

# Perinatal Alcohol Exposure in Rat Induces Long-Term Depression of Respiration after Episodic Hypoxia

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**Rationale:** Little is known about the effects of alcohol exposure during pregnancy, which is responsible for fetal alcohol syndrome and the respiratory network functions, especially respiratory network plasticity (e.g., long-term facilitation) elicited after repeated short-lasting hypoxic episodes. The mechanism of induction of respiratory long-term facilitation involves 5-HT<sub>2A/2C</sub> receptors, which also participate in the response to hypoxia. Because fetal alcohol exposure is known to reduce serotonin centrally, and synaptic plasticity in the hippocampus, we hypothesized that alcohol exposure during gestation might impair respiratory long-term facilitation after hypoxic episodes.

**Objectives:** To analyze the effects of prenatal and postnatal alcohol exposure on respiratory long-term facilitation in 5- to 7-day-old rats.

**Methods:** Respiratory frequency and amplitude were measured *in vivo* and in an *in vitro* rhythmic medullary slice before and after three hypoxia episodes or three applications of a 5-HT<sub>2A/2C</sub> receptor agonist *in vitro*. 5-HT<sub>2A/2C</sub> receptor mRNA was measured from the slice.

**Measurements and Main Results:** Alcohol exposure impaired respiratory long-term facilitation and induced long-term depression of respiration in both *in vivo* and *in vitro* models. Alcohol altered 5-HT<sub>2A/2C</sub> mRNA expression, although 5-HT<sub>2A/2C</sub> agonist efficacy was not altered in increasing rhythmic activity in slices. However, a higher concentration of 5-HT<sub>2A/2C</sub> agonist was necessary to induce transient facilitation in slices from ethanol-exposed animals, suggesting disturbances in induction and maintenance mechanisms of respiratory long-term facilitation.

**Conclusions:** Respiratory facilitation after repeated hypoxia was converted to long-term depression in rats treated with alcohol *in utero*. Alcohol exposure during pregnancy may therefore induce long-term maladaptive behavior of the respiratory system in neonates.

**Keywords:** plasticity; respiratory depression; serotonin; ethanol

Alcohol ingestion during pregnancy may induce fetal alcohol syndrome (FAS) characterized by craniofacial malformations and mental retardation, resulting in life-long impairment (1). A total of 3 to 5% of women continue to drink alcohol during pregnancy (2), and present a higher risk of abortion, fetal death, and premature delivery, and are more likely to have infants with compromised physical and mental development (3, 4). In the central nervous system (CNS), *in utero* ethanol exposure reduces long-term synaptic plasticity (LTP) in the hippocampus, which may account for memory deficits in infants born with FAS (5). The activity of serotonergic (5-HT) pathways is also

## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Alcohol depresses respiration, but little is known about the effects of chronic alcohol exposure during pregnancy on respiratory long-term facilitation (LTF), induced after repeated hypoxic episodes.

### What This Study Adds to the Field

Perinatal alcohol exposure in rats converts respiratory LTF into long-term depression. Alcohol exposure during pregnancy may therefore lead to long-term maladaptive behavior of the respiratory network in neonates in response to low oxygen.

dramatically reduced due to ethanol-induced neuronal death (6–8). Concerning the control of breathing, 5-HT neurons innervate the phrenic motoneuron pool and the respiratory brainstem neurons, including hypoglossal (XII) motoneurons (9–11), and are key players in the response to hypoxia (12, 13). XII nuclei and the nucleus tractus solitarius, which innervate tongue muscles and integrate important sensory afferents for breathing, respectively, have fewer neurons or neurons with abnormal shape after prenatal ethanol exposure (14–16). Moreover, ethanol exposure *in utero* increases the risk of sudden infant death syndrome (SIDS) in certain ethnic groups (17), and is associated with brain serotonergic system deficiency (17–19). SIDS is considered to be an inappropriate response of the newborn to chemosensory challenge induced by hypoxic episodes during sleep, resulting in recurrent apnea (20). This suggests an inability to adapt ventilation in the long term after repeated hypoxia, which is clearly a disadvantage for survival. However, episodic hypoxia, which mimics certain features of sleep apnea, induces respiratory long-term facilitation (LTF) in rodents (22). Whereas induction of this respiratory network plasticity involves 5-HT<sub>2A/2C</sub> receptor activation (22), new synthesis of brain-derived neurotrophic factor (BDNF) is both necessary and sufficient to elicit underlying mechanism (23). Interestingly, *in utero* ethanol exposure decreases BDNF protein and mRNA levels in rat brain (24), whereas BDNF is necessary for normal development of central respiratory centers (25). Ethanol exposure *in utero* could therefore affect the central serotonergic control of breathing and the normal development of the brainstem respiratory areas. In this context, our previous study showed that juvenile rats exposed to ethanol during pre- and postnatal periods have low  $\dot{V}_E$  (26). This finding, combined with the known effect of *in utero* ethanol on 5-HT and hippocampal synaptic LTP, led us to postulate that ethanol during pregnancy might impair LTF. Our study in newborn rats after perinatal ethanol exposure showed that LTF was replaced by long-term depression of respiration (LTD<sub>Res</sub>). Ethanol exposure altered mRNA levels for 5HT<sub>2A/2C</sub> receptors without abolishing the efficacy of a 5-HT<sub>2A/2C</sub>

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receptor agonist. We conclude that prenatal alcohol exposure in rats substantially disturbed the mechanisms of long-term ventilatory adaptation after repeated hypoxia.

