Opioid depression of respiration in neonatal rats

John J. Greer, Jo Ellen Carter and Zaidoon Al-Zubaidy

Division of Neuroscience, Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

1. The effects of opioid receptor agonists and antagonists on the breathing pattern of neonatal rats were studied. Three experimental approaches were taken. In the first approach, the effects of opioid agonists and antagonists on the spontaneous respiratory neural activity generated by brainstem–spinal cords isolated from neonatal rats aged 0–4 days postnatal (P0–4) maintained *in vitro* were studied. Secondly, similar studies were performed utilizing medullary slice preparations consisting of respiratory rhythm-generating regions (pre-Bötzinger complex). Thirdly, whole-body plethysmographic recordings were obtained from unanaesthetized neonatal (P0–18) rats before and after I.P. administration of opioid-receptor agonists and antagonists.

2. The μ-receptor agonists morphiceptin and DAGO (Tyr-D-Ala-Gly-[*N*MePhe]-Gly-ol), when added either to the solutions bathing the brainstems of neonatal rat brainstem–spinal cord preparations or bathing the medullary slice preparations, resulted in a naloxone-reversible, dose-dependent decrease in the frequency of respiratory rhythmic discharge.

3. The respiratory burst frequency and amplitude *in vitro* were unaffected by the addition of the δ-opioid receptor agonist DPDPE ([D-pen^{2,5}]-enkephalin) and the κ-opioid receptor agonist U50488 (*trans*-[+]-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl) benzene-acetamide) or the opioid receptor antagonist naloxone.

4. Intraperitoneal administration of the μ-opioid receptor agonist fentanyl resulted in a naloxone-reversible, dose-dependent decrease in the frequency and amplitude of breathing of unanaesthetized neonatal rats (P0–P10). I.P. administration of the δ-opioid receptor agonist DPDPE did not affect breathing of neonatal rats until the second week postnatally.

5. We conclude that opioids suppress the frequency of neonatal rat respiration by acting via μ-opioid receptors located within regions of the ventral medulla containing respiratory rhythm-generating centres (the pre-Bötzinger complex). δ-Opioid receptor activation does not affect breathing in neonatal rats until approximately the second week postnatally.

The administration of exogenous opioids has long been associated with the depression of central respiratory drive in mammals. More recently, attention has focused on the aetiological role of endogenously released opioid neurotransmitters in neonatal respiratory dysfunction. Evidence linking abnormal endogenous opioid levels with anomalies of neonate breathing patterns includes the following. (i) β-Endorphin levels are significantly elevated from normal in the brainstems of infants who have died of sudden infant death syndrome (SIDS) (Coquerel, Buser, Tayot, Pfaff, Matray & Proust, 1992). (ii) Elevated endorphin levels have been found in the cerebrospinal fluid (CSF) of infants suffering from apnoea of prematurity and obesity– hypoventilation syndrome. These patients in turn respond favourably to treatment with the opioid receptor antagonist naloxone (Blanchard & Aranda, 1992). (iii) Naloxone decreases primary apnoea in newborn rabbits (Chernick, Madansky & Lawson, 1980). (iv) Infants with a history of life-threatening events show significantly higher levels of CSF β-endorphins relative to those of control subjects (Myer, Morris, Adams, Brase & Dewey, 1987). (v) Siblings of infants with SIDS, who themselves have an increased likelihood of being afflicted by SIDS, have higher than normal levels of β-endorphins within their CSF (Shook, Watkins & Camporesi, 1990). (vi) Offspring of methadone-addicted mothers show signs of abnormalities in respiratory chemosensory responses during the first months after birth, as well as having a higher incidence of SIDS (Chavez, Ostrea, Stryker & Smialek, 1979; McCann & Lewis, 1991).

There are at least three classes of opioid receptors (μ, δ and κ) distributed widely within the spinal cord, medulla, pons and higher brain centres, all of which could potentially modulate respiratory rhythm and/or motor output (Sales,

Riche, Roques & Denavit-Saubié, 1985; Attali, Saya & Vogel, 1990; Kinney, Ottoson & Frost-White, 1990). Nerve terminals containing endorphins have been identified in medullary respiratory nuclei (Leibstein, Dermietzel, Willenberg & Pauschert, 1985) and a subpopulation of neurons in these respiratory regions of the cat responds to iontophoretic application of opioid agonists, antagonists and enkephalinase inhibitors (Denavit-Saubié, Champagnat & Zieglgänsberger, 1978; Morin-Surun, Boudinot, Fournie-Zaluski, Champagnat, Roques & Denavit-Saubié, 1992). Yet despite the abundance of correlative data regarding opioids and the depression of respiratory drive, the neuronal targets, receptor subtypes and cellular mechanisms of actions associated with opioid-mediated effects on neonatal breathing remain unclear (see Shook *et al.* 1990). An understanding of the underlying neuronal mechanisms of opioid action has potentially important implications for the diagnosis and treatment of neonatal breathing disorders as well as for the design and administration of opioid analgesic drugs. In the present study, we have addressed two fundamental questions pertaining to the depression of neonatal respiration by opioids. First, can opioids depress breathing by acting directly on neuronal receptors in rhythm-generating centres of the ventrolateral medulla? Second, which of the opioid receptor subtypes are involved in depressing neonatal breathing?

To address these issues, we have measured the changes in respiratory frequency and amplitude induced by opioid receptor agonists and antagonists in two types of *in vitro* neonatal rat preparations and in unanaesthetized neonatal rats. The first type of *in vitro* model consisted of the brainstem–spinal cord isolated from neonatal rats (Suzue, 1984; Smith, Greer, Liu & Feldman, 1990). The second type of *in vitro* preparation consisted of 500–600 μm thick slices of regions of the neonatal rat medulla which contain recently identified respiratory rhythm-generating centres (Smith, Ellenberger, Ballanyi, Richter & Feldman, 1991). Both of these preparations generate spontaneous respiratory neural discharge consisting of inspiratory and expiratory phases. Finally, measurements of the effects of opioid agonists and antagonists on the breathing patterns of unanaesthetized neonatal rats aged 0–18 days postnatal (P0–18) were performed by means of whole-body plethysmographic recordings in order to compare *in vitro* and *in vivo* responses.

