Neuroscience Letters 477 (2010) 124–128

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Urethane inhibits genioglossal long-term facilitation in un-paralyzed anesthetized rats

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ARTICLE INFO

Article history:
Received 12 March 2010
Received in revised form 17 April 2010
Accepted 20 April 2010

Keywords:
Urethane
Genioglossus muscle
Long-term facilitation
Respiratory control
Paralysis
Intermittent hypoxia

ABSTRACT

For ~3 decades, urethane has been (partially or solely) used as a successful anesthetic in numerous respiratory long-term facilitation (LTF) studies, which were performed on anesthetized, paralyzed, vagotomized and artificially ventilated animals of several different species. However, things become complicated when LTF of muscle activity is studied in un-paralyzed animals. For example, a commonly used acute intermittent hypoxia (AIH) protocol failed to induce muscle LTF in anesthetized, spontaneously breathing rats. But muscle LTF could be induced when hypoxic episode number was increased and/or anesthetics other than urethane were used. In these studies however, neither anesthetic nor paralysis was mentioned as a potential factor influencing AIH-induced muscle LTF. This study tested whether urethane inhibits AIH-induced genioglossal LTF (gLTF) in un-paralyzed ventilated rats, and if so, determined whether reducing urethane dose reverses this inhibition. Three groups of adult male Sprague–Dawley rats were anesthetized (Group 1: ~1.6 g kg^{-1} urethane; Group 2: 50 mg kg^{-1} α-chloralose +0.9–1.2 g kg^{-1} urethane; Group 3: 0.9 g kg^{-1} urethane +200–400 μg kg^{-1} min^{-1} alphaxalone), vagotomized and mechanically ventilated. Integrated genioglossus activity was measured before, during and after AIH (5 episodes of 3-min isocapnic 12% O_2, separated by 3-min hyperoxic intervals). The AIH-induced gLTF was absent in Group 1 rats (success rate was only ~1/7), but was present in Group 2 (in 10/12 rats) and Group 3 (in 11/11 rats) rats. The genioglossal response to hypoxia was not significantly different among the 3 groups. Collectively, these data suggest that urethane dose-dependently inhibits gLTF in un-paralyzed anesthetized rats.

Acute intermittent hypoxia (AIH) or repeated carotid sinus nerve (CSN) stimulation induces a persistent increase in respiratory activity, known as long-term facilitation (LTF). LTF has been identified in many animal species [9,20]. As AIH-induced LTF was also observed in snorers and obstructive sleep apnea (OSA) patients during sleep [1,2], some have speculated that LTF may be a protective mechanism, promoting upper airway stability in OSA patients after repeated apneas/hypopneas [1,3,4,10,12].

Since LTF was first reported in 1980 [19], considerable progress has been made in understanding where and how LTF is generated and maintained. An overwhelming majority of the LTF studies were conducted in urethane-anesthetized, vagotomized, paralyzed and mechanically ventilated animals. Although AIH occasionally fails to induce LTF (~1 in 10–15), urethane as a reliable and successful anesthetic has never been questioned. However, things are different when LTF of respiratory muscle activity is studied. For example, a commonly used AIH protocol (3 episodes of short-term hypoxia) failed to induce LTF in un-paralyzed spontaneously breathing rats, which was attributed mainly to a higher level of baseline $PaCO_2$ [8] or a smaller number of hypoxia episodes [21]. On the other hand, muscle LTF could be induced by AIH (or elicited by repeated carotid sinus nerve stimulation) in spontaneously breathing animals when hypoxic episode number was increased [21] or other anesthetics were used [13,18,21,22]. In all these muscle LTF studies, however, neither anesthetic nor paralysis was mentioned as a potential factor determining whether LTF of muscle activity can be induced.

The objective of the present study was to assess the effect of urethane on the genioglossal electromyogram activity (EMGgg) LTF (gLTF) in anesthetized, un-paralyzed, ventilated (to achieve normocapnia) rats. Genioglossus is the principal tongue protruder muscle and important for the control of upper airway patency [3,13,18,21]. Because so far no successful LTF study has ever been reported in solely urethane-anesthetized un-paralyzed animals and all successful LTF studies in un-paralyzed animals used other anesthetics totally or partially, we hypothesized that urethane would inhibit AIH-induced gLTF in un-paralyzed rats and this inhibition could be reversed by reducing the urethane dose.