## METHODS

### Animals

We used 5- to 7-day-old Sprague-Dawley rats (Charles River Laboratories, l'Arbresle, France) exposed to ethanol or water. Ethanol exposure consisted of drinking 10% vol/vol ethanol as the sole source of fluid for dams 1 month before mating and until the end of the lactation period (26). Experimental procedures were in accordance with guidelines for care and use of laboratory animals (European Community, law 86/609/EEC).

### Plethysmography

Breathing was measured by whole-body plethysmography (26). Neonates ( $n = 36$ ) were not anesthetized and were unrestrained, and the recording chamber temperature was maintained at 31°C. Body temperature was measured with an anal probe throughout the experiment. Breathing was recorded for 30 seconds before (normoxia), during the third hypoxia, and 90 minutes after three 3-minute hypocapnic-hypoxic episodes (11% O<sub>2</sub>), separated by 5 minutes of normoxia. Inspiration time (Ti) and expiration time (Te), V<sub>T</sub>, respiratory frequency (f), and V<sub>E</sub> were measured. More details on these methods can be found in the online supplement.

### Slice Preparation

Neonates were deeply anesthetized with ether and decapitated. The brainstem was cut serially (vibroslicer VT 1000E; Leica Microsystems, Rueil-Malmaison, France) into carbogen-gassed (5% CO<sub>2</sub>, 95% O<sub>2</sub>, pH 7.4) artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 5 mM KCl, 21 mM NaHCO<sub>3</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 30 mM D-glucose, 1.5 mM CaCl<sub>2</sub>. Rhythmic slices (750- $\mu$ m thick), obtained posterior to the facial nucleus, displayed anatomic landmarks, including XII nucleus and axon tract, inferior olive, pyramidal tract, and nucleus tractus solitarius (NTS) (27). Slices were superfused in the recording chamber with equilibrated aCSF containing 8 mM [K<sup>+</sup>]<sub>e</sub>. XII nerve rootlet activity was recorded with a glass suction electrode, filtered (1–3 kHz and 300 Hz), amplified (50,000 times), integrated ( $\zeta = 100$ –150 ms), and recorded via a CED 1401 interface (Spike 2; CED, Cambridge, UK) connected to a computer. See the online supplement for further details on rhythmic slice preparation.

### Drug

$\alpha$ -Methylserotonin maleate ( $\alpha$ -Me-5-HT), a 5-HT<sub>2A/2C</sub> receptor agonist, was obtained from Sigma Inc. (St. Quentin Fallavier, France), prepared in distilled water, dissolved in equilibrated aCSF, and bath applied via the main flow line. Washout was performed with drug-free aCSF.

### Slice Experiments

**Inducing respiratory LTF.** LTF was induced in 17 slices by switching from a reservoir containing carbogen-gassed aCSF to a reservoir containing 95% N<sub>2</sub> and 5% CO<sub>2</sub>-gassed aCSF for three 3-minute episodes, separated by 5 minutes of oxygenated aCSF (27). On 30 slices, we used three 3-minute bath applications of  $\alpha$ -Me-5-HT separated by 5-minute washouts (28).

**Control experiments.** XII burst was analyzed over the last 90 minutes of a 170-minute recording session (corresponding to the total duration of LTF experiments) in 14 slices never exposed to anoxic episodes or to  $\alpha$ -Me-5-HT. Long-term response to 9 minutes of continuous anoxia (corresponding to the cumulative duration of the 3 episodes) was performed on 10 slices.

### Measurements of Rhythmic Slice Activity

Amplitude and frequency of integrated XII burst were measured during baseline activity, anoxic episodes, 1  $\mu$ M  $\alpha$ -Me-5-HT applications, and at several time points after anoxic episodes and 1  $\mu$ M  $\alpha$ -Me-5-HT. More details on these measurements can be found in the online supplement.

