State-dependent vs. central motor effects of ethanol on breathing

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Ethanol (1.25 g/kg, intraperitoneal injection) suppresses genioglossus (GG) activity in rats and/or by state-dependent regulation of motor activity via independent mechanisms. Intraperitoneal injections of ethanol at the hypoglossal motor pool influences on sleep/arousal processes. Intraperitoneal injections of ethanol (1.25 g/kg, n = 6 rats) resulted in maximum blood levels of 125.5 ± 15.8 mg/dl, i.e., physiologically relevant levels for producing behavioral impairment in rats and humans. Ethanol decreased wakefulness, reduced sleep latency, and increased non-rapid eye movement sleep (P < 0.001, n = 10 rats) and significantly reduced postural muscle tone and electroencephalogram frequencies, consistent with sedation. Ethanol also caused a state-dependent (wakefulness only) decrease in respiratory-related GG activity (P = 0.018) but did not affect diaphragm amplitude or rate, with the magnitude of GG decrease related to baseline activity (P < 0.0002). Ethanol did not alter GG activity when applied to the hypoglossal motor pool (0.025–1 M, n = 16 isoﬂurane-anesthetized rats). In conclusion, ethanol promoted sleep and altered electroencephalogram and postural motor activities, indicative of sedation. The lack of effect on GG with ethanol at the hypoglossal motor pool indicates that the GG and postural motor suppression following systemic administration was mediated via effects on state-dependent/ arousal-related processes. These data show that ethanol can suppress GG by primary influences on state-dependent aspects of central nervous system function independent of effects on the respiratory network per se, a distinction that has not previously been identified experimentally.

Obstructive sleep apnea-hypopnea (OSAH) constitutes a common respiratory problem and significant public health burden (45, 64). Ethanol is one of the most widely used drugs in Western society, worsens obstructive sleep apnea in humans. No studies, however, have distinguished between two primary mechanisms that could mediate suppression of genioglossus (GG) activity with ethanol. We test the hypothesis that ethanol suppresses GG activity by effects at the hypoglossal motor pool and/or by state-dependent regulation of motor activity via independent mechanisms that could mediate ethanol-induced suppression of GG activity: 1) a primary state-dependent influence on motor activity via an effect of ethanol on sleep/arousal processes per se and 2) a motor suppression effect operating via an inhibitory influence of ethanol directly at the hypoglossal motor pool. The distinction between these two independent mechanisms is outlined below.

Ethanol exerts complex effects on the central nervous system, generally depressing neuronal function, at least in part via interactions with the GABA\textsubscript{A} receptor, and exerting a GABA-mimetic profile (4, 9, 18, 35). GABAergic neurons are important in the initiation and maintenance of non-rapid eye movement (non-REM) sleep (25, 32, 47), and a major component of the sedative effect of ethanol (1, 62) may be mediated by potentiation of this sleep-promoting (i.e., state-dependent) system. Accordingly, one explanation for an increased incidence and duration of disordered breathing events after ethanol (2, 23, 36, 48, 51) may be reduced arousal processes, leading to increased time spent asleep and/or in deeper sleep and, therefore, contributing to OSAH via primary state-dependent influences on the respiratory system (i.e., independent of an influence of ethanol on the hypoglossal motor pool per se).

A second explanation for how ethanol can predispose to, and worsen, obstructed breathing during sleep is that ethanol may directly suppress respiratory neuronal and motoneuronal activity, leading to suppression of respiratory muscle tone, which for the pharyngeal muscles would also increase the incidence and duration of obstructed breathing events. Such a scenario contributing to hypopneas via a central effect of ethanol on respiratory motor activity (i.e., independent of a state-dependent mechanism) is plausible, because GABA and glycine, the main inhibitory neurotransmitters in the central nervous system, exert tonic inhibitory effects at the hypoglossal motor pool in vivo (29, 37–39, 57, 58), and ethanol potentiates inhibitory GABA and glycine receptor function at this motor pool in vitro (12, 13, 53).

Accordingly, this study tests the hypothesis that systemic administration of ethanol, at a physiologically relevant dose, will suppress GG activity by effects at the hypoglossal motor pool and/or by state-dependent regulation of motor activity via independent influences on sleep/arousal processes. This is the first study to identify the ability of ethanol to suppress GG activity by primary influences on state-dependent aspects of central nervous system function independent of effects on the respiratory network per se, a distinction that has not previously been identified experimentally. Understanding the adverse impact of ethanol on breathing and recognizing the foremost influence of state-dependent modulation have physiological significance and clinical relevance and broader implications for the impact of other sedative agents on breathing.
METHODS

Procedures conformed to the recommendations of the Canadian Council on Animal Care, and the protocols were approved by the University of Toronto Animal Care Committee. Rats were housed individually, maintained on a 12:12-h light-dark cycle (lights on at 0700), and had free access to food and water.

Study 1: Blood Ethanol Levels in Conscious Rats

Blood ethanol levels were determined in a group of six male Wistar rats (mean body weight = 360 g, range 297–425 g) following intraperitoneal injection of ethanol [1.25 g/kg in saline, 15% (vol/vol)]. For sampling, the inner thigh over the saphenous vein was shaved. Blood samples (200 μl) were obtained from the saphenous vein via a sterile 23,75-gauge needle, with the rat being held and the hindlimb extended, immediately before (i.e., time 0) and after (30, 60, 90, 120, 180, and 240 min) ethanol injection. The needle tip was inserted into the vein and then quickly removed, and each blood sample was collected in a heparinized vial and centrifuged at 2,500 rpm. The plasma was collected, frozen, and analyzed for ethanol concentration (Analox Instruments, Lunenburg, MA).

Study 2: Effects of Ethanol on Sleep and Respiratory Motor Activity in Freely Behaving Rats

Anesthesia and surgical procedures. Experiments were performed on 10 male Wistar rats (mean body weight = 262 g, range 234–285 g). Sterile surgery was performed under anesthesia induced and maintained by inhalation of 2.5–3% isoflurane. Rats were also intraperitoneally injected with buprenorphine (0.03 mg/kg) to minimize potential postoperative pain, atropine sulfate (1 mg/kg) to minimize airway secretions, and saline (3 ml, 0.9%) for fluid loading. An anesthesia mask was placed over the snout throughout surgery, and the rats also breathed 50:50 room air-O_2. Effective anesthesia was judged by abolition of pedal withdrawal and corneal blink reflexes. During surgery, body temperature was maintained with a water pump and heating pad (T/Pump-Heat Therapy System, Gaymar, Orchard Park, NY).