METHODS

In vitro neonatal rat preparations

Brainstem–spinal cord preparations. Neonatal Sprague–Dawley rats (P0–4) were anaesthetized via inhalation of Metofane (methoxyfluorane) gas (3–4% in air), decerebrated and the neuraxis isolated by dissection in mock CSF solution at 27 °C, as previously described (Smith & Feldman, 1987). In three experiments, dorsal medullary regions were also removed; as shown previously, this does not affect respiratory neural motor discharge *in vitro* (Smith *et al.* 1990; Greer, Smith & Feldman, 1991). The neuraxis was bathed in mock CSF that contained (mM): 128 NaCl, 3·0 KCl, 1·5 $CaCl_2$, 1·0 $MgSO_4$, 24 $NaHCO_3$, 0·5 NaH_2PO_4, and 30 D-glucose equilibrated with 95% O_2–5% CO_2 at 27 °C (pH 7·4). The solutions bathing the brainstem and spinal cord were separated by a Vaseline barrier constructed at the spinomedullary junction.

Medullary slice preparations. Details of the preparation have been previously described (Smith *et al.* 1991). Briefly, 1- to 4-day-old neonatal rats were anaesthetized with Metofane and decerebrated, and the neuraxis isolated by dissection as described above. The brainstem–spinal cord was then pinned down, ventral surface upward, on a paraffin-coated block. The block was mounted in the vise of a vibratome bath (VT 1000; Pelco, St Louis, MO, USA). The brainstem was sectioned serially in the transverse plane starting from the rostral medulla to within ~200 μm of the rostral boundary of the pre-Bötzinger complex, as judged by the appearance of the inferior olive. A single transverse slice containing the pre-Bötzinger complex and more caudal reticular formation regions was then cut (500–600 μm thick), transferred to a recording chamber and pinned down onto a Sylgard elastomer. The medullary slice was bathed in physiological solution similar to that used for the brainstem–spinal cord preparation except for the K^+ concentration, which was elevated to 9–10 mM. Elevated K^+ is necessary for the maintenance of spontaneous rhythmic respiratory motor discharge in the medullary slice (Smith *et al.* 1991). Respiratory motoneuron population activities were recorded with suction electrodes applied to cut ends of XII nerve roots.

In vivo neonatal plethysmographic measurements

Whole-body plethysmographic measurements of frequency and depth of breathing were made from unanaesthetized Sprague–Dawley rats of either sex aged between embryonic day 21 (E21) and postnatal day 18 (P18). Pressure changes associated with neonatal rat breathing (produced by the warming and humidifying of inspired air and the subsequent cooling and condensation of expired air) were measured using a 150 ml whole-body plethysmograph chamber, a pressure transducer (± 0·01 PSI; model DP103, Validyne, Northridge, CA, USA) and signal conditioner (CD-15). The plethysmograph was contained within an infant incubator (model C-86, Isolette, Warminster, PA, USA) in order to maintain the ambient temperature at the approximate nest temperature of 32 °C (Fig. 4*A*). Data were stored on disk for subsequent analysis of inter-breath intervals and the relative amplitude of volume excursions associated with each breath before and after drug delivery.

Recording and analysis

Recordings of cranial (XII) and spinal (C4) motoneuron population activity *in vitro* were made with suction electrodes applied to the cut ends of cranial nerves and spinal ventral roots (Figs 1 and 3). Signals were amplified, rectified, low-pass filtered and recorded on videotape via pulse code modulation (model 3000A, Vetter, Rebersburg, PA, USA) and/or on computer via an analog–digital converter (DigiData 1200, Axon Instruments) and data acquisition software (Axotape, Axon Instruments). Mean values relative to control for the period and peak integrated amplitude of

respiratory motoneuron discharge (*in vitro*) or pressure changes (*in vivo*) were calculated for 2–3 min durations pre- and post-drug delivery. Post-drug measurements were taken 5 min (*in vitro*) and 10 min (*in vivo*) after administration of the drug being studied. In both *in vitro* and *in vivo* experiments, dose–response curves were generated by plotting the changes in breathing parameters in response to incremental doses of opioid agonists. Values of EC_{50} and ED_{50} are defined as the concentration (*in vitro*) and dosage (*in vivo*), respectively, of the drug necessary to produce 50% of the maximum mean measured response. Results were expressed as means ± standard deviation and any differences were tested using paired difference Student's *t* test (comparing data points with control values); significance was accepted at *P* values lower than 0·05.

Pharmacological agents

Drugs added to the *in vitro* bathing solution included the μ-opioid receptor agonists morphiceptin (0·1–2·5 μM) and DAGO (Tyr-D-Ala-Gly-[*N*MePhe]-Gly-ol; 0·2–1·0 μM; Peninsula Laboratories, Belmont, CA, USA); the δ-opioid receptor agonist DPDPE ([D-pen^{2,5}]-enkephalin; 0·1–2·0 μM; Research Biochemicals Inc.); the non-specific opioid receptor antagonist naloxone (1·0–2·0 μM; Sigma); the μ-opioid receptor antagonist CTOP (Cys^2,Try^3, Orn^5,Pen^7-amide; 1–2 μM; Peninsula Laboratories); and the κ-opioid receptor agonist U50488 (*trans*-[+]-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide; 0·1–4·0 μM; Research Biochemicals Inc.). All drugs used *in vitro* were dissolved in mock CSF and the pH adjusted to 7·4. Drugs administered I.P. *in vivo* included the μ-opioid agonist fentanyl HCl (0·01–0·1 mg kg^{-1}; Research Biochemicals Inc.), which readily crosses the blood–brain barrier; naloxone (25–100 μg kg^{-1}); DPDPE (0·01–0·1 mg kg^{-1}) and U50488 (1·0 mg kg^{-1}), all of which were dissolved in physiological saline (0·9% NaCl). The maximum volume of drug solutions administered was 100 μl *in vitro* (added to a 10 ml bath) and 22 μl I.P. *in vivo*. Administration of such small volumes of vehicle did not affect the respiratory parameters studied.

