ANESTHESIOLOGY

Activating α4β2 Nicotinic Acetylcholine Receptors Alleviates Fentanyl-induced Respiratory Depression in Rats

Jun Ren, Ph.D, Xiuqing Ding, M.Sc., John J. Greer, Ph.D.

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Opioid-induced respiratory depression results in part from direct activation of μ-opioid receptors expressed in the inspiratory rhythm generator located in the ventrolateral medulla, the preBötzinger Complex
- Respiratory neurons within the medulla also express nicotinic acetylcholine receptors, which are made up of five subunits, arranged symmetrically around a central pore
- Activation of the nicotinic acetylcholine receptor α4, α7, and β2 subunits increases respiratory rhythm, whereas activation of the nicotinic acetylcholine receptor α4β2 or α7 subunits induces analgesia in multiple forms of pain

What This Article Tells Us That Is New

- The nonselective nicotinic acetylcholine receptor agonist nicotine and the α4β2 nicotinic acetylcholine receptor agonist A85380, but not the α7 nicotinic acetylcholine receptor agonist PNU282987, reversed respiratory depression induced by activation of μ-opioid receptors in rats both *in vitro* and *in vivo*
- Coadministration of A85380 with fentanyl not only markedly reduced respiratory depression and apneas but also enhanced the fentanyl-induced analgesia

ABSTRACT

Background: Opioid analgesics are widely used for treatment of acute, postoperative, and chronic pain. However, activation of opioid receptors can result in severe respiratory depression. There is an unmet clinical need to develop a pharmacologic therapy to counter opioid-induced respiratory depression without interfering with analgesia. Further, additional advances to confront accidental lethal overdose with the use of fentanyl and other opioids are needed. Here, the authors test the hypothesis that activation of nicotinic receptors expressed within respiratory rhythm–generating networks would counter opioid-induced respiratory depression without compromising analgesia.

Methods: Respiratory neural discharge was measured using *in vitro* brainstem–spinal cord and medullary slice rat preparations. *In vivo*, plethysmographic recording, nociception testing, and righting reflexes were used to examine respiratory ventilation, analgesia, and sedation, respectively.

Results: The administration of nicotine, selective α4β2 nicotinic receptor agonist A85380, but not α7 nicotinic receptor agonist PNU282987, reversed opioid-induced respiratory depression in neonatal pups *in vitro* and *in vivo*. In adult rats *in vivo*, administration of A85380 (0.03 mg/kg), but not PNU282987, provides a rapid and robust reversal of fentanyl-induced decrease in respiratory rate (93.4 ± 33.7% of control 3 min after A85380 *vs.* 31 ± 20.5% of control after vehicle, n = 8 each, $P < 0.001$), without marked side effects. The coadministration of A85380 (0.06 mg/kg) with fentanyl or remifentanil markedly reduced respiratory depression and apneas, and enhanced the fentanyl-induced analgesia, as evidenced by increased paw withdrawal latency in Hargreaves plantar test (14.4 ± 2.8 s *vs.* vehicle: 11.3 ± 2.4 s, n = 8 each, $P = 0.013$) and decreased formalin-induced nocifensive duration (2.5 ± 2.4 min *vs.* vehicle: 5.4 ± 2.7 min, n = 8 each, $P = 0.029$).

Conclusions: The novel strategy of targeting α4β2 nicotinic acetylcholine receptors has the potential for advancing pain control and reducing opioid-induced respiratory depression and overdose.

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Respiratory depression and lethal overdose caused by fentanyl and other opioids is a major clinical and societal problem.[1,2] Significant populations of patients experience respiratory depression when receiving opioids postoperatively that results in the challenge of meeting sufficient analgesia while maintaining adequate ventilation. Patient-controlled analgesia for extended periods of pain relief is another critical area in which opioid-induced respiratory depression is a concern (*i.e.*, hypoxemia or accidental death). Naloxone and related opioid antagonists can counter opioid-induced respiratory depression in those patients but at the expense of losing the required analgesia. There is an unmet clinical need to develop a pharmacologic therapy to counter opioid-induced respiratory depression without interfering with analgesia. Finally, there is an epidemic of opioid-induced overdose; pharmacologic tools that could reduce the incidence will be a welcome and timely advancement.

Opioid receptors have been classified into four major types (μ,δ,κ, nociceptin/orphanin FQ peptide receptors), and it is activation of the μ-opioid receptor that causes the majority of both the opioid-induced respiratory depression

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and analgesia.[3–5] The respiratory depression results in part from direct activation of μ-opioid receptors expressed in the inspiratory rhythm generator located in the ventrolateral medulla, the preBötzinger Complex.[6,7] Respiratory neurons within the medulla, including the preBötzinger Complex, also express nicotinic acetylcholine receptors. Nicotinic receptors are made up of five subunits, arranged symmetrically around a central pore.[8] The medullary respiratory networks express nicotinic acetylcholine receptor subunits α4, α7, and β2, and activation of these receptors increases respiratory rhythm.[9–11] Further, α4β2 or α7 nicotinic acetylcholine receptor activation induces analgesia in multiple forms of acute, chronic, inflammatory, and neuropathic pain[12–17] and thus could work in concert with opioids, perhaps reducing the opioid dose necessary to achieve analgesia. Thus, we hypothesized that administration of drugs that positively enhance the activity of specific populations of nicotinic acetylcholine receptors would counter opioid-induced respiratory depression without compromising opioid-induced analgesia. Here we present data from *in vitro* and *in vivo* rat models to demonstrate that activation of α4β2 but not α7 containing nicotinic acetylcholine receptors reduces the severity, or provides a rapid and robust alleviation, of opioid-induced respiratory depression while enhancing the desired analgesia.

Materials and Methods

All experimental procedures were approved by the University of Alberta Faculty of Medicine Animal Welfare Committee (Edmonton, Alberta, Canada). Adult male and pregnant female Sprague–Dawley rats were purchased from Charles River Canada (Sherbrooke, Quebec, Canada).

Brainstem–Spinal Cord and Medullary Slice Neonatal Preparations

Neonatal (postnatal day 1 to 3) rats were anesthetized with metofane or isoflurane, decerebrated, and the brainstem–spinal cord dissected as previously reported.[18,19] The neuraxis was continuously perfused at 27° ± 1°C (5 ml/min; chamber volume, 3 ml) with modified Krebs solution that contained 128 mM NaCl, 3.0 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23.5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, and 30 mM d-glucose equilibrated with 95% O_2–5% CO_2 (pH 7.4).

A single transverse slice containing the preBötzinger Complex and more caudal reticular formation regions was then cut (700 μm thick) from brainstem–spinal cord preparations[18,19] perfused with a bathing solution identical to that used for brainstem–spinal cord preparation with the exception that the KCl concentration was increased to 9 mM to facilitate long-term generation of stable rhythm. Recordings were made with glass suction electrodes (A-M Systems, USA) placed over the fourth ventral cervical nerve roots of brainstem–spinal cord or hypoglossal nerve roots of medullary slice preparations. Signals were amplified (×5000), band pass filtered (100 Hz to 5 kHz), rectified, integrated (τ = 25 ms), then saved to a computer *via* a Digidata 1322 A/D board (sampling rate = 1 kHz) and data acquisition software AxoScope (Axon Instruments, USA). Baseline and threshold levels for bursts were set using Clampfit software. Bursts were then automatically detected so that area and frequency were measured. The inspiratory burst area (related to tidal volume *in vivo*) was calculated for the region of the burst that was above the baseline, as described in our previous study.[20]

Whole-body Plethysmographic Recordings

Measurements were performed in whole body, cylindrical transparent plexiglass plethysmographs that had one inflow and two outflow ports for the continuous delivery of fresh room air and removal of expired carbon dioxide.[19,20] The plethysmograph volumes were 80 (inner diameter: 3.8 cm, length: 7 cm) and 2,000 (inner diameter: 10.1 cm, length: 25 cm) ml for measures of respiratory parameters of neonatal and adult (390 to 460 g) rats with a flow rate of 80 ml/min and 700 ml/min, respectively, delivered from compressed air cannisters with the pressure being monitored using 0–200 ml/min and 0-1,000 ml/min gas regulators (Porter Instrument Company, USA). For newborns, the plethysmograph was contained within an infant incubator (Isolette, model C-86; Air-Shields/Dräger Medical, USA) to maintain the ambient temperature at the approximate nest temperature of 32°C. For adult infusion experiments, adult rats were anesthetized with 3% isoflurane in an induction chamber and maintained with 2% isoflurane anesthesia during tail vein cannulation (P10 size tubing directly inserted in the lateral tail vein after the surgery exposure, with both veins cannulated if needed). The chamber had an additional port to allow exteriorization of the tail (port outlet sealed with the Play-Doh to minimize pressure leakage) for IV drug infusion *via* an infusion pump (KD Scientific, USA). With the infusion approach, all drug deliveries can be performed with continuous monitoring of plethysmographic recordings without physical handling of the animal. Pressure changes were detected with a pressure transducer (model DP 103; Validyne, USA), signal conditioner (CD-15; Validyne), recorded with data acquisition software (Axoscope) *via* analog-digital board (Digidata 1322A). Signals were high pass filtered (0.01 kHz), with a sampling rate at 1 kHz. Data was rectified, integrated (τ = 80 ms) with Labchart 8 (AD Instruments Inc., USA), then exported to Clampfit (Axon Instruments) for further analysis. Baseline and threshold levels for bursts were set using Clampfit software. Bursts were then automatically detected so that frequency and tidal volume (calculated for the region of the burst that was above the baseline) were measured. A pulse oximeter (Norin 8600V, USA) was placed on the tail to monitor oxygen saturation (Sa_{O_2}) levels and heart rate in adult rats. Body temperature was measured using a rectal probe (Dual thermometer; Fisher Scientific, Canada).