With the rats supine, the ventral surface of the GG was exposed via a submental incision and dissection of the overlying geniohyoid and mylohyoid muscles. Two insulated, multistranded stainless steel wires (catalog no. AS631, Cooner Wire, Chatsworth, CA) were implanted bilaterally into the GG and secured with sutures and tissue glue. Tongue movement in response to electrical stimulation (0.4–0.8 V) was also used to confirm electrode placements. We showed in previous experiments that tongue muscle activity is markedly decreased, and almost abolished, after section of the medial branches of the hypoglossal nerve (39); therefore, our present recordings were predominantly from the GG with these electrode placements. For measurement of diaphragm electromyogram (EMG) activity, two insulated, multistranded stainless steel wires (catalog no. AS636, Cooner Wire) were sutured onto the costal diaphragm via an abdominal approach. The size, configuration, and placement of the GG and diaphragm electrodes were consistent across experiments. To further ensure adequate electrode placements during surgery, the GG and diaphragm signals were monitored on a loudspeaker (AMB audio amplifier, Grass) to document respiratory-related activity. The GG and diaphragm wires were tunneled subcutaneously to a small incision on the skull, and the submental and abdominal incisions were closed with absorbable sutures.

The rats were placed in a stereotoxic apparatus (model 962, Kopf, Tujunga, CA) with blunt ear bars. For measurement of electroencephalographic (EEG) activity, two stainless steel screws (1.5 mm diameter) attached to insulated wires (30 gauge) were positioned on the skull ~2 mm anterior and 2 mm to the right of bregma and 3 mm posterior and 2 mm to the left of bregma, respectively (16, 22). The reference electrode was placed ~5 mm anterior and 3 mm to the left of bregma (16, 22). Two insulated, multistranded stainless steel wires were also sutured onto the dorsal neck muscles for measurement of neck muscle activity (16, 22).

Data analysis. Each experiment was coded, and the experimenter was blinded to the treatment (saline or ethanol) during the analyses of sleep and respiratory motor activities. Sleep-wake states were identified visually and classified using standard criteria (22). Respiratory muscle activities within the identified sleep-wake states were measured during all periods of quiet wakefulness (>30-s duration, with these periods excluding any with body movements, including overt behaviors such as eating, drinking, or grooming), non-REM sleep (>60-s duration), and REM sleep (>30-s duration). Data were included in the analyses of respiratory activity only if they were obtained during such unequivocal and clearly defined states.
obtained during periods of active wakefulness [i.e., with movements and overt behaviors as judged by video observations, movement artifact obscuring the diaphragm EMG signal, and repetitive high-amplitude GG and neck EMG activities typifying grooming and eating (24)] were not included in the analysis of respiratory muscle activities, nor were data from transitional states (e.g., drowsiness, arousals from sleep, and transitions from non-REM to REM sleep) included. Duration of quiet wakefulness without body movements is typically shorter than duration of uninterrupted non-REM sleep, and there are typically fewer periods of REM sleep than other sleep-wake states, which accounts for analysis of >30-s periods in quiet wakefulness and REM sleep as opposed to >60-s periods in non-REM sleep.

Respiratory muscle activities were quantified as previously described (22, 57). The EMG signals were analyzed from the respective moving-time average signals (above electrical zero). The GG signal was quantified as mean tonic activity (i.e., basal activity in expiration) and respiratory-related activity (peak inspiratory activity — tonic activity). The amplitude of diaphragm activity, respiratory rate, and mean neck muscle activity were also calculated. The tonic and respiratory-related GG signals were quantified in arbitrary units and as percentage of maximum. The maximum GG level in each rat was determined from the period of recording after the animals were instrumented for the habituation period until the experiment on the following day, i.e., from ~1600 on the day before the experiment until 0930 on the following day. This period therefore corresponded to the time of day during which behavioral activity was normally high, and in each rat the time of maximum GG activity occurred in the dark phase (between 1900 and 0700), when tongue movements were characteristic of active behaviors.

As in all our previous studies in behaving rats, we prefer to illustrate and present the data in arbitrary units, because we consider it the most reliable and physiologically relevant measure for such experiments. Importantly, in contrast to humans, where behavioral tongue activity can be standardized (e.g., maximum voluntary protrusion), maximum behavioral activity in this animal model cannot be controlled. Furthermore, behavioral motor activity may originate from motoneurons different from those generating the phasic respiratory-related activity (14). Some investigators who do not work with motor nucleus to infuse artificial cerebrospinal fluid (ACSF) followed by ethanol dissolved in ACSF (22, 39). The rats, in the prone position, were then placed in a stereotaxic apparatus (model 962, Kopf), and two stainless steel screws attached to insulated wire were implanted in the skull over the frontal-parietal cortex to record the cortical EEG, as described for study 2 (22, 39). To ensure consistent positioning between rats, the flat skull position was achieved with an alignment tool (model 944, Kopf).

Microdialysis and recordings. Microdialysis probes (CMA/11 14/01, CSC, St. Laurent, QC, Canada) were targeted into the hypoglossal motor nucleus to infuse artificial cerebrospinal fluid (ACSF) followed by ethanol dissolved in ACSF (n = 10 rats) or continuous ACSF for the same time period (n = 6 rats, time control experiments). The probes were placed 13.7 ± 0.12 (SE) mm posterior to bregma, 0.2 ± 0.04 mm lateral to the midline, and 10.1 ± 0.12 mm ventral to bregma. The rats were allowed to stabilize for ≥30 min before any interventions. The microdialysis probes were 240 μm diameter, with a 1-mm cuprophane membrane. Each probe was connected to fluorinated ethylene-propylene Teflon tubing (0.12 mm ID), which was, in turn, connected to a 1.0-ml syringe via a zero dead space switch (Uniswitch, BAS, West Lafayette, IN). The probes were continually flushed with ACSF at a flow rate of 2.1 μl/min via a syringe pump and (CMA/Microdialysis Systems, Inc., MI). The composition of ACSF (mM) was 125 NaCl, 3 KCl, 1 KH₂PO₄, 2 CaCl₂, 1 MgSO₄, 25 NaHCO₃, and 30 n-glucose. The electrical signals were amplified and filtered as described for study 2. Each signal, along with blood pressure [DT-XX transducer (Ohmeda, Madison, WI) and PM-1000 amplifier (CWE)], was also recorded on computer as described for study 2.

