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Opioid-resistant respiratory pathway from the preinspiratory neurones to abdominal muscles: *in vivo* and *in vitro* study in the newborn rat

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We report that after spontaneous breathing movements are stopped by administration of opioids (opioid-induced apnoea) in neonatal rats, abdominal muscles continue to contract at a rate similar to that observed during periods of ventilation. Correspondingly, *in vitro* bath application of a μ opioid receptor agonist suppresses the activity of the fourth cervical root (C4) supplying the diaphragm, but not the rhythmic activity of the first lumbar root (L1) innervating the abdominal muscles. This indicates the existence of opioid-resistant rhythmogenic neurones and a neuronal pathway transmitting their activity to the abdominal motoneurones. We have investigated this pathway by using a brainstem–spinal cord preparation of the neonatal rat. We identified bulbospinal neurones with a firing pattern identical to that of the L1 root. These neurones were located caudal to the obex in the vicinity of the nucleus retroambiguus. Resting potentials ranged from −49 to −40 mV (mean ± S.D. −44.0 ± 4.3 mV). The mean input resistance was 315.5 ± 54.8 MΩ. The mean antidromic latency from the L1 level was 42.8 ± 4.4 ms. Axons crossed the midline at the level of the cell body. The activity pattern of the bulbospinal neurones and the L1 root consisted of two bursts per respiratory cycle with a silent period during inspiration. This pattern is characteristic of preinspiratory neurones. We found that 11 % of the preinspiratory neurones projected to the area where the bulbospinal neurones were located. These preinspiratory neurones were found in the rostral ventrolateral medulla close (200–350 μm) to the ventral surface at the level of the rostral half of the nucleus retrofacialis. Our data suggest the operation of a disynaptic pathway from the preinspiratory neurones to the L1 motoneurones in the *in vitro* preparation. We propose that the same pathway is responsible for rhythmic activation of the abdominal muscles during opioid-induced apnoea in the newborn rat.

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An increased level of a μ opioid receptor agonist in the cerebrospinal fluid suppresses respiratory movements. Opioid-related cessation of inspiratory efforts may protect against entry of amniotic fluid into the upper respiratory pathways of a fetus immediately before birth and during the course of delivery (Jansen & Chernick, 1983, 1991) but is hazardous afterwards. Abnormally high levels of endogenous opioid peptides are found in infants with a history of an 'apparent life-threatening event' and in siblings of victims of the sudden infant death syndrome (Myer *et al.* 1987; Storm *et al.* 1994). Sensitivity to μ opioid receptor agonists is highest in the early stages of postnatal development and decreases with maturation (Bragg *et al.* 1995; Windh & Kuhn, 1995). Under identical experimental conditions, a μ opioid receptor agonist, fentanyl, reduces the frequency of breathing by 75 % in neonates, but less than 50 % in adult rats of the same strain (Colman & Miller, 2001).

In humans and other mammalian species, in both adults and neonates, exposure to exogenous opioids, as a consequence of therapeutic administration or opiate dependence, leads to pathological breathing (Shook *et al.* 1990; Greer *et al.* 1995; Ballanyi *et al.* 1997). Recognizing which subpopulations of the respiratory neurones are most sensitive to opioids may help us to understand the mechanism of opioid-induced respiratory depression. If these neurones exhibit a distinct phenotype, they may be targeted with drugs capable of reversing respiratory depression without affecting desired analgesic effects of opioids (Ballanyi *et al.* 1997; Sahibzada *et al.* 2000).

In the present study, we report that abdominal muscle EMG discharges (EMG_{ABD}) remain rhythmic despite suppression of inspiratory activity by opioids in the newborn rat *in vivo*. We have sought to identify the origin of the opioid-resistant rhythm and the pathway

transmitting it to L1 motoneurones, by using the *in vitro* brainstem–spinal cord preparation.

Premotoneurones supplying abdominal motoneurones are localized in the caudal part of the ventral respiratory group (VRG), within or close to the nucleus retroambiguus in adult cats, ferrets and monkeys (Miller *et al.* 1985; Arita *et al.* 1987; Sasaki *et al.* 1994; Iscoe, 1998; Boers & Holstege, 1999; Billig *et al.* 2000; Vanderhorst *et al.* 2000). One may assume that the location of the abdominal premotoneurones is the same in the newborn rat.

The location of neurones providing excitatory, respiratory-related input to the premotoneurones is not known. Abdominal premotoneurones integrate inputs subserving both respiration and non-respiratory behaviours such as speech (or vocalization), coughing, sneezing, vomiting, straining, defecation and postural adjustments (Iscoe, 1998). Studies using trans-synaptic neuronal tracers (Holstege & Kuypers, 1982, 1987; Iscoe, 1998; Billig *et al.* 2000) labelled several groups of neurones in the medulla and pons which project to the premotoneurones, but at present it is not possible to determine which one of them is involved in respiratory function of the abdominal muscles.

In vitro, after addition of morphine, or a selective μ opioid receptor agonist, to the artificial cerebrospinal fluid, all preinspiratory neurones continue to fire regularly, and many of these bursts are no longer followed by the inspiratory C4 root activity (Takeda *et al.* 2001). Since activity of both the abdominal muscles and the preinspiratory neurones is resistant to opioids and, as shown below, their discharge patterns are similar, we postulated that the preinspiratory neurones may contribute to the activation of the abdominal muscles. Therefore, using the brainstem–spinal cord preparation, we tested the hypothesis that the preinspiratory neurones supply abdominal motoneurones either directly or via bulbospinal neurones located in the caudal VRG.

