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Buprenorphine Substitution Ameliorates Spontaneous Withdrawal in Fentanyl-Dependent Rat Pups

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ABSTRACT

Iatrogenic physical dependence has been documented in human infants infused i.v. with fentanyl or morphine to maintain continuous analgesia and sedation during extracorporeal membrane oxygenation and mechanical ventilation. Many infants are slowly weaned from the opioid. However, this approach requires extended hospital stays. Little is known about the potential benefits of substitution therapy to prevent abstinence. Therefore, the hypothesis was tested that s.c. and p.o. buprenorphine substitution would ameliorate spontaneous withdrawal in fentanyl-dependent rat pups. Analgesia in the tail-flick test was used to indicate behaviorally active doses of buprenorphine in opioid-naïve postnatal day 17 rats. Other postnatal day 14 rat pups were surgically implanted with osmotic minipumps that infused saline (1 μL/h) or fentanyl (60 μg/kg/h) for 72 h. Vehicle or buprenorphine was administered s.c. or p.o. before the initiation of spontaneous withdrawal brought about the removal of the osmotic minipumps. The major withdrawal signs of wet-dog shakes, jumping, wall climbing, forepaw tremor, and mastication were counted during a 3-h period of withdrawal. The major scored sign, scream on touch, was assessed every 15 min for 3 h. Injection of naloxone after the 3-h observation did not reveal any residual dependence. Subcutaneous buprenorphine administration significantly ameliorated all signs of withdrawal. Surprisingly, p.o. buprenorphine was nearly as efficacious as the s.c. route of administration. These results indicate that buprenorphine substitution therapy may be effective in fentanyl-dependent human infants. **(*Pediatr Res* 49: 50–55, 2001)**

Abbreviations:
NAS, neonatal abstinence syndrome
ECMO, extracorporeal membrane oxygenation
P14, P17, postnatal day 14, postnatal day 17
%MPE, percentage of maximum possible effect

Today, many infants receive fentanyl or morphine by i.v. administration to provide continuous analgesia and sedation during ECMO and mechanical ventilation (1–6). Considerable evidence indicates that iatrogenic physical dependence can develop in these infants (1–3, 7–14). Physical dependence to morphine and fentanyl was characterized by the presence of spontaneous withdrawal signs when the drug was discontinued. These signs are much like those reported in infants born to opioid-dependent mothers [for reviews see Finnegan (15) and Finnegan and Kaltenbach (16)]. Studies that used the NAS developed by Finnegan *et al.* (17), demonstrated a highly positive correlation between opioid dose and NAS, and length of infusion and NAS (1, 8, 12, 13). Fifty to 84% of neonates removed from fentanyl within a 24-h period exhibited opioid withdrawal, and 48% exhibited signs with morphine withdrawal (1, 8, 12).

A review of the literature indicates that no standards have been developed to detoxify fentanyl- or morphine-dependent infants. In some medical centers, they are weaned from the opioid. Reductions in infusion dose are based on withdrawal scores obtained every 2 to 4 h (1–3, 7, 10). However, this approach requires extended hospital stays. In one center, infants removed from ECMO were infused an additional 21 and 14 d with tapered doses of fentanyl and morphine, respectively, to reverse dependence (3). Others have recommended a more rapid tapering of dose (1, 2, 10). Yet, Franck *et al.* (3) comment that rapid tapering of dose can increase the severity of withdrawal, necessitating an increase in the overall requirement of sedatives, thus delaying discharge from the hospital. Nevertheless, all agree that weaning schedules must be carefully monitored to avoid recurring abstinence, consequent dehydration, and inadequate nutrition.