Protocol and data analyses. Interventions were performed during steady-state periods with predominantly high-voltage and low-frequency EEG activity. In a group of 10 rats, the microdialysis probes were perfused with ACSF for ≥30 min and then with 25, 50, 100, 300, and 1,000 mM ethanol, each for 30 min. To examine whether any of the potential changes in GG activity were caused by effects of time, i.e., independent of ethanol, further experiments were performed in a separate group of six rats, in which repeated switches to perfusion of (0.5–2 Hz) activity was also calculated as a relative index of EEG activation (16).

Study 3: Ethanol at the Hypoglossal Motor Pool and Effects on GG Activity

Anesthesia and surgical procedures. Local application of ethanol to the hypoglossal motor pool was studied in isoflurane-anesthetized male Wistar rats because of the wide range of doses required to span the range previously tested in vitro at this same motor pool (12, 13, 53). Such a range would not have been possible to replicate over the course of an experiment in a conscious rat for any given sleep-wake state, not least because of the added complexity of the spontaneous changes in behavioral state and motor activities that typify the behaving preparation. Also, importantly, if ethanol were exerting its physiological actions to suppress hypoglossal motor output to GG muscle via an effect at the hypoglossal motor pool, then this would also be expected to be documented in the anesthetized preparation, inasmuch as glycine and GABA inhibitory mechanisms are operative under such conditions (29, 39).

Accordingly, 16 rats (mean body weight = 293 g, range 260–327 g) were anesthetized with isoflurane (2–3%) and given atropine (1 mg/kg) to minimize airway secretions. After the onset of surgical anesthesia, the rats were tracheotomized and the femoral artery and vein were cannulated. The rats spontaneously breathed 50:50 room air-O₂ throughout the experiments. Core body temperature was monitored with a rectal probe and maintained at 36–38°C with a heating pad (TC-1000 Temperature Controller, CWE). The rats received continuous intravenous fluid (0.4 ml/h) containing 7.6 ml of saline, 2 ml of 5% dextrose, and 0.4 ml of 1 M NaHCO₃. Bipolar electrodes were inserted into the GG and costal diaphragm for EMG recordings (22, 39). The rats, in the prone position, were then placed in a stereotaxic apparatus (model 962, Kopf), and two stainless steel screws attached to insulated wire were implanted in the skull over the frontal-parietal cortex to record the cortical EEG, as described for study 2 (22, 39). To ensure consistent positioning between rats, the flat skull position was achieved with an alignment tool (model 944, Kopf).
ACSF into the hypoglossal motor nucleus were performed (i.e., “sham interventions”) over the same time course as the drug interventions. For ACSF or ethanol delivered to the hypoglossal motor nucleus, measurements were taken over 1-min periods at the end of each 30-min drug or sham intervention. GG and diaphragm responses were measured from the moving average signals above electrical zero, as described in previous experiments (22, 39) and for the above-described experiments during sleep (study 2). In practice, there was no tonic GG activity under anesthesia; therefore, only data for respiratory-related activity are reported.

Tests of function of hypoglossal motor nucleus and histology. At the end of each experiment, 10 mM serotonin (creatinine sulfate complex) was applied to the hypoglossal motor nucleus as a positive control to confirm that it was still functional and able to respond to manipulation of neurotransmission, as judged by the expected increase in GG activity (24). At the end of each study, the rats were overdosed with isoflurane. The rats were then perfused intracardially with 40 ml of 0.9% saline followed by 40 ml of 10% formalin, and the brain was removed and fixed in 10% formalin. Medullary regions containing the hypoglossal motor nucleus were blocked and transferred to a 30% sucrose solution for cryoprotection. The tissue was cut in 50-μm sections using a cryostat (model CM1850, Leica, Nussloch, Germany). Sections were mounted and stained with neutral red, and the lesion sites left by the microdialysis probes were recorded on a corresponding standard cross section using a stereotaxic atlas of the rat brain (43).

Statistical Analysis

The statistical test used for each analysis is indicated where appropriate. For all comparisons, differences were considered significant if the null hypothesis was rejected at P < 0.05. Where post hoc comparisons were performed after ANOVA with repeated measures (ANOVA-RM), Bonferroni’s corrected P value was used to infer statistical significance. Data were tested for normality using the Kolmogorov-Smirnov test, and if data were not normally distributed, analyses were performed on the logarithmically transformed data (7). Analyses were performed using SigmaStat (SPSS, Chicago, IL). Values are means ± SE unless otherwise indicated.

RESULTS

Study 1: Blood Ethanol Levels in Conscious Rats

Figure 1 shows the changes in blood ethanol levels over time following intraperitoneal injection of ethanol. Blood ethanol increased to a maximum of 125.5 ± 15.8 mg/dl 30 min after injection. Statistical analysis confirmed the significant effect of time on blood ethanol concentration (F0,36 = 50.68, P < 0.001, 1-way ANOVA-RM). Post hoc analyses showed that blood ethanol concentration remained significantly elevated above baseline levels at all time points up to 2 h after injection (all t5 > 6.40, P < 0.001, Bonferroni’s t-test comparisons with the preinjection control; Fig. 1). Blood ethanol levels were not significantly different from baseline at 3 h (t5 = 6.40, P = 0.475) or 4 h (t5 = 1.82, P = 1.000) after injection. Although no formal behavioral motor tests were conducted in this experiment, the rats appeared to be sedated, as judged by less exploration and slower movements around the recording chamber, especially in the 1st h. The rats still showed such signs of sedation and reduced activity when the blood samples were obtained at 60 and 90 min after injection, observations consistent with behavioral studies (17). More detailed analysis of the effects of ethanol on sleep, postural and respiratory motor activities, and the EEG signal are included in results of study 2.