## Statistical Analysis

Differences in baseline activity, animal body weight, and temperature were determined with unpaired Student's *t* test. The *in vivo* response to hypoxia and the response at 90 minutes after hypoxic episodes were analyzed with paired Student's *t* test for each animal and with unpaired Student's *t* test between populations. *In vitro* response of XII burst amplitude to anoxia and to  $\alpha$ -Me-5-HT was tested with paired Student's *t* test. Then, an unpaired Student's *t* test was applied for each episode between populations. The response during the 90 minutes after hypoxic episodes was analyzed at several time points by two-way repeated-measure analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) *post hoc* test. Data are presented as mean ( $\pm$  SEM) for each group and expressed as percent variation of baseline values recorded prior to stimulation. A *P* value of less than 0.05 was considered significant. Additional details on statistics are provided in the online supplement.

## Reverse Transcriptase–Polymerase Chain Reaction Experiments for 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> Receptors

Total RNA was isolated using the RNA Insta-Pure System (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions in 19 slices not used for electrophysiology but identified as rhythmic. Total RNA (1  $\mu$ g) was reverse transcribed, and 1/12 of the reaction product was amplified by polymerase chain reaction. Glyceraldehyde phosphate dehydrogenase was used as internal standard (29). DNA products of amplification were analyzed by gel electrophoresis stained with ethidium bromide. Data are presented as the target:glyceraldehyde phosphate dehydrogenase mRNA ratio, and statistical analysis was performed with unpaired Student's *t* test. A *P* value of less than 0.05 was considered significant. See the online supplement for more details on reverse transcriptase–polymerase chain reaction experiments.

## RESULTS

### *In Vivo* Experiments

Animal body weight and body temperature before and throughout experiments was similar between groups ( $P > 0.05$ ; Table 1). During normoxia, *f* was 18% lower in the ethanol group ( $P < 0.05$ ) due to a 30% increase in Te ( $P < 0.05$ ). V<sub>T</sub> was 30% higher in the ethanol group ( $P > 0.05$ ), whereas V<sub>E</sub> was not significantly increased (+11%; Table 1). Both groups of animals ( $n = 6$  for each) responded to hypoxia with a similar ( $P > 0.05$ ) increase in V<sub>T</sub> (control, 213.8  $\pm$  39%,  $P < 0.05$ ; ethanol, 144.0  $\pm$  74.0,  $P < 0.05$ ) and V<sub>E</sub> (control, 249.0  $\pm$  74.0%,  $P < 0.05$ ; ethanol, 209.0  $\pm$  43.1%,  $P < 0.05$ ) and no change in frequency. At 90 minutes after three hypoxic episodes (11% O<sub>2</sub>), V<sub>T</sub> and V<sub>E</sub> were significantly increased in the control group, by 117% and 75%, respectively (Figures 1A and 1B), whereas, in the ethanol group, V<sub>T</sub> was decreased by 47% ( $P < 0.001$ ) and V<sub>E</sub> was decreased by 52% ( $P < 0.001$ ). R<sub>f</sub> decreased only in the control group (–15%;  $P < 0.01$ ). Significant differences were observed between groups for V<sub>T</sub> and V<sub>E</sub> ( $P < 0.01$  for both) but not for frequency. Episodic hypoxia, therefore, induced LTF on respiratory amplitude in control animals, whereas ethanol-treated animals showed an LTD<sub>Res</sub>.

### Induction of LTF with Anoxic Episodes *In Vitro*

Before anoxia, XII burst frequency was similar between groups (ethanol group, 12.2  $\pm$  0.9 bursts/min,  $n = 9$ ; control group, 14.5  $\pm$  1.0 bursts/min,  $n = 8$ ;  $P > 0.05$ ). During anoxia, the increase in XII burst amplitude during the first and the third episode was similar within each population (control first episode, 116.0  $\pm$  5.8% vs. third episode, 116.5  $\pm$  3.9%,  $P > 0.05$ ; ethanol first episode, 126.1  $\pm$  5% vs. third episode, 121.4  $\pm$  5.6%,  $P > 0.05$ ). No significant difference was observed between the groups for the first and the third episode ( $P > 0.05$  for both). After anoxic episodes, XII burst amplitude in the control group increased

**TABLE 1. PLETHYSMOGRAPHY DURING NORMOXIA: BODY WEIGHT, BODY TEMPERATURE, AND BREATHING PARAMETERS DURING NORMOXIA IN CONTROL AND ETHANOL-EXPOSED GROUPS**

	Control (n = 12)	Ethanol (n = 12)
Body weight, g	18.9 ± 0.6	18.9 ± 0.9
Body temperature before hypoxia, °C	31.4 ± 0.5	31.4 ± 0.2
Body temperature at end of experiment, °C	32.4 ± 0.4	32.2 ± 0.5
f, cycles/min	175.4 ± 7.7	143.4 ± 6.2*
Ti, ms	95 ± 4	108 ± 8
Te, ms	265 ± 16	344 ± 26†
V <sub>T</sub> , ml/100 g	1.788 ± 0.241	2.342 ± 0.261
V <sub>E</sub> , ml/min/100 g	296 ± 31	330 ± 37

Definition of abbreviations: f = respiratory frequency; Te = expiratory time; Ti = inspiratory time.