It should be noted that our plethysmograph is effective for studying respiratory frequency (f_R) and detection of apneas. An apnea is defined as the absence of airflow (pressure changes) for a period equivalent or greater than two complete respiratory cycles. Our whole-body plethysmographic system provided semiquantitative measurements of tidal volume (V_T, ml/g) and minute ventilation ($V_E = f_R \times V_T$: ml min^{-1} g^{-1}), from which we report changes relative to the control state.[19,21]

Nociception Testing and Righting Reflex

Two nociceptive tests were performed: (1) Hargreaves plantar test: Thermal nociception was measured with a plantar test apparatus (Ugo Basile, Comerio VA, Italy),[19,21] consisting of an infrared heat source (with heat setting at 70 W) positioned directly beneath the hind paw, 20 mm below the plexiglass panel. When the rat perceived pain and withdrew its paw, the instrument automatically detected the withdrawal latency to the nearest 0.1 s. The heat stimulus was automatically terminated if a withdrawal response was not observed within 20 s of its onset to avoid the tissue damage. (2) Formalin test: A dilute solution of formalin (50 μl, 1.5% formalin diluted in saline) was injected into the intraplantar region of the right hind paw, followed by assessment of nocifensive behaviors (licking/lifting/flinching of the injected paw) in the second phase (20 to 40 min; reflecting inflammation) of the assay.[22] A simple sum of time spent on licking/lifting is a recognized assessment of formalin-induced nocifensive behaviors.[23]

Sedation (loss of righting reflex) is defined as the rat's inability to right itself into the prone position after the animal was placed supine.[21] The duration of sedation was defined as the time interval from the beginning of fentanyl administration to the recovery of righting reflex. Figure 1 provides a graphic outline of the experimental protocol for preadministration of A85380 (0.06 mg/kg, neck subcutaneously) with fentanyl (20 μg/kg more than 400 s, IV infusion) and measures of nociception and righting reflex.

Pharmacologic Agents

Fentanyl citrate was from Sandoz (Boucherville, Canada); [D-Ala2,N-Me-Phe4,Gly5-ol]enkephalin (DAMGO) and 2-hydroxypropyl-β-cyclodextran were from Sigma (Canada); (−)-nicotine (tartrate) was from Cayman Chemical (USA); other nicotinic acetylcholine receptor targeting drugs were from Tocris (USA). Dihydro-beta-erythroidine hydrobromide (DHβE), A85380 dihydrochloride, and methyllycaconitine citrate were dissolved in saline, and PNU282987 was dissolved in 33% 2-hydroxypropyl-β-cyclodextran 0.4% saline solution. The doses of nicotinic acetylcholine receptor targeting agents were based on extensive literature with these compounds in rodent studies.[11,13,16,24–26]

Statistics

Data are expressed as mean ± SD (Sigmaplot 11 Systat Software Inc., USA). No statistical power calculation was conducted before the study. The sample size was based on our previous experience with these experimental protocols. Rats were randomly assigned to groups using block randomization sequence and tested in sequential order. Specifically, for two group (one dose drug *vs.* vehicle) comparisons, one of two rats (housed in the same cage) was randomly assigned to the vehicle group, with the other assigned to the drug treatment group. For comparison of three groups (vehicle and two doses of drug test), each of three animals was randomly assigned to each group. We reported and analyzed all the data. Blind testing was used where one person administered the drug and the second person ran nociception testing, rated behavior, and analyzed the data without knowledge of drug administration. Respiratory parameters were calculated over an average of 2 min *in vitro* recordings and 1 min of continuous recordings *in vivo*. The respiratory parameters f_R, V_T,

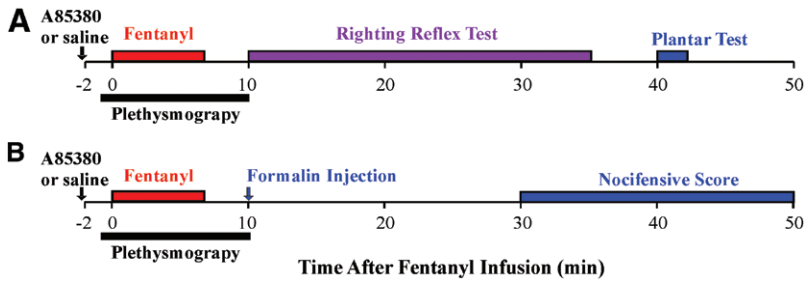

Fig. 1. Graphic outline of the experimental protocols to evaluate the effects of preadministration of A85380 or vehicle (saline) on fentanyl-induced respiratory depression, analgesia, and sedation. A85380 (0.06 mg/kg, neck, subcutaneously) or saline was administrated approximately 2 min before fentanyl (20 μg/kg more than 400 s, intravenous infusion). (*A*) In one set of animals, the righting reflex testing started 10 min after administration of fentanyl, and then the animal was removed from plethysmography for thermal nociception testing 40 min after administration of fentanyl. (*B*) In another set of animals, formalin was administered 10 min after administration of fentanyl. Nocifensive response was scored 30–50 min after administration of fentanyl. A85380, A85380 dihydrochloride.

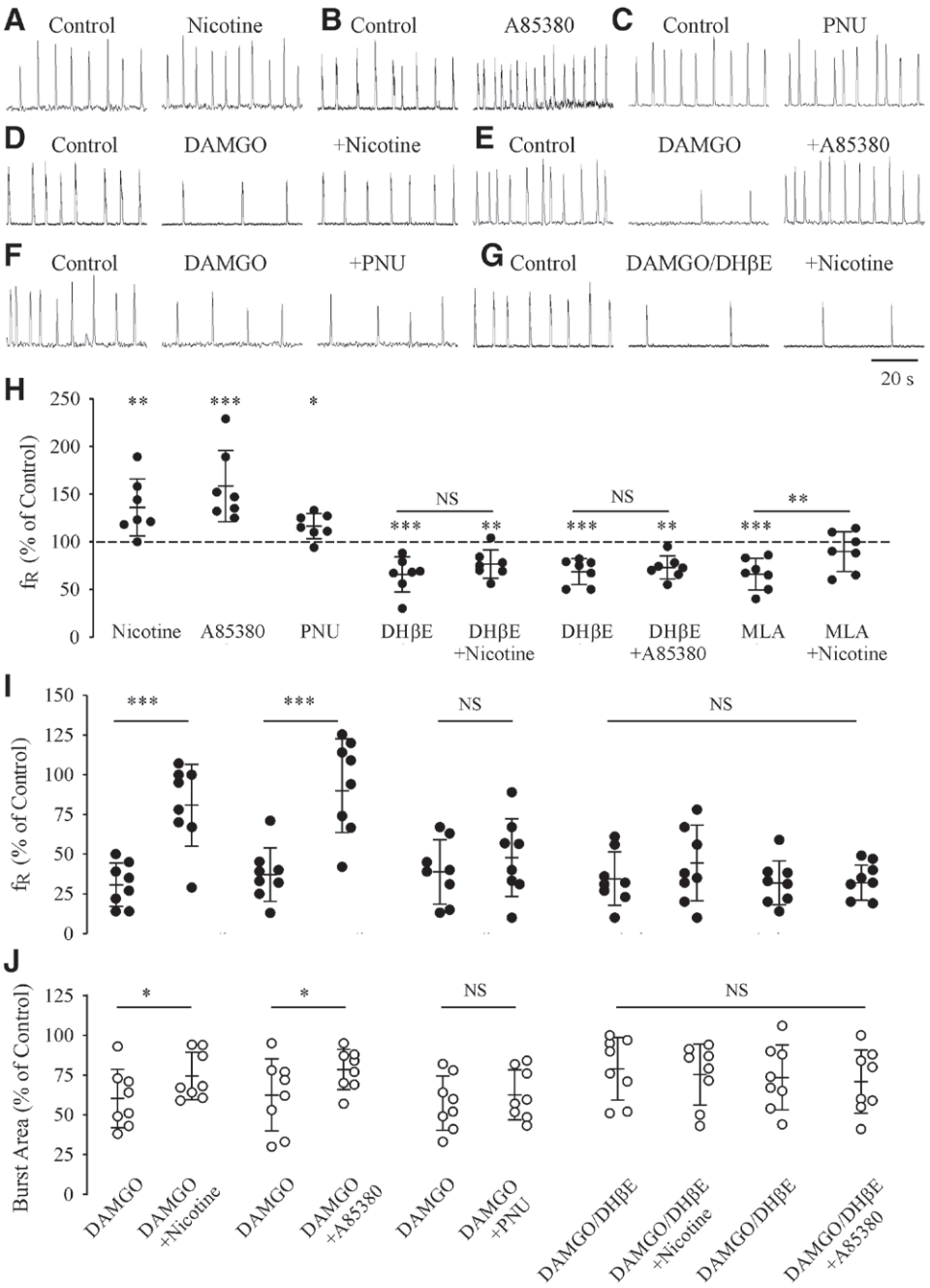

Fig. 2. The effects of α4β2 and α7 nicotinic acetylcholine receptor targeting agents on respiratory rhythm generated by neonatal rat brainstem–spinal cord *in vitro* preparation (postnatal day 1 to 3). (*A–C*) Bath application of nicotine (600 nM, *A*) or A85380 (25 nM, *B*) markedly increased baseline respiratory frequency (f_R) without effects on respiratory burst area. PNU282987 (30 μM, *C*) slightly increased baseline f_R. (*D–F*) DAMGO (200 nM) suppressed f_R and burst area (calculated for the region of the burst that was above the baseline), the effects were reversed by a subsequent application of nicotine (600 nM, *D*), A85380 (25 nM, *E*), but not by PNU282987 (30 μM, *F*). (*G*) Coadministration of DAMGO and α4β2 antagonist DHβE (400 nM) suppressed f_R and burst area; the effects were no longer affected by a subsequent application of nicotine (600 nM). (*H–J*) Population data (mean and SD) showing the effects of nicotinic acetylcholine receptor agonists and antagonists on baseline f_R (relative to control before nicotinic receptor drugs, *H*), DAMGO-induced respiratory depression (f_R relative to control before DAMGO, *I*; burst area relative to control before DAMGO, *J*). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, statistically significant difference; ns: *P* > 0.05, no significant difference; compared after *versus* before drug application (paired *t* test for application of one drug; using one-way repeated measures ANOVA followed by Holm–Sidak method for the sequential application of two drugs). n = 7 to 8 animals each group. DAMGO, D-Ala2, N-MePhe4, Gly-ol-enkephalin; DHβE, dihydro-beta-erythroidine hydrobromide; MLA, methyllycaconitine citrate; PNU, PNU282987.

