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Ventilatory long-term facilitation is evident after initial and repeated exposure to intermittent hypoxia in mice genetically depleted of brain serotonin

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Abstract

Our study was designed to determine if central nervous system (CNS) serotonin is required for the induction of ventilatory long-term facilitation (LTF) in intact, spontaneously breathing mice. Nineteen tryptophan hydroxylase 2-deficient (Tph2^{-/-}) mice, devoid of serotonin in the CNS, and their wild-type counterparts (Tph2^{+/+}) were exposed to intermittent hypoxia each day for 10 consecutive days. The ventilatory response to intermittent hypoxia was greater in the Tph2^{+/+} compared with the Tph2^{-/-} mice (1.10 ± 0.10 vs. 0.77 ± 0.01 ml min⁻¹.percent⁻¹ oxygen; $P = 0.04$). Ventilatory LTF, caused by increases in breathing frequency, was evident in Tph2^{+/+} and Tph2^{-/-} mice following exposure to intermittent hypoxia each day; however, the magnitude of the response was greater in the Tph2^{+/+} compared with the Tph2^{-/-} mice (1.11 ± 0.02 vs. 1.05 ± 0.01 normalized to baseline on each day; $P = 0.01$). The magnitude of ventilatory LTF increased significantly from the initial to the final days of the protocol in the Tph2^{-/-} (1.06 ± 0.02 vs. 1.11 ± 0.03 normalized to baseline on the initial days; $P = 0.004$) but not in the Tph2^{+/+} mice. This enhanced response was mediated by increases in tidal volume. Body temperature and metabolic rate did not account for differences in the magnitude of ventilatory LTF observed between groups after acute and repeated daily exposure to intermittent hypoxia. We conclude that ventilatory LTF, after acute exposure to intermittent hypoxia, is mediated by increases in breathing frequency and occurs in the absence of serotonin, although the magnitude of the response is diminished. This weakened response is enhanced following repeated daily exposure to intermittent hypoxia, via

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DISCLOSURES

All authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Author contributions: S.H., M.A-P., D.M.F., D.M.K., and J.H.M. conception and design of research; S.H., N.H., and J.H.M. performed experiments; S.H., N.H., and J.H.M. analyzed data; S.H., D.M.K., and J.H.M. interpreted results of experiments; S.H., N.H., and J.H.M. prepared figures; S.H. and J.H.M. drafted manuscript; S.H., N.H., M.A-P., D.M.F., D.M.K., and J.H.M. edited and revised manuscript; S.H., N.H., M.A-P., D.M.F., D.M.K., and J.H.M. approved final version of manuscript.

increases in tidal volume, to a similar magnitude evident in $Tph2^{+/+}$ mice. Thus the magnitude of ventilatory LTF following repeated daily exposure to intermittent hypoxia is not dependent on the presence of CNS serotonin.

Keywords

intermittent hypoxia; long-term facilitation; spontaneously breathing mice

Long-term facilitation (LTF) is characterized by an increase in respiratory motor activity that is sustained for up to 60 min following exposure to intermittent hypoxia (28, 29, 32, 33). Phrenic nerve LTF has been observed in a variety of reduced preparations, following acute and repeated daily exposure to intermittent hypoxia (28, 29, 32, 33). Likewise, ventilatory LTF has been recorded in spontaneously breathing, unanesthetized animals (6, 34, 39, 46, 50), including healthy humans (8, 14, 16, 24, 44, 51) and humans with spinal cord injury (48) or sleep apnea (13, 24, 44). Measures of phrenic nerve activity or minute ventilation indicate that LTF, induced by acute and repeated daily exposure to moderate-intermittent hypoxia, is abolished by the administration of serotonergic antagonists (3, 12, 26, 31). These findings have led to a widespread belief that phrenic nerve and ventilatory LTF are induced by stimulation of raphe neurons and the release of serotonin onto phrenic motoneurons. This idea has received compelling support from studies that revealed that the binding of 5-hydroxytryptamine (5-HT-serotonin) to 5-HT₂ receptors on phrenic motoneurons activates a cascade of cellular events, termed the Q pathway (4, 10, 36, 40, 52). This cellular pathway is emerging as a mechanistic foundation for the increase in synaptic strength between medullary bulbospinal neurons and phrenic motoneurons.

Despite these findings, serotonin may not be solely responsible for initiating phrenic or ventilatory LTF, since there is some evidence that other neuromodulators may contribute to the induction of this phenomenon. Norepinephrine may contribute to the induction of LTF, given that selective serotonergic receptor antagonists (i.e., ketanserin), which abolish LTF, also antagonize α_1 adrenergic receptors (7). This possibility is supported further by findings that showed that the pharmacological activation of α_1 receptors induced phrenic (9) and hypoglossal (35) motoneuron facilitation. Thus acute exposure to moderate intensities of intermittent hypoxia could result in the release and binding of norepinephrine to α_1 adrenergic receptors, which in turn, activates the same cellular pathway (i.e., the Q pathway), triggered by binding of serotonin to 5-HT₂ receptors (10, 40). Additional evidence also suggests that a separate cellular pathway, referred to as the S pathway, activated via the binding of adenosine to receptors on phrenic motoneurons, may impair phrenic nerve LTF (21, 36) following exposure to moderate-intermittent hypoxia.

Notwithstanding the results obtained using pharmacological interventions, it remains to be established whether serotonin is or is not the sole mediator of ventilatory LTF in spontaneously breathing animals following exposure to moderate-intermittent hypoxia. Thus in the present investigation, tryptophan hydroxylase 2-deficient ($Tph2^{-/-}$) mice (49), devoid of serotonin in the central nervous system (CNS), and their wild-type (WT) counterparts ($Tph2^{+/+}$) were exposed to intermittent hypoxia each day for 10 consecutive days to

establish if serotonin is the sole neuromodulator responsible for the initiation of ventilatory LTF. We hypothesized that the lack of serotonin would have its greatest effect on the magnitude of ventilatory LTF following acute exposure to intermittent hypoxia and that the absence of serotonin would be inconsequential following repeated daily exposure to intermittent hypoxia.