Drug substitution offers the hope of allowing neonates and infants to be rapidly and safely removed from i.v. fentanyl or morphine. Drug substitution could eliminate the risk of spon-

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taneous withdrawal, as well as significantly reduce the length of hospital stays required by the weaning regimens. Nonopioids substituted at one time or another include benzodiazepines, phenobarbital, phenothiazines, and clonidine. Based on the few studies conducted in neonates, most nonopioid drugs lack significant therapeutic advantages over opioid substitution therapy. Although benzodiazepines, barbiturates, and phenothiazines provide sedation during withdrawal, they do not control the vomiting, diarrhea, and other autonomic signs, thereby increasing the risk of dehydration and electrolyte abnormalities. Furthermore, they add psychotropic effects to an already difficult clinical condition (18, 19). In one medical letter, oral clonidine was effective in two infants (20). Yet clinical trials remain to be conducted within the larger population to prove clonidine's safety and efficacy.

In addition, little is known about the safety and efficacy of methadone and other opioid substitutes. Methadone substitution p.o. was effective in treating iatrogenic fentanyl dependence in three infants (21). However, the physical dependence from the methadone itself required an additional 4 to 6 wk of care. Buprenorphine is a partial *mu*-opioid receptor agonist that effectively ameliorates withdrawal in adult opioid abusers (22), and has the efficacy of methadone in reducing illicit opioid use during maintenance (23–25). To our knowledge, buprenorphine has not been substituted in opioid-dependent infants to ameliorate withdrawal after removal from fentanyl or morphine.

This laboratory has developed and characterized a neonatal and infant rat model of fentanyl tolerance and physical dependence (26, 27). Dependence has been demonstrated with naloxone-precipitated withdrawal. This manuscript presents a characterization of spontaneous fentanyl withdrawal in rat pups. With this model, the hypothesis was tested that s.c. and p.o. buprenorphine substitution significantly ameliorates spontaneous withdrawal in fentanyl-dependent rat pups.

METHODS

Source of rat pups. Nulliparous female Sprague-Dawley rat dams were purchased from Zivic-Miller (Zedianople, PA, U.S.A.). Litter sizes were culled to 10 pups per dam (five females and five males). The animals were allowed food and water *ad libitum* and were housed at the Virginia Commonwealth University School of Medicine animal care facilities with a 12-h light-dark cycle (0700-light, 1900-dark). The Institutional Animal Care and Use Committee at Virginia Commonwealth University approved the experiments.

Tail-flick test of analgesia. The tail-flick test of radiant heat nociception was used to measure the analgesic effects of buprenorphine. Male and female rats were randomly distributed to receive vehicle or buprenorphine doses. Baseline tail-flick latencies were obtained from opioid-naïve P17 rat pups before administering vehicle or buprenorphine. The heat stimulus was adjusted to yield baseline latencies of 3 to 4 s, with a 10-s cut-off (26–29). Vehicle or buprenorphine was injected s.c. or gavaged p.o. For time-course experiments, test latencies were obtained every 15 min to measure the peak time-effect of buprenorphine. The data were transformed to the %MPE according to the method of Harris and Pierson (30). This was calculated as %MPE = [(test latency − baseline latency)/(10 − baseline latency)] × 100. Time-course data were analyzed using two-factor (drug treatment and time) repeated measures ANOVA. A significant drug treatment × time interaction led to *post hoc* analysis with Tukey's test, comparing the vehicle and buprenorphine groups at each time point. At the peak time-effect, dose-response curves were constructed by administering increasing doses of buprenorphine. These data were transformed into %MPE values and analyzed with one-factor ANOVA followed by Tukey's test.

Surgical implantation of osmotic minipumps. Alzet osmotic minipumps were surgically implanted as previously described in detail (26, 27). Briefly, P14 rat pups were anesthetized with 0.5% methoxyflurane (minimal alveolar concentration = 0.23%) for implantation of Alzet 1003D osmotic minipumps (Alza Corp, Palo Alto, CA, U.S.A.) that deliver at a rate of 1 μL/h. Within each litter of five female and five male rats, two were anesthetized but remained naïve, whereas eight were randomly assigned to receive saline- or fentanyl-filled pumps. The surgical incision was closed with Vetbond Tissue Adhesive (3M Animal Care Products, St. Paul, MN, U.S.A.), and the area was swabbed with 10% povidone iodine (General Medical, Prichard, WV, U.S.A.). Fentanyl citrate concentrations were adjusted to deliver at a rate of 60 μg/kg/h. This infusion rate was based on a series of implantation trials that led to the initial characterization of fentanyl tolerance and dependence in P17 rat pups (27).