and V_E were reported as means relative to control values (*i.e.*, before opioid administration). The nature of the hypothesis testing is two-tailed. We first ran the normality test (Shapiro-Wilk) and equal variance test (Brown-Forsythe). For those data that failed either the normality test or equal variance test, nonparametric statistics (conducted with the Mann–Whitney Rank Sum Test) were applied. For those data that passed both tests, parametric statistics were used with (1) paired *t* test for one group before and after one treatment; (2) independent *t* test for two groups (*e.g.*, comparison of effects of A85380 *vs.* saline on the fentanyl-induced sedation); (3) ANOVA for multiple groups followed by multiple comparisons (conducted with the Holm-Sidak method). One-way ANOVA, one-way repeated measures ANOVA, and two-way repeated measures ANOVA were used. For two-way repeated measures ANOVA, factors are between-subjects; for one-way repeated measures ANOVA, factors are within-subjects. $P < 0.05$ is taken as statistically significant difference; n refers to the number of animals, with animal as the unit of analysis for statistical tests. The significance of changes in respiratory parameters *in vitro* by drug application was examined with paired *t* test for application of one drug, or one-way repeated measures ANOVA followed by Holm–Sidak method for the sequential application of two drugs (figs. 2 and 3). For *in vivo* fentanyl experiments, the significance of changes in f_R, V_T, V_E, and Spo_2 after treatment was compared by using two-way repeated measures ANOVA (drug × time; Holm–Sidak method; figs. 4, 5, and 6). The significance of changes in remifentanil-induced apneas and decrease in V_E was compared by using one-way ANOVA (Holm-Sidak method) in three groups coadministrated with two doses of A85380 *versus* saline (fig. 7). The significance of changes in the nociceptive responses was compared by using one-way ANOVA (Holm-Sidak method) among saline-, fentanyl-, A85380-, and fentanyl with A85380–treated groups (fig. 8). The experiments were conducted between 10 AM and 5 PM. Animals were euthanized with an overdose of pentobarbital upon completion of experiments.

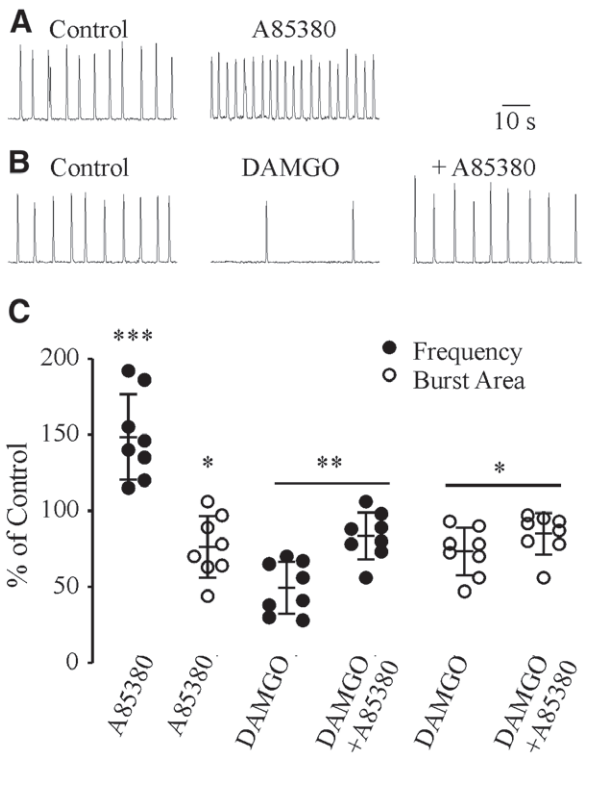

Fig. 3. The effects of A85380 on respiratory rhythm generated by the neonatal rat medullary slice preparations. (*A*) Bath application of A85380 (25 nM) increased baseline respiratory frequency and decreased baseline respiratory burst area. (*B*) Bath application of DAMGO (200 nM) suppressed respiratory frequency and burst area; the effects were reversed by a subsequent application of A85380 (25 nM). (*C*) Population data (mean and SD). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, statistically significant difference, compared after *versus* before drug administration, with paired *t* test for application of one drug; one-way repeated measures ANOVA followed by Holm–Sidak method for the sequential application of two drugs. n = 8 animals each group. DAMGO, D-Ala2, N-MePhe4, Gly-ol-enkephalin.

Results

Potentiation of Baseline Respiratory Rhythm and Alleviation of DAMGO-induced Respiratory Depression *In Vitro via* Activation of α4β2 Nicotinic Acetylcholine Receptors

We started the *in vitro* analyses by examining the effects of drug application to media bathing brainstem–spinal cord preparations that generate spontaneous inspiratory motor activity (fig. 2). Nicotine (600 nM, fig. 2A), a nonselective agonist of nicotinic acetylcholine receptor, caused an increase of baseline f_R, at concentrations above 200 nM. The selective α4β2 nicotinic acetylcholine receptor agonist A85380 (25 nM, fig. 2B) increased baseline f_R at concentrations above 5 nM, whereas activation of the α7 nicotinic acetylcholine receptor with the agonist PNU282987 (30 μM, fig. 2C) resulted in a very modest increase in f_R. The μ-opioid receptor agonist DAMGO (200 nM) was used to induce respiratory depression *in vitro*. There was a clear reversal of DAMGO-induced respiratory depression (decrease in f_R and burst area) by subsequent application of nicotine (600 nM, fig. 2D) or A85380 (25 nM, fig. 2E), but not by PNU282987 (30 μM, fig. 2F). The effects of nicotine and A85380 on baseline and DAMGO-induced respiratory depression were blocked by pre-application of the α4β2 nicotinic acetylcholine receptor antagonist dihydro-β-erythroidine (400 nM, figs. 2, G and H), but not by the α7 nicotinic acetylcholine receptor antagonist methyllycaconitine (400 nM, fig. 2H). Consistent with a previous study,[11] f_R was slower in the presence of Dihydro-β-erythroidine (400 nM, fig. 2H) or methyllycaconitine