METHODS

Animals and Protocol

All experiments were approved by the Wayne State University Institutional Animal Care and Use Committee. The experiments were performed on male C57BL/6-129Sv Tph2^{+/+} and Tph2^{-/-} mice. Each group was comprised of 19 mice. The methodology used to breed the Tph2^{-/-} mice in our facility has been described previously (2, 22, 49). Our published work has confirmed that serotonin neurons remain intact, whereas CNS serotonin is abolished in the Tph2^{-/-} mice (2, 22, 49). The Tph2^{+/+} and Tph2^{-/-} mice were housed individually in a controlled environment (6 AM lights on; 6 PM lights off). Temperature and humidity were held within a controlled range, and mice were allowed to eat and drink ad libitum.

A diagram outlining the protocol is shown in Fig. 1. Unanesthetized and unrestrained mice were initially acclimated to plethysmography chambers (model PLY4211; Buxco Research Systems, Wilmington, NC) for 4 h on 4 days leading up to the start of the protocol. Thereafter, the protocol was completed over 10 consecutive days. On each day of the protocol, mice were acclimated to the plethysmography chambers for 50 min beginning at ~7 AM. Subsequently, ventilatory parameters were recorded over a 45-min baseline interval. During the acclimation and baseline periods, the mice breathed room air. Subsequently, the mice were exposed to 12 × 4-min hypoxic episodes (10% oxygen, balance nitrogen), interspersed with 4-min recovery periods. The final recovery period following the 12th hypoxic episode was 45 min in duration. During the recovery periods, the mice breathed room air. The laboratory temperature was maintained at 22–24°C.

Plethysmography

Minute ventilation, tidal volume, breathing frequency, inspiratory time, expiratory time, ventilatory drive (i.e., tidal volume/inspiratory time), and carbon dioxide (CO₂) production were obtained using whole-body, unrestrained plethysmographs (model PLY4211; Buxco Research Systems). Room air or a hypoxic gas mixture (10% oxygen, balance nitrogen) was pumped through the plethysmographs at a rate of 1 l/min, using a bias flow supply (model BFL0100; Buxco Research Systems). Gases were allowed to mix in a metabolism distribution reservoir (model PLY4000; Buxco Research Systems) before entering the plethysmograph chambers. Ventilatory parameters were recorded using a modular accessory unit (model MAX1500; Buxco Research Systems) and preamplifiers (model MAX2275; Buxco Research Systems). The fractional concentration of CO₂ was measured using a CO₂ gas analyzer (model AUT4499; Buxco Research Systems). All parameters were analyzed and recorded using FinePointe software (Buxco Research Systems).

Telemetry

Telemetry transmitters (model TA-F10; Data Sciences International, St. Paul, MN) were surgically implanted into the abdominal cavities of a subset of Tph2^{+/+} ($n = 4$) and Tph2^{-/-} ($n = 4$) mice to measure core body temperature throughout the intermittent hypoxia protocol. Before surgery, mice were anesthetized with an injection of ketamine (80 mg/kg ip) and xylazine (10 mg/kg ip). A supplemental dose (i.e., one-fourth of the original dose) was given if needed. Thereafter, a 1.5- to 2-cm incision was made in the skin and ventral midline of the abdominal muscle fascia, and the transmitter was placed in the peritoneal cavity. The abdominal wall was closed using a 4-0 absorbable suture with a simple interrupted pattern, and the abdominal skin was closed using wound clips. Following surgery, buprenorphine (0.1 mg/kg sc) was administered every 10–12 h and carprofen (5.0 mg/kg sc) once daily for 2 days to reduce postoperative pain. After the mice exhibited normal weight gain, the protocol was initiated.

In a separate set of experiments, Tph2^{+/+} ($n = 5$) and Tph2^{-/-} ($n = 5$) mice were anesthetized with isoflurane. Thereafter, transponders (model IPTT-300; BioMedic Data Systems, Seaford, DE) were implanted subcutaneously between the scapulae of the mice using a syringe-like device with a sterilized disposable needle. Two days after implantation, temperature was recorded. After 30 min of baseline recording at room temperature (22°C), animals were subjected to a cold room (4°C) for 4 h and then returned back to ambient temperature. Temperatures were recorded (model DAS-5001; BioMedic Data Systems) every 15 min throughout the 4-h period.

Data Collection and Analysis

Minute ventilation, tidal volume (11), breathing frequency, inspiratory time, expiratory time, ventilatory drive (i.e., tidal volume/inspiratory time), CO₂ production, and temperature in a subset of mice were measured on each day of the protocol. Minute ventilation, tidal volume, and CO₂ production were normalized to animal weight in grams. During data collection, the ventilatory parameters were averaged using segments comprised of 25 breaths, and CO₂ production was measured for 30 s every 2 min. As a consequence of the limited sampling frequency, CO₂ production measures were not recorded during exposure to hypoxia but were obtained for baseline and recovery measures. Telemetric measures of temperature were recorded every 10 s throughout the intermittent hypoxia protocol. Movements and corresponding times were monitored and recorded throughout each study to eliminate data points associated with movement, thereby excluding the effect of physical activity on minute ventilation during data collection.

Following data acquisition, segments (i.e., baseline, hypoxic episodes, and recovery), with a minimum of 40% of data points recorded during quiet periods, were used in the analysis. This criterion was implemented to ensure that the value representing a given segment was obtained from a sufficient sample size. Average values of the physiological parameters were obtained from the final 2 min of each hypoxic episode and recovery period, with the exception of the end-recovery period. The final 2 min were used to ensure that the gases from the previous episode had been “flushed out” of the plethysmographs. The hypoxic ventilatory response was calculated as the change in minute ventilation divided by the

change in fractional concentration of oxygen. Hypoxic tidal volume and breathing frequency responses were also calculated. Average values for each physiological parameter were determined for 15-min segments from the baseline and endrecovery periods on each day. Given that the measures for each segment were similar, the segmented baseline data were combined, as was the end-recovery data, for purposes of presentation and statistical analysis.

All physiological parameters were presented as absolute values and as a fraction of baseline values. Measured values from end-recovery periods, following exposure to intermittent hypoxia, were normalized to: 1) within-session baseline values (i.e., within a given day) to examine the acute effect of intermittent hypoxia on the measured physiological parameters and 2) baseline values measured on the initial days to examine the cumulative effect of repeated daily exposure to intermittent hypoxia. Data are presented as an average of the initial 2 days compared with the average of the final 2 days.

