Respiratory depression in rats induced by alcohol and barbiturate and rescue by ampakine CX717

Jun Ren, Xiuqing Ding, and John J. Greer

Department of Physiology, Centre for Neuroscience, University of Alberta, Edmonton Alberta, Canada

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Barbiturate use in conjunction with alcohol can result in severe respiratory depression and overdose deaths. The mechanisms underlying the additive/synergistic actions were unresolved. Current management of ethanol-barbiturate-induced apnea is limited to ventilatory and circulatory support coupled with drug elimination. Based on recent preclinical and clinical studies of opiate-induced respiratory depression, we hypothesized that ampakine compounds may provide a treatment for other types of drug-induced respiratory depression. The actions of alcohol, pentobarbital, bicuculline, and the ampakine CX717, alone and in combination, were measured via 1) ventral root recordings from newborn rat brain stem-spinal cord preparations and 2) plethysmographic recordings from unrestrained newborn and adult rats. We found that ethanol caused a modest suppression of respiratory drive in vitro (50 mM) and in vivo (2 g/kg ip). Pentobarbital induced an ~50% reduction in respiratory frequency in vitro (50 μM) and in vivo (28 mg/kg for pups and 56 mg/kg for adult rats ip). However, severe life-threatening apnea was induced by the combination of the agents in vitro and in vivo via activation of GABA_A receptors, which was exacerbated by hypoxic (8% O_2) conditions. Administration of the ampakine CX717 alleviated a significant component of the respiratory depression in vitro (50–150 μM) and in vivo (30 mg/kg ip). Bicuculline also alleviated ethanol-/pentobarbital-induced respiratory depression but caused seizure activity, whereas CX717 did not. These data demonstrated that ethanol and pentobarbital together caused severe respiratory depression, including lethal apnea, via synergistic actions that blunt chemoreceptive responses to hypoxia and hypercapnia and suppress central respiratory rhythmogenesis. The ampakine CX717 markedly reduced the severity of respiratory depression.