RESULTS

Effects of opioid receptor agonists *in vitro*

The μ-receptor agonist DAGO (0·2–1·0 μM; $EC_{50} \approx 320$ nM; $n = 8$), when added to the solution bathing the brainstem, caused a depression in the respiratory burst frequency generated by *in vitro* preparations isolated from neonatal rats (Fig. 1). Respiratory rhythm returned to control frequency upon subsequent application of either the broad-based opioid receptor antagonist naloxone (1 μM) or the μ-receptor specific antagonist CTOP (1–2 μM). The μ-selective opioid receptor agonist morphiceptin (0·1–2·5 μM) also resulted in a dose-dependent decrease in the frequency of rhythmic respiratory bursts (Fig. 2; $EC_{50} \approx 410$ nM; $n = 16$). Note that no attempt was made to study any age-dependent changes in μ-receptor-mediated depression of respiratory frequency *in vitro*. The

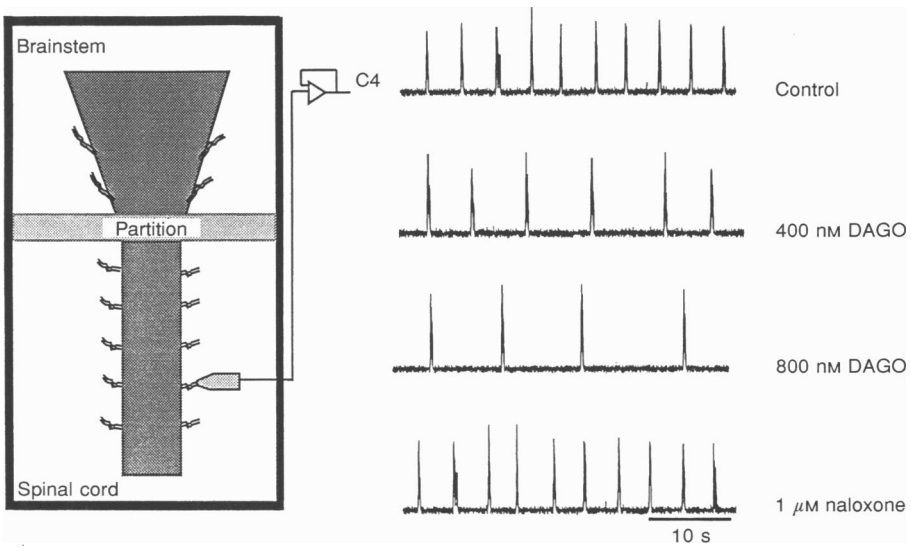

Figure 1. μ-Opioid receptor-mediated depression of spontaneous respiratory burst frequency generated by neonatal rat (P1) brainstem–spinal cord *in vitro*

Left panel depicts recording chamber with isolated neonatal rat brainstem–spinal cord showing arrangement of recording electrode on cervical (C4) ventral roots. Right panel shows rectified and filtered signals from suction electrode recordings of inspiratory motor discharge from the fourth cervical ventral root (C4). The bathing medium was partitioned at the spinomedullary junction and drugs were added selectively to either the brainstem or spinal cord. The addition of the μ-opioid receptor agonist DAGO to the solution bathing the brainstem results in a dose-dependent, naloxone-reversible suppression of respiratory burst frequency.

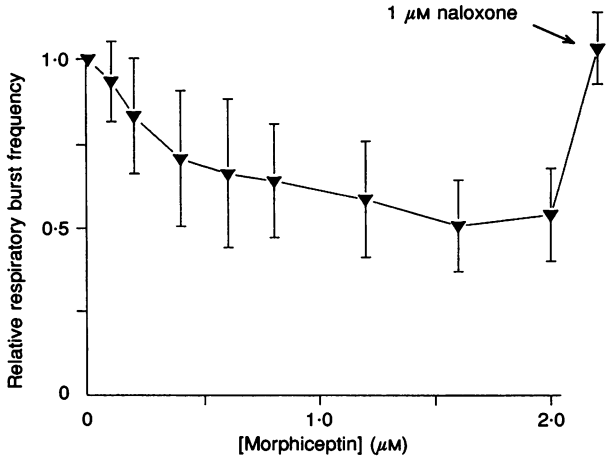

Figure 2. μ-Opioid receptor activation induced a dose-dependent decrease in respiratory frequency in the neonatal brainstem–spinal cord *in vitro*

Morphiceptin concentration–response curve showing respiratory burst frequency plotted as the fraction of control frequency *vs.* concentration of morphiceptin in solution bathing the brainstem. A statistically significant depression of respiratory frequency was induced by as little as 200 nM morphiceptin. Naloxone (1 μM) or CTOP (not shown) antagonized the action of morphiceptin. Data were derived from tissue isolated from neonatal animals aged between 0 and 4 days postnatally (P0, $n = 3$; P1, $n = 6$; P2, $n = 3$; P3, $n = 2$; P4, $n = 2$).