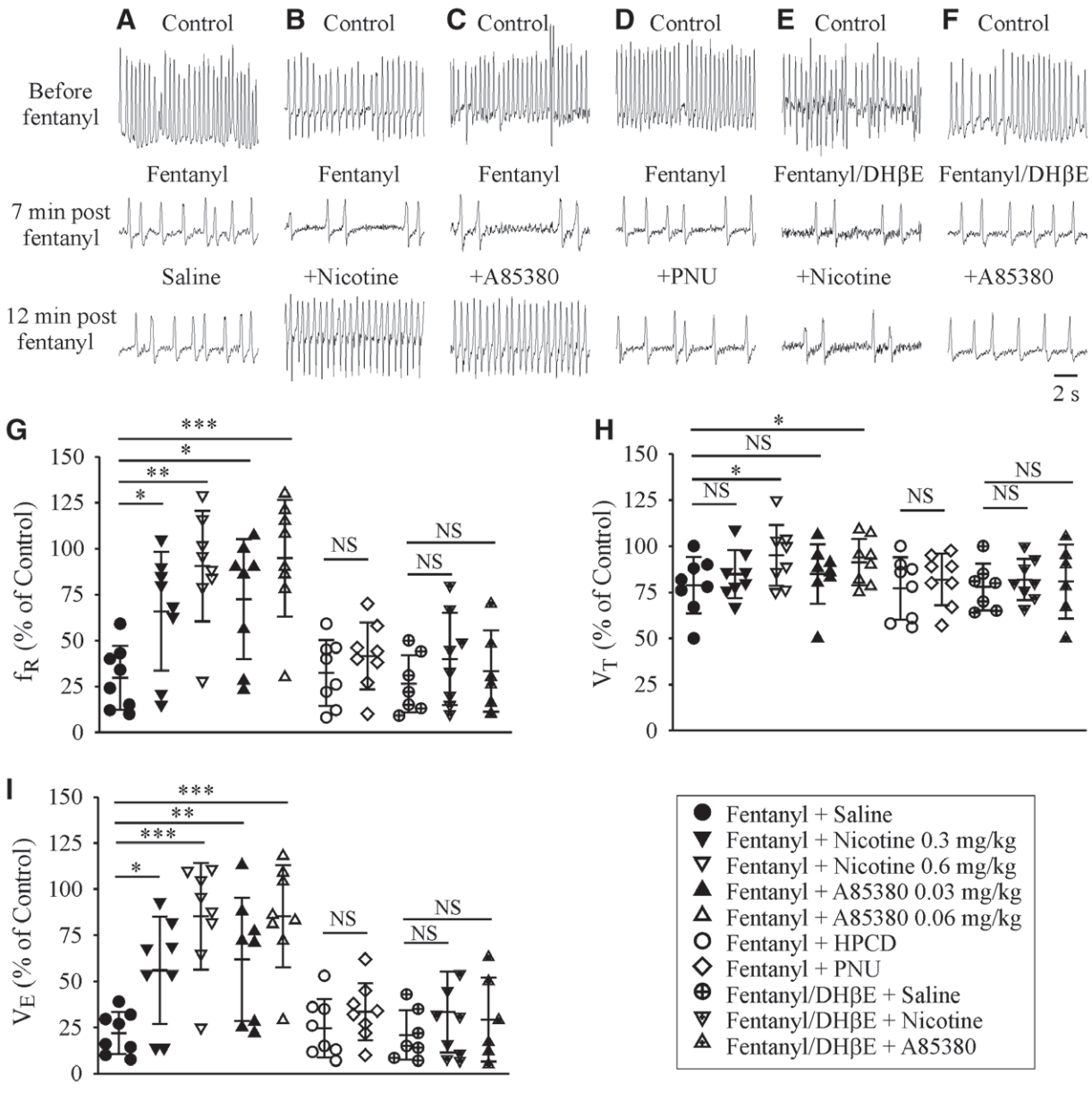

Fig. 4. Effects of α4β2 and α7 nicotinic acetylcholine receptor targeting agents on fentanyl-induced respiratory depression in newborn rats. (*A–F*) Representative whole-body plethysmographic recordings from six postnatal day 3 pups. All drugs tested were administered subcutaneously (sc) in the posterior neck region. Administration of fentanyl (35 μg/kg, subcutaneously, *A–D*) or coadministration with DHβE (6 mg/kg, subcutaneously, *E* and *F*) caused a marked depression of respiratory frequency and a mild depression of tidal volume within 7 min after fentanyl administration. Subsequent administration of saline had no effect on fentanyl-induced respiratory depression (*A*). Nicotine (0.6 mg/kg, subcutaneously, *B*) and A85380 (0.06 mg/kg, subcutaneously, *C*), but not PNU282987 (20 mg/kg, subcutaneously, *D*) reversed fentanyl-induced respiratory depression. However, neither nicotine (0.6 mg/kg, subcutaneously, *E*) nor A85380 (0.06 mg/kg, subcutaneously, *F*) had any effect on respiratory depression induced by fentanyl coadministrated with DHβE (6 mg/kg, subcutaneously). Respiratory variables were presented and measured before fentanyl administration (*top traces*), 7 min after fentanyl (*middle traces*), and 12 min after fentanyl administration (*bottom traces*). (*G–I*) Population data (mean and SD) showing respiratory frequency (f_R), tidal volume (V_T), and minute ventilation (V_E) at 12 min after fentanyl (4 to 5 min after administration of nicotinic acetylcholine receptor agonists and antagonists). Respiratory parameters were calculated over an average of 1 min of continuous recordings relative to control before fentanyl administration. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, statistically significant difference; ns: $P > 0.05$, no significant difference in compared groups, using two-way repeated measures ANOVA (Holm–Sidak method). DHβE, Dihydro-beta-erythroidine hydrobromide; HPCD, 2-hydroxypropyl-β-cyclodextran; PNU, PNU282987.

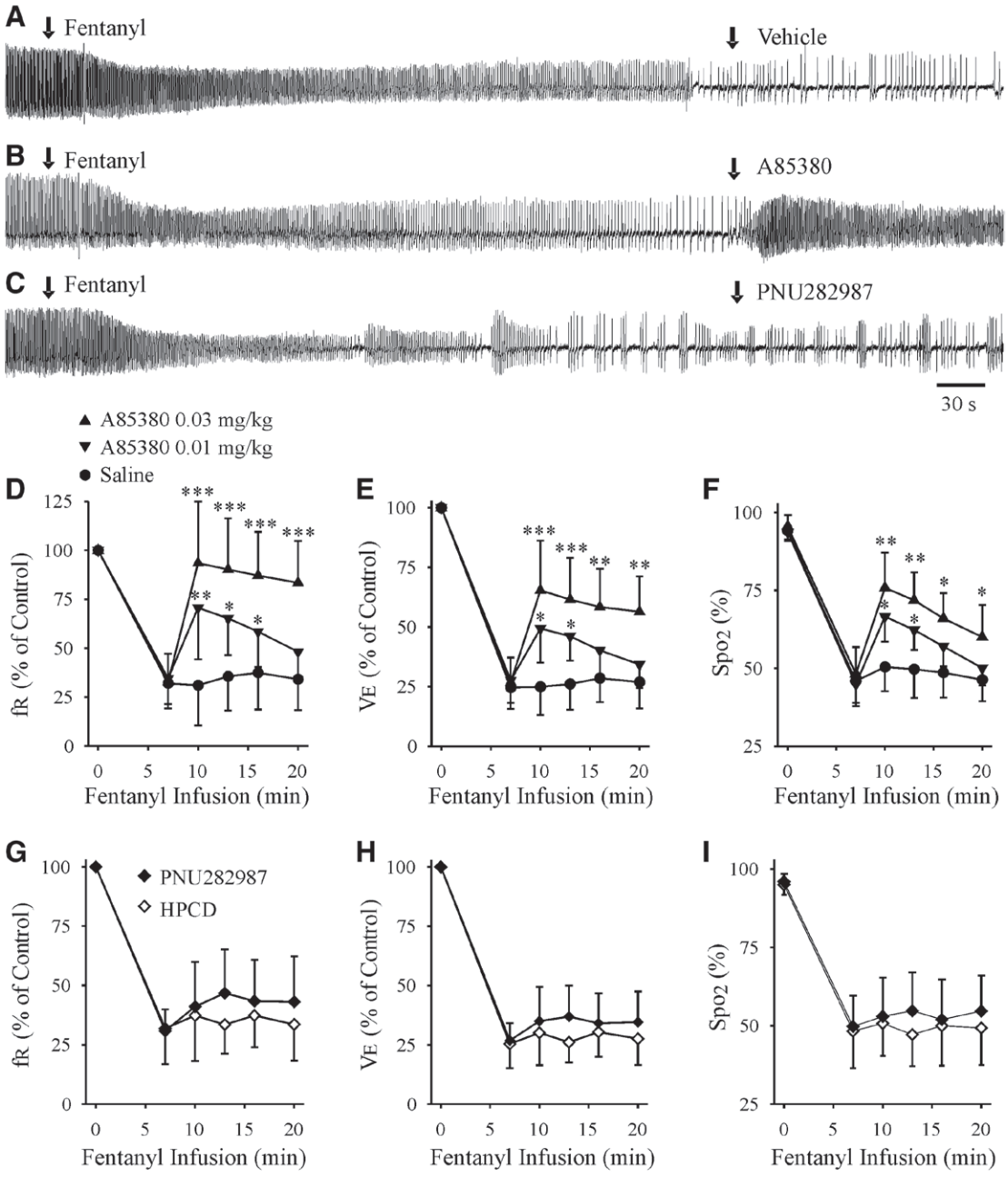

Fig. 5. A85380, but not PNU282987, reversed fentanyl-induced decrease of respiratory rate and oxygen saturation (Sao_2) in adult rats. (*A–D*) Representative whole-body plethysmographic recordings from four adult rats. Administration of fentanyl (60 μg/kg over 20 min, intravenous [IV] infusion) caused a marked decrease of respiratory rate within 7 min of the infusion. The fentanyl-induced decrease of respiratory frequency was not affected by subsequent administration (IV) of saline (*A*), but was diminished by A85380 (0.03 mg/kg, IV, *B*). Subsequent administration of PNU282987 (10 mg/kg, IV, *C*) had no effect on fentanyl-induced decrease of respiratory depression. (*D–F*) Population data (mean and SD) showing the time course of changes of respiratory parameters (*D*: frequency, f_R; *E*: minute ventilation: V_E; relative to control before fentanyl administration) or arterial oxygen saturation (Spo_2, *F*), with IV bolus injection of A85380 (0.01 mg/kg, or 0.03 mg/kg IV) or saline 7 min after starting fentanyl IV infusion. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, statistically significant difference, using two-way repeated measures ANOVA (Holm–Sidak method). (*G–I*) Population data (mean and SD) showing the time course of changes of respiratory parameters (*G*: f_R; *H*: V_E; relative to control before fentanyl administration) or Spo_2 (*I*), with IV bolus injection of PNU282987 (1 to 10 mg/kg), or HPCD 7 min after starting fentanyl IV infusion. There is no significant difference between PNU282987- *versus* HPCD-treated groups, using two-way repeated measures ANOVA (Holm–Sidak method). HPCD, 2-hydroxypropyl-β-cyclodextran. n = 8, 8, and 6 animals each data point for f_R, V_E, and Spo_2, respectively.