MATERIALS AND METHODS

In vitro brain stem-spinal cord preparations. All experimental procedures were approved by University of Alberta Animal Welfare Committee. Neonatal Sprague-Dawley (SD) rats aged postnatal day 0 (P0) to P2 were anaesthetized with metofane and decerebrated, and the brain stem-spinal cord was dissected according to procedures previously described (11, 37). The neuraxis was continuously perfused at 27 ± 0.5°C (5 ml/min; volume of 3 ml) with modified Kreb’s solution that contained (in mM): 128 NaCl, 3.0 KCl, 1.5 CaCl_2, 1.0 MgSO_4, 24 NaHCO_3, 0.5 NaH_2PO_4, and 30 d-glucose equilibrated with 95% O_2-5% CO_2 (pH 7.4). Recordings from the fourth ventral cervical nerve roots were amplified, rectified, low-pass filtered, and recorded using an analog-to-digital converter (Axon Instruments Digidata 1200; Molecular Devices, Sunnyvale, CA) and data acquisition software (Axon Instruments). Plethysmographic measurements. Measurements from unrestrained newborn and adult male SD rats were performed in whole-body plethysmographs that had inflow and outflow ports for continuous delivery of gases (room air, 8% oxygen, or 5% CO_2) and removal of expired carbon dioxide. The plethysmograph volumes were 260 and 2,000 ml for neonatal (P7–P8) and adult (300–400 g) male rats with a flow rate of 2 l/min and 10 l/min, respectively. Pressure changes...
were recorded with a pressure transducer (model DP 103, Validyne, Northridge, CA), signal conditioner (CD-15, Validyne), and analog-to-digital board (pClamp, Axon Instruments). Our plethysmographic recording setup is effective for studying respiratory frequency (fR) and detection of apneas. However, it is not suitable for precise quantification of tidal volume (VT) but rather changes relative to control values (i.e., pre- and post-drug delivery). The physical principle underlying whole-body plethysmography is the detection of pressure changes in the chamber resulting from the heating and humidification of inspired gas. However, VT (ml/g) measurement may also be influenced by gas compression effects related to the airway resistance. Because of these limitations, whole-body plethysmography only provides semi-quantitative measurements of VT and detection of changes relative to control state (before drug). Minute ventilation (VE; ml·min⁻¹·g⁻¹) equals fR × VT, providing semi-quantitative measurements. Therefore, relative value (to control), but not absolute value for VT and VE are provided. The chemoreceptive responses (5 min, after 30 s of gas exchange equilibration) to changed O₂ or CO₂ were made by switching from room air to hypoxia (8% O₂) or hypercapnea (5% CO₂) with a flow rate of 10 l/min. It took ~30 s for gas exchange within the large chamber, as confirmed with Oxycheck instrumentation (Critikon, Tampa, FL). Furthermore, a pulse oximeter (Norin 8600V, Plymouth, MN) was used for monitoring oxygen saturation levels. Rectal temperature was measured before drug and after ethanol and/or pentobarbital administration (model 8402-10 thermistor, Cole-Parmer Instrument). However, VT (ml/g) measurement may also be influenced by gas compression effects related to the airway resistance. Because of these limitations, whole-body plethysmography only provides semi-quantitative measurements of VT and detection of changes relative to control state (before drug). Minute ventilation (VE; ml·min⁻¹·g⁻¹) equals fR × VT, providing semi-quantitative measurements. Therefore, relative value (to control), but not absolute value for VT and VE are provided. The chemoreceptive responses (5 min, after 30 s of gas exchange equilibration) to changed O₂ or CO₂ were made by switching from room air to hypoxia (8% O₂) or hypercapnea (5% CO₂) with a flow rate of 10 l/min. It took ~30 s for gas exchange within the large chamber, as confirmed with Oxycheck instrumentation (Critikon, Tampa, FL). Furthermore, a pulse oximeter (Norin 8600V, Plymouth, MN) was used for monitoring oxygen saturation levels. Rectal temperature was measured before drug and after ethanol and/or pentobarbital administration (model 8402-10 thermistor, Cole-Parmer Instrument). For newborns, the plethysmograph and air pump were contained within an infant incubator (Isolette, model C-86; Air-Shields/Dräger Medical, Hatboro, PA) to maintain the ambient temperature at the approximate nest temperature of 32°C and relative humidity at ~50% (20, 30). The fresh air is also warmed up to 32°C before entering the plethysmographical recording chamber to avoid the “cool down” effect of rapid air flow on the pups’ body temperature. The dose of ethanol (2 g/kg ip) tested is twice the blood alcohol concentration limits (0.08%) for driving in U.S., Canada, and United Kingdom. Pentobarbital (28–56 mg/kg ip) is based on previous studies of patients with acute intoxication of pentobarbital at the range of 2–72 µg/ml plasma concentration (10).

**Pharmacological agents.** Bicuculline and strychnine (from Sigma Canada) were dissolved in DMSO and pentobarbital (Nembutal or Euthanyl, 240 mg/ml; Bimeda-MTC, Cambridge, ON), and/or ethanol (anhydrous ethyl alcohol) was injected intraperitoneal into the left peritoneal cavity of rats. Application of ethanol (50 mM) or pentobarbital (50 µM) caused a partial depression in the frequency of rhythmic respiratory discharge, with a maximum suppression occurring within 5–40 min of perfusion. Co-administration of ethanol (50 mM) and pentobarbital (50 µM) severely suppressed the respiratory frequency and completely blocked the respiratory activity within 10–40 min of perfusion in three of five preparations. The depression of respiratory frequency persisted for 10–60 min after washout of ethanol-pentobarbital from the bathing medium. There was no significant effect on respiratory amplitude with application of ethanol or pentobarbital. Population data (Fig. 1D) show the respiratory frequency suppressed by 50 mM ethanol alone (76 ± 9% of control; n = 5), 50 µM pentobarbital alone (48 ± 9% of control; n = 5), and after co-administration (13 ± 10% of control; n = 5).

