0031-3998/91/3002-0202$03.00/0
PEDIATRIC RESEARCH

Vol. 30, No. 2, 1991
Printed in U.S.A.

The Effects of Selective mu_1 Opiate Receptor Blockade on Breathing Patterns in the Fetal Lamb

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ABSTRACT. The effects of naloxone and naloxonazine (an irreversible mu_1 antagonist) administration on fetal breathing movement (FBM) patterns under control, physiologic conditions were studied in 10 fetal lambs with chronically implanted electromyogram electrodes in the diaphragm. Neither naloxone (6 mg/h) nor naloxonazine (34 mg) had any effect on the total number of diaphragmatic electromyogram bursts per hour, mean instantaneous breathing rate, or incidence of breathing. However, naloxonazine caused a more fragmented FBM pattern, as indicated by a significant increase in both the number of apneas and pauses per hour, along with decreased epoch duration. In addition, naloxonazine caused a significant reduction in the stability or regularity of the breathing rate. Naloxone had no effects on the dynamic pattern of the FBM. These results suggest that endogenous opiate peptides play a tonic role at the mu_1 receptor to maintain both the continuity and stability of the FBM pattern in late gestation. (*Pediatr Res* 30: 202–206, 1991)

Abbreviations

EC_OG, electrocorticogram
EMG, electromyogram
FBM, fetal breathing movement
FBR, fetal breathing rate
GABA, γ-aminobutyric acid

It has been proposed that elevated levels of endogenous opiate peptides may be associated with increased incidence of apnea in early development. Several investigators have reported increased plasma and cerebrospinal fluid levels of immunoreactive β-endorphin in infants with sleep apnea (1–4) and apnea of prematurity (5). Furthermore, administration of the opiate antagonist naloxone has been reported to reduce the number of apneas in infants with sleep apnea who had high CSF endorphin levels (3). Similar success has been reported in a subsequent study with the long-acting opiate antagonist, naltrexone (1). However, other studies reported no significant change in respiratory pattern or occurrence of apnea after opiate antagonist treatment in infants with apnea of prematurity (5) or infants at risk for sudden infant death syndrome (6).

These conflicting findings may, in part, be explained by the lack of selectivity of naloxone and naltrexone for the various opiate receptor subtypes. The usefulness of nonselective antagonists in studying the role of endogenous opiate peptides in regulation of ventilatory control is questionable, especially if different receptor subtypes mediate opposing effects. Although naloxone is often considered to be a mu-antagonist, its affinity for the mu site is only 4- to 10-fold that for the delta and kappa sites (7). Current pharmacologic and receptor-binding studies further suggest that there are two subtypes of mu receptors: the mu_1 and mu_2 receptor (8). Recent findings show that the mu_1 receptor is involved in respiratory stimulation (9), whereas the mu_2 receptor may mediate respiratory depression in the adult (10). In view of these findings, use of opiate antagonists more selective than naloxone would be more appropriate to study the role of endogenous opiates in ventilatory control in early development.

Our report compares the effects of naloxone and naloxonazine, a highly selective, irreversible mu_1 antagonist (11), on FBM patterns *in utero* in an animal model. The unanesthesized fetal lamb model was used because its FBM pattern is characterized by the presence of prolonged apneas, and there is abundant evidence that exogenously administered opiates and opiate peptides can profoundly affect the FBM pattern (12–15).

MATERIALS AND METHODS

Animal preparation. All studies were carried out in the unanesthesized fetal lamb, inasmuch anesthesia has been shown to influence the action of opiates. Ten fetal lambs greater than 120 d of gestation were used in this study (term being 145 ± 2 d). Fetal lambs were surgically instrumented for long-term intrauterine recording of FBM and EC_OG activity. For monitoring of FBM, a polyvinyl catheter was placed in the fetal trachea for continuous monitoring of tracheal pressure changes, and stainless steel electrodes were implanted in the diaphragm through an incision in the thoracic wall. For recording EC_OG activity, four size 0-80 stainless steel screw electrodes were implanted in the parietal bone (two electrodes on either side of the midline) and cemented with dental acrylic. Details of the surgical procedure have been described previously (12, 16). In addition, polyvinyl catheters were placed in the fetal inferior vena cava and distal aorta for drug administration and determination of arterial blood gases, respectively (BMS3Mk2 blood gas analyzer, Radiometer, Copenhagen, Denmark; 37°C). The catheters and electrodes were tunneled s.c., exteriorized on the maternal flank, and stored in a pouch.