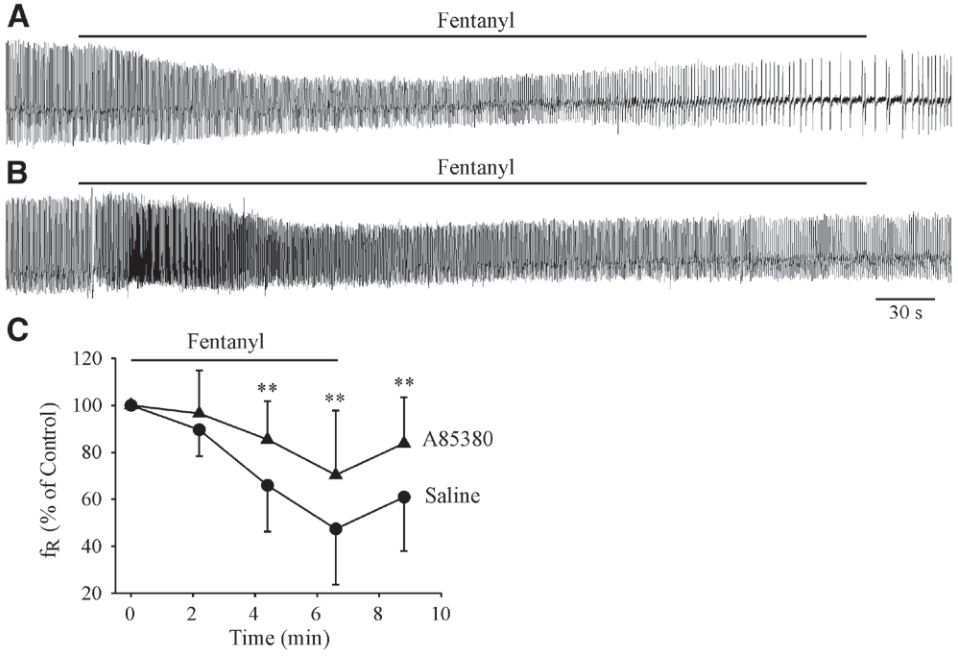

Fig. 6. Preadministration of A85380 alleviates fentanyl-induced respiratory depression in adult rats. (*A* and *B*) Representative whole-body plethysmographic recordings from two adult rats. (*A*) Two minutes after saline administration (neck subcutaneously), administration of fentanyl (20 μg/kg over 400 s, IV) caused a marked depression of respiratory frequency and minute ventilation. (*B*) Preadministration of A85380 (0.06 mg/kg, subcutaneously) approximately 2 min before fentanyl administration reduced the fentanyl-induced decrease of respiratory frequency. (*C*) Population data (mean and SD) showing respiratory frequency (f_R) relative to control prior to fentanyl administration. $^{**}P < 0.01$, statistically significant difference in two groups, using two-way repeated measures ANOVA (Holm–Sidak method). n = 16 animals each data point.

(400 nM, fig. 2H), indicating tonic excitation of respiratory rhythm by endogenous activation of α4β2 and α7 nicotinic acetylcholine receptors. The above nicotinic acetylcholine receptor targeting agents had no significant effects on inspiratory burst area (data not shown).

We then used the medullary slice preparation (fig. 3) that contains the preBötzinger Complex and a population of XII motoneurons that discharge during the inspiratory phase of the respiratory cycle.[27] This allows for a more direct assessment of drug action at the level of the preBötzinger Complex. Bath application of A85380 (25 nM) increased f_R, whereas PNU282987 (30 μM, data not shown) had no effect on baseline respiratory activity. DAMGO (200 nM) caused a suppression of f_R that was alleviated by A85380 (25 nM), but not by PNU282987 (30 μM, data not shown).

Countering Fentanyl-induced Respiratory Depression *In Vivo* in Neonatal Rats by Activation of α4β2 Nicotinic Acetylcholine Receptors

The next stage was to examine these compounds *in vivo* (all drugs administered *via* neck subcutaneously) in rat pups of similar age as those used *in vitro*. Neither nicotine (0.3 to 0.6 mg/kg, subcutaneously), A85380 (0.03 to 0.06 mg/kg, subcutaneously), PNU282987 (1 to 20 mg/kg, subcutaneously) nor DHβE (6 mg/kg, subcutaneously) significantly altered baseline V_E (data not shown). Consistent with our previous studies,[19] fentanyl (35 μg/kg, subcutaneously) induced a marked decrease in f_R (basal f_R: 123.4 ± 24.5 breaths/min), and minor decrease in V_T, and the effects lasted for ~20 min. At ~7 min after administration of fentanyl, subsequent administration of vehicle (saline: fig. 4A or 2-hydroxypropyl-β-cyclodextran) had no effect on respiratory depression. Nicotine (0.3 to 0.6 mg/kg, subcutaneously, fig. 4B) and A85380 (0.03 to 0.06 mg/kg, subcutaneously, fig. 4C) alleviated fentanyl-induced respiratory depression (decrease in f_R, V_T, V_E) in a dose-dependent manner, whereas there was no reversal by PNU282987 (1 to 20 mg/kg, subcutaneously, fig. 4D). Coapplication of DHβE (6 mg/kg, subcutaneously) with fentanyl prevented the alleviation of fentanyl-induced respiratory depression by subsequent administration of nicotine (fig. 4E) or A85380 (fig. 4F). Population data are shown in fig. 4, G–I.

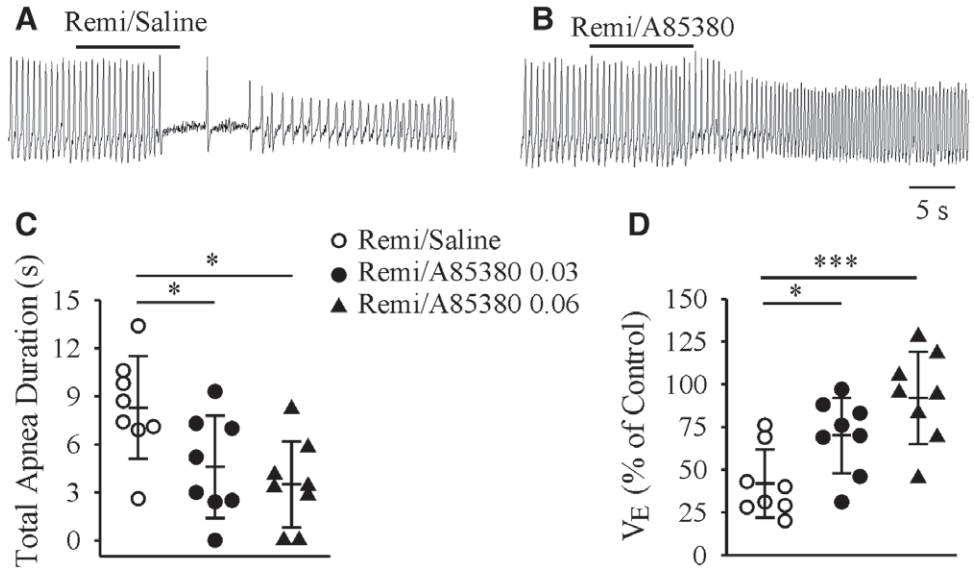

Fig. 7. Coadministration of A85380 alleviates remifentanil-induced respiratory depression in adult rats. (*A* and *B*) Representative whole-body plethysmographic recordings from two adult rats. A bolus of remifentanil (5 μg/kg IV bolus over 20 s, coadministrated with saline) caused marked apneas and decreased minute ventilation (V_E) in the first minute (*A*). Coadministration of A85380 (0.06 mg/kg, IV) with remifentanil markedly reduced the remifentanil-induced apneas and decrease in V_E (*B*). (*C–E*) Population data (mean and SD). *$P < 0.05$, ***$P < 0.001$, statistically significant difference in compared groups, using one-way ANOVA (Holm–Sidak method). n = 8 animals each group.

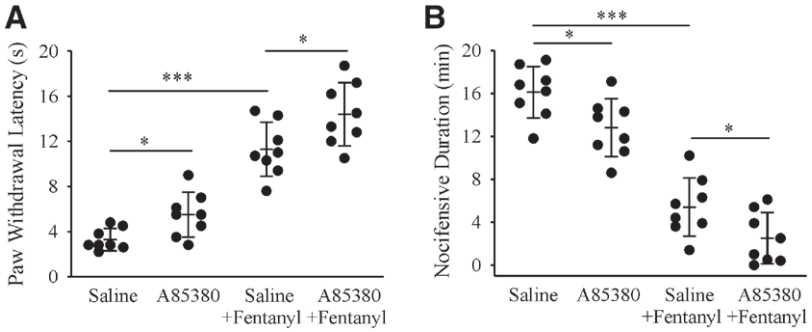

Fig. 8. A85380 induces basal analgesia and enhances fentanyl-induced analgesia in adult rats. (*A*) Effects of A85380 (0.06 mg/kg, neck subcutaneously) on paw withdrawal latency in response to thermal stimuli, measured at 42 min after A85380 or saline with or without subsequent fentanyl administration (20 μg/kg more than 400 s intravenous [IV] infusion). (*B*) Effects of A85380 administration (0.06 mg/kg, neck subcutaneously) on the time spent engaging in nociceptive behaviors (licking and lifting) 20 to 40 min after formalin administration, measured at 32 to 52 min after A85380 or saline with or without subsequent fentanyl administration (20 μg/kg more than 400 s IV infusion). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, statistically significant difference in compared groups, using one-way ANOVA (Holm–Sidak method). n = 8 animals each group.