Study 2: Effects of Ethanol on Sleep and Respiratory Motor Activity in Freely Behaving Rats

Effects of ethanol on sleep-wake regulation. The effects of ethanol on sleep and wakefulness were determined from the data obtained in the first 2 h after injection, i.e., when blood ethanol levels were significantly elevated above baseline (Fig. 1). Ethanol significantly reduced sleep latency, as judged by the time from the onset of injection to the time of the first sustained (60-s) period of non-REM sleep (23.3 ± 1.3 min for ethanol vs. 37.6 ± 3.9 min for saline, P = 0.005, paired t-test). The latency to the first sustained (30-s) period of REM sleep was also shortened after ethanol injection (49.3 ± 3.4 min for ethanol vs. 71.4 ± 8.6 min for saline, P = 0.033, paired t-test). These latencies include the 15-min period following the onset of injection to the beginning of data recording (see METHODS). Figure 2 shows an example and group data for the effects of ethanol on sleep-wake regulation. Ethanol had significant effects on overall sleep-wake states (F3,27 = 9.43, P < 0.001, 2-way ANOVA-RM), in that it significantly reduced the duration of active wakefulness (i.e., periods with movements and overt behaviors; t3 = 4.46, P < 0.001, post hoc Bonferroni’s paired t-test; Fig. 2B) and increased the duration of non-REM sleep (t3 = 4.21, P < 0.001; Fig. 2B). Overall, the durations of quiet wakefulness (i.e., periods without body movements) and REM sleep were unchanged by ethanol (both t3 < 0.18, P > 0.861; Fig. 2B).

These effects of ethanol on the distribution of sleep-wake states were due to significant influences on the duration of the sleep-wake episodes, rather than the number of episodes (F3,27 = 4.85, P = 0.008 (for durations) and F3,27 = 1.13, P = 0.353 (for number of episodes), 2-way ANOVA-RMs; Fig. 2, C and D). Further analyses showed that ethanol significantly reduced the duration of active wakefulness and increased the duration of non-REM sleep episodes (both t3 > 2.32, P < 0.026, post hoc Bonferroni’s paired t-tests; Fig. 2D).

Effects of ethanol on respiratory motor activity. The effects of ethanol on respiratory motor activity were also determined from the data obtained in the first 2 h after injection, i.e., when blood ethanol levels were significantly elevated above baseline (Fig. 1). Moreover, data were included in these analyses only if they were obtained during unequivocal and clearly defined
Fig. 2. A: distribution of sleep-wake states over time in a rat infused with saline and the same rat treated with ethanol on a different day. B: group data for effects of ethanol on percentage of active wakefulness (i.e., periods with movements and overt behaviors), quiet wakefulness (i.e., periods without body movements), non-rapid eye movement (non-REM) sleep, and REM sleep. C and D: number of episodes of each sleep-wake state and their average duration. Values were obtained from data collected in the first 2 h after injection, i.e., when blood ethanol levels were significantly elevated above baseline (Fig. 1). Values are means ± SE (n = 10 rats). *Significantly different (P < 0.05) from saline.
states of quiet wakefulness, non-REM sleep, and REM sleep (see METHODS). A total of 11,972 5-s epochs (i.e., a total of 16.63 h of data) were included in the analysis of respiratory motor activity: 1,632 of these epochs were from periods of quiet wakefulness, 8,439 were from non-REM sleep, and 1,901 were from REM sleep. Of these epochs, 5,123 and 6,849 were obtained with saline and ethanol, respectively. Figure 3 shows an example and group data for the effects of systemically administered ethanol or saline on respiratory motor activity.

**RESPIRATORY-RELATED GG ACTIVITY.** Ethanol had significant effects on respiratory-related GG activity, depending on the prevailing sleep-wake state ($F_{2,18} = 5.07, P = 0.018$, 2-way ANOVA). Figure 3 shows an example and group data for the effects of systemically administered ethanol or saline on respiratory motor activity.

![Graphs and diagrams showing respiratory-related GG activity](image-url)

**Fig. 3.** Effects of systemically administered ethanol and vehicle (saline) on respiratory motor activity across sleep-wake states are shown. A: electroencephalogram (EEG) and neck electromyogram (EMG) signals. Genioglossus (GG) and diaphragm (DIA) signals are displayed as their moving-time averages (MTA) in arbitrary units (AU). Increasing levels of muscle activity during inspiration are indicated by arrows. Baseline of the integrator (i.e., electrical zero) is shown for GG MTA. B–I, group data. *Significantly different ($P < 0.05$) from saline. #Significant difference between respective sleep-wake states. Values are means ± SE ($n = 10$ rats). Mean values for each individual rat were first calculated from the population of values for all 5-s epochs during each sleep-wake state in the first 2 h following ethanol or saline administration. Means from each individual rat in each condition were averaged to yield grand means for the group. For correlations in F and G, sleep-wake states are shown as (wakefulness) • (non-REM sleep), and ■ (REM sleep), with 3 states per animal for 10 animals and overlap obscuring some of the symbols; solid lines indicate correlations, and dashed lines show 95% confidence intervals. Some individual values for reduction in GG activity with ethanol are negative, because, in these instances, GG activity was increased compared with saline controls. There were significant positive relationships between the level of respiratory and tonic GG activities recorded with vehicle within a rat and the magnitude of decrease observed in response to ethanol: $r = 0.627, P = 0.0002$; $r = 0.701, P = 0.00002$ for respiratory (F) and tonic (G) GG activities, respectively (Pearson product moment correlations).
ANOVA-RM). Compared with the saline controls, ethanol significantly reduced respiratory-related GG activity in wakefulness ($t_0 = 2.32$, $P = 0.028$, post hoc Bonferroni’s paired t-test; Fig. 3B), but this effect was not observed in non-REM or REM sleep (both $t_0 < 1.38$, $P > 0.181$; Fig. 3B). This same state-specific suppression of respiratory-related GG activity by ethanol was also observed when the data were quantified and analyzed as a percentage of maximum activity ($F_{2,18} = 6.88$, $P = 0.006$, 2-way ANOVA-RM), with the suppression also observed in wakefulness only ($t_0 = 2.87$, $P = 0.008$, post hoc Bonferroni’s paired t-test; Fig. 3C). As shown in Fig. 3C, the actual values of GG activity normalized to percentage of maximum are relatively low in magnitude, in agreement with a previous study using the same normalization (55). These values normalized to percentage of maximum are low, not because the actual levels of GG activity are minimal, e.g., there is clear and appreciable activity, including respiratory modulation (Fig. 3A), but, rather, because high levels of motor activity are recorded during periods of behavioral activation (24) to which the values are normalized.