Seventy-two hours later, the pumps were surgically removed to allow the P17 rats to undergo spontaneous fentanyl withdrawal. During brief methoxyflurane anesthesia, the implantation site was swabbed with 10% povidone iodine and washed with 70% ethanol USP. Once dry, the initial incision was opened with sterile scissors, and the osmotic minipump was removed with sterile forceps. The s.c. space that housed the osmotic minipump was closed with Vetbond Tissue Adhesive, and swabbed with iodine.

Assessment of spontaneous withdrawal. Naïve, vehicle pump- and fentanyl pump-implanted P17 rat pups were injected s.c. with vehicle or buprenorphine 10 min before the pumps were removed. Anesthesia and pump removal took about 5 min. Other groups were administered vehicle or buprenorphine p.o. 55 min before pump removal. The rats were then observed for signs as described in previous studies in this laboratory (26, 27, 29). The rats were placed in pairs in individual Plexiglas chambers for the observations. Pairing reduces isolation stress that can confound withdrawal data (26, 27, 29). Quantitative signs of spontaneous withdrawal were counted continuously and divided into 15-min intervals during the 3-h observation period. Scored signs were noted as either absent or present when tested within each 15-min interval. At the end of 3 h, the animals were injected with naloxone and observed another 15 min for residual dependence. Based on the low frequency of signs within each 15-min observation, we decided to calculate the average number of signs exhibited over the 3-h observation.

Both quantitative and scored signs were obtained and analyzed statistically. Quantified signs that could be counted were

wet-dog shakes, spontaneous jumping, wall climbing, forepaw tremors, and mastication. These signs were subjected to ANOVA comparing responses between vehicle pump- and fentanyl pump-implanted rats injected with vehicle or buprenorphine s.c. or p.o. A significant F value led to *post hoc* analysis with Tukey's test to determine which withdrawal signs were statistically significant. The scored sign, scream on touch, was elicited every 15 min by gently prodding each animal's trunk with the eraser end of a pencil. This sign was subjected to χ^2 analysis.

Drugs and supplies. Fentanyl citrate (Research Biochemicals International, Natick, MA, U.S.A.) was dissolved in sterile pyrogen-free isotonic saline (Baxter Healthcare Corp., Deerfield, IL, U.S.A.). Buprenorphine HCl (National Institute on Drug Abuse, Bethesda, MD, U.S.A.) has a limited solubility of approximately 1.0 to 1.5 mg/mL in distilled water, and less in isotonic saline. Walker *et al.* (31) demonstrated that solutions of approximately 0.1% lactic acid, which greatly increase the solubility of buprenorphine, elicit no apparent discomfort when injected s.c. in animals. Therefore, all buprenorphine doses were dissolved in 0.1% lactic acid in distilled water. Alzet 1003D osmotic minipumps were filled with either isotonic saline or fentanyl.

RESULTS

Analgesic effects of s.c. and p.o. buprenorphine. Little is known about the behavioral effects of buprenorphine in rat pups. To conduct substitution studies, it was necessary to discover the peak time-effect and active range of buprenorphine doses. Time-course studies reveal that s.c. buprenorphine (1.0 mg/kg) administration elicited significant analgesia compared with vehicle-injected rat pups (drug-treatment × time interaction $F_{5,45} = 4.5$; $p = 0.002$; Fig. 1*A*). Analgesia was significant at 15 min, reached a peak at 30 min, and was near baseline by 90 min. Buprenorphine injected s.c. appears to be a partial agonist (Fig. 1*B*). Doses >1.0 mg/kg did not elicit greater analgesia. Maximum analgesia was limited to 44.8% MPE at 1 mg/kg. Analgesia was significant with the 0.1, 1.0, and 5.0 mg/kg doses (one-factor ANOVA $F_{6,34} = 6.2$; $p < 0.001$). ED_{50} values were incalculable because the response did not exceed 50% MPE.