In the ethanol group, f was lower due to an increased Te. Data are presented as mean ± SEM.

† P < 0.05 between groups.

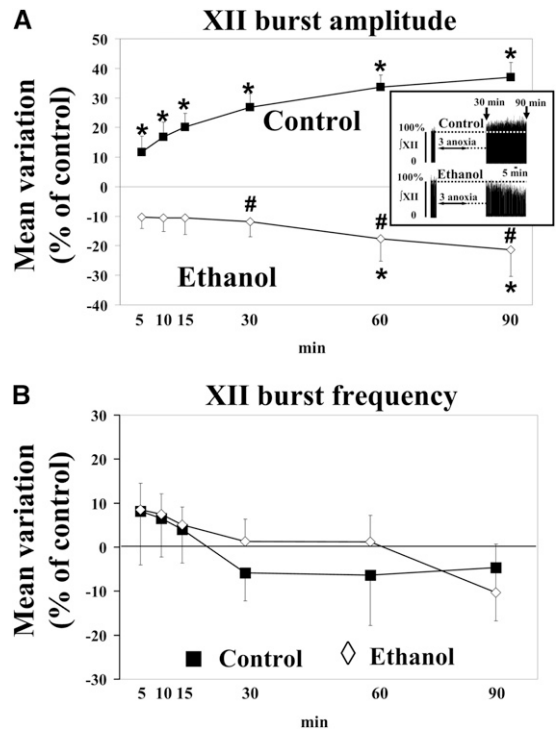
\* P < 0.01 between groups.

significantly at all postanoxia time points (P < 0.05, SNK), and reached +37 ± 5% at 90 minutes (Figure 2A and insert). In the ethanol group, XII burst amplitude decreased continuously to reach -21.4 (± 8.9)% at 90 minutes after anoxia (Figure 2A and insert). The percent decrease was significant at 60 and at 90 minutes postanoxia (P < 0.05, SNK). ANOVA analysis revealed a time effect (P = 0.05), a group effect (P < 0.001), and a significant difference between groups from 30 to 90 minutes (P < 0.001). However, no significant change in frequency during the 90-minute postanoxia period was observed within or between groups (Figure 2B).

**LTF/LTD<sub>Res</sub> In Vitro with α-Methyl-5-HT**

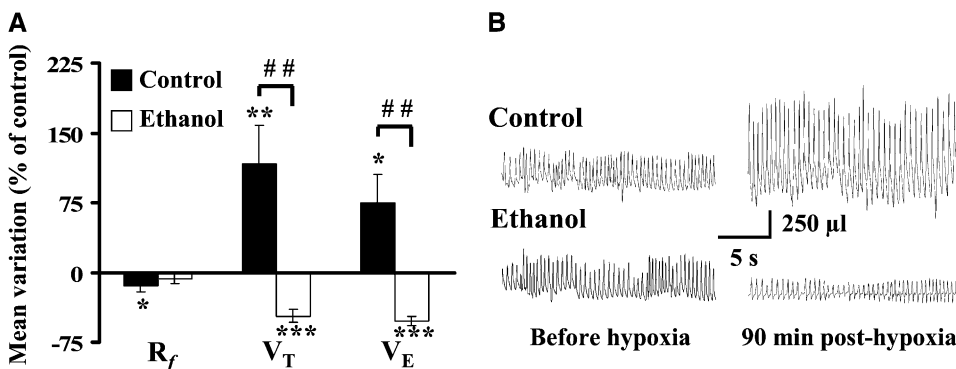
LTF induction requires activation of 5-HT<sub>2A/2C</sub> receptors (22). The absence of LTF after episodic anoxia suggests that ethanol affected serotonergic neurotransmission. We therefore tried to induce LTF by episodic bath application of α-Methyl-5-HT to slices.

**1 μM α-Methyl-5-HT.** In the control group, LTF was elicited and maintained for the 90 minutes of analysis (Figure 3A [a] and Figure 3B [b]; n = 7), with a significant increase of XII burst amplitude by 41% (± 8.5%) at 5 minutes and 29.3% (± 8.7%) at 90 minutes. ANOVA indicated a time effect (P = 0.002) and significant percentage changes at all time points after drug application (P < 0.05, SNK). In contrast, in the ethanol group (Figure 3A [b] and Figure 3B [b], n = 8), XII burst amplitude did not change until 15 minutes after drug application and then