Countering Fentanyl-induced Respiratory Depression *In Vivo* in Adult Rats by α4β2 Nicotinic Acetylcholine Receptor Agonists

We examined the effects of α4β2 and α7 nicotinic acetylcholine receptor agonists to counter fentanyl-induced respiratory depression in adult rats *in vivo*. Fig. 5 shows representative plethysmographic recordings of adult rats breathing during a 20-min IV infusion of 60 μg/kg fentanyl. Similar to past studies,[19,21] this paradigm caused a marked suppression of f_R (more than 50% decrease; basal f_R: 87.8 ± 11.2 breaths/min) in most rats within 7 min after fentanyl administration. Subsequent injection of vehicle (fig. 5A) did not change the course of fentanyl action. In contrast, subsequent injection of nicotine (0.1 to 0.3 mg/kg, IV, fig. 5B) reversed the fentanyl-induced f_R decrease and

the reversal lasted 5 to 10 min. Injection of A85380 (0.01 to 0.03 mg/kg, IV, fig. 5C) dose-dependently reversed the fentanyl-induced f_R decrease and at the highest dose the reversal persisted beyond the duration of the fentanyl infusion. PNU282987 (1 to 10 mg/kg, IV, fig. 5D) had no effect on the fentanyl-induced respiratory depression. Population data showed the fentanyl-induced decrease in respiratory frequency, minute ventilation, and oxygen saturation dose-dependently alleviated by subsequent administration of A85380 (fig. 5, E, F, and G, respectively), but not by subsequent administration of PNU282987 (fig. 5, H, I, and J, respectively). The reversal of fentanyl-induced decrease in f_R by A85380 (0.03 mg/kg, IV) was rapid (median onset of effect: 11.1 s, interquartile ranges: 7.6 s, n = 8) and comparable with the reversal caused by naloxone (0.3 mg/kg, median: 10.1 s, interquartile ranges: 8.6 s, n = 4, Mann–Whitney Rank Sum test, $P = 0.683$). Fentanyl-induced decrease of body temperature at the end of the 20-min infusion of 60 μg/kg fentanyl was not significantly different with saline (median: −1.1°C, interquartile ranges: −0.9°C, n = 6) or A85380 (median: −1.2°C, interquartile ranges: −0.8°C, n = 6, Mann–Whitney Rank Sum test, $P = 0.589$) treatments.

We then examined the effects of A85380 to prevent fentanyl-induced decrease of f_R in adult rats. Vehicle (saline) or A85380 (0.06 mg/kg, neck subcutaneously) was injected 2 min before fentanyl administration (20 μg/kg, 400 s IV infusion, fig. 6). It caused a marked f_R decrease in most vehicle treated animals. Administration of A85380 reduced the severity of fentanyl-induced respiratory depression. Note that the 0.06 mg/kg dose administered is approximately the EC_{50} based on previous rat studies of A85380.[13,16] Consistent with those studies, we did not observe behavioral side effects.

Fentanyl also caused a decrease in V_T *in vivo*. Our previous study[18] indicates that the reduced tidal volume is most likely in part attributable to decreased drive to respiratory motoneurons and a larger component results from fentanyl-induced muscle rigidity and rib cage stiffness. The rigidity that occurs in rats is a well-documented phenomenon, possibly involving striatal μ-opioid receptors.[28] A85380 did not appear to reduce the muscle rigidity and thus there is no reversal of V_T in adult rats. In contrast, in P3 pups, where fentanyl-induced muscle rigidity is much less severe, there was a reversal of the V_T depression (fig. 4) after nicotine or A85380.

Prevention of Remifentanil-induced Apnea *In Vivo* in Adult Rats by α4β2 Nicotinic Acetylcholine Receptor Agonist

The opioid remifentanil induces a particularly strong respiratory depression that is short acting. It has proven to be more difficult to counter by either a low dose of naloxone[29–31] or an ampakine (recent clinical trial).[32] Thus, to further assess the potency of A85380 we tested it against remifentanil-induced respiratory depression (fig. 7). Coadministration of remifentanil (5 μg/kg more than 20 s) and saline induced marked apneas and decreased V_E during the first minute after injection and then recovered after 2 min. Coadministration of remifentanil (5 μg/kg, IV) and A85380 (0.03 to 0.06 mg/kg, IV) markedly reduced the incidence of apnea and reduced the depression of V_E that normally occurred immediately after remifentanil injection.

Fentanyl-induced Sedation Alleviated in Adult Rats by α4β2 Nicotinic Acetylcholine Receptor Agonist

In addition to respiratory depression, unintended sedation is another serious opioid-induced adverse event which contributes to patient morbidity and increased length of hospitalization.[33] Although the underlying mechanisms are not fully understood, opioid-induced sedation is thought to involve the anticholinergic activity of opioids.[34] Thus, we tested the hypothesis that activation of α4β2 by A85380 (0.06 mg/kg, subcutaneously) would reduce the sedation induced by fentanyl (20 μg/kg more than 400 s IV infusion). The sedation (loss of righting reflex) was modestly, but significantly, shortened in the A85380 (16.2 ± 3.6 min after fentanyl, n = 8, independent *t* test, $P = 0.039$) *versus* vehicle treated group (20.5 ± 4.0 min, n = 8).

Fentanyl-induced Analgesia in Adult Rats Enhanced by α4β2 Nicotinic Acetylcholine Receptor Agonist

We assessed the effects of A85380 (0.06 mg/kg, subcutaneously) on baseline nociception in adult rats (fig. 8). First, we measured the paw withdrawal latency to thermal stimuli before and 42 min after A85380 or saline administration. There was a marked increase in paw withdrawal latency after treatment with A85380; whereas there was no change in the vehicle group. Second, we performed a formalin test that scored time spent engaging in nociceptive behaviors (licking plus lifting of injured paw) 20 to 40 min after formalin injection. Formalin was administrated 12 min after A85380 or saline. The A85380-treated group had a decreased nocifensive response relative to the saline group. A85380-induced basal analgesia in both tests was consistent with a previous study.[13]

We then assessed the effects of A85380 (0.06 mg/kg, subcutaneously, 2 min before fentanyl) on fentanyl (20 μg/kg over 400s IV infusion)-induced analgesia in adult rats. Fentanyl administration (pretreatment with saline) induced marked analgesia as measured by the thermal and formalin tests, whereas pretreatment of A85380 further increased fentanyl-induced analgesia in both tests (fig. 8). Collectively, these data indicate that fentanyl-induced analgesia was enhanced by A85380.

Discussion

Activation of α4β2 Nicotinic Acetylcholine Receptor Rapidly Alleviates Opioid-induced Respiratory Depression

We have determined that the nonselective nicotinic acetylcholine receptor agonist nicotine and α4β2 nicotinic acetylcholine receptor agonist A85380, but not the α7 nicotinic acetylcholine receptor agonist PNU282987, reverses respiratory depression induced by activation of μ-opioid receptors *in vitro* and *in vivo*. The effects of both nicotine and A85389 were blocked by the α4β2 nicotinic acetylcholine receptor specific antagonist DHβE. A85380 had a rapid onset of reversing fentanyl-induced respiratory depression. Further, coadministration (IV) of A85380 with remifentanil markedly reduced the potent respiratory depression. These results demonstrated that activation of α4β2 nicotinic acetylcholine receptor alleviates opioid-induced respiratory depression. These results also pointed to an important role played by the medullary respiratory networks in the effects of αα4β2 nicotinic acetylcholine receptor agonists on alleviating opioid-induced respiratory depression. The conclusion is supported by the fact that the medullary respiratory network expresses nicotinic acetylcholine receptor subunits α4 and β2 and activation of α4β2 receptors increases respiratory rhythm.[9–11] Additionally, α4β2 nicotinic acetylcholine receptors are present in the carotid bodies[35,36] and they may be involved in the effects of nicotine and A85380 on opioid-induced respiratory depression *in vivo*, although that possibility was not addressed in this study. The relative timing of the onset of action of fentanyl, remifentanil, and A85380 were similar so that when delivered concomitantly there is the potential for minimizing the degree of respiratory depression or apneas induced by opioids on their own. Also of importance is the fact that the effective dose for preventing or alleviating opioid-induced respiratory depression did not induce hyperventilation or obvious side effects. This is consistent with previous studies of A85380 and other α4β2 agonists in rats[12,13,16] and humans.[37] The reason for the lack of effect of activating α4β2 nicotinic acetylcholine receptors on baseline breathing *in vivo* is unclear, but it may be attributable to the fact that nicotinic acetylcholine receptor agonists act presynaptically to enhance the release of both inhibitory and excitatory neurotransmitters released within brainstem respiratory nuclei[38] and thus the net response is neutral under baseline conditions. *In vitro*, A85380 and nicotine did increase baseline activity, but there is minimal tonic release of inhibitory neurotransmitters affecting respiratory rhythm in those neonatal preparations.[39]

Opioids suppress respiratory activity by presynaptic inhibition of excitatory drive and direct hyperpolarization of preBötzinger Complex neurons.[6,7,40–42] Electrophysiologic *in vitro* studies will be necessary to determine the mechanisms by which activation of α4β2 nicotinic acetylcholine receptors alters the actions of opioids at the cellular and synaptic level.

Interestingly, a past study demonstrated that elevating the overall levels of acetylcholine by administration of anticholinesterases alleviated opioid-induced respiratory depression in rabbits.[43] That more blunt approach leads to the broad activation of multiple classes of muscarinic and nicotinic acetylcholine receptors, but the results are consistent with our findings with selective α4β2 nicotinic acetylcholine receptor activation.

The timing of the onset of A85380 action in reversing respiratory depression in response to moderate to severe nonlethal doses of fentanyl was similar to naloxone. Both are water soluble and thus amenable to IV and intramuscular injection. The advantage of A85380 over naloxone in clinical settings would obviously be the maintenance of the desired analgesia and sedation when the respiratory depression would be deemed to be mild. In a rescue situation from an overdose in a nonclinical setting, loss of analgesia would not be a consideration. Naloxone administration provides a very potent reversal of respiratory depression and will be the primary course of action in a nonclinical setting. However, a potential advantage of A85380 *versus* naloxone could be the maintenance of sedation in cases where it would be deemed advantageous not to have the subject becoming fully alert and mobile immediately after injection of the rescue agent. Further, the half-life of naloxone is approximately 15 to 30 min and thus retainment of the subject beyond the initial arousal is required to ensure a second naloxone administration to guard against the return of opioid-induced respiratory depression.[29–31] In comparison, the half-life of A85380 in humans is 7.2 h,[44] and thus a single dose would be sufficient.