Buprenorphine gavaged p.o. elicited a different quality of analgesia than buprenorphine s.c. Significant analgesia was delayed 60 min after the p.o. administration of 1 mg/kg buprenorphine ($F_{7,70} = 5.2$; $p < 0.001$; Fig. 1*A*). Furthermore, the 1.0, 10.0, and 50 mg/kg doses elicited significant analgesia, but the response was not dose-related despite testing a 2000-fold range of doses ($F_{7,45} = 3.4$; $p = 0.006$; Fig. 1*B*).

Subcutaneous buprenorphine ameliorates spontaneous withdrawal in fentanyl-dependent rat pups. Vehicle or buprenorphine was injected s.c. before removing the osmotic minipumps. In Table 1, vehicle pump-implanted rats exhibited no signs of withdrawal caused by endogenous opioid release. None were expected, because naloxone failed to precipitate withdrawal in vehicle pump-implanted P17 rats in an earlier study (27). However, fentanyl pump-implanted rats injected s.c. with vehicle exhibited significant quantitative and scored withdrawal signs during 3 h. Withdrawal began within 5 min of removing the pump and diminished by the third hour of observation. Concerns about residual dependence were addressed by injecting naloxone (1 mg/kg, s.c.) immediately after 3 h. No precipitated withdrawal signs were noted. This was further verified by the absence of withdrawal to an additional 5 mg/kg naloxone dose injected 30 min later. Buprenorphine (1

