Ampakine CX717 Protects against Fentanyl-induced Respiratory Depression and Lethal Apnea in Rats

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Background: The use of fentanyl as a potent analgesic is contradicted by marked respiratory depression among a subpopulation of patients. The commonly used approach of reversing fentanyl-induced respiratory depression with μ-opiate receptor antagonists such as naloxone has the undesirable effect of blocking analgesia. Here, the authors report a clinically feasible pharmacological solution for countering fentanyl-induced respiratory depression *via* a mechanism that does not interfere with analgesia. Specifically, to determine if the ampakine CX717, which has been proven metabolically stable and safe for human use, can prevent and rescue from severe fentanyl-induced apnea.

Methods: Plethysmographic recordings were performed from young and adult rats. Varying doses of fentanyl were administered either intraperitoneally or intravenously to induce moderate to life-threatening apneas. CX717 was administered either before or after fentanyl administration. In addition, phrenic nerve recordings were performed from *in situ* working heart brainstem preparations from juvenile rats.

Results: Preadministration of CX717 markedly attenuated fentanyl-induced respiratory depression. Postadministration of CX717 rescued animals from a lethal dose of fentanyl. Significantly, CX717 countered fentanyl-induced depression of respiratory frequency without suppressing analgesia. The effective dose of CX717 was in the range deemed safe on the basis of clinical trials examining its efficacy for cognitive disorders. *In situ*, fentanyl-induced depression in respiratory frequency and amplitude was alleviated by CX717.

Conclusions: CX717 is an agent that enhances the safety of using opiate drugs while preserving the analgesic effects. This advancement could significantly improve pain management in a variety of clinical settings.

FENTANYL is a widely used and effective opiate analgesic for the treatment of acute, postoperative, and chronic pain.[1] However, fentanyl and other μ-opiate receptor agonists suppress respiratory activity through direct actions on neurons within the respiratory rhythm generating center, the preBötzinger complex (preBötC).[2,3] There is a varied susceptibility to fentanyl-induced respiratory depression among individuals.[4] Predicting which patients are most sensitive, however, is difficult, although older age, obesity, diseases affecting the respiratory or cardiovascular system and sleep apnea are some of the risk factors for fentanyl-induced respiratory depression. Extended periods of patient-controlled analgesia is another major area in which opioid-induced respiratory depression is problematic.[5] Therefore there has been a long-felt need to develop a method that allows the analgesic power of opioids to be harnessed without significantly depressing respiratory function. Naloxone and related opioid antagonists are currently used to counter respiratory depression in an emergency setting. However, those agents reverse analgesia. Patients are thus experiencing significant pain and clinicians are left trying to find a balance of partial analgesia with manageable respiratory depression. Here, we demonstrate that the Ampakine CX717 prevents severe fentanyl-induced respiratory depression and fatal apneas in rats without interfering with analgesia. Importantly, CX717 is metabolically stable and has been deemed safe in primate studies and clinical trials for cognitive disorders.[6–8] Thus, this study has the potential to have an immediate impact in clinical settings.

CX717 is a member of the ampakine family of compounds that modulate amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors by increasing the duration of glutamate-induced AMPA receptor-gated inward currents.[9] Ampakines bind to an allosteric site on the AMPA receptor to modulate the kinetics of deactivation (channel closing and transmitter dissociation) and desensitization.[10] Glutamate-mediated neurotransmission, acting *via* AMPA receptors, is a critical component for the generation of respiratory rhythm within the preBötC.[11–13] We hypothesize that accentuation of AMPA receptor-mediated conductances with ampakines will counter depressive actions of opioids acting within the preBötC.

Materials and Methods

Plethysmographic Recording Methods

All experimental procedures were approved by University of Alberta Faculty of Medicine Animal Welfare Committee (Edmonton, Alberta, Canada). Measurements from male Sprague-Dawley rats were performed in whole-body, plexiglass plethysmographs (260 and 2,000 ml volume for postnatal day [P]17–18 and adult rats, respectively) that had inflow and outflow ports for the continuous delivery of a steady flow of fresh air and removal of expired carbon dioxide. Pressure changes were recorded with a pressure transducer (model DP

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103; Validyne, Northridge, CA), signal conditioner (CD-15; Validyne), and an analogue-digital board (pClamp; Axon Instruments, Molecular Devices, Sunnyvale, CA).