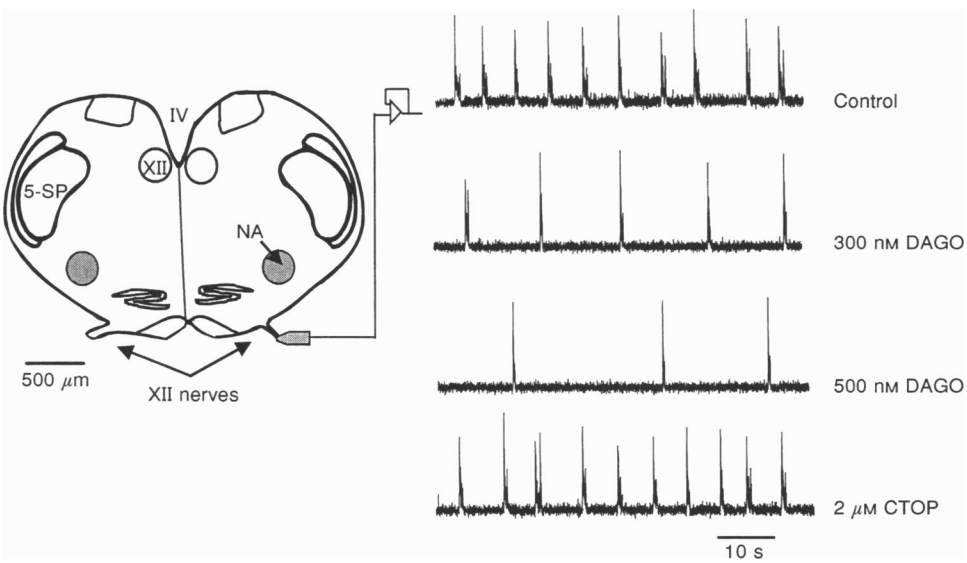

Figure 3. μ-Opioid receptor activation induced a dose-dependent decrease in respiratory frequency in the neonatal medullary slice preparation

The drawing on the left depicts the neonatal rat medullary slice preparation showing the arrangement of recording electrodes on hypoglossal (XII) cranial roots. The right panel shows rectified and filtered signals from suction electrode recordings of inspiratory motor discharge from the hypoglossal cranial nerve (XII). The spontaneous respiratory burst frequency and the burst duration were decreased by the μ-opioid receptor agonist DAGO and reversed by the μ-opioid receptor antagonist CTOP. NA, nucleus ambiguous; XII, hypoglossal motor nucleus; IV, fourth ventricle; 5-SP, spinal trigeminal nucleus.

amplitude (1·08 ± 0·14) and duration (1·04 ± 0·06) of integrated spinal (C4) discharge, relative to control, were not significantly changed by the addition of μ-receptor agonists to medium bathing the brainstem. The morphiceptin-induced inhibition of respiratory rhythm persisted when dorsal regions (i.e. the dorsal respiratory group) of the brainstem were removed ($n = 3$). The amplitude (0·925 ± 0·07) and duration (0·98 ± 0·06) of integrated spinal (C4) discharge were not significantly changed by the addition of μ-receptor agonists to medium bathing the spinal cord. Naloxone (2 μM; $n = 4$), when added on its own to solutions bathing the neonatal spinal cord and brainstem, did not alter the respiratory burst frequency (1·05 ± 0·08) or the amplitude (0·98 ± 0·06) and duration (1·02 ± 0·03) of integrated respiratory motoneuron discharge recorded from spinal axons.

Figure 3 illustrates the effects of the μ-receptor agonist DAGO on the spontaneous respiratory discharge generated by the medullary slice preparation. As with the brainstem–spinal cord preparation, DAGO ($EC_{50} \approx$ 340 nM; $n = 4$) and morphiceptin ($EC_{50} \approx 415$ nM; $n = 4$) depressed respiratory burst frequency in a dose-dependent manner and were antagonized by naloxone and CTOP. The integrated amplitude (1·09 ± 0·14) of hypoglossal motor nerve discharge was not significantly affected by addition of μ-receptor agonists to the bathing medium, but the burst duration was significantly shortened (0·92 ± 0·05).