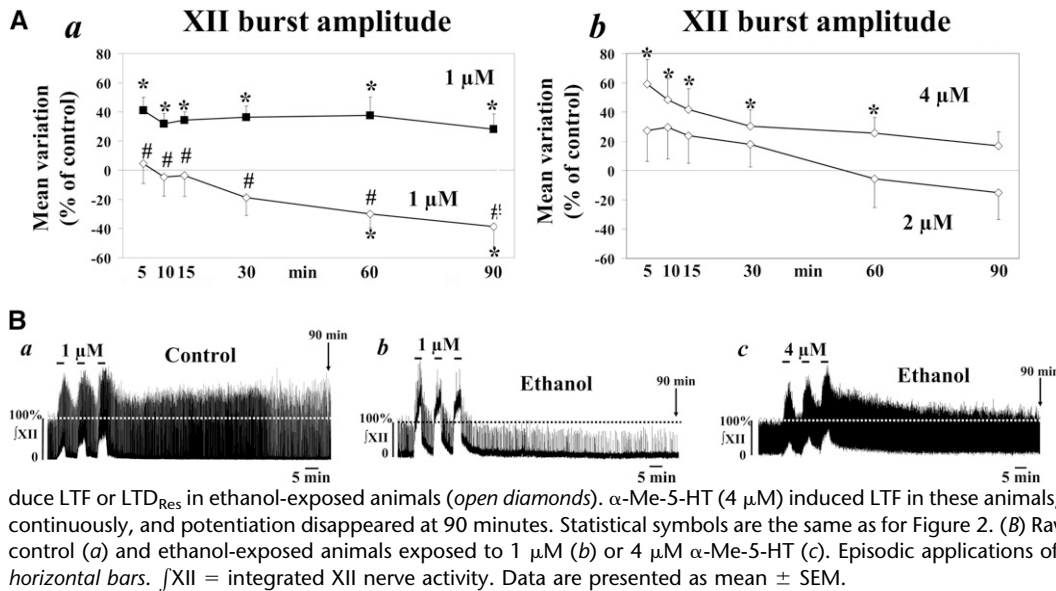
**Figure 2.** *In vitro* experiments. (A) Measurements of hypoglossal (XII) burst amplitude showed that the control group developed long-term facilitation (LTF) with a continuous increase in amplitude over the 90-minute postanoxic episodes (three 3-min episodes of anoxia). In contrast, ethanol-exposed animals developed long-term depression of respiration (LTD<sub>Res</sub>), as XII burst amplitude decreased continuously. Differences between populations were significant from 30 to 90 minutes (#P < 0.05; analysis of variance; \*P < 0.05 compared with control value before anoxia). Inset shows XII rootlet raw recordings before and after anoxic episodes in a control and an ethanol-exposed animal. Only the portion between 30 and 90 minutes is shown, corresponding to a significant difference between populations. The dotted horizontal line indicates basal XII burst amplitude as measured before stimulation. ∫XII = integrated XII rootlet activity. (B) Variation in XII burst frequency over the 90-minute postanoxic episodes. In both populations, the frequency remained stable along the recording, and no significant differences were observed within or between groups. Data are presented as mean ± SEM.

decreased continuously to reach -40.2 ± 11.7% at 90 minutes. Measurements of XII burst amplitude at 60 and 90 minutes were significant (P < 0.05, SNK). XII burst amplitude decreased by 40% during the 90-minute recording. Differences



**Figure 1.** *In vivo* experiments. (A) Breathing measurements by plethysmography 90 minutes after three 3-minute hypoxic episodes (11% O<sub>2</sub>). Control animals showed long-term facilitation (LTF) with an increase in V<sub>T</sub> and V<sub>E</sub>. The ethanol group showed long-term depression of respiratory activity (LTD<sub>Res</sub>) with decreased V<sub>T</sub> and V<sub>E</sub>. Differences between groups were significant only for V<sub>T</sub> and V<sub>E</sub> (#P < 0.01; analysis of variance). Respiratory frequency (R<sub>f</sub>) was decreased only in the control group (\*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001). (B) Raw traces of breathing recordings for the two groups before and after hypoxia. Inspiration corresponds to an upward inflection. Data are presented as mean ± SEM.

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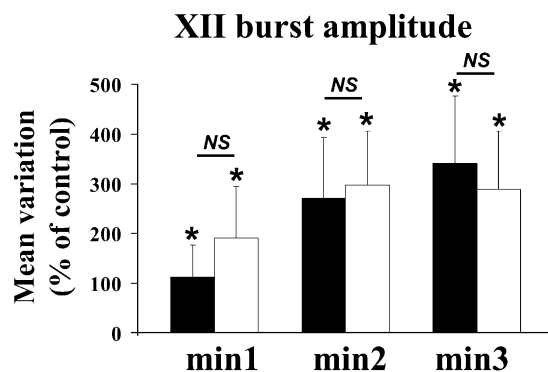