For intravenous infusion experiments, adult rats (300–370 g) were anesthetized with 3% isoflurane in an induction chamber and maintained under 2% isoflurane anesthesia during tail vein cannulation (P10 size tubing). Animals were then placed within a whole-body plethysmograph that has been further modified to allow exteriorization of the tail for drug infusion (KD Scientific infusion pump, Hollistan, MA). A pulse oximeter (Norin 8600V, Plymouth, MN) was placed on the tail to monitor oxygen saturation levels.

In Situ *Working Heart Brainstem Preparations and Recordings*

Juvenile male Sprague-Dawley rats (P22–24, 55–80 g) were anesthetized with isoflurane, submerged in ice-cold oxygenated perfusate, decerebrated, and transected caudal to the diaphragm, and the descending aorta was cannulated (less than 8 min). The torso and brainstem were then transferred to a recording chamber, where the descending aorta was cannulated with a double lumen cannula (one line to deliver perfusate, the second to monitor blood pressure) and perfused with saline (bubbled with 95% O_2-5% CO_2) at a flow rate sufficient to generate and maintain arterial pressure at 60 mmHg. Once perfusion was initiated and arterial pressure was stabilized, the animal was gradually warmed to 32°C by heating the perfusate. The preparation was then allowed to stabilize for 1 h, during which time the left phrenic nerve was dissected to monitor inspiratory activity (frequency and burst amplitude). After the 1-h stabilization period, baseline respiratory output was recorded, and the various drugs (fentanyl and CX717) were added directly to the perfusate. Respiratory rhythm in perfused, *in situ* preparations was monitored from the phrenic nerve, which was placed over two platinum hook electrodes. Signals were amplified, rectified, low-pass filtered, and recorded to a computer by using an analog–digital converter (Axon Instruments Digidata 1200) and data acquisition software (Axon Instruments AxoScope).

Pharmacological Agents

CX717 (provided by Dr. Mark Varney, Ph.D., CEO Cortex Inc., Irvine, CA) was dissolved in a 10% 2-hydroxypropyl-β-cyclodextran (HPCD; Sigma, Oakville, Ontario, Canada) 0.45% saline solution for all experiments (intraperitoneal and IV). Fentanyl citrate (130 μg/kg; Sandoz, Boucherville, Quebec, Canada) was injected into the left abdomen of P17–18 rats with a 23-gauge needle while the animal remained lightly anesthetized under isoflurane. This gives a stable background respiratory rate similar to that observed in calm or sleeping animals.[14,15] Intravenous drug infusions of varying

doses of fentanyl[16,17] or CX717 commenced after approximately 5 min of flowing room air through the plethysmograph chamber to remove the residual ambient isoflurane.

Nociception Testing and Righting Reflex

Thermal nociception was measured by a modification of a previously reported method.[18] Briefly, the plantar test apparatus (Ugo Basile, Comerio VA, Italy) consisted of a movable infrared heat source that was positioned directly beneath the hind paw, 20 mm below the chamber floor. When the rat perceived pain and withdrew its paw, the instrument automatically detected the withdrawal latency to the nearest 0.1 s. Withdrawal latencies were recorded 10 min before and after drug administration. The heat stimulus was automatically terminated if a withdrawal response was not observed within 20 s of its onset to avoid the tissue damage. Further measure of analgesia in adult rats was performed by examining responses to tail pinching with forceps at 5-min intervals. The sedation state was assessed by monitoring the rat's ability to right itself into the prone position.

Data Analysis

Data are expressed as mean ± SEM. EC_{50} was calculated using the Hill equation: $E = E_{max}/[1+(EC_{50}/C)^n]$, where E is the drug effect, E_{max} is the maximal effect of CX717 tested in alleviation of fentanyl-induced depression in respiratory frequency, C is the concentration of CX717, EC_{50} is the CX717 concentration producing 50% of the maximal effect, and n is the Hill coefficient. Sigmastat 3.5 (Systat Software, Chicago, IL) was used to conduct the statistical analyses. In intact animals, the significance of changes in the respiratory frequency and body temperature was evaluated by a two-way ANOVA (dose × time) using Tukey Test; whereas significance of changes in the respiratory amplitude, oxygen saturation, nociception testing, and sedation observation was evaluated by a one-way ANOVA. For *in situ* experiments, values of respiratory frequency and peak inspiratory burst amplitude were measured from the integrated nerve recording and reported as means relative to control values. There is a similar level of variances in the control (before drug application). We normalized and reported control values as 1. However, raw data were used for the analytical test. All statistical comparisons from *in situ* working heart brainstem preparations were made by one-way repeated measures ANOVA followed by Tukey. $P < 0.05$ was taken as significant difference.