Statistical Analysis

The age and weight of the mice were compared using a two-way repeated-measures ANOVA in conjunction with a Student-Newman-Keuls post hoc test (GB-STAT 8.0; Dynamic Microsystems, Silver Spring, MD). The factors in the ANOVA were mouse genotype (Tph2^{+/+} vs. Tph2^{-/-}) and days (i.e., initial vs. final days). A similar analysis was used to compare: 1) absolute baseline values of each physiological parameter, 2) the hypoxic ventilatory response among mice on the initial and final days, and 3) core body temperature during the cold challenge. The factors in the ANOVA were mouse genotype (Tph2^{+/+} vs. Tph2^{-/-}) and days (i.e., initial vs. final days) for the baseline and hypoxic ventilatory response analysis. The factors for the temperature analysis were mouse genotype (Tph2^{+/+} vs. Tph2^{-/-}) and time segment (i.e., 15-min time segments). A three-way ANOVA, in conjunction with Student-Newman-Keuls post hoc test, was used to compare absolute or standardized baseline and recovery measures of the physiological parameters along with core body temperature. The factors in the ANOVA were time (baseline vs. recovery), mouse genotype (Tph2^{+/+} vs. Tph2^{-/-}), and days (i.e., initial vs. final days). The data are presented as means $\pm$ SE, and $P < 0.05$ was considered significant.

RESULTS

The Tph2^{+/+} and Tph2^{-/-} groups were comprised of 19 male mice each. All mice in each group completed the protocol. However, on a couple of days, the number of data points recorded during inactive periods did not meet our criteria (see data analysis), and consequently, baseline values for one Tph2^{+/+} and two Tph2^{-/-} mice were not available on the final days of the protocol. Likewise, end-recovery data from one Tph2^{+/+} mouse on the final days of the protocol were absent. In addition, measures of CO₂ production were unavailable from two Tph2^{+/+} and two Tph2^{-/-} mice on the final days of the protocol, due to technical issues associated with the analyzer.

The Tph2^{+/+} mice were 15.0 ± 0.6 wk old and 26.9 ± 0.3 g on the initial day of the protocol. The Tph2^{-/-} mice were 18.7 ± 2.9 wk old and 28.7 ± 0.6 g on the 1st day of the protocol. Two of the Tph2^{-/-} mice, initially used for collection of preliminary data, were substantially older (i.e., 54 wk) than the remaining WT and knockout (KO) mice. Excluding these two

mice, the Tph2^{-/-} mice ($n = 17$) were 14.5 ± 0.4 wk old and 28.2 ± 0.5 g on the initial days of the protocol. The ages of the Tph2^{+/+} and Tph2^{-/-} mice were similar, with or without inclusion of the two older Tph2^{-/-} mice. At the onset of the protocol, the weight of the Tph2^{+/+} was less than the Tph2^{-/-} mice ($P = 0.01$), with the older mice included in the analysis. This difference was eliminated when the older mice were excluded. Despite the difference in age, the responses of the older mice to intermittent hypoxia were similar to the other KO mice; thus their data were included in the results.

Baseline Values: Initial and Final Days

Baseline measures of minute ventilation on the initial days of the protocol (Fig. 2A) were similar in the Tph2^{+/+} and Tph2^{-/-} mice. In contrast, minute ventilation on the final days (Fig. 2A) was greater in the Tph2^{-/-} compared with the Tph2^{+/+} mice ($P = 0.05$). Breathing frequency (Fig. 3A) was decreased ($P = 0.03$) in the Tph2^{-/-} compared with the Tph2^{+/+} mice on both the initial and final days, because of an increased inspiratory time ($P = 0.001$; Fig. 4A), since expiratory time (Fig. 5A) was comparable between the groups. Conversely, tidal volume (Fig. 6A) was increased (initial days, $P = 0.002$; final days, $P = 0.001$) in the Tph2^{-/-} compared with the Tph2^{+/+} mice on both the initial and final days. Baseline measures of ventilatory drive (Fig. 7A) were similar in the Tph2^{+/+} and Tph2^{-/-} mice on the initial and final days, whereas CO₂ production (Fig. 8A) was greater in the Tph2^{-/-} compared with the Tph2^{+/+} mice ($P = 0.03$).

Hypoxic Ventilatory Responses

The Tph2^{-/-} mice had a lower hypoxic ventilatory response than the Tph2^{+/+} mice on the initial days of the protocol ($P = 0.04$; Fig. 9). The response recorded from the Tph2^{-/-} mice was accompanied by a concurrent reduction in the breathing frequency response ($P = 0.01$; data not shown). In contrast, a decline in the tidal volume response was less evident ($P = 0.11$; data not shown). A reduction in the hypoxic ventilatory response from the initial to the final days of the protocol was evident in the Tph2^{-/-} and Tph2^{+/+} mice ($P = 0.001$; Fig. 9). This decline was evident in both breathing frequency (Tph2^{+/+} mice, $P = 0.001$; Tph2^{-/-} mice, $P = 0.01$) and tidal volume ($P = 0.003$) responses to hypoxia. Core body temperature was similar in the Tph2^{+/+} and Tph2^{-/-} mice during exposure to hypoxia (35.0 ± 0.15 vs. 34.6 ± 0.15 ; $n = 4$ in both groups). Likewise, core body temperature during exposure to hypoxia was similar on the initial and final days of the protocol in the Tph2^{+/+} (35.0 ± 0.15 vs. $34.7 \pm 0.19^\circ\text{C}$) and Tph2^{-/-} (34.6 ± 0.15 vs. $34.7 \pm 0.15^\circ\text{C}$) mice. In addition to these similarities, the change in temperature in the Tph2^{+/+} and Tph2^{-/-} mice during exposure to a cold challenge was similar at the start (i.e., 15-min exposure to 4°C ; -0.3 ± 0.5 vs. $-0.4 \pm 1.0^\circ\text{C}$ decrease from baseline) and end (i.e., 4-h exposure to 4°C ; -5.1 ± 1.0 vs. $-4.1 \pm 1.1^\circ\text{C}$ decrease from baseline) of the challenge.