Data in Fig. 2B show that the respiratory depression induced by ethanol-pentobarbital was significantly alleviated...
(82 ± 10% of control) by the GABA<sub>A</sub> receptor antagonist bicuculline (BIC; 3 µM, administered for 15 min). As shown in previous studies (28), BIC alone at these doses does not significantly alter baseline respiratory activity but often induced long-duration (>3 s), high-amplitude, non-respiratory, seizure-like motor discharge (Fig. 2B; observed in four of five preparations). Bath application of the glycine receptor antagonist strychnine (1 µM) did not change the respiratory depression by ethanol-pentobarbital combination (n = 3; data not shown). Note that chloride-mediated conductances in the respiratory rhythm-generating preBötC are inhibitory after embryonic day 19 (E19) in rats (28).

We then examined the effects of CX717 on reversing the respiratory depression caused by co-administration of ethanol and pentobarbital by applying two doses of CX717 (50 or 150 µM) to the bathing medium. After 15 min of exposure, the lower dose of 50 µM CX717 partially alleviated the alcohol-pentobarbital-induced depression of respiratory frequency (35 ± 9% of control; Fig. 2C), whereas 150 µM CX717 had a much more pronounced effect (80 ± 11% of control; Fig. 2, A and C). Note that a low dose of CX717 (50 µM; n = 5) did not cause a significant change of baseline respiratory frequency when added on its own to the media bathing brain stem-spinal cord preparations, whereas a higher dose (150 µM; n = 4) increased baseline respiratory frequency to 123 ± 5% of control. Neither dose induced nonrespiratory, seizure-like activity.

**In vivo newborn rat plethysmographic recordings.** The next stage of the study was to examine the interactions of ethanol and pentobarbital in vivo. Toward correlating with the in vitro studies, we first examined newborn rats in vivo. Figure 3 shows data from whole-body plethysmographic recordings from unrestrained newborn rats (P7–8). The baseline respiratory frequency in pups was 141 ± 5 breaths/min (n = 44). Administration of ethanol (2 g/kg; n = 6) alone significantly suppressed f<sub>R</sub> (81 ± 5% of control) and V<sub>E</sub> (75 ± 4% of control) without significant effects on V<sub>r</sub> (93 ± 5% of control). Pentobarbital (28 mg/kg; n = 8) alone significantly suppressed f<sub>R</sub> (53 ± 8% of control), V<sub>r</sub> (64 ± 10% of control), and V<sub>E</sub> (37 ± 11%). The combined administration of ethanol (2 g/kg) and pentobarbital (28 mg/kg) caused a severe depression of f<sub>R</sub> (11 ± 6% of control), V<sub>r</sub> (12 ± 6% of control), and V<sub>E</sub> (6 ± 3%). Ethanol-pentobarbital combination caused marked apneas interposed with periodic bursts of respiratory activity in 12 of 15 animals (Fig. 3A). The administration of either ethanol (2 g/kg; n = 6) or pentobarbital (28 mg/kg; n = 8) did not induce any fatal apneas (Table 1). In contrast, 10 of 15 newborn rats had lethal apnea after the combined administration of ethanol (2 g/kg) and pentobarbital (28 mg/kg). Administration of vehicle (10% HPCD) did not change the course of respiratory depression and lethal apneas (Fig. 3A), whereas with administration of CX717 (30 mg/kg ip), 12 of 15 animals survived when CX717 was administered within 5–30 min after ethanol and pentobarbital administration (Fig. 3, B and C; Table 1; Z-test, P = 0.026). Furthermore, ethanol-pentobarbital-induced decrease of respiratory activity (f<sub>R</sub>, V<sub>r</sub>, and V<sub>E</sub>) was significantly alleviated after administration of CX717 (30 mg/kg; Fig. 3C; P < 0.05). Consistent with a previous study (27), administration of CX717 (30 mg/kg; n = 4) alone to unanesthetised animals did not cause any seizure-like activity, marked behavioral change, or significant change in baseline respiratory parameters.