Results
Intraperitoneal Injections of Cx717 In Juvenile Rats

The first series of experiments were performed with intraperitoneal administration of fentanyl and CX717 to
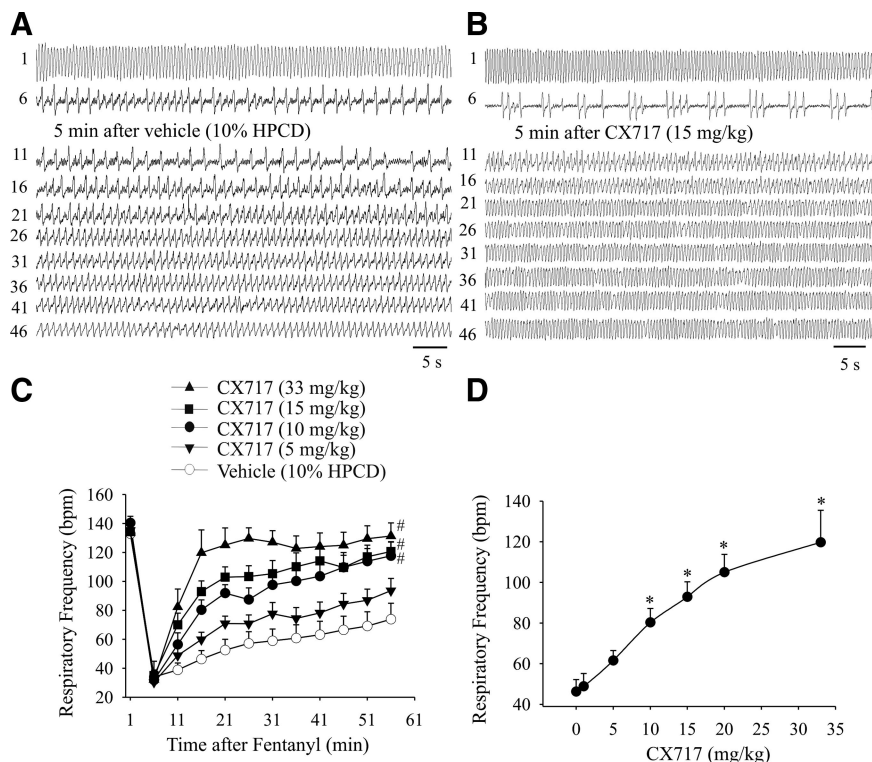
Fig. 1. CX717 (intraperitoneal) alleviates respiratory depression induced by fentanyl (intraperitoneal) in postnatal day (P)17–18 animals. (*A*) Traces shown are whole-body plethysmographic measurements of the frequency and relative depth of breathing in unrestrained rats. *Numbers to the left of traces refer to minutes after injection of 130 μg/kg fentanyl*. Injection of the vehicle HPCD (2-hydroxypropyl-b-cyclodextrin) solution 6 min after fentanyl administration had no effect on the depression of respiratory frequency. (*B*) In another P18 rat, the injection of 15 mg/kg intraperitoneal CX717 6 min after fentanyl administration alleviated a significant component of the depression of respiratory frequency. (*C*) Dose-response data showing the relative frequency of breathing after injection of vehicle or increasing doses of CX717 6 min after the injection of fentanyl; # *P* < 0.05 relative to the vehicle control group (n = 9–13 animals for each dose). (*D*) Average respiratory frequency 10 min after the injection of various doses of CX717. Significant changes of respiratory frequency relative to vehicle control were achieved at more than 5 mg/kg CX717 (intraperitoneal) with the EC50 = 10.7 ± 0.6 mg/kg. * *P* < 0.05 relative to vehicle control respiratory frequency.

generate dose-response data of CX717 efficacy. We found that the respiratory-depressing effects of intraperitoneal drug delivery of fentanyl to be more consistent in P17–18 rats (33–45 g) relative to older animals with higher body fat composition. A fentanyl dose of 130 μg/kg (intraperitoneal) induced a marked depression of respiratory frequency within 5–10 min in 19 of 24 animals tested. In the remaining 5 rats, there was only a modest (*i.e.*, <30%) decrease in respiratory frequency, and those animals were not used for analyses of CX717 effects. Figure 1 shows a plethysmographic recording from a P18 rat in response to fentanyl injection. There was a clear decrease in respiratory frequency within 6 min, and the depression persisted for 45–60 min. Our single chamber plethysmograph did not allow for the measurement of absolute tidal volume. However, we could determine that the relative amplitude of the signal was decreased from control levels in all animals after the administration of fentanyl. Figure 1B shows the reversal of respiratory frequency depression, but not amplitude, within 5 min of CX717 injection. The relative amplitude was 40 ± 4.7% (n = 8) and 42 ± 4.1% (n = 7) 10 min after fentanyl administration in the absence and presence of CX717, respectively. Figures 1C and 1D show the dose-dependent effect of CX717. There were significant alleviations of fentanyl-induced depression of respiratory frequency at concentrations greater than 5 mg/kg CX717 (EC$_{50}$ at 10 min after CX717 injection = 10.7 ± 0.6 mg/kg). Administration of the vehicle (10% HPCD) did not cause a significant change in fentanyl-induced depression of respiratory frequency.