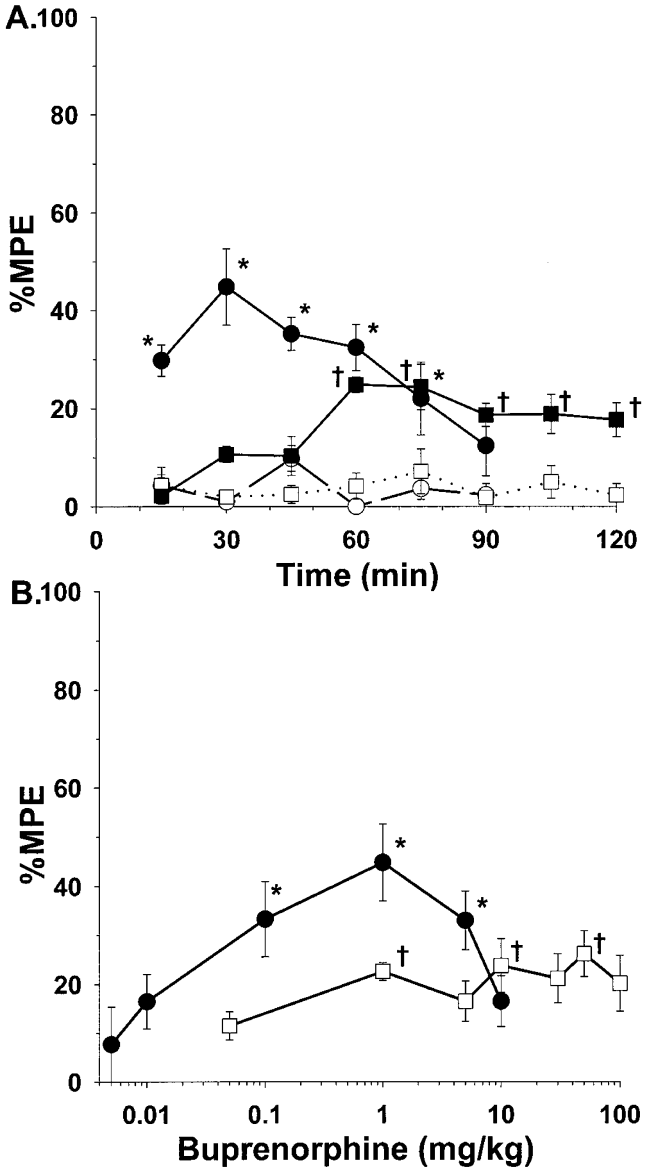

Fig. 1. *A,* time-course of buprenorphine analgesia in P17 rats. Baseline tail-flick latencies were obtained before s.c. injection of vehicle (○, dashed line) or buprenorphine (1 mg/kg, ●, solid line). Other rats were gavaged p.o. with vehicle (□, dotted line) or buprenorphine (1 mg/kg, , solid line). Test latencies were obtained over time as indicated. The data were converted into the %MPE, and analyzed as described in Methods. Each treatment consisted of six rats. *$p < 0.05$ compared with respective s.c. vehicle time point; †$p < 0.05$ compared with respective p.o. vehicle time point. *B,* dose-response curve of buprenorphine analgesia. Individual groups of rats were administered doses of buprenorphine s.c. (●) or gavaged p.o. (□) 30 and 60 min, respectively, before the tail-flick test. Each dose-response curve consisted of 36 to 42 rats. *$p < 0.05$ compared with respective s.c. vehicle value of 1.0 + 0.6%MPE; †$p < 0.05$ compared with respective p.o. vehicle value of 4.2 + 2.7%MPE.

Table 1. *Subcutaneous buprenorphine ameliorates the signs of spontaneous fentanyl withdrawal in infant rats**

	Vehicle-P + vehicle	Vehicle-P + buprenorphine	Fentanyl-P + vehicle	Fentanyl-P + buprenorphine
Quantitative signs				
Wet-dog shakes	0.0 ± 0.0	0.0 ± 0.0	21.8 ± 4.3†	3.7 ± 0.7‡
Jumping	0.0 ± 0.0	0.0 ± 0.0	11.2 ± 3.7†	0.5 ± 0.5‡
Wall climbing	0.0 ± 0.0	0.0 ± 0.0	11.7 ± 1.8†	0.0 ± 0.0‡
Forepaw tremor	0.0 ± 0.0	0.0 ± 0.0	3.7 ± 1.2†	0.0 ± 0.0‡
Mastication	0.0 ± 0.0	0.0 ± 0.0	5.2 ± 0.4†	1.2 ± 0.5‡
Scored sign				
Scream on touch	0/6	0/6	6/6§	0/6

* P17 rats received s.c. buprenorphine 10 min before removal of the osmotic minipumps. Anesthesia and pump removal took about 5 min. The animals were observed 3 h for signs of withdrawal. Quantitative signs were counted in each animal and expressed as the mean ± SEM for six rats per group. The scored sign is indicated as the number of rats exhibiting the sign to the total number of animals observed.

† $p < 0.05$ compared with vehicle-P + s.c. vehicle group, Tukey's test.

‡ $p < 0.05$ compared with the fentanyl-P + s.c. vehicle group, Tukey's test.

§ Significantly different from vehicle-P + s.c. vehicle group, χ^2, and significantly different from fentanyl-P + s.c. vehicle group, χ^2.

Abbreviation: P, pump.

mg/kg, s.c.) significantly ameliorated all the signs of withdrawal, thus demonstrating its potential utility in substitution therapy.

Per os buprenorphine ameliorates withdrawal in fentanyl-dependent rat pups. Vehicle or buprenorphine was gavaged p.o. before removing the osmotic minipumps. In Table 2, vehicle pump-implanted rats exhibited no signs of withdrawal, whereas fentanyl pump-implanted rats gavaged p.o. with vehicle exhibited significant quantitative and scored withdrawal signs during 3 h. The signs diminished in frequency during the third hour of observation. However, forepaw tremor was not significantly increased in this group, making it impossible to compare with the buprenorphine-gavaged group. Concerns about residual dependence after 3 h were addressed by injecting naloxone, as described earlier. Naloxone failed to precipitate any additional withdrawal in the rat pups. The 1.0 mg/kg buprenorphine p.o. dose was chosen to compare the efficacy of enteral and parenteral routes of administration. Buprenorphine p.o. appeared to be nearly as effective as s.c. buprenorphine. Buprenorphine significantly ameliorated nearly all the signs, but only reduced mastication in a nonsignificant fashion ($p = 0.09$).