**Figure 3.**  $\alpha$ -Methylserotonin maleate ( $\alpha$ -Me-5-HT) episodic applications and long-term facilitation (LTF)/long-term depression of respiration (LTD<sub>Res</sub>) induction *in vitro*. (A) Mean variations in hypoglossal (XII) burst amplitude during the 90-minute postanoxic period compared with control values in control and ethanol-exposed animals. (a)  $\alpha$ -Me-5-HT (1  $\mu$ M) induced sustained LTF in control animals (closed squares), whereas LTD<sub>Res</sub> was induced in ethanol-exposed animals (open diamonds). (b)  $\alpha$ -Me-5-HT (2  $\mu$ M) did not induce LTF or LTD<sub>Res</sub> in ethanol-exposed animals (open diamonds).  $\alpha$ -Me-5-HT (4  $\mu$ M) induced LTF in these animals, but XII burst amplitude decreased continuously, and potentiation disappeared at 90 minutes. Statistical symbols are the same as for Figure 2. (B) Raw recordings of XII nerve activity in control (a) and ethanol-exposed animals exposed to 1  $\mu$ M (b) or 4  $\mu$ M  $\alpha$ -Me-5-HT (c). Episodic applications of the drug correspond to the three horizontal bars. [XII] = integrated XII nerve activity. Data are presented as mean  $\pm$  SEM.

between populations were significant ( $P < 0.001$ ) between 5 and 90 minutes after stimulus ( $P < 0.05$ , SNK). These results suggest a difference in sensitivity of 5-HT<sub>2A/2C</sub> receptors between the two populations. However, mean XII burst amplitude measured during the 3-minute application of 1  $\mu$ M  $\alpha$ -Me-5-HT was significantly increased in both groups, with no significant difference between groups (Figure 4).

**2 and 4  $\mu$ M  $\alpha$ -Me-5-HT.** Neither LTF nor LTD<sub>Res</sub> were elicited with 2  $\mu$ M  $\alpha$ -Me-5-HT in the ethanol group (Figure 3A [b],  $n = 5$ ;  $P = 0.08$ ). At 4  $\mu$ M (Figure 3A [b], Figure 3B [c],  $n = 10$ ), a significant, long-lasting (60-min) increase in XII burst amplitude was induced with  $+59.2 (\pm 16.9) \%$  at 5 minutes (time effect;  $P < 0.001$ ). However, a continuous 43% decrease in amplitude was observed over the recording time, and respiratory facilitation disappeared at 90 minutes, indicating that LTF was not maintained despite successful induction.

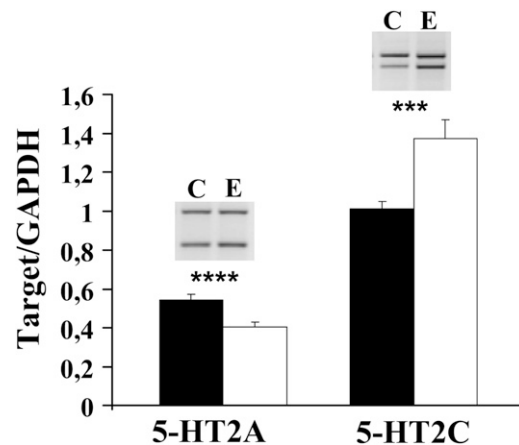
**5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> mRNA levels after ethanol exposure.** In ethanol-exposed animals ( $n = 8$ ; Figure 5), baseline 5-HT<sub>2A</sub> receptor mRNA levels were lower ( $P < 0.001$ ) and 5-HT<sub>2C</sub> receptor mRNA levels were higher ( $P = 0.003$ ) compared with the control group ( $n = 11$ ).



**Figure 4.** Mean variation in hypoglossal (XII) burst amplitude during the 3-minute (min1, min2, and min3) episodic applications of 1  $\mu$ M  $\alpha$ -methylserotonin maleate. Both populations responded in a similar way (control, closed bars; ethanol, open bars) with an increase in XII burst amplitude ( $*P < 0.05$  compared with baseline values), but no significant differences were observed between groups. NS = non-significant. Data are presented as mean  $\pm$  SEM.

### Control Experiments

Tests were performed to determine whether LTD<sub>Res</sub> recorded *in vitro* from ethanol-exposed animals was not due to spontaneous degradation of rhythmic activity and whether LTD<sub>Res</sub> was specific to the episodic anoxic stimulus. No significant change in XII burst amplitude was observed during the last 90 minutes of a 170-minute recording session in either the ethanol ( $n = 8$ ;  $P = 0.9$ ) or control groups ( $n = 6$ ). Differences between groups were not significant ( $P = 0.98$ ). Rhythmic activity was therefore stable in the two groups. Moreover, a single 9-minute episode of anoxia failed to elicit either LTF or LTD<sub>Res</sub> in either group (control,  $n = 4$ ; ethanol,  $n = 6$ ;  $P > 0.05$  for both). Changes in amplitude were not significantly different between groups. See Figures E1 and E2 in the online supplement for both experiments.