We then tested the ability of a preadministration of CX717 to counter fentanyl-induced respiratory depression. Figure 2 shows that 15 mg/kg CX717 (intraperitoneal) was very effective at minimizing fentanyl-induced depression of respiratory frequency when delivered 2 min before fentanyl injections. In a further set of experiments, we noted a similar block of fentanyl-induced depression of respiratory frequency when CX717 and fentanyl were coadministered in a cocktail (data not shown). Thus, the two compounds have a similar onset of action time course.

CX717 did not significantly alter fentanyl-induced analgesia. The mean response time of paw withdrawal to a thermal stimulation in control (10% HPCD, n = 5), CX717 alone (15 mg/kg, n = 5), fentanyl (130 μg/kg, n = 7), and preadministration of fentanyl (130 μg/kg) 2 min before CX717 (15 mg/kg, n = 7) were 6.7 ± 0.9 s, 6.6 ± 0.8 s, more than 20 s, and more than 20 s, respectively. Further, the level of sedation induced by fentanyl was not altered by CX717. Specifically, animals did not regain response to tail pinching before 40 min after fentanyl administration. With the relatively high dose of fentanyl used, all animals showed clear muscle rigidity, as reported in previous studies.[19–22]

Intravenous Administration of CX717 In Adult Rats

Having determined the dose-response characteristics of CX717, we performed a further study using older rats (300–370 g) and evaluated the effects of IV infusions. Administration of CX717 before, concomitant with, or
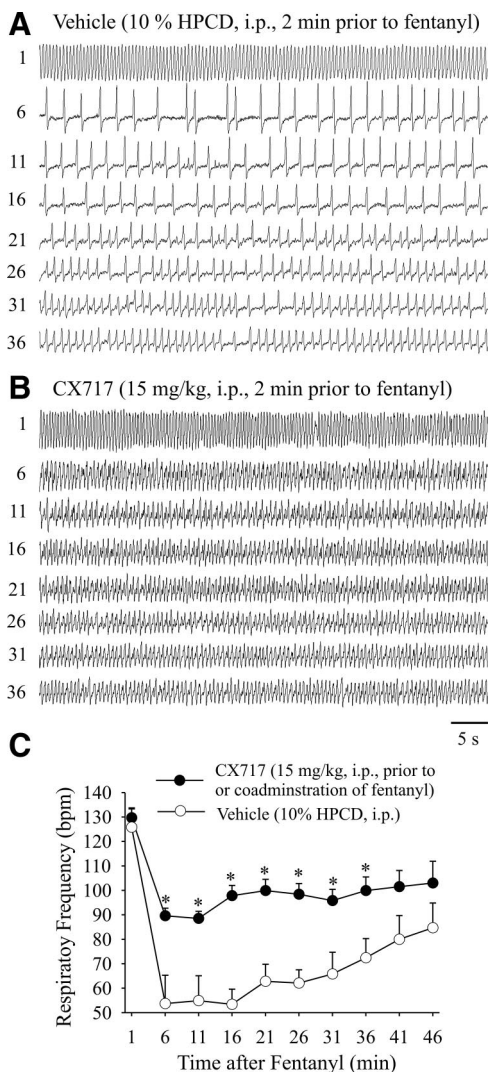
Fig. 2. Preadministration of CX717 (intraperitoneal [IP]) prevents respiratory depression induced by fentanyl in postnatal day (P17)–18 rats. (*A*) Preadministration of the vehicle HPCD (2-hydroxypropyl-b-cyclodextrin) solution (intraperitoneal) had no effect on the fentanyl-induced suppression of respiratory frequency in a P17 rat. (*B*) In another P17 rat, administration of CX717 (15 mg/kg, intraperitoneal) 2 min before the fentanyl administration markedly alleviated depression of respiratory frequency. (*C*) Population data showing the time course of changes of respiratory frequency in response to fentanyl administration after preadministration of vehicle or CX717. * *P* < 0.05 relative to vehicle control respiratory frequency (n = 9 for each group).