Acute Responses to Intermittent Hypoxia

As the absolute measures indicate, minute ventilation was significantly greater during recovery compared with baseline in the Tph2^{+/+} and Tph2^{-/-} mice ($P = 0.001$) on the initial and final days (Fig. 2A). When standardized to baseline measures on a given day, the increase was greater in the Tph2^{+/+} compared with the Tph2^{-/-} mice ($P = 0.001$; Fig. 2B). This enhancement was unlikely related to differences in metabolic rate, since this measure

was not different between baseline and recovery on the initial or final days in both groups (Fig. 8, *A* and *B*). Alterations in breathing frequency contributed to the enhancement of minute ventilation following exposure to intermittent hypoxia. More specifically, as indicated by the absolute measures, breathing frequency increased during recovery compared with baseline in the $Tph2^{+/+}$ and $Tph2^{-/-}$ animals on the initial and final days ($P = 0.03$; Fig. 3*A*). The increase, however, was more prominent in the $Tph2^{+/+}$ compared with the $Tph2^{-/-}$ mice. This was clearly evident when breathing frequency measures during recovery were standardized to baseline values recorded on the initial or final days in the $Tph2^{+/+}$ and $Tph2^{-/-}$ mice ($P = 0.001$; Fig. 3*B*). The increase in breathing frequency following exposure to intermittent hypoxia on the initial and final days was due to a reduction in both inspiratory ($P = 0.0001$; Fig. 4, *A* and *B*) and expiratory ($P = 0.0001$; Fig. 5, *A* and *B*) time in the $Tph2^{+/+}$ mice. Conversely, the decrease in breathing frequency solely was due to a reduction in inspiratory time ($P = 0.0001$) in the $Tph2^{-/-}$ mice (Fig. 4, *A* and *B*). The decrease in inspiratory time was similar in the $Tph2^{+/+}$ and $Tph2^{-/-}$ mice when recovery values were standardized as a fraction of baseline on the initial or final days (Fig. 4*B*). In contrast, expiratory time was decreased in the WT compared with the KO mice, as evinced by the absolute ($P = 0.001$; Fig. 5*A*) and standardized ($P = 0.002$; Fig. 5*B*) data. Absolute (Fig. 6*A*) and standardized (Fig. 6*B*) measures showed that tidal volume did not increase significantly following acute exposure to intermittent hypoxia on the initial days in the $Tph2^{+/+}$ and $Tph2^{-/-}$ mice. Increases in tidal volume were evident during the recovery periods compared with baseline on the final days of the protocol, although these increases did not manifest as being statistically significant. However, the increase in tidal volume contributed significantly to the enhanced ventilatory drive during recovery on the final days compared with the initial days in the $Tph2^{+/+}$ and $Tph2^{-/-}$ mice, which was evident in the absolute ($P = 0.001$; Fig. 7*A*) and standardized ($P = 0.02$; Fig. 7*B*) data.

Cumulative Responses to Repeated Daily Exposure to Intermittent Hypoxia

To examine the cumulative response of minute ventilation to repeated daily exposure to intermittent hypoxia, values recorded during recovery on the initial days and baseline and recovery values on the final days were standardized relative to baseline measures obtained on the initial days. Baseline and recovery values on the final days were not affected by repeated daily exposure to intermittent hypoxia in the $Tph2^{+/+}$ mice (Fig. 2*C*). In contrast, baseline and recovery values on the final days were greater than measures obtained on the initial days in the $Tph2^{-/-}$ mice ($P = 0.03$; Fig. 2*C*). Consequently, the cumulative minute ventilation response to intermittent hypoxia, as indicated by the standardized recovery values on the final days, was similar between the $Tph2^{+/+}$ and $Tph2^{-/-}$ mice (Fig. 2*C*). The cumulative increase in minute ventilation observed in the $Tph2^{-/-}$ mice, following repeated daily exposure to intermittent hypoxia, was not due to alterations in breathing frequency, since this measure remained unchanged from the initial to final days of the protocol (Fig. 3*C*). Instead, tidal volume was significantly greater during baseline and recovery on the final compared with the initial days in the $Tph2^{-/-}$ mice ($P = 0.001$; Fig. 6*C*). Consequently, the cumulative tidal volume responses measured during baseline and recovery on the final days of the protocol were significantly greater in the $Tph2^{-/-}$ mice compared with the $Tph2^{+/+}$ mice ($P = 0.02$; Fig. 6*C*). Likewise, enhancement of the tidal volume response following repeated daily exposure to intermittent hypoxia was also manifested in the ventilatory drive

measures on the final days that were greater than measures recorded on the initial days ($P = 0.01$; Fig. 7C) and were significantly greater in the $\text{Tph2}^{-/-}$ compared with the $\text{Tph2}^{+/+}$ mice ($P = 0.004$; Fig. 7C) on the final days. The enhanced response observed in the $\text{Tph2}^{-/-}$ compared with the $\text{Tph2}^{+/+}$ mice was not related to changes in CO_2 production (Fig. 8C). Core body temperature during baseline on the initial days ($\text{Tph2}^{+/+} 36.2 \pm 0.2$ vs. $\text{Tph2}^{-/-} 36.0 \pm 0.3^\circ\text{C}$) was not significantly different from recovery values on the initial days ($\text{Tph2}^{+/+} 35.7 \pm 0.1$ vs. $\text{Tph2}^{-/-} 35.2 \pm 0.2^\circ\text{C}$) and baseline ($\text{Tph2}^{+/+} 35.8 \pm 0.1$ vs. $\text{Tph2}^{-/-} 35.6 \pm 0.1^\circ\text{C}$) and recovery values ($\text{Tph2}^{+/+} 35.7 \pm 0.2$ vs. $\text{Tph2}^{-/-} 35.3 \pm 0.1^\circ\text{C}$) on the final days in the $\text{Tph2}^{+/+}$ and $\text{Tph2}^{-/-}$ mice. Moreover, core body temperature during baseline and recovery on the final days was similar to measures of core body temperature during recovery on the initial days in the $\text{Tph2}^{+/+}$ and $\text{Tph2}^{-/-}$ mice.