The Harvard Medical Area Standing Committee on Animals approved all experimental procedures. Experiments were mainly conducted on 3 groups of adult male Sprague–Dawley rats

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doi:10.1016/j.neulet.2010.04.047

(250–350 g, colony CDIGS, Charles River, Wilmington, MA), which were anesthetized with urethane alone (Group 1: $n = 33$), urethane + α-chloralose (Group 2: $n = 12$) and urethane + alphaxalone (Group 3: $n = 11$), respectively (all 3 anesthetics from Sigma-Aldrich, Natick, MA, USA).

Group 1 rats were anesthetized initially with isoflurane in a closed chamber and then injection of urethane (~1.6 g kg^{-1}, i.p.). The depth of anesthesia was adjusted so that the animal failed to show a reflex withdrawal of the hind paw to a strong pinch. The trachea was cannulated, and the rat was mechanically ventilated (Harvard Apparatus Inc., Holliston, MA, USA). The inspired gas mixture was 50% O_2 (N_2 balance) during baseline and other non-hypoxia periods. Bilateral vagotomy was performed at the mid-cervical level. End-tidal CO_2 partial pressure ($P_{ET}CO_2$) was monitored in the expired line of the ventilator circuit using a flow-through capnograph (Novametrix; Wallingford, CT, USA). $P_{ET}CO_2$ values obtained with this method are close (usually within 1–2 mmHg) to arterial CO_2 partial pressure ($PaCO_2$). The CO_2-apneic threshold was defined as the $P_{ET}CO_2$ at which respiratory rhythmic activity resumed from hypocapnic apnea. $P_{ET}CO_2$ was maintained at 3 mmHg above the threshold by manipulating the inspire CO_2, and ventilator's rate (its ratio to respiratory frequency was set at about 3:2) and/or tidal volume. Rectal temperature was maintained at 37.0–37.5 °C with a servo-controlled heat blanket and a heating lamp. Before AIH, a supplemental dose of urethane (0.16 g kg^{-1}) was given to provide stable anesthesia for the remainder of the experiment. At the end of the experiments, rats were sacrificed by a lethal dose of urethane (3.2 g kg^{-1}).

Group 2 rats were also anesthetized initially with isoflurane and then injection of α-chloralose (50 mg kg^{-1}, i.p.) plus urethane (0.9–1.2 g kg^{-1}, i.p.). Other preparation procedures were the same as those in Group 1 rats.

Group 3 rats were first anesthetized with isoflurane and then injected with urethane (0.9 g kg^{-1}, i.p.). This dose usually was not sufficient to have the rat fully anesthetized. Thus, isoflurane (1–2% in 30% O_2; balance N_2) was added via a facemask. After the trachea was cannulated, isoflurane was delivered via the tracheal cannula. Inspired gas mixture was 30% O_2 (N_2 balance) during baseline and other non-hypoxia periods. A femoral venous catheter was inserted for fluid administration. A femoral arterial catheter was placed to monitor arterial blood pressure (Statham Pressure Transducer, P23-id) and to withdraw arterial blood samples for blood gases and pH analysis (Opti CCA-TS, Osmetech, Roswell, Georgia) with correction for rectal temperature. $P_{ET}CO_2$ was maintained at 4 mmHg above the CO_2-apneic threshold by manipulating the ventilator's rate and tidal volume. Before AIH, the anesthetic was slowly converted to alphaxalone (200–400 μg kg^{-1} min^{-1} in DMSO solution, i.v.) and the isoflurane was gradually withdrawn in 10–20 min.

EMGgg activity was recorded by two insulated fine silver wires inserted into genioglossus muscle via the floor of the mouth. The EMGgg activity was filtered (300–10,000 Hz), amplified (2000–10,000×, BMA-200 AC/DC Bioamplifier, CWE Inc., Ardmore, PA, USA), full-wave rectified and integrated (Paynter Filter, BAK Electronics, Mount Airy, MD; time constant: 100 ms). The integrated signals were digitized and acquired with computer software (LabView 8.0, National Instruments), and analyzed with a customized program developed in our laboratory. This program determined the amplitude and timing of integrated EMGgg activity, from which the minute EMGgg activity could be calculated.