With the vehicle control, respiratory-related GG activity was normally decreased from wakefulness to non-REM and REM sleep (both $t_0 > 3.66$, $P < 0.004$; Fig. 3, B and C). Because of the aforementioned suppression effects of ethanol, however, this normal suppression of GG activity from wakefulness to non-REM and REM sleep was not observed in the presence of ethanol (both $t_0 < 1.71$, $P > 0.296$; Fig. 3, B and C).

TOUGH GG ACTIVITY. There was also a significant effect of sleep-wake state on tonic GG activity ($F_{2,18} = 21.02$, $P < 0.001$, 2-way ANOVA-RM), with tonic activity also significantly decreasing from wakefulness to non-REM and REM sleep (both $t_0 > 5.09$, $P < 0.001$, post hoc Bonferroni’s paired t-tests; Fig. 3D). However, for the group, there was no independent effect of ethanol on tonic GG activity compared with the saline controls ($F_{1,0} = 0.48$, $P = 0.505$, 2-way ANOVA-RM), and there were no effects of ethanol that were specific to any particular sleep-wake state ($F_{2,18} = 2.26$, $P = 0.133$, 2-way ANOVA-RM; Fig. 3D).

The results and conclusions are the same when tonic GG activity was also analyzed as percentage of maximum and measured in arbitrary units (cf. Fig. 3D with Fig. 3E). There was a significant effect of sleep-wake state on tonic GG activity ($F_{2,18} = 21.80$, $P < 0.001$, 2-way ANOVA-RM), with tonic activity also significantly decreasing from wakefulness to non-REM and REM sleep (both $t_0 > 5.09$, $P < 0.001$, post hoc Bonferroni’s paired t-tests; Fig. 3E). There was also no independent effect of ethanol on tonic GG activity compared with the saline controls ($F_{1,0} = 0.06$, $P = 0.815$, 2-way ANOVA-RM), and there were no effects of ethanol that were specific to any particular sleep-wake state ($F_{2,18} = 2.11$, $P = 0.150$, 2-way ANOVA-RM; Fig. 3E).

MAGNITUDE OF ETHANOL EFFECTS ON GG ACTIVITY VARIES WITH BASELINE ACTIVITY. The significant suppression of respiratory GG activity by ethanol in wakefulness, but not sleep (Fig. 3, B and C), may have been due to the already low GG activity in sleep, such that it was more difficult to detect a change. Accordingly, we sought to determine whether the magnitude of suppression of respiratory-related GG activity observed with ethanol varied with the level of the respiratory GG signal during the vehicle control. Figure 3F shows the significant positive relationship between the level of respiratory GG activity recorded with vehicle within a rat and the magnitude of decrease observed in response to ethanol ($r = 0.627$, $P = 0.0002$, Pearson product moment correlation). Similarly, there was a statistically significant relationship between the level of baseline tonic GG activity with vehicle and the magnitude of decrease in response to ethanol ($r = 0.701$, $P = 0.00002$, Pearson product moment correlation; Fig. 3G).

DIAPHRAGM ACTIVITY AND RESPIRATORY RATE. There were no effects of ethanol or sleep-wake state on the amplitude of diaphragm activity ($F_{1,0} = 0.94$, $P = 0.357$, and $F_{2,18} = 2.53$, $P = 0.108$, respectively, 2-way ANOVA-RMs; Fig. 3H). Nor were there any effects of ethanol on diaphragm amplitude that depended on the prevailing sleep-wake state ($F_{2,18} = 1.90$, $P = 0.178$, 2-way ANOVA-RM). However, respiratory rate did vary with sleep-wake state ($F_{2,18} = 32.22$, $P < 0.001$, 2-way ANOVA-RM), with respiratory rate in REM sleep being significantly increased compared with non-REM sleep and wakefulness (both $t_0 > 6.93$, $P < 0.001$; Fig. 3J). Nevertheless, there were no differences in respiratory rate between the ethanol and saline conditions ($F_{1,0} = 1.42$, $P = 0.264$, 2-way ANOVA-RM), nor was any effect of ethanol dependent on the prevailing sleep-wake state ($F_{2,18} = 0.55$, $P = 0.587$, 2-way ANOVA-RM; Fig. 3I). Overall, therefore, the significant effects of systemically administered ethanol on respiratory motor activity were confined to the GG muscle during wakefulness: the GG having dual respiratory and nonrespiratory (including postural) functions.

EFFECTS OF ETHANOL ON POSTURAL MOTOR tone and EEG activity. NECK EMG activity. There was a significant effect of sleep-wake state on neck EMG activity ($F_{2,18} = 20.43$, $P < 0.001$, 2-way ANOVA-RM), with neck EMG being significantly decreased in non-REM and REM sleep compared with wakefulness (both $t_0 > 5.21$, $P < 0.001$, post hoc Bonferroni’s paired t-tests; Fig. 4A). Ethanol also had a significant effect on neck EMG activity that depended on sleep-wake state ($F_{2,18} = 5.00$, $P = 0.019$, 2-way ANOVA-RM). Post hoc analyses showed significantly reduced neck EMG activity in wakefulness with ethanol compared with saline control ($t_0 = 4.12$, $P < 0.001$, Bonferroni’s paired t-test; Fig. 4A), but this effect was not observed in non-REM or REM sleep (both $t_0 < 1.11$, $P > 0.278$; Fig. 4A). The changes in neck EMG activity with wakefulness were not significantly correlated with the changes in respiratory-related or tonic GG activities in wakefulness ($P = 0.059$ and 0.662, respectively, Pearson product moment correlations, $n = 10$ for each comparison).

