locomotion, wall climbing, stretching, and less time spent quietly huddling with littermates (16,20).

The effects of methadone and pair feeding on USV were complex. The chronic treatments altered vocalizations independently of acute naltrexone or saline treatment; pups given the higher dose of methadone increased crying over time, whereas the pairfed controls decreased crying over time. During withdrawal precipitated by naltrexone, vocalizations increased significantly only for the high methadone dose group. Although some studies have reported increased USVs following acute treatment with opiate antagonists in normal pups (5,17,19), most have not (2,4,7,21). Likewise, we failed to find an increase in either the untreated or pair-fed controls. Companion-tested pups administered naltrexone, but not saline, showed increased USVs, but the increase was significant only for the pair-fed group. Clearly, the role that opiate antagonists play in ultrasonic vocalizations is not fully understood (1). Nonetheless, because these vocalizations are altered by chronic administration of opiates, although in a manner not yet fully characterized, they may be a useful metric in the analysis of the effects of chronic opiate administration in infants.

For the measures used here, there was no evidence of passive abstinence; no effects on locomotor activity or USVs were observed for the methadone pups administered saline. Thus, these abstinence signs, similar to observations reported for morphine (2,16), may only be detected when precipitated with an opioid antagonist. We did not measure methadone concentrations in the pups so the lack of passive abstinence signs could also be related to low plasma levels that produced only weak physical dependence. Or if passive abstinence does occur in week-old rats, the tests and procedures used here may not be sufficiently sensitive to reveal the effects. However, the increase in USVs and locomotor activity seen here contribute to current literature characterizing the opiate withdrawal syndrome in infant rats. Together, these studies demonstrate that increased activity, characterized by age specific behaviors, and perhaps USVs, afford sensitive behavioral indicators of abstinence in the young rat. Detailed description of the developmental course of opiate withdrawal is important because it is increasingly likely that even neonates experience an abstinence syndrome. The consequences of infant withdrawal on normal development and adult behavior are not known.

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