Withdrawal signs observed but not quantified in fentanyl-dependent rats. Other signs of spontaneous withdrawal were observed, but were impractical to quantify because of their low frequency of incidence. The behaviors were noted throughout the 3-h observation in all fentanyl-dependent rat pups given vehicle s.c. or p.o. Table 3 estimates the severity of signs observed and the effect of buprenorphine on these infrequent signs.

DISCUSSION

Subcutaneous and p.o. buprenorphine analgesia. Buprenorphine is a partial agonist that binds with high affinity to *mu*- and *kappa*-opioid receptors (32). At low doses, analgesia is mediated through *mu*-opioid receptor activation. For example, the irreversible *mu*-opioid receptor antagonist β-funaltrexamine significantly blunted buprenorphine analgesia in mice (33). Furthermore, buprenorphine analgesia was significantly reduced in *mu1*-opioid receptor–deficient CXBK mice (34). At higher doses, buprenorphine acts as a *mu*-opioid receptor antagonist and can block morphine analgesia and precipitate withdrawal in morphine-dependent rodents (35, 36). Our time-course studies indicate that s.c. buprenorphine had a more rapid onset of analgesia than p.o. buprenorphine (*i.e.* 15 min *versus* 60 min, respectively). This is not surprising when one considers the delays in gastrointestinal transit and absorption that occur with many drugs. Interestingly, buprenorphine is subject

Table 2. *Per os* buprenorphine ameliorates the quantitative signs of spontaneous fentanyl withdrawal*

	Vehicle-P + vehicle	Vehicle-P + buprenorphine	Fentanyl-P + vehicle	Fentanyl-P + buprenorphine
Quantitative signs				
Wet-dog shakes	0.0 ± 0.0	0.0 ± 0.0	17.0 ± 2.0†	4.2 ± 0.9‡
Jumping	0.0 ± 0.0	0.0 ± 0.0	7.8 ± 4.0†	0.0 ± 0.0‡
Wall climbing	0.0 ± 0.0	0.0 ± 0.0	11.0 ± 5.0†	0.5 ± 0.3‡
Forepaw tremor	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.7	3.2 ± 1.7
Mastication	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 1.1†	2.3 ± 1.0
Scored sign				
Scream on touch	0/6	0/6	6/6§	1/6

* P17 rats received buprenorphine p.o. 55 min before removal of the osmotic minipumps. Anesthesia and pump removal took about 5 min. The animals were then observed 3 h for signs of withdrawal. Quantitative signs were counted in each animal and expressed as the mean ± SEM for six rats per group. The scored sign is indicated as the number of rats exhibiting the sign to the total number of animals observed.

† $p < 0.05$ compared with vehicle-P + p.o. vehicle group.

‡ $p < 0.05$ compared with the fentanyl-P + p.o. vehicle group.

§ Significantly different from vehicle-P + p.o. vehicle group, χ^2, and significantly different from fentanyl-P + s.c. vehicle group, χ^2.

to first-pass metabolism and is converted into norbuprenorphine (37, 38). Norbuprenorphine has one fourth the analgesic activity of buprenorphine in rats (39). Thus, it is tempting to speculate that the limited analgesic properties of p.o. buprenorphine represent the predominant effect of norbuprenorphine. Alternatively, s.c. buprenorphine administration avoids first-pass metabolism and appeared to produce a partial agonist effect in P17 rat pups. This is consistent with reports of partial agonist analgesia exhibited in adult rodents assessed with the tail-flick test (32, 33). Like this study, the dose-response curves were described as bell-shaped, with the highest doses eliciting less analgesia. Future studies need to be conducted to reveal doses that antagonize fentanyl analgesia, as well as those that precipitate withdrawal during fentanyl dependence.