EEG ACTIVITY. As expected, there was a significant effect of sleep-wake state on the ratio of high-frequency (20–30 Hz) to low-frequency (0.5–2 Hz) EEG power (i.e., $\beta_2$-to-$\delta_1$ ratio; $F_{2,18} = 117.53$, $P < 0.001$, 2-way ANOVA-RM), with significant differences observed between all pairs of states (all $t_0 > 7.52$, $P < 0.001$, post hoc Bonferroni’s paired t-tests; Fig. 4B). For example, the $\beta_2$-to-$\delta_1$ ratio was minimal in non-REM sleep, indicating significantly reduced EEG power in the faster ($\beta_2$) frequency range and/or increased power in the slower ($\delta_1$) frequency range (Fig. 4B). Importantly, further analyses showed that ethanol also significantly modulated the EEG signal, as judged by the $\beta_2$-to-$\delta_1$ ratio, with this effect being dependent on the prevailing sleep-wake state ($F_{2,18} = 5.55$, $P = 0.013$, 2-way ANOVA-RM). Compared with the saline control, ethanol significantly reduced the $\beta_2$-to-$\delta_1$ ratio in non-REM sleep, indicating a shift to slower-frequency EEG activity ($t_0 = 3.63$, $P = 0.001$, post hoc Bonferroni’s paired t-test; Fig. 4B).
Additional analyses were performed to further determine the effects of ethanol and sleep-wake states on the power of the EEG signal in each of the different bandwidths (Fig. 4, C–H). In general, the data in Fig. 4, C–H, show that, compared with wakefulness and REM sleep, non-REM sleep was associated with significantly increased power in the $\delta_2$ and $\delta_1$ bandwidths (0.5–4 Hz) and reduced power in the $\theta$ (4–7.5 Hz) and $\beta_2$ (20–30 Hz) bandwidths. Importantly, however, ethanol significantly increased $\delta_2$ and $\delta_1$ power in non-REM sleep and decreased $\beta_1$ (13.5–20 Hz) power in non-REM sleep and wakefulness (Fig. 4, C–H), all changes consistent with a sedating effect. Further analyses showed that there was not a statistically significant relationship between the reduction in $\beta_1$ power with ethanol in wakefulness and the reductions in respiratory-related or tonic GG activities, whether the GG activities were quantified in arbitrary units or as percentage of maximum (each $r < 0.490$, each $P > 0.151$, Pearson product moment correlations). Further inspection, however, shows that the reductions in $\beta_1$ EEG power with ethanol were small, albeit consistent (Fig. 4G), likely indicating that this one EEG measure is insensitive as a sole marker of brain stem arousal state.

Study 3: Ethanol at the Hypoglossal Motor Pool and Effects on GG Activity

Sites of microdialysis. Figure 5 shows an example of a lesion site left by a microdialysis probe in the hypoglossal motor nucleus. The distribution of microdialysis sites from all the experiments in study 3 are also shown in Fig. 5, with the sites located within or immediately adjacent to the hypoglossal motor nuclei in all animals.
Effects of ethanol at the hypoglossal motor pool. In these anesthetized rats, baseline respiratory rate averaged 49.9 ± 1.9 min⁻¹, blood pressure averaged 70.7 ± 2.0 mmHg, and respiratory-related GG and diaphragm muscle activities averaged 137.7 ± 22.8 and 370.7 ± 72.8 arbitrary units, respectively. Group mean data for the effects of ethanol at the hypoglossal motor pool on GG activity and these other variables are shown in Fig. 6. Statistical analyses showed a decline in GG activity over the course of the experiment (F₅,₇₀ = 7.83, P < 0.001, 2-way ANOVA-RM) that first became statistically significant at a point corresponding to 100 mM ethanol and the same time point (i.e., 120 min) in the ACSF time-control experiment (t₁₄ = 3.18, P = 0.033, post hoc t-test; Fig. 6A). Importantly, however, there was no effect of experimental protocol per se on the change in GG activity (F₅,₇₀ = 0.36, P = 0.876, 2-way ANOVA-RM); i.e., any effect on GG activity observed in study 3 with ethanol at the hypoglossal motor pool was statistically indistinguishable from the corresponding sham time-control experiment with ACSF. As expected, given that the interventions in study 3 were performed at the hypoglossal motor pool, there were no effects of experimental protocol (i.e., ethanol or ACSF at the motor nucleus) on the amplitude of diaphragm activity, respiratory rate, or blood pressure during the course of the experiments (all F₅,₇₀ < 1.17, P > 0.334, 2-way ANOVA-RM; Fig. 6, B–D). Finally, in these anesthetized rats, the baseline ratio of high-frequency (β₂, 20–30 Hz) to low-frequency (δ₁, 2–4 Hz) activity in the EEG signal averaged 1.17 ± 0.18, and δ₁ activity averaged 9.3 ± 3.0%, of the total EEG power. Also, as expected, there were no effects of delivery of ethanol or saline to the hypoglossal motor pool on the β₂-to-δ₁ ratio or δ₁ activity, indicating no relative change in EEG activation compared with the ACSF time-control experiments with these localized interventions at the motor nucleus (F₅,₇₀ = 2.02 and 0.48, P = 0.086 and 0.785 for β₂/δ₁ and δ₁ activity, respectively, 2-way ANOVA-RM).

DISCUSSION

Major Findings and Their Significance

This study contributes to the field of physiology, in that it is the first to determine the effects of ethanol on pharyngeal and respi-
ratory pump muscle activities in combination with indexes of state-dependent arousal processes and, therefore, distinguish between the two major mechanisms that could mediate the suppression of pharyngeal (i.e., GG) motor tone: 1) a state-dependent influence on motor activity via an effect of ethanol on sleep/arousal processes and 2) a motor suppression effect operating via an inhibitory influence of ethanol directly at the hypoglossal motor pool. Overall, the results support the former state-dependent mechanism, rather than the latter mechanism involving direct effects at the motor pool, to explain the influences of ethanol on breathing in the intact organism in vivo. Previously, the physiological mechanisms underlying the adverse influence of ethanol on breathing had not been adequately addressed, because measurements of pharyngeal and respiratory pump muscle activities, combined with indexes of sleep/arousal regulation (i.e., beyond the identification of sleep-wake states) had not been performed in human (2, 8, 23, 36, 48, 51, 52, 59) or animal (3, 19, 34) studies. Importantly, the influence of ethanol, applied directly to a central respiratory motor pool, on respiratory motor activity had also not been studied in vivo to differentiate between the potential central effects of ethanol on respiratory motor activity and state-dependent effects operating via influences on sleep/arousal processes.