Fig. 3. Intravenous (IV) CX717 counters respiratory depression induced by IV fentanyl in adult rats. (*A*) Delivery of 60 μg/kg fentanyl (IV) over a 20-min infusion period caused an initial marked depression of respiratory frequency. In this particular rat, there was a partial rebound of frequency and then a subsequent further decrease of respiratory rate. Depression of respiratory frequency persisted after intraperitoneal administration of vehicle HPCD (2-hydroxypropyl-β-cyclodextrin). (*B*) In another adult rat, infusion of 15 mg/kg CX717 approximately 6 min after the commencement of the fentanyl infusion caused a rapid and marked alleviation of respiratory frequency depression. (*C*) Population data showing the time course of changes of respiratory frequency in response to fentanyl administration with the subsequent administration of vehicle or CX717. * *P* < 0.05 relative to vehicle control respiratory frequency (n = 7–9 for each group).

after fentanyl administration were performed to determine their efficacy in countering respiratory depression. In the first paradigm, 60 μg/kg fentanyl (IV) was delivered over a 20-min infusion period (fig. 3). This caused a marked suppression of respiratory frequency (>50% of control) that lasted for the duration of fentanyl infusion. In approximately 40% of those rats (*e.g.*, fig. 3A), there was a partial rebound in respiratory frequency after an initial slowing of breathing frequency. This partial rebound was typically followed by further depression of respiratory frequency as the fentanyl infusion continued.
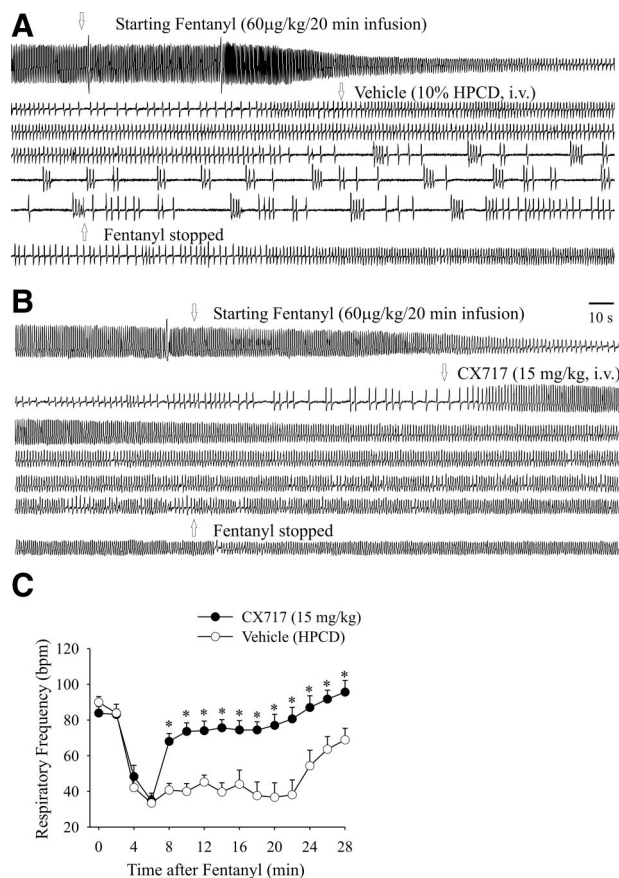
In the remaining 60% of rats, the level of respiratory depression was more constant (fig. 4A). Injection of CX717 (15 mg/kg IV; n = 7) 6 minutes after the fentanyl administration caused an increase in respiratory frequency within 1 min that lasted beyond the duration of the fentanyl infusion (fig. 3, B and C). Specifically, 10 min after administration of fentanyl, the respiratory frequency decreased from 83.9 ± 3.7 bursts/min to 44.8 ± 10.4 (n = 9) bursts/min *versus* 73.6 ± 4.8 bursts/min (n = 7) in control and CX717-treated animals, respectively. Further, oxygen saturation levels 10 min after the fentanyl injection were elevated from 59.6 ± 2.4% (vehicle group, n = 9) to 80.1 ± 4.6% (n = 7) in the presence of CX717 administration.