Activation of α4β2 Nicotinic Acetylcholine Receptor Enhances Opioid-induced Analgesia

The pain measurements demonstrated that, if anything, the analgesic effects of fentanyl were enhanced with coadministration of A85380 in two types of nociceptive tests. This is consistent with several studies showing that drugs that activate α4β2 nicotinic acetylcholine receptors also induce analgesia.[12–16] A85380-induced acute thermal antinociception is mediated supraspinally *via* augmentation of descending inhibition to the spinal cord.[13,16] This descending modulatory circuit is also an opioid-sensitive circuit[40] and may be responsible for the additive antinociceptive effects induced by the combination of fentanyl and A85380. Thus, drugs that target α4β2 nicotinic acetylcholine receptors, when coadministered with opioids, could allow for relatively lower doses of opioid to achieve the desired analgesia. That in itself would tend to reduce respiratory depression and other side effects (*e.g.*, sedation, constipation) in addition to the direct stimulatory actions

of A85380 on the respiratory network. These results pointed to important clinical potentials using coadministration of α4β2 nicotinic acetylcholine receptor agonist with opioid to minimize the degree of opioid-induced respiratory depression or apneas and to enhance desired analgesia.

Other Strategies of Countering Opioid-induced Respiratory Depression

There have been other pharmacologic approaches proposed for countering opioid-induced respiratory depression. Preclinical data have indicated that activation of serotonin receptors can overcome opioid-induced respiratory depression without loss of analgesia.[45,46] We have also demonstrated the positive effects of a serotonergic receptor agonist against fentanyl-induced respiratory depression in a rat model but at the expense of inducing marked side effects.[19] Those preclinical data were consistent with clinical trials assessing serotonergic receptor agents against opioid-induced respiratory depression that have all shown a lack of positive effect at doses below those that cause serious side-effects.[47,48] Golder *et al.*[49] have investigated the strategy of countering opioid-induced respiratory depression by pharmacologically blocking BK_{Ca}-channels on carotid bodies and central nervous system neurons. That strategy induces hyperventilation (largely resulting from increases in tidal volume) of baseline breathing in rats and humans that partially balances out hypoventilation associated with opioid-induced respiratory depression.[49–51] A recent human study demonstrated that esketamine significantly reduced respiratory depression induced by remifentanil without altering baseline breathing.[52] The effective dose of esketamine was subanesthetic and induced psychomimetic side effects in a small minority of subjects. In contrast, racemic ketamine administration actually worsened respiratory depression induced by morphine in an earlier human study.[53] Our group has shown that ampakines, drugs that positively modulate AMPA (amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, counter opioid-induced respiratory depression in rodent models.[18,20,21,54] Those findings were supported in a Phase IIa clinical trial that showed the positive effect of an ampakine in humans exposed to modest levels of respiratory depression induced by alfentanil.[49] However, an ampakine was not effective in reducing the rapid and more severe respiratory depression induced by remifentanil bolus in a more recent Phase IIa clinical trial.[32] More potent ampakines than those used in trials to date may be more effective if concerns about overexcitation of the central nervous system are overcome.[55] In addition, because of the solubility limitation of ampakines, only oral formulations are currently available for clinical trials, and they are associated with a delay in ampakines reaching plasma therapeutic levels. Preclinical work has started toward developing a water-soluble ampakine, and those data with CX1942 showed partial reversal of opioid-induced respiratory depression within several minutes after administration in a goat model.[56] Thus, the ampakine technology continues to warrant investigation but has not yet been demonstrated to adequately fulfill the unmet clinical need of rapidly and effectively countering opioid-induced respiratory depression without loss of analgesia.

Conclusion

We have demonstrated that activating α4β2 nicotinic acetylcholine receptors reduces the severity, or provided a potent and rapid alleviation, of fentanyl-induced respiratory depression. Importantly, drugs of this class do not interfere with pain suppression, and in some cases enhance analgesia. This proof-of-principle preclinical study demonstrates the potent ability of A85380 (not developed for clinical use) to counter opioid-induced respiratory depression. Further screening of α4β2 nicotinic acetylcholine receptor targeting agents deemed suitable for clinical use should be performed to pick the most suitable candidates to move forward to clinical trials. This could include full agonists, partial agonists, and positive allosteric modulators of α4β2 nicotinic acetylcholine receptors. Drugs from all three classes have been examined in preclinical and clinical trials of non–respiratory-related functions involving α4β2 nicotinic acetylcholine receptors (*e.g.*, analgesia, cognition, smoking cessation). Further preclinical work will be required to determine the specific cellular and synaptic mechanisms of action of α4β2 nicotinic acetylcholine receptor targeting drugs in the presence of μ-opioid agonists.

In this study we focused on the synthetic opioid receptor agonists fentanyl and remifentanil. These are powerful analgesics commonly used for acute and chronic pain. Fentanyl is also of particular concern in cases of accidental overdose. However, it is reasonable to assume that activation of α4β2 nicotinic acetylcholine receptors will also be effective at reducing respiratory depression induced by other drugs targeting μ-opioid receptors (*e.g.*, morphine, oxycodone).

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Competing Interests

A provisional patent application for the use of nicotinic receptor agonists for treating opioid-induced respiratory depression has been submitted by the University of Alberta (Edmonton, Alberta, Canada).

Correspondence

Address correspondence to Dr. Greer: 3-020M Katz Bldg., Department of Physiology, University of Alberta, Edmonton, AB, Canada T6G 2S2. john.greer@ualberta.ca. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Shook JE, Watkins WD, Camporesi EM: Differential roles of opioid receptors in respiration, respiratory disease, and opiate-induced respiratory depression. Am Rev Respir Dis 1990; 142:895–909
2. Seth P, Scholl L, Rudd RA, Bacon S: Overdose deaths involving opioids, cocaine, and psychostimulants - United States, 2015–2016. MMWR Morb Mortal Wkly Rep 2018; 67:349–58
3. Romberg R, Sarton E, Teppema L, Matthes HW, Kieffer BL, Dahan A: Comparison of morphine-6-glucuronide and morphine on respiratory depressant and antinociceptive responses in wild type and mu-opioid receptor deficient mice. Br J Anaesth 2003; 91:862–70
4. Greer JJ, Carter JE, al-Zubaidy Z: Opioid depression of respiration in neonatal rats. J Physiol 1995; 485 (Pt 3):845–55
5. Dietis N, Rowbotham DJ, Lambert DG: Opioid receptor subtypes: Fact or artifact? Br J Anaesth 2011; 107:8–18
6. Gray PA, Rekling JC, Bocchiaro CM, Feldman JL: Modulation of respiratory frequency by peptidergic input to rhythmogenic neurons in the preBötzinger complex. Science 1999; 286:1566–8
7. Montandon G, Qin W, Liu H, Ren J, Greer JJ, Horner RL: PreBotzinger complex neurokinin-1 receptor-expressing neurons mediate opioid-induced respiratory depression. J Neurosci 2011; 31:1292–301
8. Gotti C, Zoli M, Clementi F: Brain nicotinic acetylcholine receptors: Native subtypes and their relevance. Trends Pharmacol Sci 2006; 27:482–91
9. Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW: Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: A hybridization histochemical study in the rat. J Comp Neurol 1989; 284:314–35
10. Shao XM, Feldman JL: Pharmacology of nicotinic receptors in preBötzinger complex that mediate modulation of respiratory pattern. J Neurophysiol 2002; 88:1851–8
11. Hatori E, Sakuraba S, Kashiwagi M, Kuribayashi J, Tsujita M, Hosokawa Y, Takeda J, Kuwana S: Association of nicotinic acetylcholine receptors with central respiratory control in isolated brainstem-spinal cord preparation of neonatal rats. Biol Res 2006; 39:321–30
12. Bannon AW, Decker MW, Holladay MW, Curzon P, Donnelly-Roberts D, Puttfarcken PS, Bitner RS, Diaz A, Dickenson AH, Porsolt RD, Williams M, Arneric SP: Broad-spectrum, non-opioid analgesic activity by selective modulation of neuronal nicotinic acetylcholine receptors. Science 1998; 279:77–81
13. Curzon P, Nikkel AL, Bannon AW, Arneric SP, Decker MW: Differences between the antinociceptive effects of the cholinergic channel activators A-85380 and (+/-)-epibatidine in rats. J Pharmacol Exp Ther 1998; 287:847–53
14. Marubio LM, del Mar Arroyo-Jimenez M, Cordero-Erausquin M, Léna C, Le Novère N, de Kerchove d'Exaerde A, Huchet M, Damaj MI, Changeux JP: Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. Nature 1999; 398:805–10
15. Bitner RS, Nikkel AL, Curzon P, Donnelly-Roberts DL, Puttfarcken PS, Namovic M, Jacobs IC, Meyer MD, Decker MW: Reduced nicotinic receptor-mediated antinociception following *in vivo* antisense knockdown in rat. Brain Res 2000; 871:66–74
16. Rueter LE, Donnelly-Roberts DL, Curzon P, Briggs CA, Anderson DJ, Bitner RS: A-85380: A pharmacological probe for the preclinical and clinical investigation of the alphabeta neuronal nicotinic acetylcholine receptor. CNS Drug Rev 2006; 12:100–12
17. Umana IC, Daniele CA, McGehee DS: Neuronal nicotinic receptors as analgesic targets: It's a winding road. Biochem Pharmacol 2013; 86:1208–14
18. Ren J, Poon BY, Tang Y, Funk GD, Greer JJ: Ampakines alleviate respiratory depression in rats. Am J Respir Crit Care Med 2006; 174:1384–91
19. Ren J, Ding X, Greer JJ: 5-HT1A receptor agonist Befiradol reduces fentanyl-induced respiratory depression, analgesia, and sedation in rats. Anesthesiology 2015; 122:424–34
20. Lorier AR, Funk GD, Greer JJ: Fentanyl induced suppression of hypoglossal motoneuron activity and ampakine therapy. PLoS ONE 2010; 5(1):e8766
21. Ren J, Ding X, Funk GD, Greer JJ: Ampakine CX717 protects against fentanyl-induced respiratory