The evidence (see below) that the suppressant effect of ethanol on respiratory motor activity is operating via effects on state-dependent processes, rather than effects at the motor pool, at least for the GG muscle and at the physiological blood ethanol concentrations investigated in this study, is significant. To put the results into perspective, we consider that ethanol is one of the most widely used drugs in Western society (61) and that one serious consequence of ethanol ingestion in humans is impairment of breathing during sleep in otherwise-normal individuals and significant worsening of breathing during sleep in individuals with OSAH (2, 8, 23, 36, 48, 51, 52, 59). Moreover, OSAH is a common and serious disorder (64), with the prevalence increasing with increasing levels of obesity and with most patients remaining undiagnosed (65, 66). Accordingly, understanding the adverse impact of ethanol on breathing and recognizing the foremost influence of state-dependent modulation have physiological significance and clinical relevance and broader implications for the impact of other sedative agents on breathing. Overall, these results help identify the principle that certain agents can suppress respiratory muscle activity by primary influences on state-dependent aspects of central nervous system function independent of effects on the respiratory network per se, a distinction not previously identified experimentally in vivo for ethanol.

### Interpretation of Findings and Methodological Considerations

Ethanol was administered systemically at a concentration chosen to produce physiologically relevant blood concentrations, reaching a maximum of 125.5 ± 15.8 mg/dl and declining over the subsequent 2–3 h (Fig. 1). The ethanol dose chosen for the present studies produces impairment of motor function in behavioral testing in rats and corresponds to the blood alcohol levels that produce modest intoxication and impaired cognitive and motor behavior in humans (17, 33, 49, 50). The results showed that this dose of ethanol decreased wakefulness and increased the proportion of time spent in non-REM sleep, shortened sleep latency, and significantly reduced postural (neck) muscle tone and shifted the component frequencies in the EEG toward increased power in the lower-frequency bands and decreased power in the higher-frequency bands, all changes consistent with a sleep-promoting/sedating effect.
In addition to producing these markers of sleep promotion/reduced arousal, ethanol also caused a state-dependent (wakefulness only) decrease in GG activity but did not affect diaphragm activity or respiratory rate across any sleep-wake state. The pattern of this state-dependent decrease in GG activity after ethanol administration was also similar to that produced in the nonrespiratory, postural (neck) muscle by ethanol (cf. Fig. 3, B and C, and Fig. 3, D and E, with Fig. 4A). Furthermore, the magnitude of decrease in respiratory-related and tonic GG activities produced by ethanol was related to baseline activity without ethanol; i.e., the ethanol-induced decrease in activity was larger when baseline activity was higher. This latter observation can explain why the motor suppression produced by ethanol was most apparent in wakefulness, when the levels of activity are typically increased compared with those in sleep (Fig. 3, B and C, and Fig. 3, D and E). Moreover, wakefulness also corresponds to a time when arousal processes are most active; therefore, the suppressant effects of ethanol on the components of motor activity supported by state-dependent arousal processes would be most apparent in wakefulness. Although it may first appear that the lack of a statistically significant relationship between the reduction in $\beta_1$ power with ethanol in wakefulness and the reductions in respiratory-related or tonic GG activities may argue against this notion, it is important to emphasize that the reductions in $\beta_1$ EEG power with ethanol were small, albeit consistent (Fig. 4G), likely indicating that this one EEG measure is insensitive as a sole marker of brain stem arousal state. Moreover, ethanol may be exerting suppressant effects on deeper brain stem structures, such as components of the reticular formation and ascending arousal systems (e.g., glutamatergic, serotonergic, noradrenergic, and/or cholinergic inputs), which provide major sources of drive to the hypoglossal motor pool modulating the expression of tonic and respiratory-related motor activities (11, 20, 21, 44).

The suppressant effects of ethanol on state-dependent arousal processes were also observed in sleep, e.g., as judged by the reduced ratio of high-frequency (20–30 Hz) to low-frequency (0.5–2 Hz) EEG power (i.e., the $\beta_2$-to-$\delta_1$ ratio) with ethanol in non-REM sleep (Fig. 4). Also in non-REM sleep, ethanol led to increased power in the $\delta_2$ and $\delta_1$ frequency bands (spanning 0.5–4 Hz) and reduced power in the $\beta_1$ (13.5–20 Hz) bandwidth (Fig. 4). Given the already low motor tone in sleep, however, any ethanol-induced reductions in neck and GG muscle activities were not significant for the group in states outside wakefulness (Fig. 3, B–E, and Fig. 4A). Nevertheless, it is logical and reasonable to suggest that, in conditions when the GG is more active in sleep, the sedating effects of ethanol could suppress GG activity and/or motor excitability during sleep, as well as wakefulness.

Importantly, despite these state-dependent effects of ethanol on GG motor activity following systemic administration, local application of ethanol directly to the hypoglossal motor pool caused no change in GG activity compared with the time-control (sham) experiments. These additional sham experiments were performed because of the need to determine whether a component of the observed decrease in GG activity that occurred in the presence of ethanol at the hypoglossal motor pool was due to the effects of ethanol per se or the effects of time over which the experiments were performed. This distinction was achieved by comparing the responses to ethanol with appropriate time controls, and such comparisons showed no evidence of an ethanol-induced suppression of hypoglossal motor output to GG muscle via local effects at this motor pool, inasmuch as the changes were indistinguishable from the time controls.