We then tested whether CX717 (15 mg/kg, IV) administered before fentanyl (60 μg/kg; 20-min infusion)
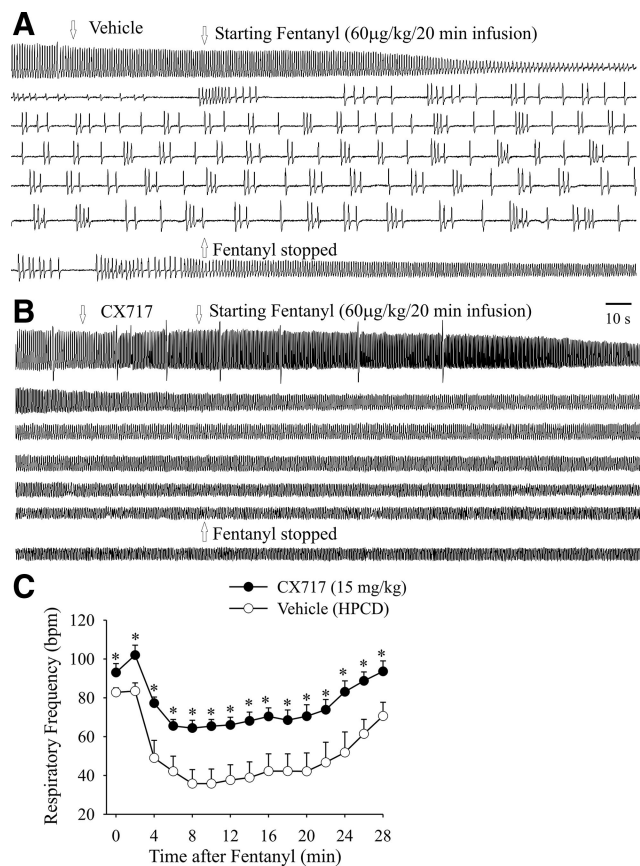
Fig. 4. Preadministration of intravenous (IV) CX717 prevents respiratory depression induced by IV fentanyl in adult rats. (*A*) The administration of 60 μg/kg IV fentanyl delivered over a 20-min infusion period produced suppression of respiratory frequency that was not affected by the preadministration of the vehicle HPCD (2-hydroxypropyl-β-cyclodextrin, IV). (*B*) In another adult rat, preadministration of CX717 (15 mg/kg, IV) markedly attenuated the fentanyl-induced depression of respiratory frequency. (*C*) Population data showing the time course of changes of respiratory frequency in response to fentanyl administration with the preadministration of vehicle or CX717. * *P* < 0.05 relative to vehicle control respiratory frequency (n = 9–10 for each group).

would prevent the fentanyl-induced respiratory depression (n = 10). Figure 4B shows that pretreatment of CX717 significantly prevented fentanyl-induced depression of respiratory frequency. Note that the initial increase in respiratory frequency after CX717 infusion was due to the countering of the minor residual respiratory depression resulting from isoflurane administration during the tail vein cannulation. Population data are summarized in figure 4C. The respiratory frequency decreased from 82.8 ± 2.3 bursts/min to 35.8 ± 7.5 bursts/min (n = 9) *versus* 65.4 ± 3.4 bursts/min (n = 9) in control and CX717-treated animals, respectively. The oxygen saturation levels 10 min after fentanyl administration were elevated from 64.6 ± 4% (n = 9) to 76.7 ± 2.4% (n = 10) with preadministration of CX717. Infusion of the vehicle (10% HPCD) did not cause a significant change in fentanyl-induced depression of respiratory frequency or oxygen saturation levels.
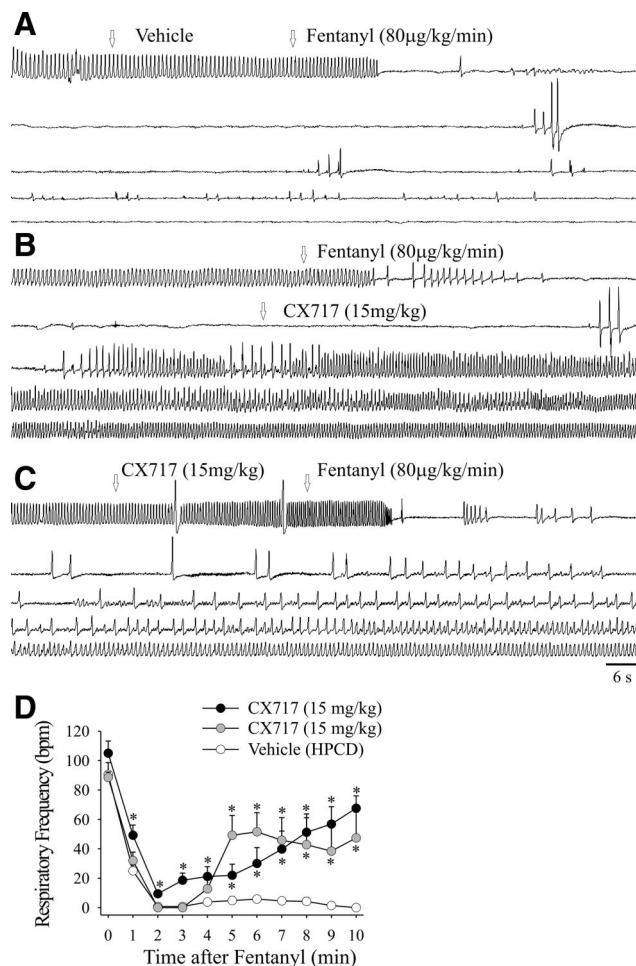