depression and lethal apnea in rats. Anesthesiology 2009; 110:1364–70
22. Dubuisson D, Dennis SG: The formalin test: A quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 1977; 4:161–74
23. Abbott FV, Franklin KB, Westbrook RF: The formalin test: Scoring properties of the first and second phases of the pain response in rats. Pain 1995; 60:91–102
24. Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, Craig CR, Collins AC, Damaj MI, Donny EC, Gardiner PS, Grady SR, Heberlein U, Leonard SS, Levin ED, Lukas RJ, Markou A, Marks MJ, McCallum SE, Parameswaran N, Perkins KA, Picciotto MR, Quik M, Rose JE, Rothenfluh A, Schafer WR, Stolerman IP, Tyndale RF, Wehner JM, Zirger JM: Guidelines on nicotine dose selection for *in vivo* research. Psychopharmacology (Berl) 2007;190(3):269–319
25. Hajós M, Hurst RS, Hoffmann WE, Krause M, Wall TM, Higdon NR, Groppi VE: The selective alpha7 nicotinic acetylcholine receptor agonist PNU-282987 [N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride] enhances GABAergic synaptic activity in brain slices and restores auditory gating deficits in anesthetized rats. J Pharmacol Exp Ther 2005; 312:1213–22
26. Damaj MI, Welch SP, Martin BR: *In vivo* pharmacological effects of dihydro-beta-erythroidine, a nicotinic antagonist, in mice. Psychopharmacology (Berl) 1995; 117:67–73
27. Funk GD, Greer JJ: The rhythmic, transverse medullary slice preparation in respiratory neurobiology: Contributions and caveats. Respir Physiol Neurobiol 2013; 186:236–53
28. Havemann U, Kuschinsky K: Further characterization of opioid receptors in the striatum mediating muscular rigidity in rats. Naunyn Schmiedebergs Arch Pharmacol 1981; 317:321–5
29. Boom M, Niesters M, Sarton E, Aarts L, Smith TW, Dahan A: Non-analgesic effects of opioids: Opioid-induced respiratory depression. Curr Pharm Des 2012; 18:5994–6004
30. Dahan A, Aarts L, Smith TW: Incidence, reversal, and prevention of opioid-induced respiratory depression. Anesthesiology 2010; 112:226–38
31. van Dorp E, Yassen A, Dahan A: Naloxone treatment in opioid addiction: The risks and benefits. Expert Opin Drug Saf 2007; 6:125–32
32. Krystal A, Lippa A, Nasiek D, Krusinska E, Purcell R: Opioids and sleep apnea: Antagonism of remifentanil-induced respiratory depression by CX1739 in two clinical models of opioid induced respiratory depression. Sleep 31st Annual Meeting of the Associated Professional Sleep Societies 2017;40:0571
33. Jungquist CR, Smith K, Nicely KL, Polomano RC: Monitoring hospitalized adult patients for opioid-induced sedation and respiratory depression. Am J Nurs 2017; 117(3 Suppl 1):27–35
34. Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R: Opioid complications and side effects. Pain Physician 2008; 11(2 Suppl):S105–20
35. Conde SV, Monteiro EC: Activation of nicotinic ACh receptors with alpha4 subunits induces adenosine release at the rat carotid body. Br J Pharmacol 2006; 147:783–9
36. Meza RC, Ortiz FC, Bravo E, Iturriaga-Vásquez P, Eugenín JL, Varas R: Functional expression of the α7 and α4-containing nicotinic acetylcholine receptors on the neonatal rat carotid body. Neurochem Int 2012; 60:115–24
37. Kimes AS, Chefer SI, Matochik JA, Contoreggi CS, Vaupel DB, Stein EA, Mukhin AG: Quantification of nicotinic acetylcholine receptors in the human brain with PET: Bolus plus infusion administration of 2-[18F]F-A85380. Neuroimage 2008; 39: 717–27
38. Pilarski JQ, Fregosi RF: Prenatal nicotine exposure alters medullary nicotinic and AMPA-mediated control of respiratory frequency *in vitro*. Respir Physiol Neurobiol 2009; 169:1–10
39. Ren J, Greer JJ: Modulation of respiratory rhythmogenesis by chloride-mediated conductances during the perinatal period. J Neurosci 2006; 26:3721–30
40. Haji A, Okazaki M, Ohi Y, Yamazaki H, Takeda R: Biphasic effects of morphine on bulbar respiratory neuronal activities in decerebrate cats. Neuropharmacology 2003; 45:368–79
41. Takeda S, Eriksson LI, Yamamoto Y, Joensen H, Onimaru H, Lindahl SG: Opioid action on respiratory neuron activity of the isolated respiratory network in newborn rats. Anesthesiology 2001; 95:740–9
42. Lorier AR, Lipski J, Housley GD, Greer JJ, Funk GD: ATP sensitivity of preBötzinger complex neurones in neonatal rat *in vitro*: Mechanism underlying a P2 receptor-mediated increase in inspiratory frequency. J Physiol 2008; 586:1429–46
43. Elmalem E, Chorev M, Weinstock M: Antagonism of morphine-induced respiratory depression by novel anticholinesterase agents. Neuropharmacology 1991; 30:1059–64
44. Ueda M, Iida Y, Mukai T, Mamede M, Ishizu K, Ogawa M, Magata Y, Konishi J, Saji H: 5-[123I]Iodo-A-85380: Assessment of pharmacological safety, radiation dosimetry and SPECT imaging of brain nicotinic receptors in healthy human subjects. Ann Nucl Med 2004; 18:337–44
45. Manzke T, Niebert M, Koch UR, Caley A, Vogelgesang S, Hülsmann S, Ponimaskin E, Müller U, Smart

TG, Harvey RJ, Richter DW: Serotonin receptor 1A-modulated phosphorylation of glycine receptor α3 controls breathing in mice. J Clin Invest 2010; 120:4118–28

46. Guenther U, Theuerkauf NU, Huse D, Boettcher MF, Wensing G, Putensen C, Hoeft A: Selective 5-HT(1A)-R-agonist repinotan prevents remifentanil-induced ventilatory depression and prolongs antinociception. Anesthesiology 2012; 116:56–64
47. Lötsch J, Skarke C, Schneider A, Hummel T, Geisslinger G: The 5-hydroxytryptamine 4 receptor agonist mosapride does not antagonize morphine-induced respiratory depression. Clin Pharmacol Ther 2005; 78:278–87
48. Oertel BG, Schneider A, Rohrbacher M, Schmidt H, Tegeder I, Geisslinger G, Lötsch J: The partial 5-hydroxytryptamine1A receptor agonist buspirone does not antagonize morphine-induced respiratory depression in humans. Clin Pharmacol Ther 2007; 81:59–68
49. Golder FJ, Hewitt MM, McLeod JF: Respiratory stimulant drugs in the post-operative setting. Respir Physiol Neurobiol 2013; 189:395–402
50. McLeod JF, Leempoels JM, Peng SX, Dax SL, Myers LJ, Golder FJ: GAL-021, a new intravenous BKCa-channel blocker, is well tolerated and stimulates ventilation in healthy volunteers. Br J Anaesth 2014; 113:875–83
51. Roozekrans M, Olofsen E, van der Schrier R, van Gerven J, Peng S, McLeod J, Dahan A: Reversal of opioid-induced respiratory depression by BK-channel blocker GAL021: A pharmacokinetic-pharmacodynamic modeling study in healthy volunteers. Clin Pharmacol Ther 2015; 97:641–9
52. Jonkman K, van Rijnsoever E, Olofsen E, Aarts L, Sarton E, van Velzen M, Niesters M, Dahan A: Esketamine counters opioid-induced respiratory depression. Br J Anaesth 2018; 120:1117–27
53. Bourke DL, Malit LA, Smith TC: Respiratory interactions of ketamine and morphine. Anesthesiology 1987; 66:153–6
54. Oertel BG, Felden L, Tran PV, Bradshaw MH, Angst MS, Schmidt H, Johnson S, Greer JJ, Geisslinger G, Varney MA, Lötsch J: Selective antagonism of opioid-induced ventilatory depression by an ampakine molecule in humans without loss of opioid analgesia. Clin Pharmacol Ther 2010; 87:204–11
55. Lynch G: Glutamate-based therapeutic approaches: Ampakines. Curr Opin Pharmacol 2006; 6:82–8
56. Haw AJ, Meyer LC, Greer JJ, Fuller A: Ampakine CX1942 attenuates opioid-induced respiratory depression and corrects the hypoxaemic effects of etorphine in immobilized goats (Capra hircus). Vet Anaesth Analg 2016; 43:528–38