If this hypothesis and a correspondence between *in vivo* and *in vitro* preparations can be confirmed, we propose that, with an increased level of opioids in the cerebrospinal fluid, EMG_{ABD} reflects the activity of the preinspiratory neurones. We assume that activity of the C4 root supplying the diaphragm reflects central inspiratory activity and that L1 root activity supplying the abdominal muscles (Fregosi *et al.* 1992) corresponds to the EMG_{ABD} activity.

To determine whether a brainstem–spinal cord preparation and the L1 activity may be used to draw conclusions about the *in vivo* changes of the EMG_{ABD} activity, we addressed three questions. (1) What is the pattern of the L1 activity in the brainstem–spinal cord preparation? (2) Does this pattern match the abdominal activity *in vivo*? (3) Does the L1 root remain active after bath application of a μ opioid receptor agonist eliminates C4 activity?

Part of this study has been presented previously in abstract form (Janczewski *et al.* 1999).

METHODS

Animals and recordings

Experiments were performed on Sprague-Dawley and Wistar neonatal rats (1–6 days old). The experimental protocols were approved by the Animal Research Committee of the Showa Medical School, which operates in accordance with Japanese Governmental Law Number 105, and by the UCLA Animal Care Committee (ARC no. 94-159-23C).

Rat pups ($n = 9$) were anaesthetized with ketamine (70 mg kg^{-1}), fentanyl citrate (0.02 mg kg^{-1}) and atropine (0.06 mg kg^{-1}) administered subcutaneously (Wixson & Smiler, 1997). The level of anaesthesia was assessed by the suppression of the withdrawal reflex, and by the absence of changes in heart rate and breathing rate in response to noxious stimuli. Rats were bivagotomized and intubated. They breathed a 1 : 3 air–oxygen mixture. Respiratory flow was measured with a pneumotachograph. Flow calibration and calculation of tidal volume (V_T) were performed as described by Wang *et al.* (1996). To record EMG_{ABD}, wire electrodes (Cooner Wire Co., Chatsworth, CA, USA) were implanted into the abdominal oblique muscles. In four pups, additional EMG electrodes were placed in the costal diaphragm to record the diaphragmatic EMG (EMG_{DIA}). Haemoglobin saturation (S_{p,O_2}) and pulse rate were measured with a pulse oximeter (Model 8600V from Nonin Medical, Inc., Plymouth, MN, USA). Administration of fentanyl citrate (0.025 mg kg^{-1} S.C.) resulted in periods of hypoventilation ($S_{p,O_2} < 90\,\%$). In some rats, shortly post-injection, a period of apnoea was associated with bradycardia. These pups were ventilated (Rodent ventilator, Harvard Apparatus, Holliston, MA, USA) until their heart rates normalized (1–2 min). About 1 h after the first dose of fentanyl the second dose (0.02 mg kg^{-1}) was administered. Naloxone (0.01 mg kg^{-1} S.C.) was injected to reverse the effects of fentanyl. Then the rats were killed with an overdose of pentobarbitone (100 mg kg^{-1} I.P.).

For *in vitro* studies, brainstem–spinal cord preparations ($n = 37$) were isolated when the rats were under deep ether anaesthesia (Suzue, 1984). Brainstem–spinal cord preparations were superfused with artificial cerebrospinal fluid containing (mM): 124.0 NaCl, 5.0 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26.0 $NaHCO_3$, 1.2 KH_2PO_4 and 30 glucose equilibrated with 95 % O_2 and 5 % CO_2, at 25–26 °C and pH 7.4.

The spinal cord was split in the lumbar and lower thoracic segments to ensure that an electrical pulse applied to one side of the spinal cord did not stimulate descending axons on the other side via axon collaterals crossing the midline at the lumbar level or due to spread of current. The activities of the C4 ventral root and the L1 ventral root were recorded through a glass suction electrode and a high-pass filter with a 0.3 s time constant. To study effects of a μ opioid receptor agonist on the L1 activity, 0.6–1.0 μM DAGO ([D-Ala(2),*N*-Me-Phe(4),Gly(5)-ol]-enkephalin) was added to the artificial cerebrospinal fluid. DAGO (also referred to as DAMGO) is a peptide and a selective μ opioid receptor agonist (Meucci*et al.* 1989), and most of the published *in vitro* work has been done using this agonist (Gray *et al.* 1999; Takeda *et al.* 2001). DAGO does not cross the blood–brain barrier; therefore, for the *in vivo* study we used the non-peptide synthetic selective μ opioid receptor agonist, fentanyl (Davis & Cook, 1986). The respective

binding affinities of these two agents to the rat μ opioid receptor are similar (Bonner *et al.* 2000).

Intracellular recordings *in vitro*

We surveyed the nucleus retroambiguus from the obex towards the decussation of the pyramids. For antidromic activation, 0.1 ms electrical pulses (0.1 ms, 10–20 μA) were delivered through a tungsten electrode. Electrodes were placed on the left and right sides of the lumbar cord. The tip of each electrode was positioned in the lower quadrant of the L1 segment. When the neurone was not activated antidromically from the L1 level, we also examined responses to T3 level stimulation. To confirm the firing pattern of respiratory neurones, extracellular recordings were always performed in cell-attached mode before whole-cell recordings were made. Then the collision test was performed. The membrane potential was recorded with a CEZ-3100 amplifier (Nihon Koden, Tokyo, Japan). Whole-cell patch clamp recordings were obtained from nine neurones in the caudal VRG. These neurones followed the L1 discharge pattern and tested positive for an antidromic spike collision. Six of these cells were stained with 1 % Lucifer Yellow. Next, a tungsten stimulating electrode was placed at the spot where the bulbospinal neurones in the caudal VRG were located. Whole-cell patch clamp recordings were made from the preinspiratory neurones in the rostral ventrolateral medulla (RVLM) (Onimaru *et al.* 1987, 1988, 1995; Takeda *et al.* 2001) and they were tested for antidromic activation from the caudal VRG area (0.1 ms, 10–20 μA). The recording method and the staining method were described previously (Onimaru & Homma, 1992; Onimaru *et al.* 1995).