DISCUSSION

The central findings of our study were that ventilatory LTF following acute exposure to intermittent hypoxia was induced in $\text{Tph2}^{-/-}$ mice; however, the magnitude of the response was reduced. On the other hand, repeated daily exposure to intermittent hypoxia enhanced the magnitude of ventilatory LTF in $\text{Tph2}^{-/-}$ mice beyond that observed in $\text{Tph2}^{+/+}$ mice so that the magnitude of ventilatory LTF was similar in the $\text{Tph2}^{+/+}$ and $\text{Tph2}^{-/-}$ mice during recovery after 10 days of exposure to intermittent hypoxia. Our results also showed that the absence of serotonin in the CNS resulted in a decrease in breathing frequency and an increase in tidal volume under baseline conditions, accompanied by a reduction in the hypoxic ventilatory response.

Methodological Considerations

Enhancement of minute ventilation and its components following exposure to acute and repeated daily intermittent hypoxia and the differences observed between the $\text{Tph2}^{+/+}$ and $\text{Tph2}^{-/-}$ mice could be the result of a number of methodological factors unrelated to the absence of CNS serotonin. The enhanced response in minute ventilation observed within a given group or across groups could be a consequence of increases in metabolic rate from baseline to recovery on a given day or from the initial to the final days. However, metabolic rate did not change from baseline to recovery on a given day or across days in the $\text{Tph2}^{+/+}$ and $\text{Tph2}^{-/-}$ mice. Measures of core temperature were similar in the $\text{Tph2}^{+/+}$ and $\text{Tph2}^{-/-}$ mice on the initial and final days, and supporting data indicated that temperature modulation was similar between the $\text{Tph2}^{+/+}$ and $\text{Tph2}^{-/-}$ mice in response to a cold challenge.

In addition to metabolic rate, it is possible that differences in arousal state between the $\text{Tph2}^{+/+}$ and $\text{Tph2}^{-/-}$ affected the magnitude of ventilatory LTF. However, we carefully documented periods of activity and inactivity throughout the protocol on each day and reported only those findings obtained during inactive periods. Despite similar periods of inactivity, it is possible that differences in arousal state still existed (quiet wakefulness vs. sleep). If this were the case, differences in the percentage of quiet wakefulness and sleep could affect the magnitude of ventilatory LTF, as reported previously for rats (46) and humans (44). However, recent analysis of electroencephalographic recordings in our laboratory revealed that the percentage of quiet wake vs. sleep was similar in $\text{Tph2}^{+/+}$ and

Tph2^{-/-} mice ($n = 12$ in each group; unpublished data). Likewise, measures of temperature during sleep in these animals were similar to values recorded during inactive baseline periods on the initial day of the present study. Thus we are confident that differences in arousal state did not account for our findings. Consequently, we believe that the differences in magnitude of ventilatory LTF following acute and repeated daily exposure were related to the absence of serotonin in the CNS.

Our results do not address if the differences observed between the Tph2^{+/+} and Tph2^{-/-} mice are directly related to the depletion of CNS serotonin in adult mice or are a consequence of developmentally hard-wired abnormalities. Although further exploration is necessary, the preponderance of evidence from published literature indicates that hard-wire abnormalities are less likely to explain our results. Gutknecht and colleagues (15) have reported that the molecular phenotype and electrophysiological properties of raphe neurons are preserved in Tph2^{-/-} mice. Likewise, Alenina and colleagues (1) did not detect significant alterations in the brain structure of Tph2^{-/-} mice using MRI. Moreover, in Lmx1b^{f/f/p} mice, who lack serotonin neurons and content, transient respiratory problems between P2 and P14 are normalized by the administration of a 5-HT_{2A} receptor agonist or with maturation (i.e., between P14 and P28) (20). Consequently, we believe that the differences in magnitude of ventilatory LTF following acute and repeated daily exposure are directly related to the absence of serotonin in the CNS during adulthood. Collectively, our findings provide a solid foundation for generating and testing additional hypotheses in future experimental investigations.

Baseline Measures before Intermittent Hypoxia

Direct measures of baseline ventilatory parameters have not been obtained previously in Tph2^{-/-} mice. Our results show that minute ventilation was similar in the Tph2^{+/+} and Tph2^{-/-} mice. Despite this similarity, inspiratory time was increased, and consequently, breathing frequency was reduced in the Tph2^{-/-} compared with the Tph2^{+/+} mice. In addition, tidal volume was increased in the Tph2^{-/-} mice. Similar baseline values were reported previously for adult Lmx1b^{f/f/p} male and female mice (19), which lack serotonin neurons and content. Likewise, increases in breathing frequency and reductions in tidal volume have been recorded from monoamine oxidase A-deficient mice with elevated levels of serotonin (5). In contrast, results from other studies using Lmx1b^{f/f/p} and *Pet-1*^{-/-} mice reported that tidal volume was unaltered when minute ventilation was unchanged or reduced relative to their WT counterpart (17, 18). These inconsistencies may be due, in part, to inherent differences in the KO models used. However, variations in tidal volume responses have been reported for Lmx1b^{f/f/p} mice (18, 19). Differences might also be due to the age and sex of the mice studied (17). Nonetheless, the consistent finding across all studies, which is independent of the KO model used, is that the absence of serotonin is accompanied by a reduction in breathing frequency (1, 5, 17–19) that is a consequence of increases in inspiratory time. The longer inspiratory time in the Tph2^{-/-} mice suggests that serotonin neurons have a crucial role in controlling inspiratory timing. Serotonin may alter inspiratory time by binding to 5-HT_{1A} receptors on glycinergic neurons. Indeed, Manzke and colleagues (27) showed that 5-HT_{1A} receptor activation on postsynaptic membranes potentiates chloride currents through GlyR α 3 glycine receptors on glycinergic neurons in the

respiratory network. These authors suggested that activation of these currents disinhibits glycine neurons that ultimately result in the activation of inspiration-terminating neurons (27). A deficit in serotonin could lead to inactivation of inspiration-terminating neurons, which could manifest as an increase in inspiratory time and a reduction in breathing frequency—evident in our $Tph2^{-/-}$ mice and other mouse models (i.e., $Lmx1b^{f/f/p}$). On the other hand, since 5-HT_{2B} and 5-HT_{2A} receptor activation stimulates breathing frequency (37), the binding of serotonin to inspiration-terminating neurons, expressing serotonin excitatory receptors, also appears to contribute to increases in breathing frequency (42).