**In vivo adult rat plethysmographic recordings.** Figure 4 shows data from whole-body plethysmographic recordings of respiratory activity from unrestrained adult rats in control and ~60 min after drug administration. The baseline breathing frequency varied among the adult rats, with an average of 134 ± 6 breaths/min (n = 39), close to an average of ~130 breaths/min (34). Ethanol (2 g/kg; n = 5) induced a significant suppression of V<sub>r</sub> (81 ± 6% of control) and V<sub>E</sub> (77 ± 8%) without a significant effect on f<sub>R</sub> (98 ± 9% of control). Pentobarbital (56 mg/kg; n = 5) administration resulted in a significant suppression of f<sub>R</sub> (80 ± 6% of control), V<sub>r</sub> (66 ± 6% of control), and V<sub>E</sub> (39 ± 3%). The combination of administering ethanol (2 g/kg) and pentobarbital (56 mg/kg)
caused a severe depression of fR (23 ± 7% of control), VT (25 ± 7% of control), and V˙E (10 ± 3%) and induced lethal apneas (in 8 of 16 animals; Table 1). Administration of vehicle (10% HPCD ip; filled rectangle) did not change the course of respiratory depression and lethal apnea induced by the combination of ethanol and pentobarbital (28 mg/kg ip). Administration of CX717 (30 mg/kg ip) rescued the animal from ethanol plus pentobarbital-induced lethal apnea. C: population data showing respiratory parameters (relative to control): frequency (fR), tidal volume (VT), and minute ventilation (V˙E) with ethanol (n = 6), pentobarbital (n = 8), ethanol + pentobarbital (n = 15), and ethanol + pentobarbital + CX717 (n = 15). *Significant difference compared with control (P < 0.05). #Significant difference compared with all other groups (P < 0.05). NS No significant difference relative to control (P > 0.05).

Table 1. Effects of drugs on survival rate of adult rats

<table>
<thead>
<tr>
<th>Age</th>
<th>Groups</th>
<th>Animals Tested</th>
<th>Animals Survived</th>
</tr>
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<tbody>
<tr>
<td>Adult</td>
<td>Ethanol (2 g/kg)</td>
<td>5</td>
<td>5*</td>
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<tr>
<td></td>
<td>Pento (56 mg/kg)</td>
<td>5</td>
<td>5*</td>
</tr>
<tr>
<td></td>
<td>Ethanol + Pento</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>CX717 (30 mg/kg) administered within 30 s of apnea after ethanol + Pento</td>
<td>13</td>
<td>12*</td>
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<tr>
<td></td>
<td>BX (2 mg/kg) administered within 60 s of apnea after ethanol + Pento</td>
<td>8</td>
<td>8*</td>
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<tr>
<td></td>
<td>CX717 (30 mg/kg) administered after 60 s of apnea after ethanol + Pento</td>
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<td>3</td>
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<tr>
<td></td>
<td>BIC (2 mg/kg) administered after 60 s of apnea after ethanol + Pento</td>
<td>4</td>
<td>2</td>
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<tr>
<td>Neonate</td>
<td>Ethanol (2 g/kg)</td>
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<td>Pento (28 mg/kg)</td>
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<td>Ethanol + Pento</td>
<td>15</td>
<td>5</td>
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<td></td>
<td>CX717 (30 mg/kg) administered after ethanol + Pento</td>
<td>15</td>
<td>12*</td>
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Pento, pentobarbital. *Significant difference from ethanol + pento co-administration group by z-test (P < 0.05).
n = 4) alone did not cause any seizure-like activity or result in significant changes of baseline respiratory parameters after CX717. However, after BIC (2 mg/kg), four of eight animals examined developed seizure-like activity and altered respiratory pattern during the seizure period.