Nevertheless, there is potential criticism of performing the experiments with local application of ethanol to the hypoglossal motor pool in anesthetized (albeit otherwise intact) rats. We used anesthetized, rather than conscious, rats for this component of the investigation, because of the impracticality of applying to the hypoglossal motor nucleus the multiple doses of ethanol that were required to span the dose range previously used in vitro (12, 13, 53). In addition, it would not have been possible to replicate controlled application of these multiple doses over the course of a single experiment in a conscious rat and to reliably obtain data in any given sleep-wake state, not least because of the complicating influence of unpredictable changes in ongoing behavioral state and spontaneous motor activities that typify the behavioral preparation. Overall, despite the potential concerns of anesthetia, it nevertheless remains reasonable to expect that if ethanol was exerting a physiological action to suppress hypoglossal motor output to GG muscle via an effect at the hypoglossal motor pool, then we would still have expected to observe such a decrease in GG activity when ethanol was locally applied even under anesthesia, and this simply did not occur. Indeed, in contrast to ethanol, local application of the sedative hypnotic drugs lorazepam (a benzodiazepine) and zolpidem (an imidazopyridine compound) to the hypoglossal motor pool causes suppression of GG activity in this anesthetized preparation in vivo (42). We also showed previously that GABA, glycine, and glutamate each exert tonic modulation effects at the hypoglossal motor pool, innervating GG muscle in this same anesthetized preparation (29, 37–39, 57, 58). This observation is also relevant because these GABA, glycine, and glutamate receptor-mediated responses have the capacity to be significantly influenced by ethanol (as the former are by lorazepam and zolpidem), influences that could reduce GG activity on the basis of in vitro studies at the hypoglossal motor pool and investigations in other brain regions (5, 12, 13, 15, 30, 53, 63). Nevertheless, there was no physiological consequence of ethanol on GG activity when locally applied to the hypoglossal motor pool, even over a wide range of applied doses (Fig. 6). Overall, our data showing a lack of effect of ethanol at the hypoglossal motor pool on GG activity do not dispute the potentiating effects of ethanol on inhibitory GABA and glycine receptor function and augmentation of inhibitory postsynaptic currents at hypoglossal motoneurons when studied in neonatal and juvenile rodent brain stem slice preparations in vitro (12, 13, 53). Rather, these data show that local application of ethanol to the hypoglossal motor pool in the adult organism in vivo, over the same dose range, does not suppress total motor outflow to the GG muscle, a difference that may relate to the older age of animals used in the present study, or that the influences observed at single motoneurons do not necessarily reflect the influence on population motor outflow as subsequently recorded by GG muscle activity.

Overall, the significant changes in sleep-wake states, sleep latencies, EEG frequencies, and postural and GG motor tone produced by ethanol when systemically administered are supportive of reduced arousal processes. In addition, the pattern of suppression of postural (neck) motor activity produced by ethanol (Fig. 4A) was similar to that observed in the tonic and
respiratory-related components of GG activity (Fig. 3, B–E), a muscle that has dual respiratory and nonrespiratory functions, whereas there was no effect on diaphragm activity (Fig. 3, H and I), a muscle that has almost solely respiratory function and is driven by the core components of the respiratory network. Coupled with the lack of effect of ethanol on GG activity with local application to the hypoglossal motor pool, these data suggest that the GG motor suppression observed with systemic administration was mediated via effects of ethanol on sleep/arousal (i.e., state-dependent) processes, rather than via a physiological effect of ethanol at the motor pool per se. This primary influence of ethanol on state-dependent inputs to the respiratory system would also explain why GG activity was suppressed by ethanol at physiologically relevant concentrations, but diaphragm activation and respiratory rate were unchanged. Several lines of evidence indicate that hypoglossal motor output is more strongly influenced by state-dependent influences than is diaphragm activity (20, 26, 28, 40, 46), with electrophysiological data from rodents showing that the source of inspiratory drive to hypoglossal motoneurons is different from the source of drive to phrenic motoneurons; predominantly from the reticular formation (lateral tegmental field) for the former and from bulbo-spinal dorsal and ventral respiratory group neurons for the latter (11, 20, 44). In the present study, ethanol-induced alterations in state-dependent drives to the hypoglossal motor pool could certainly result in reduced motor excitability and GG muscle activity, independent of a potential effect of ethanol at the motor pool per se.

Relevance of Animal Preparation and Final Conclusions

As discussed above, previous studies addressing the effects of ethanol on breathing have not attempted to separate, or distinguish between, the two major mechanisms that could mediate the suppression of pharyngeal (GG) motor tone, i.e., a state-dependent influence on motor activity via an effect of ethanol on sleep/arousal processes and/or a motor suppression effect operating via an inhibitory influence of ethanol directly at the hypoglossal motor pool, and this distinction is one of the contributions of this study. Nevertheless, to determine potential commonality of mechanisms and applicability to human studies, it is necessary to compare the results obtained in the present study with data obtained in other experiments in animals and humans. In this respect, it is also important to emphasize that these studies are performed in rats to determine physiological mechanisms of upper airway motor control and are not a model of OSAH or of the potential changes in upper airway resistance that may result from such changes in GG activity.

In the clinical literature, it has been shown that nighttime ethanol intake can increase total inspiratory resistance in normal nonsnorers and in those awake human subjects are consistent with the suppression of GG activity and lack of effect on diaphragm activity and respiratory rate observed in the present experiments. In a separate experiment in normal human subjects, ethanol also did not alter minute ventilation in wakefulness or sleep, consistent with the results of the present study, but the effects on upper airway motor tone and components of arousal state or sedation were not investigated (8).

Previous studies performed using slices of neonatal rodent medulla or the isolated brain stem-spinal cords of newborn rats in vitro showed suppression of hypoglossal nerve activity with ethanol (10, 15). However, whether that suppression occurred via effects at the motor nuclei per se or via suppression of premotor inputs was not determined, inasmuch as the ethanol was applied to the bath. Similarly, the physiological basis for the selective suppression of hypoglossal nerve activity following systemic administration of ethanol was not determined in a previous study using decerebrate or awake cats (3), inasmuch as the potential central effects of ethanol on respiratory motor activity were not differentiated from the state-dependent effects operating via influences on brain stem arousal neurons. In this respect, some of the aforementioned state-dependent inputs to the hypoglossal motor pool that provide a tonic facilitatory drive are still present in reduced (e.g., in vitro or decerebrate) animal preparations and, therefore, have the capacity to be modulated by ethanol in those preparations.

In conclusion, this study differentiates between the effects of ethanol at the hypoglossal motor pool and the effects on pharyngeal and respiratory pump muscle activities combined with indexes of sleep/arousal regulation. Together, the data support the concept of a foremost influence of ethanol on state-dependent modulation of hypoglossal motor activity, rather than an effect at the motor pool per se. Moreover, the evidence that the magnitude of decrease in respiratory-related and tonic GG activities produced by ethanol was related to baseline activity without ethanol, i.e., the ethanol-induced decrease in activity was greater when baseline activity was higher, has important clinical relevance. The implication of this observation is that individuals with high baseline levels of GG activity, such as OSAH patients, in whom this activity is thought to be required to maintain an open upper air space and adequate airflow (31), would be most susceptible to suppression of activity with ethanol, which explains the worsening of sleep disordered breathing in such individuals (2, 8, 23, 36, 48, 51, 52, 59).

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


SLEEP AND BREATHING AFTER ETHANOL