The ventilatory drive during baseline was similar in the $Tph2^{-/-}$ and $Tph2^{+/+}$ mice, because the increase in tidal volume that was evident in the KO mice occurred as a consequence of a prolonged inspiratory time. Our findings are in contrast to the reduced ventilatory drive that was reported previously for $Lmx1b^{f/f/p}$ mice in association with an increasing or unchanging tidal volume (18, 19). Our findings also revealed that baseline measures of metabolic rate, reflected in measures of CO₂ production, were greater in the $Tph2^{-/-}$ compared with the $Tph2^{+/+}$ mice. Our results are similar to findings obtained in serotonin transporter KO mice (25) but are in contrast to an unchanged or reduced metabolic rate (i.e., oxygen consumption) in $Lmx1b^{f/f/p}$ mice (18) and a reduced metabolic rate in $Pet-1^{-/-}$ mice (17).

Hypoxic Ventilatory Response

The $Tph2^{+/+}$ and $Tph2^{-/-}$ mice were exposed to hypoxia under poikilocapnic conditions; thus it is likely that the magnitude of the hypoxic ventilatory response was dampened in both groups, since hypocapnia is known to diminish the magnitude of the hypoxic ventilatory response. Nevertheless, under these conditions, the hypoxic ventilatory response was greater in the $Tph2^{+/+}$ compared with the $Tph2^{-/-}$ mice on the initial and final days of the protocol. Although we did not measure metabolism during the hypoxic episodes, core body temperature was similar in the subset of $Tph2^{+/+}$ and $Tph2^{-/-}$ mice exposed to intermittent hypoxia. Likewise, the reduced response was evident in the $Tph2^{-/-}$ mice, even though previous studies have suggested that CO₂ sensitivity is reduced in serotonin transporter KO (25) and $Lmx1b^{f/f/p}$ mice (19). Given this scenario, the reduced sensitivity to hypocapnia under poikilocapnic conditions should serve to prevent the diminution of the hypoxic ventilatory response. However, despite this favorable scenario, the hypoxic ventilatory response was depressed in the $Tph2^{-/-}$ mice. Thus we believe the reduced response could be a consequence of the absence of CNS serotonin. It is difficult to compare our results directly with previous studies that examined the link between serotonin and the hypoxic ventilatory response in mice, since we used brief episodes (i.e., 4 min) of intermittent hypoxia, whereas previous studies used sustained levels of hypoxia. Nevertheless, previous studies have reported that the hypoxic ventilatory response is reduced following the administration of a serotonergic antagonist (38). This has also been reported for $Pet-1^{-/-}$ (17) and $Lmx1b^{f/f/p}$ (18) mice. The reduced response in the KO mice was proposed to be linked to a reduced oxygen consumption and core body temperature. However, these parameters were similar in male $Pet-1^{-/-}$ and WT mice (17) during exposure to hypoxia, which suggests that reductions in serotonin alone can account for the observed depression of the hypoxic ventilatory response. In contrast, work completed in serotonin transporter KO mice reported that the

hypoxic ventilatory response was higher compared with their WT counterpart, but this increase was linked to an increase in core body temperature and oxygen consumption (25).

The mice in our study did not display progressive augmentation (28, 29) on any given day. This was somewhat surprising, given that studies have reported that unanesthetized rats, goats, ducks, and humans display progressive augmentation when administered intermittent hypoxia (28, 29). Our intermittent hypoxia protocol was not drastically different in intensity, duration, or number of episodes from other intermittent hypoxia protocols that induced progressive augmentation in other species, so it appears that mice are an exception. It is possible that a greater hypoxic intensity is required to elicit progressive augmentation in mice. In anesthetized rats, severe [partial pressure of oxygen in arterial blood (P_{aO_2}), 25–30 mmHg] but not moderate-intermittent (P_{aO_2} , 45–50 mmHg) hypoxia is required to elicit progressive augmentation (36).

Our results also showed that the hypoxic ventilatory response decreased from the initial to the final days of the protocol in both groups of mice. This decline occurred throughout the protocol in an exponential fashion (results not shown). A variety of factors might be responsible for the decline, including a decrease in metabolism or a progressive decline in CO_2 levels, although similar measures of core body temperature on the initial and final days suggest that alterations in metabolic rate were not responsible for the decline. Another possibility is that the sensitivity to hypoxia decreased over the 10-day protocol. This decline has been observed in some species exposed to chronic sustained hypoxia (45) but differs from results that indicated that chronic intermittent hypoxia typically augments the hypoxic ventilatory response (13, 26, 41). Further investigation is required to determine the mechanisms responsible for this decline.

Long-Term Facilitation

Response to acute exposure to intermittent hypoxia—Our results showed that minute ventilation increased following exposure to intermittent hypoxia on a given day in the $Tph2^{+/+}$ and $Tph2^{-/-}$ mice. The increase was due to a surge in breathing frequency, which was a consequence of a reduction in inspiratory time in both groups of mice, as well as a reduction in expiratory time in the $Tph2^{+/+}$ mice. No increases in tidal volume were evident. Consequently, the increase in ventilatory drive that was evident was principally a result of reductions in inspiratory time. Thus alterations in respiratory pattern formation at the brain stem level contributed significantly to inducing LTF following acute exposure to intermittent hypoxia. Our results coincide with previous findings that have reported that breathing frequency, rather than tidal volume, contributes to ventilatory LTF following acute exposure to intermittent hypoxia in unanesthetized mice (23, 47). Similar findings have been reported in other animals (32), although more recent studies in unanesthetized rats have indicated that the contribution of tidal volume to ventilatory LTF may be dependent on the maintenance of CO_2 levels and arousal state (46). Even though ventilatory LTF was evident in the $Tph2^{+/+}$ and $Tph2^{-/-}$ mice, the magnitude of the response was greater in the $Tph2^{+/+}$ mice.