**Effects on core temperature and oxygen saturation in adult rats.** Table 2 shows data from adult rats in control and 50 min after drug administration on the effects of core temperature and oxygen saturation. Ethanol (2 g/kg ip) administration did not significantly change core temperature and oxygen saturation levels, whereas pentobarbital (56 mg/kg ip) alone and with ethanol (2 g/kg ip) caused significant decreases in body temperature and oxygen saturation levels compared with the animals before drug administration. Note that the oxygen saturation and body temperature were markedly lower after ethanol-pentobarbital co-administration relative to control. The enhanced respiratory activity in the presence of CX717 (30 mg/kg ip) helped maintain body temperature and oxygen saturation levels.

**Responses to hypoxia and hypercapnia in adult rats.** $V_\text{E}$ was measured during hypoxia (5-min exposure to 8% $O_2$) or hypercapnia (5-min exposure to 5% $CO_2$) and reported relative to that observed in control room air conditions. Data was compared after 40-min exposure to vehicle, ethanol, and/or pentobarbital, at a time when no lethal apneas were induced by co-administration of ethanol-pentobarbital. Figure 5A shows representative plethysmographic recordings in response to hypoxia under various conditions. The group data is shown in Fig.
Exposure to hypoxia caused an increase in $\dot{V}_E$ to 128 ± 6 and 165 ± 10% of control in the untreated rats ($n = 4$) during minutes 1 and 5 of exposure (after 30 s of gas exchange equilibration), respectively. Ethanol (2 g/kg; $n = 5$) alone did not significantly change the responses to hypoxia at either time point. Pentobarbital (56 mg/kg; $n = 5$) alone caused a blunted response to hypoxia during the last minute of hypoxic exposure ($\dot{V}_E$ increased to 116 ± 11%) but did not change the response to minute 1 of hypoxia. Co-administration of ethanol (2 g/kg) and pentobarbital (56 mg/kg) caused a significantly blunted response to hypoxia in minute 1 ($P = 0.013$ vs. control group) and terminal apnea ($P < 0.001$ vs. control group) by minute 5 in all eight rats tested. Preadministration with CX717 (30 mg/kg) ~10 min before the hypoxic challenge in rats co-administered ethanol and pentobarbital significantly alleviated the respiratory depression during minutes 1 and 5 and ultimately prevented lethality in three of eight rats.

Figure 5B includes the data for responses to hypercapnia. All groups of animals tested had no significant difference in response to hypercapnia during minute 1 of 5% CO$_2$. At minute 5 of hypercapnia, pentobarbital (56 mg/kg; $P = 0.006$ vs. control group; $n = 4$) alone or co-administered with ethanol (2 g/kg; $P = 0.004$ vs. control group; $n = 4$) caused a blunted response to hypercapnia, whereas ethanol (2 g/kg; $n = 4$) did not change the response to hypercapnia compared with control group. The blunted response to hypercapnia after ethanol-pentobarbital administration was alleviated by pre-administration of CX717 (30 mg/kg; $P = 0.037$ vs. ethanol-pentobarbital group; $n = 3$).

**DISCUSSION**

Most overdoses of depressant medications arise from mixtures of drugs, commonly involving alcohol, barbiturates, or opiates. In this study, we demonstrated that ethanol and pentobarbital have a synergistic action when co-administered and cause severe respiratory depression, including lethal apnea. There are likely to be several mechanisms underlying the synergistic action of the drugs. Data from the in vitro brain stem-spinal cord preparation provide insights on drug interactions independent of supramedullary centers and peripheral afferent input to the respiratory rhythm-generating center. Ethanol, at the concentrations administered to the bathing medium, caused a modest suppression of respiratory frequency in vitro without a significant change in amplitude, consistent with a previous in vitro study (5). The concentration of pentobarbital administered caused an ~50% reduction of frequency, which is also consistent with previous studies using a similar in vitro preparation (7, 38). The administration of both agents together led to a pronounced suppression of respiratory frequency, includ-
neurons would counter the suppressing actions of alcohol and modulation of AMPA receptors within the preBötC network. Specifically, pentobarbital enhances GABA-mediated chloride currents by increasing the duration of ionophore opening at low doses, and at high doses it stimulates GABA_A receptors directly in the absence of GABA. Ethanol binds to a distinct site on the GABA_A receptor (41). At the single channel level, ethanol enhances the frequency of GABA-mediated channel opening events and mean open time. There is also evidence for a presynaptic facilitation of GABA release by ethanol at some synapses. The net functional implication is that the combinatory exposure to barbiturates and ethanol results in a strong synergistic increase in GABA_A receptor-mediated conductance. This would result in inhibition of respiratory neurons at multiple sites, including those located within the preBötC.