All signals were stored digitally with hardware and software from Axon Instruments Inc. (Foster City, CA, USA). Statistical significance was determined by one-way ANOVA followed by the Bonferroni multiple-comparison test. $P < 0.05$ was considered significant. All results are expressed as means ± S.D.

RESULTS

Abdominal muscle activity in response to fentanyl *in vivo*

Administration of a selective μ opioid receptor agonist, fentanyl, resulted in repetitive periods of apnoea. During these periods, EMG_{ABD} bursts continued at a pace similar to that observed during periods of effective ventilation. Thus, all respiratory cycles could be divided into two classes: (1) effective cycles, when inspiratory and expiratory muscles were activated; and (2) apnoeic cycles, when inspiratory muscles failed to contract and only expiratory muscles were activated. Out of 18 EMG_{ABD} expiratory bursts shown in Fig. 1, 61 % (11/18) were not associated with inspiratory activity. The number of consecutive apnoeic cycles separating effective ones varied from one to several. During effective cycles, abdominal activity typically took the form of two distinct bursts. The first burst started before inspiration and rapidly terminated at the beginning of inspiration. The second started 0.47 ± 0.04 s later, immediately after inspiration. Sometimes the first burst was prolonged and the second brief or absent.

During the apnoeic cycles, the EMG_{ABD} activity always took the form of a single burst. There was no inspiratory

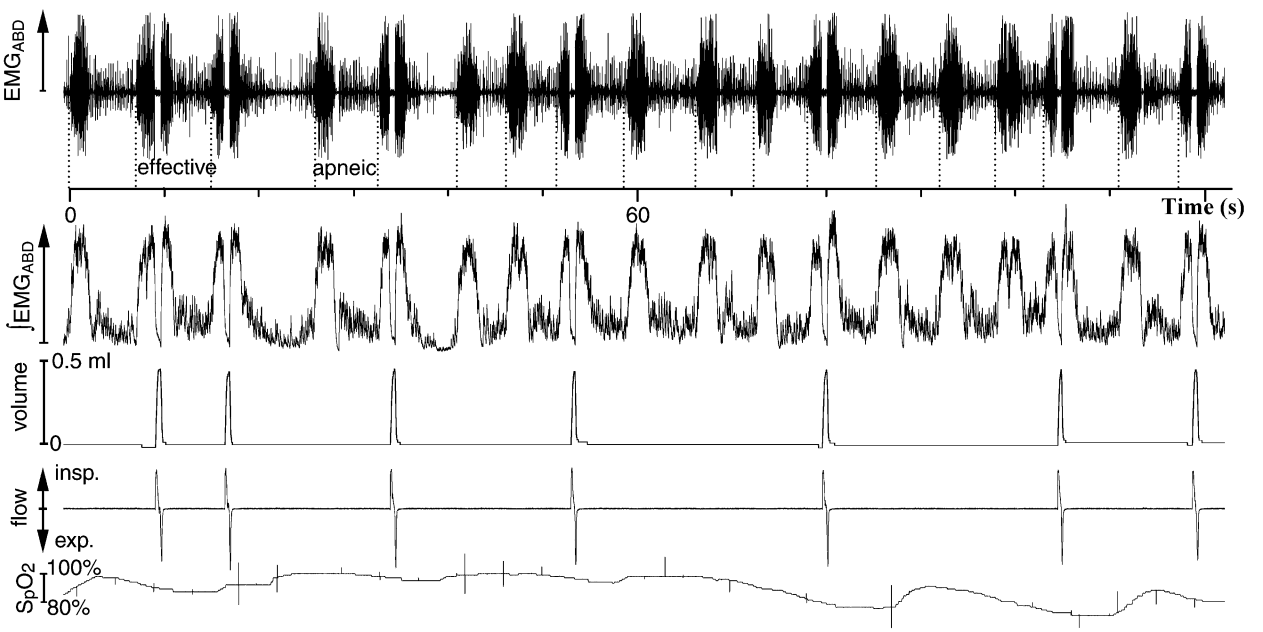

Figure 1. Abdominal muscle activity and inspiratory activity in a 5-day-old rat after administration of fentanyl

An absence of respiratory flow during apnoeic cycles indicates that neither the diaphragm nor the inspiratory intercostal muscles contract during these cycles. Due to an integration process, the S_{p,O_2} signal lags behind other signals. Each inspiration has an effect on haemoglobin oxygen saturation. This is particularly clear after three consecutive apnoeic cycles. Traces, from top to bottom: raw and integrated abdominal EMG; tidal volume; tracheal flow; percentage haemoglobin saturation.