Study design. To allow ample time for recovery from the surgical procedure, all drug studies were performed at least 5 d after surgery. Studies were carried out with the ewe standing or lying in a cart with free access to food and water throughout the study. All studies were performed at the same time of the day (0900 to 1700 h). In each animal, a 2- to 3-h control period

Received December 11, 1990; accepted March 25, 1991.
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Supported in part by the National Institute on Drug Abuse (DA02475). H.H.S. is the recipient of a Research Scientist Development Award (DA00100) from the National Institute on Drug Abuse (Alcohol, Drug Abuse and Mental Health Administration).

recording was obtained for analysis immediately before naloxone or naloxonazine administration.

In five animals, naloxone was infused i.v. to the fetus at a rate of 6 mg/h for 3 h. This dose of naloxone had previously been shown to block the respiratory stimulation caused by low doses of morphine (12). The effects of naloxone on the FBM pattern were analyzed only for the duration of the naloxone infusion due to the short duration of action of naloxone, with a reported half-life of approximately 30 min in rats and 60 min in humans (17).

In another five animals, naloxonazine (a generous gift from Dr. Gavril Pasternak, Memorial Sloan-Kettering Cancer Center, New York, NY) was administered i.v. to the fetus at a dose of 34 mg over a period of 50 min. This dose was calculated based on the effective doses reported for rodents (10–15 mg/kg) (9, 10). The effect of naloxonazine on the FBM pattern during a 3-h recording was obtained and assessed at only 24 h after naloxonazine infusion to avoid its acute effects of reversible, nonselective binding to receptor subtypes other than mu_1 (10).

Data acquisition and processing. Diaphragmatic EMG (bandpass filtered, 100 Hz to 1 kHz), tracheal pressure, and the EC_OG signal (bandpass filtered, 1–100 Hz) were recorded on a Gould 2800S analog recorder (Gould Inc., Cleveland, OH). The amplified, filtered signals were also recorded concurrently onto FM tape (TEAC XR-310, Teac Corp., Montebello, CA) for storage and off-line analysis. Analog to digital conversion of the diaphragmatic EMG signal was accomplished with a board (DT-2801A; Data Translation, Marlboro, MA) resident in an AST 386 microcomputer. Data were digitized either on-line or off-line at a rate of 256 Hz and stored in binary format. Automated recognition of diaphragmatic bursts was accomplished using a template recognition algorithm (18).

Analysis of FBM pattern. The effects of naloxone and naloxonazine were assessed by total diaphragmatic EMG burst number and mean instantaneous breathing rate. The effect on EMG burst pattern was analyzed using a slight modification of the epoch analysis method by Rey *et al.* (19) for fetal breathing analysis in the primate. A breathing epoch was defined as a series of consecutive EMG bursts whose interburst intervals were all less than 6 s, with a minimum of three bursts occurring within 9 s. The end of an epoch was defined as the time of occurrence of the last burst when fewer than three bursts were detected in 9 s. Thus, an epoch could contain as few as three EMG bursts. Apneas were defined as the interburst intervals of greater than 10 s, and periods that could not be classified as apneas or epochs were defined as respiratory pauses. The incidence of breathing was calculated as [(sum of all epoch durations/total time) × 100%]. The stability or the regularity of the breathing pattern was assessed by examining the rate of change in successive breathing rates. A group of breathing bursts was defined as a cluster of stable breathing rates if the difference between successive breathing rates was less than 20%. A minimum of three breathing bursts were required to fulfill the criteria. Each stable cluster was then characterized for cluster length, number of bursts, and the average instantaneous rate. In addition, the percentage of total breathing bursts that occurred in stable clusters was also determined.

Statistical analysis. Due to the relatively large interanimal variation in ventilatory characteristics, the effects of naloxone and naloxonazine were compared with control predrug values with each animal serving as its own control. The nonparametric Wilcoxon signed ranks test for paired data was used to determine statistical significance. All data are presented as mean ± SEM.

RESULTS

Because of the large variation in breathing characteristics among animals, it was necessary to use each animal, before drug treatment, as its own control. The two groups of animals used for naloxone and naloxonazine studies were of similar gestational ages and had similar blood gas characteristics under control conditions (Table 1).