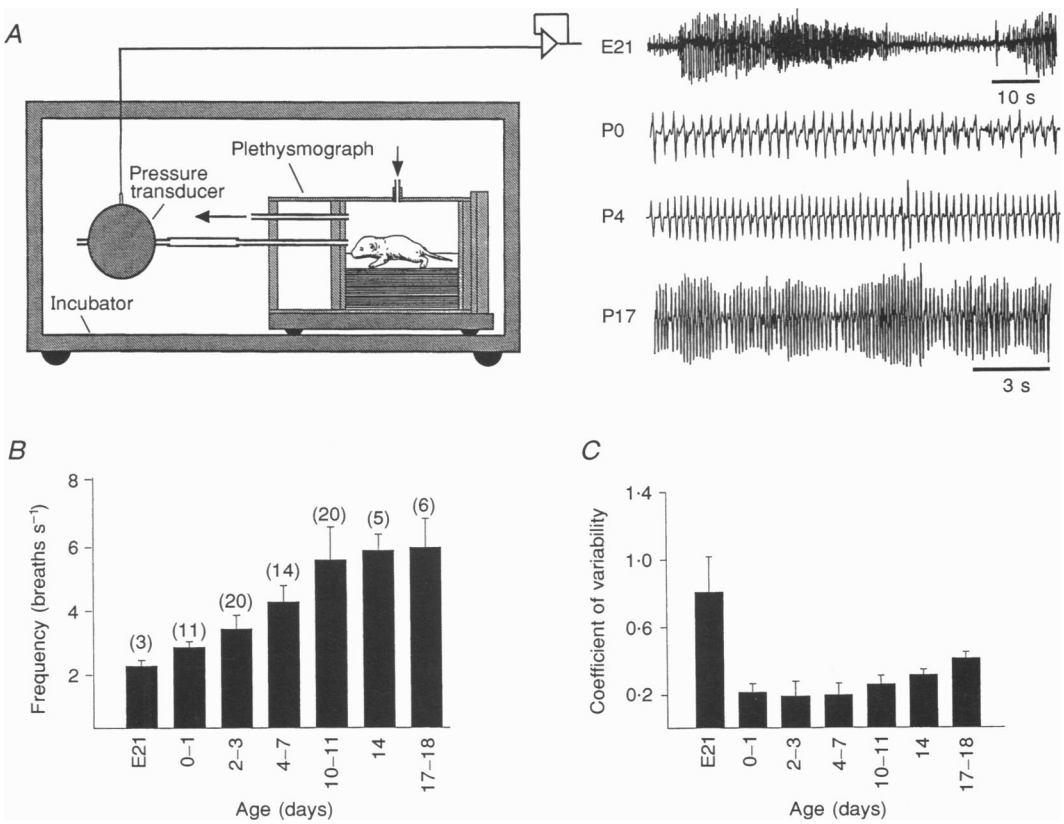

Figure 4. Plethysmographic measurements of perinatal rat breathing

A, whole-body plethysmographic measurements of the relative frequency and depth of breathing were made from unanaesthetized Sprague–Dawley rats between ages E21 (delivered 1 day prematurely) and postnatal day 18. Left panel depicts the plethysmograph contained within an infant incubator in order to maintain the ambient temperature at the approximate nest temperature of 32 °C. Right panel shows examples of plethysmographic recordings from animals of different ages. Upward deflection of the plethysmographic trace represents the inspiratory phase of the respiratory cycle. Note that the top trace (recorded from the E21 rat) has a different time scale to the remaining traces. A longer time scale was chosen to show the tremendous variability in the amplitude and frequency of breathing pattern in animals born prematurely (recordings were made 1·5 h after caesarian delivery). *B* and *C*, population data for the mean frequency (numbers of animals in parentheses) and coefficient of variability (standard deviation/mean), respectively, of breathing in perinatal rats of varying ages.

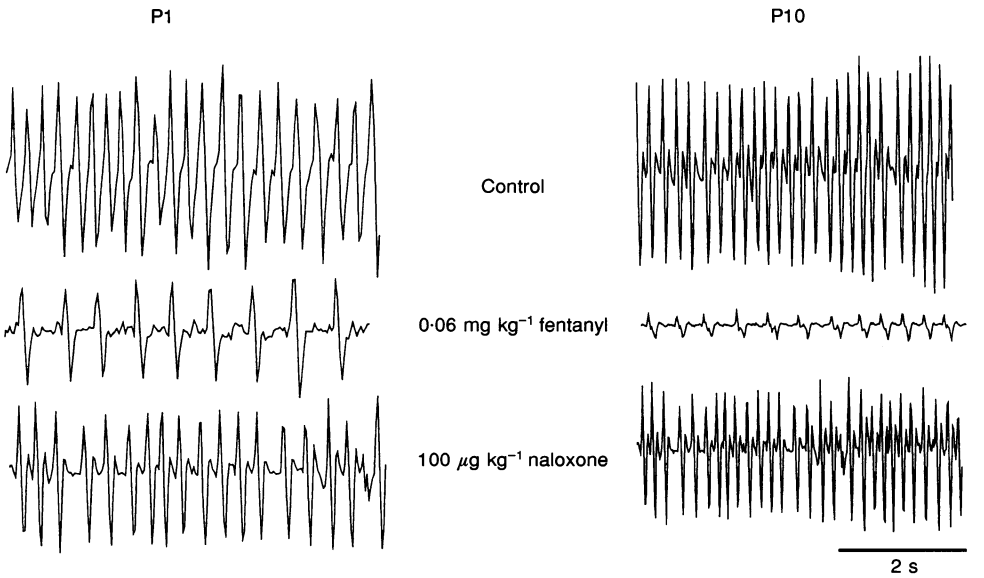

Figure 5. Plethysmographic recordings illustrating the μ-opioid receptor-mediated depression of neonatal rat breathing parameters

The μ-opioid receptor agonist fentanyl, administered I.P. to unanaesthetized rats aged 1 day postnatal (P1; left traces) and 10 days postnatal (P10; right traces) results in a naloxone-reversible depression of breathing frequency and amplitude. μ-Opioid receptor-mediated depression of breathing parameters is significantly increased with age.

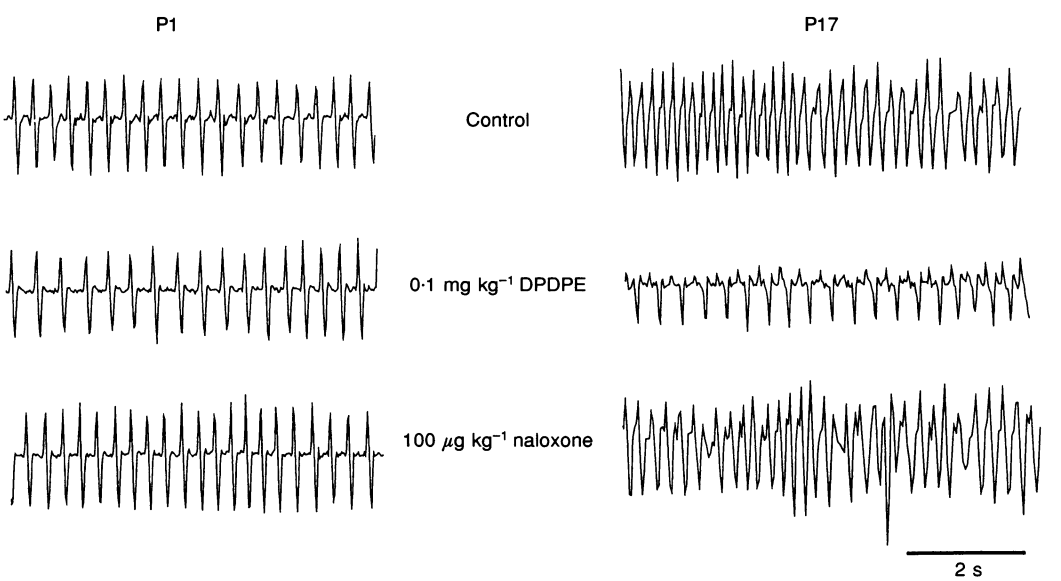

Figure 6. Plethysmographic recordings illustrating the δ-opioid receptor-mediated depression of neonatal rat breathing

The δ-opioid receptor agonist DPDPE, administered I.P. to unanaesthetized rats aged 1 day postnatal (P1; left traces) and 17 days postnatal (P17, right traces) did not affect breathing in young animals but had significant effects on older animals. DPDPE-induced perturbations of breathing were antagonized by naloxone.