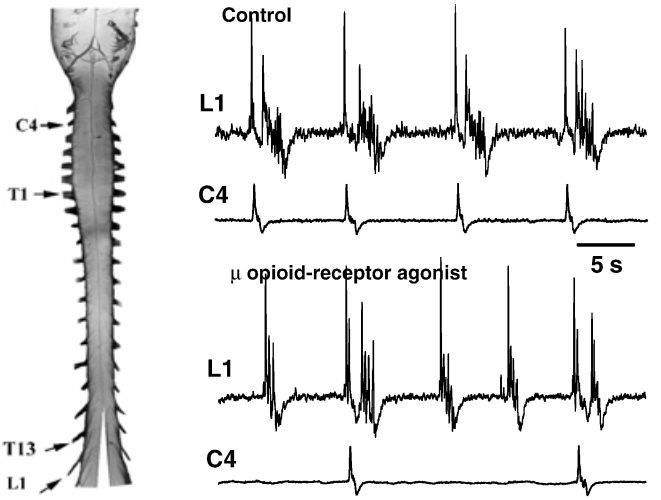

Figure 2. Activity of the C4 root and the L1 root in the brainstem–spinal cord preparation

A, a control recording. *B*, a response to the μ opioid receptor agonist. Note that in the absence of C4 activity, the L1 activity is transformed into a single burst. A model for the mechanism of this transformation is shown in Fig. 6.

flow and blood oxygen saturation gradually dropped. A single breath with 75% oxygen had the capacity to reverse this trend and temporarily increase haemoglobin saturation. Depression of breathing was most severe shortly after administration of fentanyl. The number of respiratory cycles without inspiratory effort was significantly higher 10–20 min after injection of fentanyl (42.4 ± 7.1%) than it was 30–40 min post-injection (8.8 ± 4.2%). The rates of EMG_{ABD} bursts were similar (8.9 ± 1.7 *vs.* 8.5 ± 0.9 min^{-1}) during the above periods.

In four rats, the EMG_{DIA} was simultaneously recorded. There was no inspiratory flow in the absence of EMG_{DIA}, indicating that accessory inspiratory muscles were inactivated together with the diaphragm. During periods of apnoea, in 56% of pups low-amplitude tonic EMG_{ABD} discharges continued throughout expiration (Fig. 1).

Apnoeic cycles disappeared spontaneously 50–60 min post-injection. They could be restored at will by an additional dose of fentanyl. Administration of a μ opioid receptor antagonist, naloxone, promptly eliminated all apnoeic cycles.

Characteristics of the L1 and C4 root activity *in vitro*

The firing pattern of the L1 root *in vitro* consisted of two distinct bursts bracketing C4 root activity (see Fig. 2). The duration of the burst preceeding inspiratory activity varied from 0.1 to 0.37 s. The silent period in between the first and second burst lasted for 0.8–1.7 s. The second burst lasted for 2.7–4.3 s. Double bursts appeared at a rate of

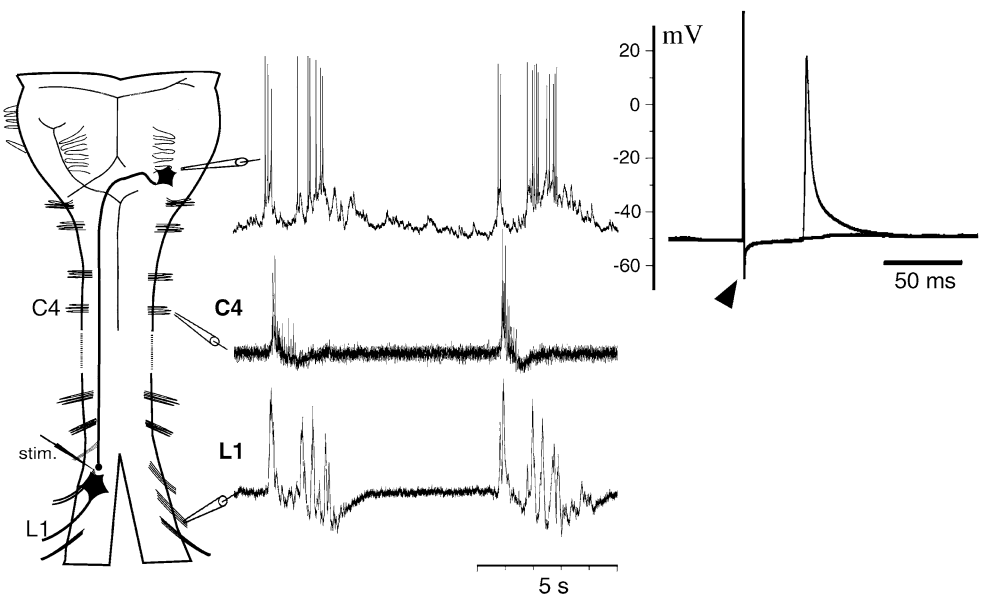

Figure 3. Activity of a bulbospinal neurone supplying L1 root motoneurones

The left panel shows activity of the twice-bursting bulbospinal neurone, together with activity of the L1 and C4 root. The right panel shows a response to the antidromic stimulation with sub- and suprathreshold current. The arrow indicates the time of stimulation. The antidromic action potential was not preceded by an EPSP. The threshold current was constant and the antidromic latency (37 ms) was constant at various stimulus strengths.

7.8 ± 1.5 min^{-1} (n = 8; period 8.6 ± 0.9 s). The pattern of the L1 activity after application of the μ opioid receptor agonist was strikingly similar to that of the EMG_{ABD} *in vivo*. More than 60 % of L1 bursts were not associated with a C4 burst. The L1 burst rate was 8.8 ± 0.8 min^{-1} (n = 8). During apnoeic cycles the L1 activity took the form of a single burst. During cycles associated with the C4 root activity, the typical pattern of L1 activity consisted of two bursts per cycle. Transiently, before complete suppression of inspiratory activity, the C4 burst appeared at the end of a single prolonged L1 burst.