**Figure 5.** Reverse transcriptase polymerase chain reaction experiments in rhythmic slices. 5HT<sub>2A</sub> receptor mRNA levels were lower in ethanol-exposed animals (open bars) than in control animals (closed bars) (\*\*\*\* $P < 0.001$ ). 5HT<sub>2C</sub> mRNA levels were higher (\*\*\* $P = 0.003$ ). The upper trace represents target mRNA; the lower trace represents glyceraldehyde phosphate dehydrogenase. C = control animals; E = ethanol-exposed animals. Data are presented as mean  $\pm$  SEM.

## DISCUSSION

The most striking result of this study was the induction of LTD<sub>Res</sub> instead of LTF after episodic hypoxia *in vivo* or anoxia *in vitro* in neonatal rats treated with chronic ethanol exposure during gestation and lactation. To our knowledge, this is the first report describing LTD<sub>Res</sub> instead of LTF after repeated hypoxia in an experimental model resembling a human pathology (e.g., FAS). However, other examples of LTD<sub>Res</sub> have been described in various experimental conditions (30–32). In the context of the present study, *in utero* ethanol exposure was shown to alter synaptic plasticity (i.e., LTP) in the hippocampus (5), and to convert synaptic LTD into LTP in the cerebellum (33). Importantly, our model of ethanol exposure induced blood alcohol levels in rat dams (29) that correspond to those observed in pregnant human drinkers and can be considered to reflect moderate alcohol intake (34). These results therefore suggest that long-term synaptic/network plasticity in the CNS is extremely sensitive to early chronic ethanol exposure.

The response to more intense stimulation (more anoxic episodes *in vitro* or severe hypoxia *in vivo*) was not tested, but LTD<sub>Res</sub> was observed both *in vivo* and *in vitro*, suggesting that it represents the physiological response of ethanol-treated animals to episodic hypoxia under our experimental conditions. Although *in vitro* anoxia-induced respiratory network plasticity may share only some of the mechanisms elicited by serotonin-dependent respiratory plasticity, LTD<sub>Res</sub> shared features in common with LTF insofar as it was specific to intermittent anoxia (22) and was mimicked by episodic applications of 5-HT<sub>2A/2C</sub> receptor agonist (28, 31). Furthermore, both LTF and LTD<sub>Res</sub> were unrelated to baseline rhythm frequency. *In vivo*, baseline *f* was different between groups, whereas *in vitro* rhythm frequency was similar, and LTF was always observed in control animals and LTD<sub>Res</sub> in ethanol-exposed animals. In addition, LTD<sub>Res</sub> was not due to an altered response to hypoxia/anoxia after ethanol exposure or to spontaneous degradation with time of the activity recorded *in vitro*. Plethysmography experiments revealed that V<sub>T</sub> was markedly affected, suggesting that respiratory drive was preferentially increased/decreased over time. Moreover, the clear correspondence between LTF/LTD<sub>Res</sub> on spontaneous breathing and on XII nerve rootlet suggests that respiratory plasticity might affect all types of inspiratory nerves in a similar way. Hypocapnic hypoxia was tested *in vivo* because it induces LTF in awake adult rats (35) and avoids the influence of CO<sub>2</sub> in LTF (36). If repeated hypocapnia contributed to LTD<sub>Res</sub>, we probably underestimated the magnitude of LTF in control animals, although it was already relatively large. Very little is known about LTF in neonatal rats, but the magnitude of LTF decreases during adult life (37, 38), indicating that neonatal rats might present an even larger LTF than young rats. Differences in metabolic rate between populations could also participate in long-term ventilatory response, but this seems unlikely as: (1) ethanol-exposed animals probably compensated for their low *f* by the 30% increase in V<sub>T</sub> in normoxia; and (2) body temperature was similar for all animals before and throughout the experiments. This would suggest a sufficient oxygenation under normoxic conditions and that changes in metabolic rate played a minor role in the long-term response. Finally, the lower spontaneous *f* measured in ethanol-exposed neonatal rats extends the results of our previous study on juvenile rats after similar ethanol exposure (26), revealing that ethanol-induced breathing disturbances are probably present since birth.

Serotonergic neurons play an important role in the respiratory response to hypoxia (12, 13), and 5-HT accumulates in the adult bulbar respiratory network *in vivo* during hypoxia. Lack