Fig. 5. Administration of 15 mg/kg intraperitoneal (IP) CX717 reverses/prevents fentanyl-induced lethal apnea in adult rats. (*A*) Administration of a high dose of fentanyl (80 μg/kg) over a short period (1 min) induced a profound, lethal respiratory depression. Vehicle (HPCD; 2-hydroxypropyl-β-cyclodextrin) injection before fentanyl administration had no significant effects on depression of respiratory frequency. (*B*) Administration of CX717 (15 mg/kg) within 1 min after the fentanyl infusion reduced the period of apnea and induced the recovery of respiratory rhythm. (*C*) Preadministration of 15 mg/kg IP CX717 before the fentanyl infusion reduced the severity of fentanyl-induced apnea and depression of respiratory frequency and prevented death. (*D*) Population data showing the time course of changes of respiratory frequency in response to fentanyl administration with pre- or postadministration of vehicle or CX717. * *P* < 0.05 relative to vehicle control respiratory frequency (n = 5 for each group).

Figure 5A shows data from an experiment in which 80 μg/kg fentanyl (n = 5) was infused rapidly. This administration protocol induced profound apnea that is typically lethal. Injection of CX717 (15 mg/kg IV; n = 5) 30–60 s after the onset of fentanyl-induced apnea led to a marked rebound of respiratory frequency in all five animals tested (fig. 5B). Preadministration of 15 mg/kg CX717 (n = 5) immediately before delivering the fentanyl bolus minimized the period of apnea and all of the animals survived (fig. 5C). Population data are summarized in figure 5D.

During thermal nociceptive testing, the mean response time of paw withdrawal in control (10% HPCD), CX717 (15 mg/kg, IV), fentanyl (60 μg/kg, IV), and fentanly-CX717 conditions were 6.1 ± 1.1 s, 6.3 ± 1.4 s, more than 20 s, and more than 20 s, respectively (n = 4–5 each group). The time to a positive response to tail clamping after fentanyl administration was not significantly altered by CX717, with 43 ± 1.2 min (n = 5) *versus* 42 ± 1.2 min (n = 5) in control and CX717-treated animals, respectively.

Body temperature decreased by 1.2 ± 0.1°C (n = 7) and 1.8 ± 0.2°C (n = 4) after 20 and 50 min, respectively, in response to a 20-min infusion of 60 μg/kg fentanyl. This decrease in body temperature was 1.0 ± 0.1°C at both time points in the presence of CX717. The increased respiratory frequency and associated muscle activity in the presence of CX717 presumably helped maintain body temperature. Varying degrees of fentanyl-induced muscle rigidity were evident in all animals tested.

In Situ *Working Heart Brainstem Data in Juvenile Rats*

We used an *in situ* experimental model that allowed for easy access to phrenic nerve recordings in an unanesthetized preparation. We were particularly interested in determining if CX717 alleviated the fentanyl-induced decrease in the amplitude of phrenic nerve discharge toward testing the hypothesis that the lack of return of tidal volume *in vivo* was primarily the result of increased airway and ribcage stiffness. Figure 6A shows a representative example of phrenic nerve discharge recorded from a P23 rat *in situ* preparation. Administration of 10 nM fentanyl to the perfusate produced a significant decrease in respiratory frequency and phrenic burst amplitude that was completely countered by subsequent administration of 100 μM CX717. Population data are presented in figure 6B.