Twenty-four h after the administration of naloxonazine, the distribution of instantaneous FBR was distinctly different from that under control conditions (Fig. 1A). After naloxonazine administration, there was an increase in the occurrence of slower breathing rates and a loss of the dominant rates. Naloxone administration did not change the FBR distribution (Fig. 1B).

Neither naloxone nor naloxonazine administration significantly altered the overall breathing activity (Table 2). Neither

Table 1. *Gestational ages and control blood gases of naloxone and naloxonazine groups**

Group	*n*	Gestational age	pH	P_{CO_2} (mm Hg)†	P_{O_2} (mm Hg)
Naloxone	5	128 ± 2	7.4 ± 0.01	42.3 ± 1.5	33.8 ± 1.0
Naloxonazine	5‡	131 ± 2	7.4 ± 0.01	42.3 ± 2.0	28.5 ± 2.4

* Values are mean ± SEM. Comparison between the gestational ages and control blood gases between the naloxone and naloxonazine group were made using the independent samples *t* test.

† 1 mm Hg = 0.1333 kPa.

‡ Although five animals were used for the naloxonazine study, blood gases could only be obtained for four of those animals.

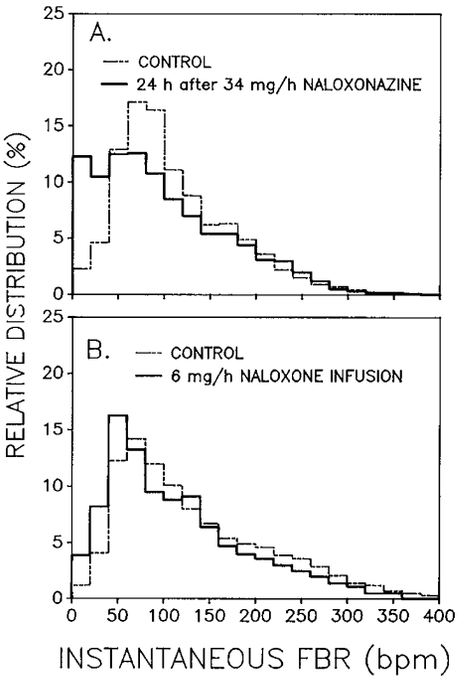

Fig. 1. Histograms illustrating the change in relative distribution of instantaneous FBR in two representative animals after naloxonazine (*A*, before and 24 h after 34 mg naloxonazine) or naloxone administration (*B*, before and during 6 mg/h naloxone infusion).

Table 2. *Effects of naloxone and naloxonazine on fetal breathing activity**

Drug	*n*	No. of bursts/h	Instantaneous rate (min^{-1})
Control	5	2520 ± 372	135 ± 9
Naloxone	5	2340 ± 606	128 ± 8
Control	5	2340 ± 84	115 ± 7
Naloxonazine	5	1740 ± 390	102 ± 11

* Values are mean ± SEM. Values for naloxone were obtained during 6 mg/h infusion. Values for naloxonazine were obtained 24 h after administration of 34 mg of naloxonazine. All values were compared to control predrug values using the Wilcoxon signed ranks test.

drug had an effect on the number of diaphragmatic EMG bursts per hour or the average instantaneous rate. Naloxone did not affect the continuity of the burst pattern, as seen by its lack of effect on the organization of the burst pattern (Table 3). However, naloxonazine resulted in a 2-fold increase in both the number of apneas and the number of pauses per hour. The increases were statistically significant compared with control predrug values ($p < 0.05$). In addition, there was a concomitant decrease in epoch duration after naloxonazine administration ($p < 0.05$), resulting in a more fragmented, less continuous breathing pattern. However, the overall incidence of breathing was not significantly changed by either drug. The changes in breathing pattern after naloxonazine administration were not accompanied by any significant changes in average blood pH, P_{CO_2}, or P_{O_2}. The blood gases could only be analyzed for four of the five animals in the naloxonazine group because the catheter in the 5th animal failed to remain patent. Control blood gases for the animals in the naloxonazine group were pH = 7.4 ± 0.01, P_{CO_2} = 42.3 ± 2.0 mm Hg (5.6 kPa), and P_{O_2} = 28.5 ± 2.4 mm Hg (3.8 kPa), and remained unchanged after naloxonazine administration with values of pH = 7.33 ± 0.02, P_{CO_2} = 43.4 ± 2.3 mm Hg (5.8 kPa), and P_{O_2} = 24.6 ± 2.9 mm Hg (3.3 kPa).