In the absence of a μ opioid receptor agonist in the bath, apnoeic cycles were rare (< 2 % of all cycles).

Properties of bulbospinal neurones and the RVLM neurones

We have found twice-bursting neurones with respective patterns matching those of the L1 root in the ventrolateral medulla caudal to the obex (i.e. the caudal VRG region). Long-lasting intracellular recordings were obtained from nine such neurones. In six of them, an action potential was induced by stimulation of the contralateral L1 segment (Fig. 3). The spike latency for each neurone was constant at various stimulus strengths and the intracellular spike was not preceded by EPSPs, indicating that these neurones were antidromically activated from the contralateral L1 level. The average antidromic latency was 42. 8 ± 4.4 ms (n = 6). In six other bulbospinal neurones that were recorded extracellularly, the antidromic latency was 40.3 ± 4.4 ms. When the stimulating current was increased by 40 %, two of these neurones could also be activated from the ipsilateral side. Since the distance from the stimulating electrode to the cell body was 23.6 ± 1.3 mm, the conduction velocity was estimated to be 0.57 ± 0.06 m s^{-1} (from 0.50 to 0.66 m s^{-1}). The resting potential ranged from −49 to −40 mV (44.0 ± 4.3 mV). The input membrane resistance was 315.5 ± 54.8 MΩ. Action potentials were generated after a depolarization of

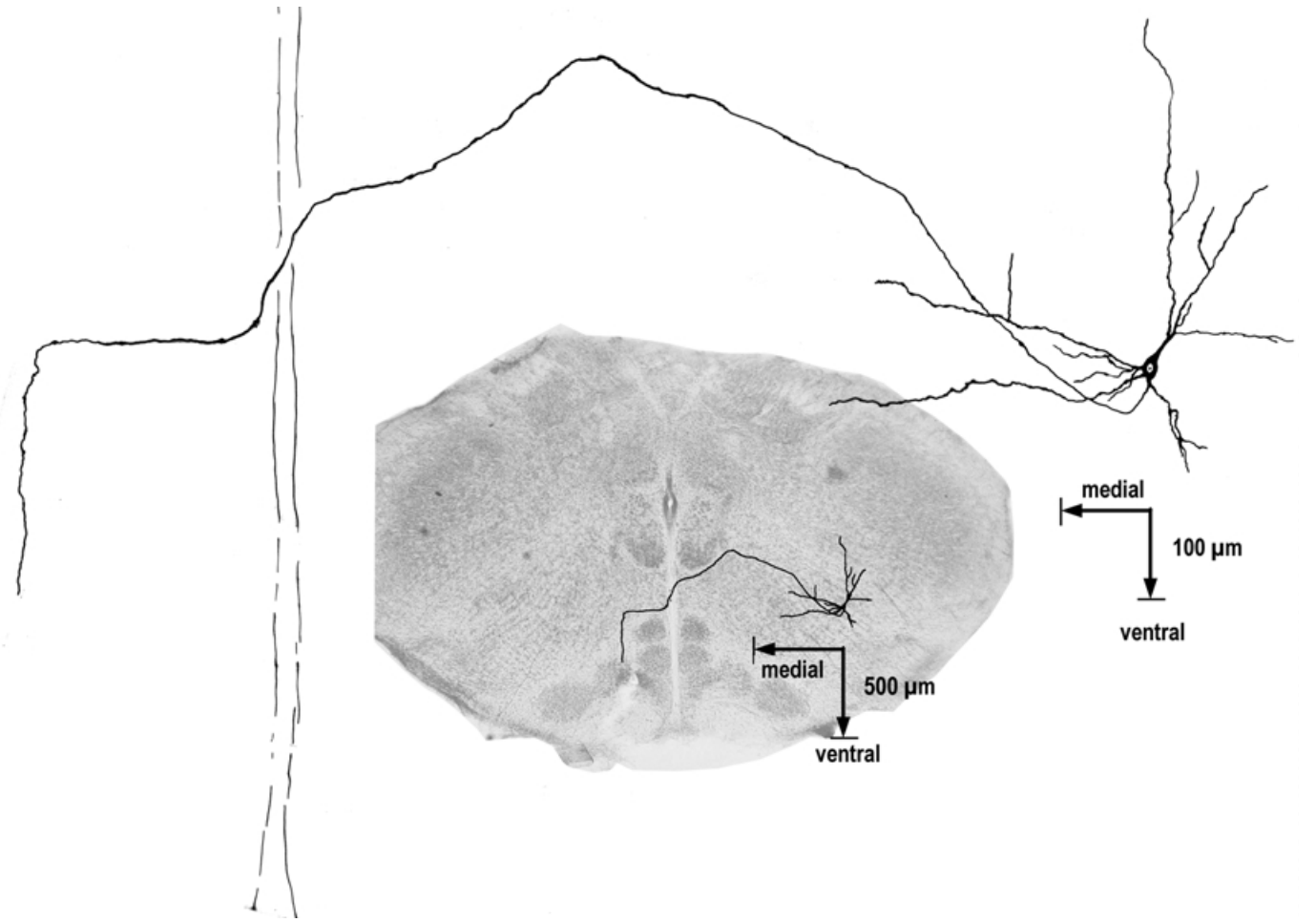

Figure 4. Transverse section through the rat medulla caudal to the obex, showing reconstruction of an electrophysiologically identified, intracellularly filled, twice-bursting bulbospinal neurone projecting to the L1 level

Twice-bursting neurones that could be antidromically activated at the L1 level had a soma diameter of 10–30 μm, and their locations coexisted with, or were just ventral to, the nucleus retroambiguus. Axons crossed the midline at the same rostrocaudal level as the cell bodies. The primary dendrites projected ventromedially and dorsolaterally.