Baseline EMGgg activity was measured at ~60 min after completion of the surgical preparation. Following the baseline measurement, rats were exposed to 5 episodes of 3-min isocapnic hypoxia (12% O_2), separated by 3-min intervals of hyperoxia (50% O_2 in Groups 1 and 2, 30% O_2 in Group 3). The EMGgg activity was then measured during and up to 60 min after the AIH to determine hypoxic genioglossal responses (HGR) and gLTF, respectively. In some Group 3 rats, arterial blood samples (~0.3 ml) were drawn during baseline, hypoxic exposure and at 45 min post-hypoxia to ensure an isocapnic condition.

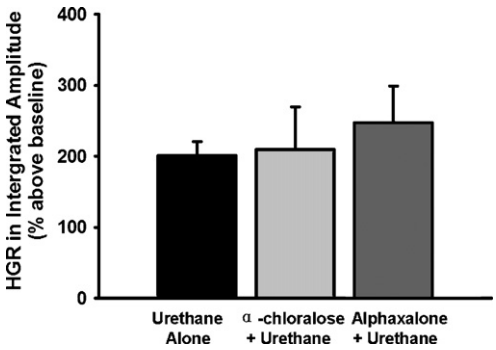

Fig. 1. The effect of different urethane doses on the hypoxic genioglossal response (HGR) in peak amplitude of integrated genioglossal electromyogram activity. The HGR data were obtained from Group 1 (urethane alone, $n = 33$), Group 2 (α-chloralose + urethane, $n = 12$) and Group 3 (alphaxalone + urethane, $n = 11$) rats. These data were the average HGR to 5 episodes of 3-min isocapnic hypoxia (12% O_2), calculated as a percentage increase above the baseline (% above baseline) and expressed as means ± SE. There were no significant differences between any 2 of the 3 groups (all $P > 0.393$).

Integrated EMGgg activity was averaged in ~1 min bins at each target time point. HGR data were collected during the last minute of the 3-min hypoxia exposure when the amplitude of integrated EMGgg activity had reached a plateau, and were averaged over five hypoxia episodes since there was no significant difference among these episodes. Variables determined in each time point included peak amplitude of integrated EMGgg activity (arbitrary units), EMGgg burst frequency (bursts min^{-1}) and minute EMGgg activity (peak amplitude × burst frequency). Changes from baseline in the peak amplitude and minute activity were normalized as a percentage of the baseline (%baseline). Changes from baseline in the burst frequency used absolute units (bursts min^{-1}).

For LTF, the within-group increases from baseline and between-group differences in baseline and the post-hypoxia peak amplitude (also in the burst frequency and minute activity) were statistically analyzed by a two-way ANOVA with repeated measures, followed by the Student–Newman–Keuls post hoc test (SigmaStat version 3.1, Jandel Corporation, San Rafael, CA, USA). For the apneic threshold and HGR, a one-way ANOVA was used to statistically analyze the differences between- and within-groups. $P < 0.05$ was considered significant. All values are expressed as means ± SE.

Although the one-way ANOVA revealed a significant group effect ($F_{2,53} = 3.789$; $P = 0.03$), the post hoc tests showed no significant differences (all $P > 0.05$) in the CO_2-apneic threshold for EMGgg activity between any 2 of the 3 groups (Group 1: 41.6 ± 0.6 mmHg, $n = 33$; Group 2: 44.7 ± 1.4, $n = 12$; Group 3: 41.5 ± 0.8, $n = 11$). The HGR was also not significantly different among the 3 groups in the peak amplitude (Fig. 1), burst frequency (Group 1: 11.6 ± 2.8 bursts min^{-1}; Group 2: 19.8 ± 1.8; Group 3: 14.8 ± 5.3) or minute activity (Group 1: 308.2 ± 39.8% above baseline; Group 2: 428.6 ± 89.6%; Group 3: 363.1 ± 70.1%), as the one-way ANOVA revealed an insignificant overall group effect in the peak amplitude ($F_{2,50} = 0.428$; $P = 0.654$), burst frequency ($F_{2,50} = 1.313$; $P = 0.278$) and minute activity data ($F_{2,50} = 1.056$; $P = 0.356$).

The AIH induced a persistent increase in the peak amplitude of integrated EMGgg activity in both Group 2 and Group 3 rats but not in Group 1 rats (Figs. 2 and 3A). The two-way ANOVA revealed a significant interaction effect ($F_{8,212} = 11.656$; $P < 10^{-6}$) between the group factor (three levels: Group 1, Group 2 and Group 3) and time factor (5 levels: baseline, 15, 30, 45 and 60 min post-hypoxia), and a significant overall group effect ($F_{2,212} = 20.461$; $P < 10^{-6}$) in peak