Discussion

Data from studies using *in vitro* and *in vivo* models support the hypothesis that the basic rhythm underlying breathing arises from a specific region of the ventrolateral medulla, the preBötC.[23–25] The precise mechanism underlying the generation of rhythmogenesis within the preBötC has not been resolved.[26] However, the neurotransmitter glutamate, acting *via* AMPA receptors, is a critical component for the generation of respiratory rhythm within the preBötC.[11–13] Fentanyl induces depression of respiratory frequency in part by direct actions at μ-opiate receptors expressed on neurons within the preBötC.[2,3] Thus, mechanistically, a significant component of the CX717 effect can be explained by accentuation of AMPA receptor-mediated glutamater-
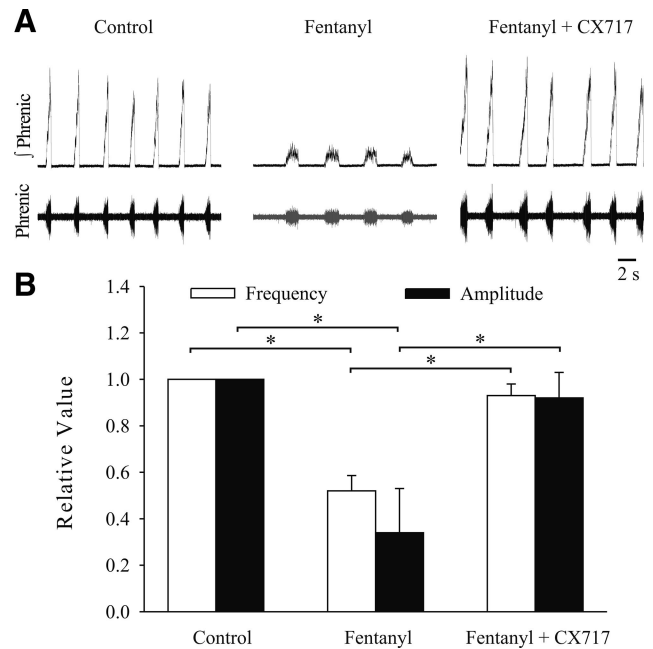

Fig. 6. CX717 counters fentanyl-induced depression of respiratory frequency and amplitude generated by perfused heart *in situ* preparations. (*A*) Recording of raw and integrated phrenic nerve discharge in a postnatal day (P)23 rat. (*B*) Population data (n = 5) showing the relative effects (compared with control) of fentanyl and CX717 (in the continued presence of fentanyl) on amplitude and frequency of integrated phrenic nerve discharge. Control values normalized to one. *Open bars* = frequency; *solid bars* = amplitude. *$P < 0.05$, significant difference.

gic excitation that will counteract the μ-opioid receptor-mediated suppression of preBötC neuronal excitability. CX717 may also be acting *via* additional neuronal populations in which AMPA-mediated conductances play an important role. This would include neurons of the nucleus of the solitary tract, retrotrapezoid nucleus, raphe complex, and pontine respiratory nuclei. All of these provide modulatory synaptic drive that regulates preBötC rhythmogenesis.[26] In clinical studies, CX717 has shown efficacy in reducing inattentiveness and hyperactivity in adults with attention deficit-hyperactivity disorder.[27] The dose in this study was 800 mg twice-a-day or approximately 11 mg/kg twice-a-day. The doses required to reverse opiate-induced respiratory depression in rats are consistent with efficacious doses in previous clinical studies. Importantly, the doses of CX717 used to alleviate or reverse both moderate and severe fentanyl-induced respiratory depression did not interfere with analgesia.

The fentanyl-induced suppression of plethysmographic pressure change amplitude was not significantly reversed by CX717. This is despite the fact that AMPA receptor activation plays a role in excitatory inspiratory drive to cranial and spinal motoneurons.[11,12] In contrast, recordings from *in vitro* brainstem-spinal cord and medullary slice preparations show that ampakines produces a clear reversal of opioid-induced suppression of inspiratory motoneuron population discharge amplitude.[28] We

hypothesized that the discrepancy likely results from the fact that fentanyl, particularly at the high doses used in this study, cause changes in upper airway and rib cage compliance *in vivo*[19,22]. Recordings directly from the phrenic nerve of the *in situ* preparation clearly demonstrated that CX717 alleviated fentanyl-induced respiratory frequency and amplitude of motor discharge. *In vivo*, the fentanyl-induced stiffness is thought to be, in part, mediated *via* α-2 adrenergic receptors.[20,21] Ampakines would not directly affect that aspect of fentanyl-induced properties. Lower doses of fentanyl used in humans typically alter respiratory frequency to a greater degree relative to amplitude[29] and thus should be very amenable to reversal by CX717. Further, unlike earlier studies of AMPA receptor modulators,[30,31] CX717 readily crosses the blood-brain barrier, is metabolically stable and does not produce significant unwarranted side effects. Hence, CX717 is an agent that enhances the safety of using opiate drugs while preserving the analgesic effects. This advancement could significantly improve pain management in a variety of clinical settings.

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