10–15 mV. During spontaneous bursts, the activity of the whole L1 root followed that of bulbospinal neurones with a latency of 72 ± 24 ms. Bulbospinal neurones recorded intracellularly were filled with Lucifer Yellow and reconstructed. They had a diameter of 10–30 μm, and their locations coexisted with, or were just ventral to, the nucleus retroambiguus (Paxinos *et al.* 1999). Initially, axons projected dorsally and slightly rostrally to the cell bodies; next, they turned caudally and ventrally, and then turned medially toward the decussation of the medial lemniscus to cross the midline at the same rostrocaudal level as the cell bodies. The primary dendrites projected ventromedially and dorsolaterally (see Fig. 4).

The activity of the bulbospinal neurones started before, was absent during, and resumed after inspiration (Fig. 3). At the location of these bulbospinal neurones, we found three neurones exhibiting the same twice-bursting firing pattern as the bulbospinal neurones, but responding to electrical stimulation neither from the L1 level nor the T3 level. In the caudal VRG, we also found seven inspiratory neurones and four tonic expiratory neurones. Five of these inspiratory neurones, like all of the tonic expiratory neurones, could not be activated from the L1 level. The remaining two inspiratory neurones responded to stimulation of the contralateral L1 segment with latencies of 42 and 45 ms, respectively.

Electrical stimulation applied at the location of the bulbospinal neurones elicited an orthodromic activation of the L1 root and antidromic action potential in the preinspiratory neurones (5/44 = 11.4 %). The preinspiratory neurones driving bulbospinal neurones were located in the ipsilateral RVLM, 200–350 μm below the ventral surface of the medulla at the level of the rostral half of the retrofacial nucleus. Their firing pattern was the same as that of the L1 root (see Fig. 5). Their resting membrane potential was −45.5 ± 2.1 mV and their input resistance was 450.5 ± 43 MΩ. Neither their membrane properties nor their locations were different from those of the remaining 39 neurones, which did not project to the caudal VRG. None of the RVLM neurones could be activated from the L1 level.

In seven preparations, a midline section was performed between the obex and the C1 segment. The section consistently resulted in an instant cessation of L1 activity but not C4 activity.

DISCUSSION

This study shows that administration of a selective μ opioid receptor agonist suppresses inspiratory activity, while rhythmic expiratory activity of the abdominal muscles/L1 root continues, in the neonatal rat *in vivo* and *in vitro*. Differences between the effect of an opioid-receptor agonist, morphine, on inspiratory motor activity and its effect on expiratory motor activity were previously investigated in adult rabbits by Howard & Sears (1991). These authors reported that morphine-induced apnoea was associated with tonic firing of intercostal expiratory motoneurones. The magnitude of the tonic expiratory activity could be substantially increased by an increase of the end-tidal CO_2. Even if they were intense, tonic discharges did not become phasic before inspiratory activity resumed (Howard & Sears, 1991).

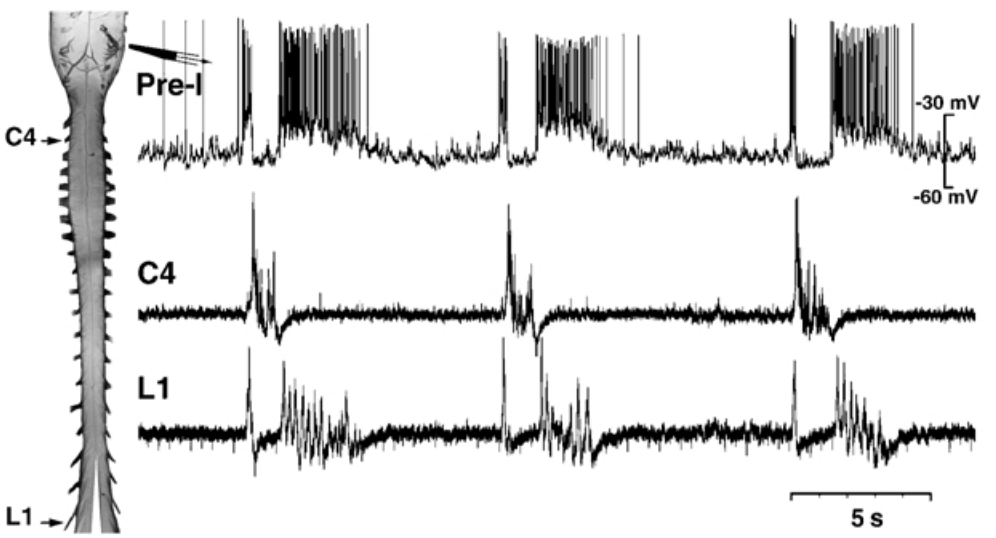

Figure 5. Activity of the preinspiratory neurone projecting to the nucleus retroambiguus and traces of the C4 and L1 root activity

Five out of 44 preinspiratory neurones projected to the nucleus retroambiguus and none projected directly to the L1 level. These five neurones were found close (200–350 μm) to the ventral surface at the level of the rostral half of the nucleus retrofacialis.

Rhythmic activity of the abdominal muscles during apnoeas of unknown aetiology is observed in infants and other young children (Southall *et al.* 1985). These authors argued that inspiratory efforts, normally associated with expiratory activity, were absent, rather than that they remained undetected because of an upper-airway obstruction.