Addition of the agonists of δ-opioid receptor subtypes (DPDPE; 0·1–2·0 μM) to the solution bathing the brainstem–spinal cord ($n = 5$) preparation did not affect the burst frequency (0·96 ± 0·06), amplitude (1·03 ± 0·04) or duration (1·02 ± 0·03) of integrated respiratory motoneuron discharge. Similarly, addition of the κ-receptor agonist (U50488; 0·1–4·0 μM; $n = 3$) did not affect the burst frequency (1·01 ± 0·04), amplitude (0·95 ± 0·08) or duration (1·03 ± 0·2) of integrated respiratory motoneuron discharge.

Respiratory pattern generated by unanaesthetized neonatal rats *in vivo*

Before assaying the effects of opioids on neonatal rat respiration, we first measured the normal breathing patterns of animals of varying ages (E21–P18). The baseline frequency of breathing in unanaesthetized neonatal rats increased approximately 3-fold during the first 3 weeks postnatally (Fig. 4). Animals born 1 day premature had a very unstable breathing pattern with wide fluctuations in the amplitude and frequency of breathing.

Effects of opioid-receptor agonists *in vivo*

The frequency and amplitude of breathing in neonatal (P0–10) rats were decreased (within < 2 min) in a dose-dependent fashion by I.P. administration of the μ-opioid receptor agonist fentanyl hydrochloride (0·01–0·1 mg kg^{-1}; Fig. 7). The perturbations of breathing induced by fentanyl were antagonized within 10 min by I.P. injections of naloxone (100 μg kg^{-1}). The magnitude of the depressant actions of fentanyl increased with age during the first 10 days postnatally (Figs 5, 7 and 8).

Administration of the δ-opioid receptor agonist DPDPE (0·01–0·1 mg kg^{-1}) did not perturb the breathing pattern of neonatal rats during the first week postnatally. However, the frequency and amplitude of breathing were reduced in a dose-dependent fashion in rats during the second and third week postnatally (Figs 6, 7 and 8). Naloxone (100 μg kg^{-1}), administered I.P., antagonized the actions of DPDPE.

Administration of the κ-opioid receptor agonist U50488 (1 mg kg^{-1}) to P4 ($n = 3$) and P18 ($n = 3$) neonates did not significantly perturb the frequency (1·02 ± 0·09; 1·06 ± 0·6), amplitude (1·08 ± 0·11; 0·97 ± 0·04) and duration (1·01 ± 0·02; 0·99 ± 0·01) of breathing. Similarly, administration of naloxone (25–100 μg kg^{-1} I.P.) to P3 ($n = 5$) or P10 ($n = 2$) neonates *in vivo* did not significantly change the frequency (1·06 ± 0·07), amplitude (0·97 ± 0·07) or duration (0·98 ± 0·03) of breathing relative to control levels.

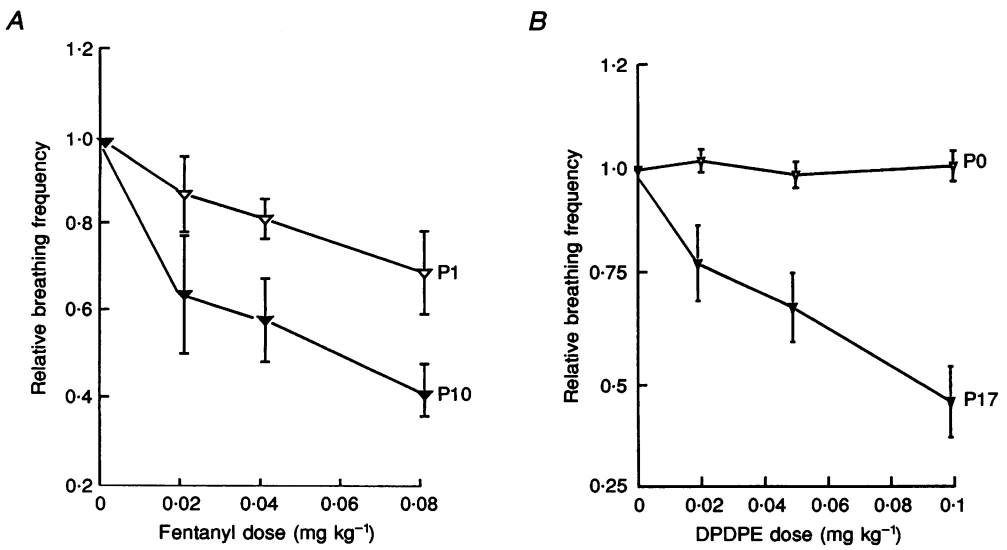

Figure 7. Opioid receptor antagonist concentration–response curves

Changes of breathing frequency induced by I.P. injections of fentanyl to rats aged 1 day postnatal (P1, $n = 5$) or 10 days postnatal (P10, $n = 7$) (*A*) and DPDPE to rats aged 0 (P0, $n = 4$) or 17 days postnatal (P17, $n = 5$) (*B*). Breathing frequency (expressed as a fraction of the control frequency) is plotted *vs.* the dose of drug administered. A significant depression of respiratory frequency was induced in P1 and P10 neonatal rats by 0·02 mg kg^{-1} fentanyl. In contrast, a significant DPDPE-induced depression of respiration was not produced until the second week postnatally.