of LTF induction, which requires activation of 5-HT<sub>2A/2C</sub> receptors (30), could then reflect either a reduced release of 5-HT during anoxia, because of a loss of 5-HT neurons after ethanol exposure (6–9), or changes in 5-HT postsynaptic receptor sensitivity or numbers (39, 40). However, postsynaptic 5-HT<sub>2A/2C</sub> receptors are present and functional after ethanol exposure, and no changes in agonist efficacy were observed. 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> respective mRNA levels were decreased and increased after ethanol exposure, suggesting that the number of 5-HT<sub>2A</sub> receptors was reduced, whereas the 5-HT<sub>2C</sub> receptor number was increased, leading to a similar short-term response during  $\alpha$ -Me-5-HT application as that in control animals. Despite the similar short-term responses to 5-HT<sub>2A/2C</sub> receptor agonist, 1  $\mu$ M  $\alpha$ -Me-5HT induced LTF in control animals (28), whereas it reproduced anoxia-induced LTD<sub>Res</sub> in ethanol-exposed animals, suggesting that a 5-HT mechanism could also be involved in LTD<sub>Res</sub>. Altogether, these findings support the idea that the polarity of plasticity within the respiratory network (facilitation or depression) is not dependent on the response to each stimulus but is, rather, related to the functional consequences at the network level of the successive stimulation. These results, therefore, support the finding that LTF magnitude is unrelated to the response to  $\alpha$ -Me-5-HT in rhythmic slices (28). Indeed, LTF induction in slices from the ethanol group required higher concentrations of agonist than in control slices. In slices from the ethanol group, increasing the concentration of  $\alpha$ -Me-5-HT to 4  $\mu$ M elicited a transient facilitation that disappeared at 90 minutes after stimulus. Consequently, induction of LTF was dependent on the level of 5-HT receptor activation, and the transient facilitation suggests a deficiency of maintenance mechanism(s). In addition, the differences in the long-term effects of repeated application of  $\alpha$ -Me-5-HT between animal populations suggest that ethanol probably altered mechanism(s) downstream of the 5-HT<sub>2</sub> receptor, which is/are probably linked to BDNF production (23). However, it cannot be ruled out that LTF was transient because of a specific, but yet unknown, mechanism. This may explain why the decrease in XII burst amplitude over time was similar with 1 and 4  $\mu$ M  $\alpha$ -Me-5-HT.

The cellular mechanisms involved in LTF have not been fully elucidated. Apart from the need for 5-HT<sub>2A/2C</sub> receptors, and possibly a neosynthesis of BDNF in the spinal cord (i.e., phrenic motoneuron pool [23]),  $\alpha$ -Me-5-HT-induced LTF on XII motoneurons in neonatal rat rhythmic slices included a potentiation of  $\alpha$ -amino-3-hydroxy-5-methylisooxal-4-propionate (AMPA) receptor current and a permissive role for group 1 metabotropic-type glutamate receptors (28). Thus, our data suggest that perinatal alcohol exposure altered facilitation of glutamatergic neurotransmission elicited by 5-HT<sub>2A/2C</sub> receptor activation. Indeed, because the network response to 5-HT<sub>2A/2C</sub> agonist was not changed after ethanol exposure, we may envisage that LTD<sub>Res</sub> was due to alteration in the second messenger cascade activation. For example, a lower protein kinase C (PKC) activity might be responsible for a lower phosphorylation of AMPA receptors. At last, we cannot rule out that ethanol exposure disturbed BDNF levels. Because BDNF is known to play a critical role in synaptic plasticity in the CNS via an increase in N-methyl-D-aspartate (NMDA)/AMPA receptor activity by inducing subunit phosphorylation (41, 42), we can hypothesize that a low level of BDNF during LTD<sub>Res</sub> could result in low NMDA/AMPA receptor phosphorylation. Alternatively, protein phosphatase activity could be increased during LTD<sub>Res</sub> because of a low level of BDNF (43). However, these assumptions need further investigation, including measurements of BDNF levels in rhythmic slices during LTD<sub>Res</sub>.

Although the physiologic role of respiratory plasticity is not yet clear, LTF is considered to be a protective mechanism for breathing in response to episodic hypoxia (22, 23, 28). LTF is present in humans with obstructive sleep apnea (OSA), upper airway flow limitations, or snoring (44). If LTF increases XII muscle tone after episodic hypoxia, it might help in reducing the frequency and severity of airway obstruction, whereas phrenic nerve LTF might assist with alveolar ventilation (i.e., oxygenation). On the other hand, a lower activation of XII motoneurons can induce collapse of upper airways, especially during sleep, thereby increasing the incidence of OSA and episodic hypoxia. Interestingly, drinking alcohol during pregnancy increases the risk for SIDS in newborns (3, 4, 17), and SIDS is associated with abnormal serotonergic neurotransmission (16–19, 21). Repeated low-oxygen episodes occur in sleep apneas, although with frequencies and durations that may be different from those observed in this study. We nevertheless suggest that the new form of respiratory plasticity reported here may have an impact on SIDS, as induction of LTD<sub>Res</sub> would either increase OSA severity or reduce the capacity to recover from repeated hypoxic episodes, such as those occurring during sleep. A better understanding of the cellular mechanisms of LTD<sub>Res</sub> would be useful to more clearly understand the long-term effects of drinking alcohol during pregnancy on respiratory adaptive mechanisms, particularly after repeated hypoxia in newborns.

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