Opioid-induced apnoea in the newborn rat provides an example of an apnoea associated with rhythmic activation of expiratory muscles. This could not result from upper-airway obstruction, because in our experiments the upper airway was bypassed with a tracheal cannula. There was virtually no inspiratory flow in the absence of diaphragmatic EMG activity, indicating that not only the diaphragm but also the thoracic accessory inspiratory muscles were inactivated during an opioid-induced period of apnoea. The longest period of apnoea was typically the first one after injection of the opioid agonist, when its concentration in the cerebrospinal fluid must have been at its highest. Afterwards, neural circuits responsible for triggering inspiratory activity failed repeatedly, leading to periods of apnoea separated by one or a few inspiratory efforts. However, even these infrequent inspirations enabled the rats to survive because they increased haemoglobin saturation (Fig. 1). In contrast to the low and variable rate of the inspiratory activity, the rate of EMG_{ABD} bursts was constant throughout the experiment. The typical EMG_{ABD}/L1 pattern associated with inspiratory activity consisted of two separate bursts bracketing inspiration. Two bursts were always replaced by a single burst during periods of apnoea.

The aforementioned patterns of the abdominal muscle activity in response to opioids can be explained by assuming that motoneurones supplying abdominal muscles receive excitatory drive from the preinspiratory neurones (Onimaru *et al.* 1987, 1988), as shown schematically in Fig. 6.

The preinspiratory neurones, also called 'biphasic expiratory neurons' (Smith *et al.* 1990) are the only ones known to fire two separate bursts during a single respiratory cycle. This pattern matches that of the EMG_{ABD}/L1 activity. Forty per cent of the preinspiratory neurones continue to generate rhythmic bursts after being synaptically isolated from other cells, which indicates that they possess intrinsic rhythmogenic properties (Onimaru *et al.* 1989, 1995). All preinspiratory neurones studied by Takeda *et al.* (2001) were opioid resistant. They did not change their bursting rate in the presence of morphine and selective μ and κ receptor agonists. Remarkably, their activity was transformed into a single burst in every cycle when C4 activity ceased.

Generation of the two-burst pattern is not possible without inhibitory synaptic inputs from inspiratory neurones. The first burst is formed because preinspiratory activity is abruptly terminated at the beginning of inspiration by a strong (up to 30 mV) $GABA_A$- and/or glycine receptor-mediated inhibition (Onimaru *et al.* 1990, 1997; Brockhaus & Ballanyi, 1998). This inhibition ceases at the end of inspiration, leading to the second burst, which either results from a resetting of the preinspiratory neurones or is a continuation of the first burst (Onimaru *et al.* 1997). Thus, a transformation of two bursts into a single one occurs whenever inspiratory neurones are inhibited or synaptic contacts between them and the preinspiratory neurones are interrupted (Onimaru *et al.* 1990, 1997; Brockhaus & Ballanyi, 1998; Takeda *et al.* 2001). Inspiratory neurones that inhibit the preinspiratory neurones are located in the VRG, rostral to the obex (Onimaru *et al.* 1997).

Data of Takeda *et al.* (2001) indicate that opioids lead to apnoea by inhibiting inspiratory neurones via pre- and postsynaptic mechanisms. These authors found that out of 66 inspiratory neurones in the RVLM, none fired action

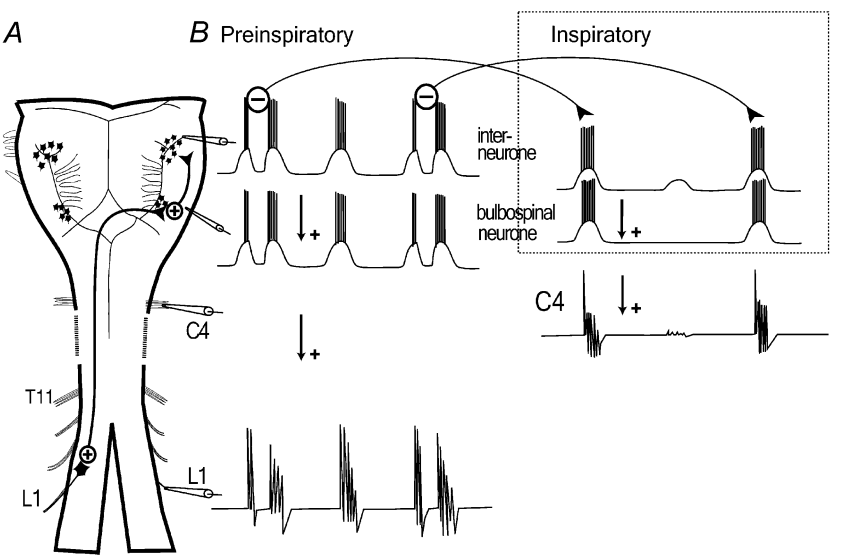

Figure 6. Model for the generation of the L1 activity after administration of opioids

A, the diagram of the ventral aspect of the brainstem–spinal cord preparation showing a disynaptic pathway from the preinspiratory neurones to the L1 motoneurones. *B*, the activity of the preinspiratory neurones driving the twice-bursting bulbospinal neurones in the cVRG. Inside the dotted rectangular there are activities of the inspiratory neurones shaping the twice bursting pattern of the preinspiratory neurones (Onimaru *et al.* 1990; Ballanyi *et al.* 1999) and bulbospinal inspiratory neurones driving the C4 root. The plus and minus signs indicate synaptic excitation and inhibition, respectively. Arrows indicate the direction in which excitation/inhibition is transmitted.

potentials in the absence of C4 activity. This is consistent with the opinion of Howard & Sears (1991) that an opioid-induced apnoea does not result from a depression of the phrenic nerve motoneurones or bulbospinal neurones which supply them, but rather from an inhibition of neurones that are a source of inspiratory drive. In the absence of inspiratory activity, rhythmic volleys of action potentials, generated by preinspiratory neurones, continue (Takeda *et al.* 2001).