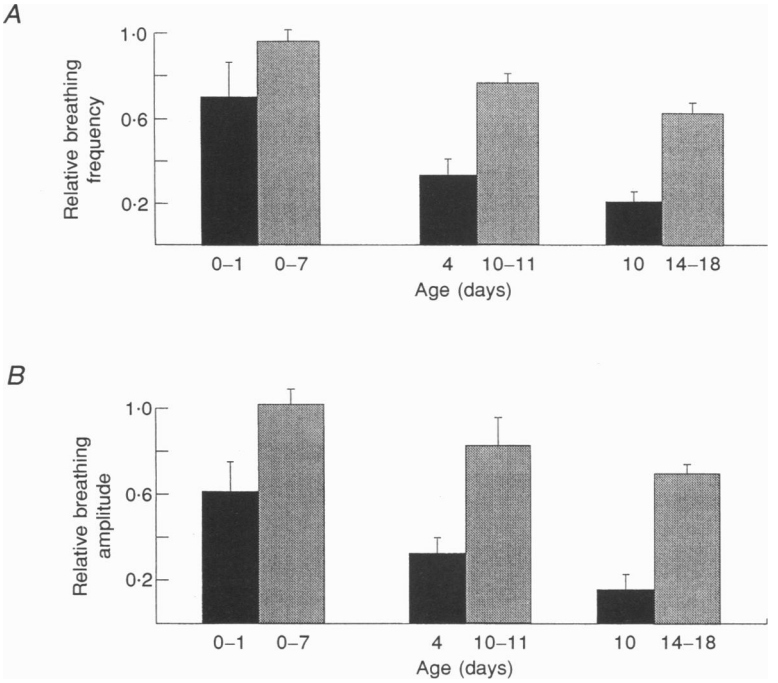

Figure 8. Summary of changes in breathing amplitude and frequency induced by fentanyl and DPDPE

Intraperitoneal injection of the μ-receptor agonist fentanyl (80 μg kg^{-1}; ■) and the δ-receptor agonist DPDPE (0·1 mg kg^{-1}; ▩) in neonatal rats of differing ages. Frequency (*A*) and amplitude (*B*) are plotted as a fraction of the control parameters. A significant age-dependent increase in the depression of respiratory parameters was induced by fentanyl between animals aged P0–1 and P4 and by DPDPE between animals aged P0–7 and P10–11. Further reductions in breathing parameters, which were not statistically significant, were induced by fentanyl between animals aged P4 and P10 and by DPDPE between animals aged P10–11 and P14–18.

DISCUSSION

The burst frequency of spontaneous respiratory motoneuron discharge is depressed in neonatal rats *in vitro* by activation of μ-opioid receptors on neurons located in the region of the ventral medulla containing the proposed respiratory rhythm-generating centre (pre-Bötzinger complex). The magnitude of the depression of breathing frequency and amplitude induced by μ-opioid receptor activation *in vivo* increases postnatally. δ-Opioid receptor activation does not affect breathing in neonatal rats until approximately the second week postnatally.

Sites of action of opioid receptor-mediated depression of respiration

The utilization of isolated brainstem–spinal cord and medullary slice preparations allowed us to address whether or not opioids were capable of depressing respiratory burst frequency and burst amplitude by acting directly on neuronal receptors located in proposed medullary respiratory rhythm-generating centres and respiratory premotoneuron and motoneuron populations. Perturbations of respiratory rhythm generation were analysed following application of selective opioid-receptor agonists and antagonists to the solution bathing the brainstem. These experiments demonstrated that the site of μ-receptor-induced respiratory depression resides within the medulla. Moreover, by removing the dorsal portions of the medulla, we ascertained that the respiratory depression was not mediated via receptors located dorsally. Further delineation of the site of μ-opioid receptor-mediated respiratory depression *in vitro* was obtained by experiments utilizing medullary slice preparations (Smith *et al.* 1991; Funk, Smith & Feldman, 1993). These preparations generate spontaneous respiratory oscillations in hypoglossal motoneuron populations, allowing analysis of the effects of opioid receptor perturbations on respiratory rhythmic motor discharge. The μ-opioid receptor-mediated depression of respiratory frequency observed in the medullary slice experiments was similar to that seen in the brainstem–spinal cord preparations. Thus

the mechanisms of the depression of respiratory burst frequency *in vitro* occurred specifically via receptors located at the level of the medulla where respiratory rhythm-generating centres are thought to exist.

In the case of the whole-animal studies, I.P. application of drugs is non-selective with regard to which neurons within the CNS are being targeted. Nevertheless, the *in vivo* studies did allow for comparison of the overall effects of opioid receptor perturbation *in vitro* and in the intact, unanaesthetized animal. Moreover, with *in vivo* studies, the developmental timeframe for studying the role of opioid receptors in the perturbation of breathing was extended beyond animals aged postnatal day 4 (after which the viability of the respiratory network within *in vitro* preparations is poor). The drugs administered I.P. access the central nervous system by readily crossing the blood–brain barrier, which is not fully mature in the rat until postnatal day 30 (Crook, Kitchen & Hill, 1992). The results from the *in vivo* studies concerning the role of κ-, μ- and δ-receptor-mediated depression of respiratory frequency during the first week postnatally corroborated those obtained from *in vitro* studies. However, there was a difference between the *in vitro* and *in vivo* studies with regard to the μ-receptor-induced depression in amplitude of the breathing parameters being measured. In the unanaesthetized animal, the amplitude as well as the frequency of breathing was depressed by μ-receptor agonists. The lack of amplitude depression of integrated motor nerve discharge *in vitro* suggests that activation of μ-receptors prenatally and until day 4 postnatally does not interfere with transmission of the basic inspiratory drive to cranial and spinal motoneuron populations. *In vivo*, the reduced amplitude of pressure changes generated by breathing movements may have been due to changes in the behavioural state of the animal rather than a direct decrease in respiratory neuronal drive. For instance, there could be a depression of conditioning or modulating inputs onto premotoneuron and/or motoneuron populations from areas of the nervous system which are inactive *in vitro*. This could include several sources, such as peripheral and central afferent pathways, as well as intramedullary and supramedullary pathways which converge on respiratory brainstem and/or spinal nuclei.