Figure 2*A* shows a representative polygraph recording of diaphragmatic EMG and EC_OG obtained during the control period, and Figure 2*B* shows the recording 24 h after the administration of 34 mg of naloxonazine. The episodes of FBM after naloxonazine treatment are clearly more fragmented, frequently being interrupted by many short pauses and apneas, compared with the control period. The fragmentation in the breathing pattern was observed within an episode of low-voltage fast activity EC_OG and did not appear to be associated with similar fragmentation in the EC_OG pattern.

Table 3. *Effects of naloxone and naloxonazine on organization of the burst pattern**

Drug	*n*	No. of apneas/h	No. of pauses/h	Epoch length (s)	Incidence of FBM (%)
Control	5	37 ± 8	25 ± 6	99 ± 29	55 ± 5
Naloxone	5	38 ± 9	27 ± 9	101 ± 37	55 ± 11
Control	5	22 ± 6	14 ± 4	209 ± 92	52 ± 8
Naloxonazine	5	43 ± 3†	31 ± 4†	92 ± 5†	51 ± 8

* Values are mean ± SEM. Values for naloxone were obtained during 6 mg/h infusion. Values for naloxonazine were obtained 24 h after administration of 34 mg of naloxonazine. All values were compared with control predrug values using the Wilcoxon signed ranks test.

† $p < 0.05$.

The increase in short pauses and apneas during episodes of breathing are more apparent in the instantaneous FBR time series shown in Figure 3*A* and *B*. Closer examination of a 20-min breathing episode segment in the control recording, between 0 and 20 min (Fig. 4*A*), and of a 20-min breathing episode after naloxonazine treatment, between 70 and 90 min (Fig. 4*B*), showed that naloxonazine clearly caused a more fragmented FBM pattern.

Under control conditions, an average of 30% of the bursts in the naloxone group and 47% of the bursts in the naloxonazine group occurred in stable clusters. Large variability in instantaneous rates were found between stable clusters. The stable clusters tended to be of short duration and, on average, lasted only about 4 s and comprised only about five bursts per cluster. Naloxone did not significantly affect the stability or regularity of breathing rates. However, naloxonazine resulted in a significant reduction in both the percentage of bursts in clusters and the percentage of time occupied by stable clusters (Table 4). This was due to a slight but insignificant decrease in the number of clusters per hour. No significant changes were detected in the number of bursts per cluster nor in the average cluster length after naloxonazine administration.

DISCUSSION

It has been a common belief that endorphins play an important role in the control of breathing, inasmuch as exogenously administered opiate peptides have been demonstrated to modulate breathing patterns in unanesthesized adult animals (20–23). However, administration of naloxone generally failed to affect breathing patterns or respiratory response to hypoxia and hypercapnia in adult animals and humans (24, 25), except when used in very high doses (26). Thus, the role of physiologic levels of endorphins on ventilatory control in the adult remains unclear.

Evidence for a role of endorphins in regulation of breathing pattern appears to be stronger for the perinatal period. Levels of immunoreactive β-endorphin are significantly higher in cerebrospinal fluid of infants with infant apnea compared with age-matched control nonapneic infants; treatment with naloxone or naltrexone significantly reduces the number of apneas and respiratory pauses (1, 3). Opiate antagonists have also been found to modulate breathing pattern during hypoxia and hypercapnia in newborn rabbits (27, 28), newborn piglets (29, 30), and fetal sheep (31, 32) and to reverse neonatal depression caused by fetal asphyxia (33). However, opiate antagonist administration did