We propose that the match between the above-described properties of the preinspiratory neurones and the EMG_{ABD}/L1 motor output indicates that the preinspiratory neurones contribute to the excitation of the L1 motoneurones. This concept, however, requires that there be a pathway from the preinspiratory neurones to the L1 level; therefore, we searched for that pathway. We found that about 11 % of the preinspiratory neurones ($n = 44$) in the RVLM project to the caudal VRG and that none project directly to the lumbar motoneurones.

In the caudal VRG, within or just ventral to the nucleus retroambiguus (Paxinos *et al.* 1999), we identified bulbospinal neurones with a firing pattern matching that of the preinspiratory neurones and the L1. The location of bulbospinal neurones with the twice-bursting pattern overlaps the location of other bulbospinal neurones supplying abdominal motoneurones in adults (Miller *et al.* 1985; Arita *et al.* 1987; Sasaki *et al.* 1994; Iscoe, 1998; Boers & Holstege, 1999; Billig *et al.* 2000; Vanderhorst *et al.* 2000). The axons of the twice-bursting bulbospinal neurones cross the midline at the level of the cell body. Only 25 % of the twice-bursting neurones in the vicinity of the nucleus retroambiguus do not project to the L1 level.

This is the first study to examine respiratory-related L1 activity in the brainstem–spinal cord preparation. The pattern of the L1 activity differed from that reported with regard to the caudal thoracic roots, which are active during both inspiration and expiration (Smith *et al.* 1990). Thoracic roots supply both inspiratory and expiratory muscles; therefore, axons supplying expiratory muscles may have the twice-bursting activity pattern, which adds to the inspiratory activity of axons supplying inspiratory muscles. This speculation is supported by the observation that the EMG activity of the expiratory intercostal muscles *in vitro* (Iizuka, 1999) sometimes resembles the activity of the whole L1 root.

Bainton *et al.* (1978) reported that a midline section extending from the obex to the C1 level selectively eliminated expiratory activity in spinal nerves in the cat. We have observed the same effect in the rat, which indicates that the two-burst pattern originates in the medulla and is transmitted via a pathway crossing the midline below the obex. The activity of the abdominal motoneurones represents a sum of drives from many different sources (Iscoe, 1998); therefore, the percentage contribution of the drive of the preinspiratory neurones to the total abdominal EMG_{ABD}/L1 activity may change with experimental conditions. Even though a drive originating from the activity of the preinspiratory neurones appears to be the sole controller of the L1 activity in the brainstem–spinal cord preparation, it may generate only a small fraction of the total motor L1 output in the intact rat. The brainstem–spinal cord preparation lacks the pons and afferent inputs, and is studied at 25–26 °C. Such *in vitro* conditions may be especially favourable for drives from the preinspiratory neurones, which remain active in the absence of any feedback and are located in the medulla.

Studies on cats (Bainton *et al.* 1978; Bainton & Kirkwood, 1979) have demonstrated that during a period of apnoea, the expiratory motoneurones show CO_2-dependent tonic discharge. When CO_2 builds up, as during opioid-induced apnoea, expiratory tonic activity intensifies (Howard & Sears, 1991). In more than half of the neonatal rats, twice-bursting activity was superimposed on tonic expiratory activity, which is consistent with data of Howard & Sears (1991) showing that tonic expiratory activity is resistant to opioids.

Presumably, after administration of opioids in the newborn rat, abdominal motoneurones were receiving constant CO_2-dependent excitation, which kept their membrane potential close to the threshold level, and rhythmic excitation originating from the preinspiratory neurones. At the same time, other rhythmic drives were depressed. The state-dependent domination by the drive from the preinspiratory neurones over all other drives normally integrated by abdominal motoneurones may be the primary reason for the remarkable match between the EMG_{ABD} pattern observed after administration of opioids *in vivo* and the L1 pattern observed *in vitro*.

It remains an open question why a brief, decrementing activation of the abdominal muscles just before inspiration may be more efficient than a typical active expiration, which consists of augmenting expiratory activity starting after the postinspiratory phase and continuing until the next inspiration (Bianchi *et al.* 1995). Considering the low respiratory rate resulting from administration of opioids, one may speculate that active expiration at the end of a prolonged expiratory pause would serve no purpose. However, a strong, brief activation of the abdominal muscles compresses the abdominal contents and shifts the diaphragm rostrally into the thorax, which stretches the diaphragm and increases its curvature. A high position of the diaphragm increases the mechanical efficiency of the subsequent contraction (DeTroyer & Loring, 1985).

In summary, we have shown that the brainstem–spinal cord preparation is an adequate model for studying

bulbospinal pathways to spinal motoneurones and determining some sources of the drives to these motoneurones. In the caudal VRG we have identified bulbospinal twice-bursting neurones that have the same burst pattern as the L1 root and we have shown that some preinspiratory neurones in the RVLM send axons to the caudal VRG. We propose that this pathway is responsible for the rhythmic contractions of the abdominal muscles observed during opioid-induced apnoea.

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