δ-Opioid receptor depression of breathing transpired after the first week postnatally and therefore was not accessible for studies utilizing *in vitro* models. Thus we could not discern whether the depression of respiratory frequency was directly due to the actions of neuronal receptors located within the pre-Bötzinger complex or indirectly due to the the actions of neuronal receptors from distant centres which converge onto this region to modulate respiratory rhythm.

Mechanisms of opioid receptor-mediated depression of respiration

κ-Receptors were not involved in the suppression of respiration in neonatal rats. Activation of κ-opioid receptors did not alter the burst frequency or integrated amplitude of respiratory motoneuron discharge *in vitro* or *in vivo*. This result is in agreement with previous studies using primate and adult rat models, which indicated that κ-receptors are not involved in respiratory depression (Shook *et al.* 1990).

μ-Receptor activation resulted in a depression of respiratory burst frequency at the earliest age studied (P0). The cellular mechanisms underlying respiratory rhythmogenesis are currently unknown and thus one cannot target a specific neuronal population to determine which membrane properties are being modulated by the actions of opioid μ-receptor agonists. However, previous studies from other parts of the CNS have shown that inhibitory actions of μ-opioid receptor activation are mediated via G protein-regulated mechanisms at pre- and/or postsynaptic membranes. Presynaptically, opioids acting at μ-receptors can diminish Ca^{2+}-dependent neurotransmitter release directly, either by interfering with the cellular mechanisms underlying vesicular exocytosis or by inhibiting voltage-dependent Ca^{2+} channels, and indirectly, by activating K^+ channels and thereby raising the threshold and shortening the duration of action potentials (Schroeder, Fischbach, Zheng & McCleskey, 1991; Capogna, Gähwiler & Thompson, 1993). Postsynaptically, μ-opioid agonists have been shown to activate at least two different types of membrane potassium conductances, one of which is an inward rectifier (Williams, North & Tokimasa, 1988; Wimpey & Chavkin, 1991). The slowing of respiratory rhythm by μ-opioid agonists observed in the present study could be accounted for by either pre- or postsynaptic events. The oscillatory frequency of respiratory rhythm generated *in vitro* is determined by the level of excitatory conditioning input from endogenously released excitatory amino acids (Greer, Smith & Feldman, 1991; Funk *et al.* 1993). Reduction of this conditioning synaptic drive by lowering the levels of excitatory amino acid release from presynaptic terminals would result in the decreased respiratory rhythm observed. Likewise a reduction in postsynaptic neuronal excitability of neurons responsible for respiratory rhythmogenesis could also result in depressed rhythmogenesis.

Basis for developmental profile of opioid receptor-mediated effects on breathing

The expression of endogenous opioid transmitters in nerve terminals contacting medullary respiratory neurons and the expression of opioid receptors specifically on identified respiratory neurons have yet to be described for the perinatal rat. However, studies from other regions of the

developing rat CNS illustrate that endogenous opioids, endorphins, enkephalins and dynorphins are all expressed prenatally (Hammer & Hauser, 1992). The μ-, κ- and δ-opioid receptors exhibit differential perinatal development profiles. In the rat, μ- and κ-receptors are present at birth, whereas δ-receptors appear during the second week postnatally (Spain, Roth & Coscia, 1985; Kitchen & McDowell, 1986). Thus, the delayed onset of δ-opioid receptor agonist depression of breathing could be explained by a delayed expression of receptors in respiratory rhythm-generating centres. The increased magnitude of μ- and δ-opioid receptor-mediated depression of respiration with age could reflect increasing transmitter release, increasing receptor density and/or increased efficacy of intracellular transduction mechanisms mediating opioid action. There may also be an age-dependent increase in the role of opioid receptor-mediated depression of neurons in synaptic pathways which converge onto and modulate the activity of medullary respiratory rhythm-generating centres and respiratory premotoneuron and motoneuron populations.

Effects of perturbing endogenous opioids with naloxone

Administration of naloxone has been widely used in an attempt to discern whether endogenous opioids act to affect breathing, either tonically or under specific conditions where the respiratory system is challenged (e.g. hypoxia, obstructive pulmonary disease). The general conclusion from these studies is that the antagonizing actions of endogenous opioids with naloxone are species and state dependent. A naloxone-mediated enhancement of normal respiratory drive is consistently seen in cats, dogs and rabbits whereas in rats and humans, opioid depression of breathing seems to participate only during certain ventilatory challenges (Olson, 1987; Shook *et al.* 1990; Akiyama *et al.* 1993). As was the case with studies of the effects of naloxone on adult rats at rest (Steinbrook *et al.* 1984), naloxone did not have a noticeable effect on respiratory parameters measured in the unanaesthetized neonatal rat. Similarly, naloxone was also without effect on its own *in vitro*.

Conclusions

We conclude that opioids suppress the frequency of neonatal rat respiration by acting via μ-opioid receptors located within regions of the ventral medulla containing respiratory rhythm-generating centres (pre-Bötzinger complex). The magnitude of respiratory depression increases postnatally. δ-Opioid receptor activation does not affect breathing in neonatal rats until approximately the second week postnatally.

Correlative opioid receptor-mediated mechanisms, if present within the brainstem of human neonates, could explain how elevated CSF opioid levels associated with SIDS and certain types of infant apnoea depress respiratory drive (i.e. via a direct suppression of neuronal activity within respiratory rhythm-generating centres). Moreover, a similar ontogeny of opioid receptor-mediated effects in the human infant would translate into an increased susceptibility to respiratory depression by endogenously released opioids during the first few months postnatally.

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