The ventilatory LTF that was induced in each group could be a consequence of differences in metabolic rate or arousal state between baseline and recovery. Likewise, these factors

could also be responsible for the difference in magnitude between groups. However, we believe this to be unlikely, as outlined above (see Methodological Considerations). Instead, we believe our findings indicate that ventilatory LTF can be induced following acute exposure to intermittent hypoxia in the absence of CNS serotonin. Thus other neuromodulators likely have a role in inducing ventilatory LTF in spontaneously breathing mice exposed to moderate forms of intermittent hypoxia. One possible candidate is norepinephrine, since recent studies using reduced preparations indicated that $\alpha 1$ -adrenergic receptor activation can induce motoneuron facilitation, independent of serotonergic receptor activation (9, 35). Moreover, published findings indicate that $\alpha 1$ -adrenergic and serotonin receptors may converge on a common pathway (i.e., Q pathway) to induce LTF following exposure to moderate hypoxia (10). These neuromodulators may interact with each other to elicit a maximum response to intermittent hypoxia, as indicated by the reduction in magnitude of ventilatory LTF in $Tph2^{-/-}$ mice. Thus the presence of neuromodulators, in addition to serotonin, may be necessary to evoke a maximum response following acute exposure to intermittent hypoxia.

Cumulative response to repeated daily exposure to intermittent hypoxia—To examine the effect of repeated daily exposure to intermittent hypoxia on the magnitude of ventilatory LTF, we normalized the baseline and recovery data measured on the final days to baseline on the initial days. Repeated daily exposure to intermittent hypoxia did not have a cumulative effect on the magnitude of ventilatory LTF in the $Tph2^{+/+}$ mice. Although enhancement of ventilatory LTF was ambiguous, absolute measures of tidal volume were greater on the final compared with the initial days. Likewise, the increase in tidal volume manifested itself in the ventilatory drive, which was greater on the final compared with the initial days in the $Tph2^{-/-}$ mice. The reasons that ventilatory LTF did not manifest clearly in the $Tph2^{-/-}$ mice remain to be determined but could be related to the intensity and duration of the intermittent hypoxic protocol. For example, previous protocols that used repeated daily exposures to intermittent hypoxia in mice (43) and rats (30, 31) to initiate ventilatory LTF were applied for longer periods of time on a given day (i.e., 12 h) and for longer than 10 days. However, this cannot be the sole reason for the rather mild response to repeated daily exposure in the $Tph2^{-/-}$ mice, since a more robust response was evident in the $Tph2^{-/-}$ mice (see subsequent paragraph).

The effect of repeated daily exposure on ventilatory LTF was clearly evident in the $Tph2^{-/-}$ mice, since normalized minute ventilation during baseline and recovery on the final days was greater compared with the initial days. In contrast to the enhanced breathing frequency response that occurred concomitantly with ventilatory LTF following acute exposure to intermittent hypoxia, the cumulative increase in minute ventilation was the consequence of increases in tidal volume that were evident during baseline and recovery on the final compared with the initial days. This increase, coupled with an unchanging inspiratory time, reflected an enhanced ventilatory drive during baseline and recovery on the final compared with the initial days in the $Tph2^{-/-}$ mice. In addition to exceeding measures on the initial days, the normalized minute ventilation, tidal volume, and ventilatory drive in the $Tph2^{-/-}$ mice also exceeded the magnitude of these parameters in the $Tph2^{+/+}$ mice on the final days of the protocol. Consequently, although the magnitude of ventilatory LTF was less in the

Tph2^{-/-} compared with the Tph2^{+/+} mice on a given day, this weakened response was enhanced following repeated daily exposure to intermittent hypoxia. Consequently, a similar magnitude in ventilatory LTF was evident in Tph2^{+/+} and Tph2^{-/-} mice on the final days of recovery compared with baseline on the initial days. Previous studies completed in mice (43) and rats (31) are in agreement with our findings, since repeated daily exposure to intermittent hypoxia resulted in concomitant increases in tidal volume and minute ventilation. It is also interesting to note that this response was unlike the initiation of ventilatory LTF following acute exposure to intermittent hypoxia, which was mediated by increases in breathing frequency (31), a result that is similar to our findings.

Our results suggest that the magnitude of ventilatory LTF, following repeated daily exposure to intermittent hypoxia, may not be dependent on the presence of CNS serotonin. Likewise, the enhanced tidal volume response indicates that spinal mechanisms may be responsible for the ventilatory LTF observed following repeated exposure to intermittent hypoxia. The neuromodulator(s) and pathways responsible for the enhanced response in the Tph2^{-/-} mice remain to be identified. However, Devinney and colleagues (10) have recently proposed a variety of hypothetical mechanisms that could explain the enhanced response. One possible scenario is that the absence of serotonin released the S pathway from Q-pathway inhibition, which allowed other neuromodulators that activate the S pathway (e.g., adenosine) to enhance ventilatory LTF.

Conclusion

Our findings showed that ventilatory LTF in unanesthetized, spontaneously breathing mice can be induced following exposure to acute intermittent hypoxia, despite the absence of serotonin in the CNS. However, the magnitude of the response was reduced in the absence of serotonin. Therefore, the optimal response to acute exposure to intermittent hypoxia may reflect an interaction between serotonin and other neuromodulators. Moreover, enhancement of ventilatory LTF following repeated daily exposure to intermittent hypoxia was greatest in the absence of CNS serotonin. Consequently, the cumulative magnitude of ventilatory LTF following repeated daily exposure to intermittent hypoxia was independent of CNS serotonin. Overall, our findings suggest that other neuromodulators, besides serotonin, contribute to initiating ventilatory LTF in unanesthetized, spontaneously breathing mice.

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Fig. 1.

Protocol. *Top:* mice were acclimated to plethysmograph chambers before the onset of the protocol. Thereafter, mice were exposed to the intermittent hypoxia protocol each day for 10 consecutive days. *Bottom:* the intermittent hypoxia protocol consisted of a 50-min acclimation period, followed by a 45-min baseline period. The mice were then exposed to 12 $\times$ 4-min hypoxic episodes, interspersed with normoxic recovery periods. Following the final hypoxic episode, the physiological parameters were measured for 45 min.

Fig. 2.