Characterization of spontaneous fentanyl withdrawal. The signs of spontaneous withdrawal in Tables 1 and 2 were nearly identical to those observed in fentanyl-dependent rat pups during naloxone-precipitated withdrawal (27). Two signs not observed during spontaneous withdrawal were diarrhea and rhinorrhea. Interestingly, several signs observed at low frequency could be unique to spontaneous withdrawal (Table 3). These were splayed hindlimbs, wide stance, walking on toes, arched back, sniffing, and head shakes. Naloxone-precipitated withdrawal usually diminishes within 25 min because of its short duration of effect (27). These unique signs might be observed if the longer-acting antagonist naltrexone is used instead. However, the fact that these rat pups underwent spontaneous withdrawal supports our previously published conclusions that chronic fentanyl administration leads to dependence.

Efficacy of buprenorphine in ameliorating spontaneous fentanyl withdrawal. Both s.c. and p.o. buprenorphine were nearly equally efficacious in ameliorating spontaneous fentanyl withdrawal. There was a remarkable contrast between vehicle- and buprenorphine-injected animals. Buprenorphine reduced both the quantitative and scored signs, and outwardly the animals looked like opioid-naïve rats. Both p.o. and s.c. buprenorphine appear to have long durations of action, inasmuch as the withdrawal signs did not recur during the 3-h observation. In adult rodents, buprenorphine has a long half-life and a long analgesic effect because of its slow dissociation from *mu*-opioid receptors (39–41). This has enabled opioid-dependent human adults to receive buprenorphine once daily or on alternate days (42, 43). In contrast, the onset of action to ameliorate withdrawal could have differed by both routes of administration, as was observed with analgesia. However, in this study the timing of the buprenorphine injections was based on the analgesia data. Thus, p.o. buprenorphine could have been less efficacious if spontaneous withdrawal had been initiated immediately after gavaging the buprenorphine. More research is still needed to establish the onset of action of different routes of buprenorphine administration, including i.v. administration.

The National Institute on Drug Abuse is currently investigating the potential benefits of sublingual buprenorphine tablets. It is assumed that transmucosal absorption contributes to the majority of the effects of buprenorphine. Interestingly, buprenorphine in this study was gavaged directly into the stomach, which prevented possible transmucosal absorption in the mouth. This suggests that the active metabolite norbuprenorphine contributed to the actions of p.o. buprenorphine. It would be reasonable to examine the efficacy of norbuprenorphine in a future study.

The clinical basis for substitution therapy needs to include establishment of the effective dose range of buprenorphine and determination of its onset and duration of action by different routes of administration. On a practical matter, human infants are unlikely to tolerate sublingual tablet administration. However, a p.o. liquid formulation might be given as mouth drops or added to an infant's formula and still be effective. In addition, a p.o. formulation could significantly reduce hospital stays by allowing the parents to administer the buprenorphine at home.

In summary, buprenorphine may prove to be superior to other opioid substitutes. Buprenorphine has several unique properties that methadone lacks. Most significantly, buprenorphine has a low dependence liability in human adults after prolonged therapy (44). Buprenorphine also causes limited respiratory depression because of its partial agonist activity at *mu*-opioid receptors (45, 46). This could be important because some individuals experience chronic lung diseases such as bronchopulmonary dysplasia long after their removal from ECMO and mechanical ventilation (47, 48). The respiratory depressant properties of methadone could exacerbate respiratory problems, whereas buprenorphine might be a safer alternative. Buprenorphine is commercially available, and its pharmacokinetic and pharmacologic effects i.v. have been characterized in human neonates (49). Hopefully further research and clinical trials will be stimulated by these results.

Table 3. *Behavioral withdrawal signs observed but not quantified in fentanyl-dependent rats given vehicle or buprenorphine s.c. or p.o.**

Signs	Vehicle	Buprenorphine
Splayed hindlimbs	+++	−
Wide stance	+++	−
Walking on toes	++	−
Arched back	+++	−
Abdominal stretching	++++	+
Sniffing	++	−
Head shakes	++	+

* Quantitative or scoring analyses were impractical for other signs observed at frequencies below those reported in Tables 1 and 2. The number of positive symbols provides an estimate of the severity of signs. Buprenorphine either blocked all signs (indicated by a negative sign), or reduced the sign (indicated by a positive sign).

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