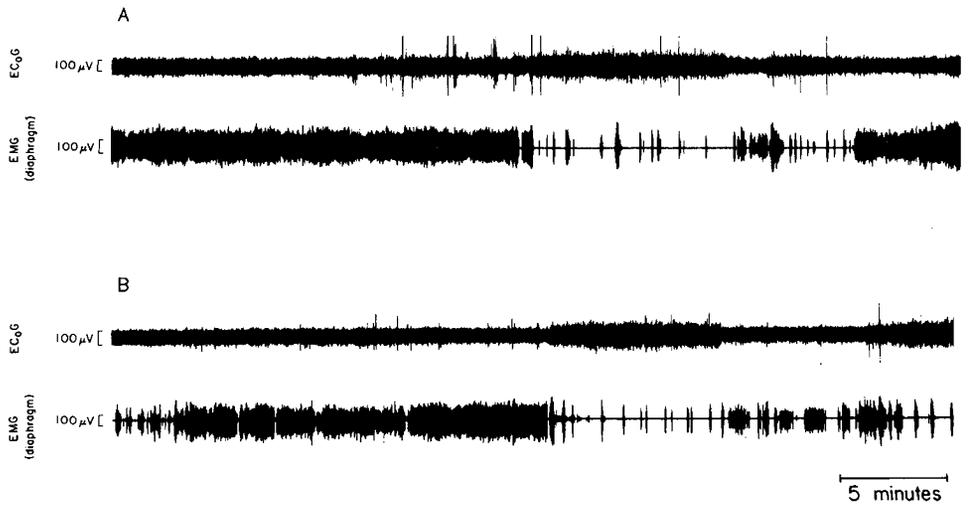

Fig. 2. Polygraph tracings showing effects of naloxonazine on FBM and EC_OG patterns in the fetal lamb. Each panel represents 40 min of recording. *A*, control. *B*, 24 h after administration of naloxonazine (34 mg, i.v.).

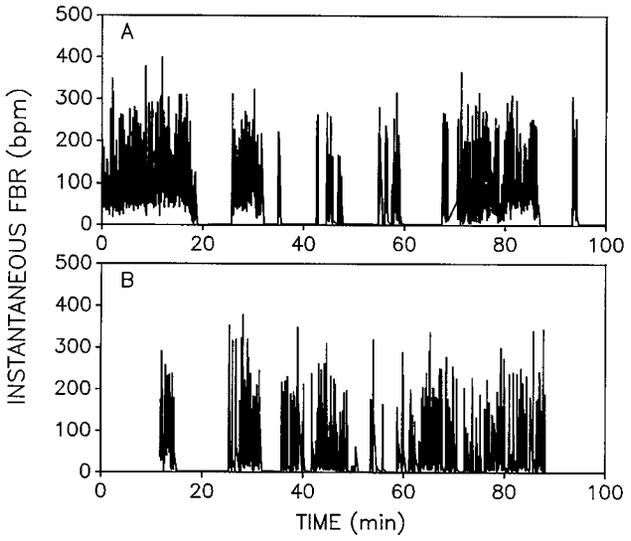

Fig. 3. Time series of instantaneous FBR from the same animal. (*A*) before, and (*B*) 24 h after administration of 34 mg naloxonazine.

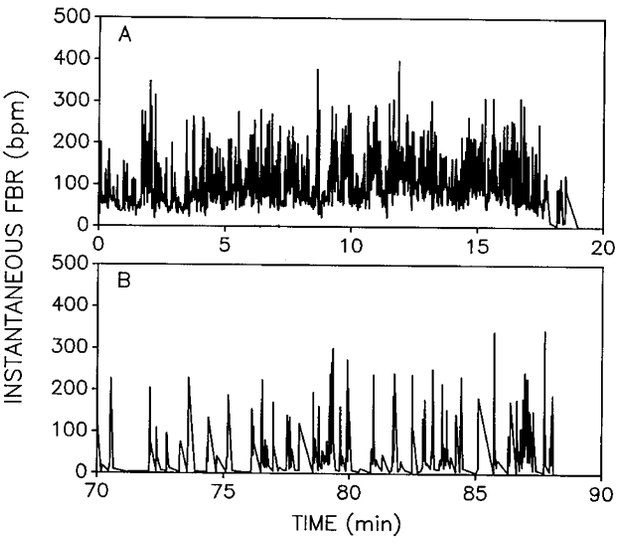

Fig. 4. Each panel represents 20 min of the time series shown in Figure 3*A* and *B*. *A*, control, showing a breathing episode from 0 to 20 min. *B*, 24 h after 34 mg of naloxonazine, showing a breathing episode from 70 to 90 min.

Table 4. *Effects of naloxone and naloxonazine on stability of FBR**

Drug	*n*	% Bursts in clusters	% Time in clusters	No. of clusters/h
Control	5	30 ± 3	9 ± 2	180 ± 30
Naloxone	5	32 ± 9	11 ± 5	180 ± 72
Control	5	47 ± 6	15 ± 3	198 ± 18
Naloxonazine	5	27 ± 4†	8 ± 2†	114 ± 30

* Values are mean ± SEM. Values for naloxone were obtained during 6 mg/h infusion. Values for naloxonazine were obtained 24 h after administration of 34 mg of naloxonazine. All values were compared to control predrug values using the Wilcoxon signed ranks test.