Absolute and normalized measures of minute ventilation. *A*: average values for minute ventilation recorded during baseline and recovery on the initial and final days of the protocol in the tryptophan hydroxylase 2 wild-type (Tph2^{+/+}) and Tph2-deficient (Tph2^{-/-}) mice. *B*: average values for minute ventilation recorded during recovery and normalized to baseline values (dashed, horizontal line) on each day in Tph2^{+/+} and Tph2^{-/-} mice. This normalization procedure was completed to examine the acute effect of intermittent hypoxia on a given day of the protocol. *C*: average values for minute ventilation obtained from Tph2^{+/+} and Tph2^{-/-} mice during baseline and recovery on the final days were normalized to baseline values (dashed, horizontal line) on the initial days of the protocol. This normalization procedure was completed to examine the cumulative effect of intermittent hypoxia over the 10-day protocol. §significantly different from baseline; *significantly different from Tph2^{+/+} mice on a given day; †significantly different from initial days.

Fig. 3.

Absolute and normalized measures of breathing frequency. *A*: average values for breathing frequency recorded during baseline and recovery on the initial and final days of the protocol in the Tph2^{+/+} and Tph2^{-/-} mice. *B*: average values for breathing frequency recorded during recovery and normalized to baseline values (dashed, horizontal line) on each day in Tph2^{+/+} and Tph2^{-/-} mice. This normalization procedure was completed to examine the acute effect of intermittent hypoxia on a given day of the protocol. *C*: average values for breathing frequency obtained from Tph2^{+/+} and Tph2^{-/-} mice during baseline and recovery on the final days were normalized to baseline values (dashed, horizontal line) on the initial days of the protocol. This normalization procedure was completed to examine the cumulative effect of intermittent hypoxia over the 10-day protocol. §significantly different from baseline; *significantly different from Tph2^{+/+} mice on a given day.

Fig. 4.

Absolute and normalized measures of inspiratory time. *A*: average values for inspiratory time recorded during baseline and recovery on the initial and final days of the protocol in the Tph2^{+/+} and Tph2^{-/-} mice. *B*: average values for inspiratory time recorded during recovery and normalized to baseline values (dashed, horizontal line) on each day in Tph2^{+/+} and Tph2^{-/-} mice. This normalization procedure was completed to examine the acute effect of intermittent hypoxia on a given day of the protocol. *C*: average values for inspiratory time obtained from Tph2^{+/+} and Tph2^{-/-} mice during baseline and recovery on the final days were normalized to baseline values (dashed, horizontal line) on the initial days of the protocol. This normalization procedure was completed to examine the cumulative effect of intermittent hypoxia over the 10-day protocol. §significantly different from baseline; *significantly different from Tph2^{+/+} mice.

Fig. 5.

Absolute and normalized measures of expiratory time. *A*: average values for expiratory time recorded during baseline and recovery on the initial and final days of the protocol in the Tph2^{+/+} and Tph2^{-/-} mice. *B*: average values for expiratory time recorded during recovery and normalized to baseline values (dashed, horizontal line) on each day in Tph2^{+/+} and Tph2^{-/-} mice. This normalization procedure was completed to examine the acute effect of intermittent hypoxia on a given day of the protocol. *C*: average values for expiratory time obtained from Tph2^{+/+} and Tph2^{-/-} mice during baseline and recovery on the final days were normalized to baseline values (dashed, horizontal line) on the initial days of the protocol. This normalization procedure was completed to examine the cumulative effect of intermittent hypoxia over the 10-day protocol. §significantly different from baseline; *significantly different from Tph2^{+/+} mice on a given day.

Fig. 6.

Absolute and normalized measures of tidal volume. *A*: average values for tidal volume recorded during baseline and recovery on the initial and final days of the protocol in the Tph2^{+/+} and Tph2^{-/-} mice. *B*: average values for tidal volume recorded during recovery and normalized to baseline values (dashed, horizontal line) on each day in Tph2^{+/+} and Tph2^{-/-} mice. This normalization procedure was completed to examine the acute effect of intermittent hypoxia on a given day of the protocol. *C*: average values for tidal volume obtained from Tph2^{+/+} and Tph2^{-/-} mice during baseline and recovery on the final days were normalized to baseline values (dashed, horizontal line) on the initial days of the protocol. This normalization procedure was completed to examine the cumulative effect of intermittent hypoxia over the 10-day protocol. *significantly different from Tph2^{+/+} mice on a given day; †significantly different from initial days.

Fig. 7.

Absolute and normalized measures of ventilatory drive. **A:** average values for ventilatory drive recorded during baseline and recovery on the initial and final days of the protocol in the Tph2^{+/+} and Tph2^{-/-} mice. **B:** average values for ventilatory drive recorded during recovery and normalized to baseline values (dashed, horizontal line) on each day in Tph2^{+/+} and Tph2^{-/-} mice. This normalization procedure was completed to examine the acute effect of intermittent hypoxia on a given day of the protocol. Note that ventilatory drive during recovery was greater than baseline on a given day in both groups. **C:** average values for ventilatory drive obtained from Tph2^{+/+} and Tph2^{-/-} mice during baseline and recovery on the final days were normalized to baseline values (dashed, horizontal line) on the initial days of the protocol. This normalization procedure was completed to examine the cumulative effect of intermittent hypoxia over the 10-day protocol. §significantly different from baseline; *significantly different from Tph2^{+/+} mice; †significantly different from initial days.

Fig. 8.

Absolute and normalized measures of carbon dioxide (CO₂) production. *A*: average values for CO₂ production recorded during baseline and recovery on the initial and final days of the protocol in the Tph2^{+/+} and Tph2^{-/-} mice. *B*: average values for CO₂ production recorded during recovery and normalized to baseline values (dashed, horizontal line) on each day in Tph2^{+/+} and Tph2^{-/-} mice. This normalization procedure was completed to examine the acute effect of intermittent hypoxia on a given day of the protocol. *C*: average values for CO₂ production obtained from Tph2^{+/+} and Tph2^{-/-} mice during baseline and recovery on the final days normalized to baseline values (dashed, horizontal line) on the initial days of the protocol. This normalization procedure was completed to examine the cumulative effect of intermittent hypoxia over the 10-day protocol. *significantly different from Tph2^{+/+} mice.

Fig. 9.

Hypoxic ventilatory response. Average values for the hypoxic ventilatory response measured on the initial and final days in the Tph2^{+/+} and Tph2^{-/-} mice. *significantly different from Tph2^{+/+} mice on a given day; †significantly different from initial days.