The data from newborn and adult rat in vivo studies were consistent with the in vitro results; the combination of ethanol and pentobarbital, at doses that on their own cause partial respiratory depression, resulted in pronounced apneas that could be lethal. In vivo, the combinatorial actions of alcohol and pentobarbital will be beyond simple depression of medullary respiratory rhythm-generating centers. There may be compromised degradation of each class of compound when both are present in the circulation due to interference with liver function (33). Alcohol, opiates, and perhaps other sedative agents can indirectly modulate respiratory drive via effects on state-dependent/arousal-related processes (24, 25, 39). Furthermore, data in this study demonstrate a marked suppression of hypoxia- and hypercapnia-induced regulatory reflexes in the presence of alcohol and pentobarbital. The fact that exposure to what is normally a well tolerated level of hypoxia was lethal in all cases when ethanol and pentobarbital were administered was particularly striking. These marked accentuated inhibitory actions of hypoxia may include direct effects of alcohol- and pentobarbital on chemoreceptors, neuronal circuitry that processes reflex input (45), and accentuation of the suppression of respiratory drive in the medulla in response to elevated GABA release with hypoxia (13, 19, 44). There was also a blunting of the reflex stimulatory response to hypercapnia in the presence of pentobarbital on its own or in conjunction with alcohol. Collectively, the functional consequences of these drug-induced alterations in chemosensitivity are that there is less protection against the hypoventilation and apneas induced by the central actions of the agents.

The fact that administration of bicuculline counteracted a significant component of the alcohol-pentobarbital-induced respiratory depression via blocking of GABA_A receptor-mediated effects was informative regarding mechanism. However, bicuculline also induces nonrespiratory, seizure-like neuronal activity that would negate clinical use. The administration of the ampakine CX717 was also effective for partially alleviating the drug-induced respiratory depression, and it has been shown to be well tolerated at therapeutic doses. We propose that part of the CX717 mechanism of action is via positive allosteric modulation of AMPA receptors within the preBötC network. Increased current flow through the AMPA receptor on those neurons would counter the suppressing actions of alcohol and pentobarbital via both GABA-mediated conductances and their additional inhibitory actions directly at AMPA receptors (8, 18, 40, 43). This is similar to the mode of action proposed for the ampakine-mediated alleviation of opiates acting, in part, by suppressing preBötC excitability via binding to μ-opiate receptors (27, 30). In addition, it is likely that CX717 provides positive respiratory drive via action at other brain stem and supramedullary neuronal structures that modulate breathing.

In conclusion, these data suggest that exposure to both alcohol and barbiturates can lead to a much more profound suppression of breathing than with either agent in isolation. Furthermore, ampakine compounds, which also counter opiate-induced respiratory depression, can alleviate GABA_A receptor-mediated suppression of central respiratory drive and thus may offer a novel pharmacological approach to counter a wider range of drug-induced central hypoventilation and apnea.

GRANTS

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DISCLOSURES

Ampakines were provided by Cortex Pharmaceuticals.

AUTHOR CONTRIBUTIONS

Author contributions: J.R. and X.D. performed experiments; J.R. and X.D. analyzed data; J.R. and J.J.G. interpreted results of experiments; J.R. and J.J.G. prepared figures; J.R. and J.J.G. drafted manuscript; J.R. and J.J.G. edited and revised manuscript; J.R., X.D., and J.J.G. approved final version of manuscript; J.J.G. conception and design of research.

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