† $p < 0.05$.

not affect the apnea of prematurity (5), nor the recovery of asyphyxiated newborn infants during resuscitation (34).

Although naloxone appears to be ideal for investigating the role of endogenous opioid peptides because it freely crosses the blood-brain barrier and has no apparent agonist actions of its own, a growing concern with the use of naloxone is its lack of selectivity for the various opiate receptor subtypes. Although naloxone is considered to have some selectivity for the mu receptor, its affinity for the mu site is only 4- to 10-fold greater than for the delta site and slightly more for the kappa site (7). Because there is substantial evidence to suggest that each opiate receptor subtype mediates different and possibly opposing actions in the same physiologic system, nonselective blockade may similarly affect opposing actions and thus show no net effect. In addition, it is not possible to study the role of each receptor subtype using a nonselective opiate antagonist such as naloxone or naltrexone.

Recent pharmacologic, biochemical, and receptor binding studies support the presence of two subtypes of mu receptors: the mu_1, which binds both opiates and most enkephalins with similar very high affinity, and the mu_2, which preferentially binds morphine (8). Paakkari *et al.* (9) recently demonstrated, using the conscious adult rat, that naloxonazine, an irreversible mu_1 antagonist (11) was able to antagonize the respiratory stimulation produced by low doses of dermorphin, a highly selective mu agonist. Naloxonazine had no effect on the respiratory depression produced by dermorphin at higher doses. In fact, naloxonazine potentiated the respiratory depression at higher doses of dermorphin. The results of Paakkari *et al.* are in accordance with the concept that mu_2 receptors mediate respiratory depression (10) and mu_1 receptors mediate respiratory stimulation. We have recently demonstrated that the stimulation of FBM by low doses of morphine can also be abolished by naloxonazine pretreatment (18). This led us to propose that endogenous opiate peptides may play a role in maintaining the normal fetal breathing pattern via their actions at the mu_1 receptor.

When naloxonazine was administered to fetal lambs under normal physiologic conditions, it significantly increased the number of apneic periods and respiratory pauses, resulting in a more discontinuous or fragmented FBM pattern without affecting the overall incidence of breathing. In addition, naloxonazine significantly decreased the overall stability or regularity of the breathing rate. It appears that the fragmentation in the FBM pattern occurs within an episode of low-voltage fast activity and is not simply a reflection of a fragmented EC_0G pattern.

Although FBM pattern is generally only characterized by the overall incidence of breathing, these results illustrate the importance of a more quantitative analysis of the dynamic pattern. The dose of naloxonazine used in this study was based on effective doses reported in rodents (9, 10). In addition, we have found this dose to be adequate for blocking the respiratory stimulation effects of low doses of morphine in the fetal lamb (18). The response to naloxonazine was only tested 24 h after administration, inasmuch as it has been shown that [H^3]naloxonazine can label a number of opiate-binding sites with a potency similar to naloxone. However, a portion of this binding is not freely reversible and appears to correspond to the mu_1 site (11). With an elimination of half-life of less than 3 h (35), it can be expected that reversible binding of naloxonazine to other opiate receptor subtypes should be minimal at 24 h, and only the irreversible binding to the mu_1 site would be present.

In contrast to naloxonazine, continuous administration of naloxone to the fetal lamb had no significant effect on the continuity or stability of the breathing pattern. Similarly, previous studies on the effects of naloxone in the fetal sheep did not show any significant effect on the incidence of FBM (31, 32). The dose of naloxone used in this study has previously been shown to be adequate for blocking the effects of exogenously administered opiates (12). Although it is possible that higher doses of naloxone may be required to block the effects of endogenous opiates, we did not use higher doses due to the concern of nonopiate actions of naloxone, such as to the respiratory depressant neurotransmitter, GABA (36). The dose of naloxone we used was much lower than those that have been postulated to antagonize GABA. Furthermore, we did not observe seizures

during naloxone infusion, which provides additional evidence that GABA was not affected.

These results suggest that endogenous opiate peptides play a tonic role at the mu_1 receptor to maintain both the stability and the continuity of the ventilatory pattern in early development. Contrary to the general belief that apnea should be treated with opiate antagonists, these results suggest that mu_1 antagonism may actually further exacerbate the incidence of apneas. Furthermore, these results demonstrate that a lack of effect with naloxone in any system does not necessarily imply that endogenous opioid peptides do not play a regulatory role, and more selective antagonists should be used.

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