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Enhanced non-eupneic breathing following hypoxic, hypercapnic or hypoxic-hypercapnic gas challenges in conscious mice

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Abstract

C57BL6 mice display non-eupneic breathing and spontaneous apneas during wakefulness and sleep as well as markedly disordered breathing following cessation of a hypoxic challenge. We examined whether (1) C57BL6 mice display marked non-eupneic breathing following hypercapnic or hypoxic-hypercapnic challenges, and (2) compared the post-hypoxia changes in non-eupneic breathing of C57BL6 mice to those of B6AF1 (57BL6 dam × A/J sire) and Swiss-Webster mice, which display different ventilatory responses than C57BL6 mice. C57BL6 mice displayed marked increases in respiratory frequency and non-eupneic breathing upon return to room-air after hypoxic (10% O_2, 90% N_2), hypercapnic (5% CO_2, 21% O_2, 74% N_2) and hypoxic-hypercapnic (10% O_2, 5% CO_2, 85% N_2) challenges. B6AF1 mice displayed less tachypnea and reduced non-eupneic breathing post-hypoxia, whereas Swiss-Webster mice displayed robust tachypnea with minimal increases in non-eupneic breathing post-hypoxia. These studies demonstrate that non-eupneic breathing increases after physiologically-relevant hypoxic-hypercapnic challenge in C57BL6 mice and suggest that further studies with these and B6AF1 and Swiss-Webster mice will help define the genetics of non-eupneic breathing.

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Conflicts of Interest
None

Keywords

Non-eupneic breathing; hypoxic-hypercapnic gas challenge; C57BL6 mice; B6AF1 mice; Swiss-Webster mice

1. Introduction

Sleep-related breathing disorders during sleep states and periods of wakefulness affect 9-14% of the US population (Dempsey et al., 2010). Studies in normal and transgenic mice have been used widely as models of sleep apnea (see Yamauchi et al., 2010). The C57BL6 mouse is a common inbred strain that is widely used in ventilatory and pulmonary function studies (Soliz et al., 2008, 2009) and to produce mice lacking genes for numerous functional proteins (Kline et al., 2002; Liu et al., 2004; Duling et al., 2006; Palmer et al., 2013b). The C57BL6 mouse was presumably chosen to be a model with “normal” physiology and indeed they display many normal traits (see Tankersley et al., 2002a; Campen et al., 2004, 2005; Tewari et al., 2013; Palmer et al., 2013a,b; Gaston et al., 2014). For example, resting systemic and pulmonary arterial blood pressures and cardiovascular responses upon challenges with hypoxic (HX), hypercapnic (HC) and hypoxic-hypercapnic (H-H) gas mixtures are representative of those observed in “normal” mice and rats (Campen et al., 2004, 2005; Tewari et al., 2013). Moreover, the ability of HC to modulate the effects of HX on arterial blood pressure (HX elicits pronounced depressor response, HC elicits a minor pressor response, H-H elicits a minimal response) appears to be intact in C57BL6 mice (Campen et al., 2004, 2005).

Following exposure of mice to a HX challenge, the return to room-air results in respiratory patterns classified as short-term potentiation (STP), in which ventilation remains elevated (Powell et al., 1998) or post-hypoxic frequency decline (PHFD) in which breathing frequency falls below baseline (Dick and Coles, 2000). One mechanism for initiation and propagation of unusual breathing patterns is the disturbed balance between STP and PHFD with the dominance of PHFD (i.e., absence of STP and expression of PHFD) promoting disordered breathing (Powell et al., 1998; Yamauchi et al., 2008a,b,c; Strohl, 2003; Younes, 2008; Yamauchi et al., 2010). Eupnea (quiet breathing) is the term for normal ventilation (see Mitchell et al., 1975). Eupnea reflects the output of a pontomedullary neuronal circuit that drives inspiratory motor neurons (St-John and Paton, 2004; Garcia et al., 2011). During eupneic breathing, neural output to respiratory muscles is regular, with rhythmic bursts of motor nerve activity to the diaphragm and external (but not internal) intercostal muscles causing inspiration. Expiration during eupneic breathing is passive and occurs via simple elastic recoil of the lungs (Mitchell et al., 1975; St-John and Paton, 2004; Garcia et al., 2011). In contrast, disordered breathing is characterized by abnormal breaths (disturbed balance of inspiratory and expiratory volume per breath) during rest, and the presence of recurrent spontaneous apneas (pauses), sighs and sniffs, and irregular breaths due to abrupt movements such as grooming and locomotor activity (Han and Strohl, 2000; Han et al., 2001, 2002; Price et al., 2003; Strohl, 2003; Yamauchi et al., 2007, 20008a,b,c, 2010; Chai et al., 2011; Gillombardo et al., 2012; Strohl et al., 2012).

The mechanisms responsible for post-HX disordered breathing have received considerable attention. At present, evidence is in favor of disturbances in central signaling (Wilkinson, 1997; Strohl, 2003) including the pons (Coles and Dick, 1996; Dick and Coles, 2000) rather than processes within the carotid bodies (Vizek et al., 1987; Brown et al., 1993) although it is evident that carotid body chemoafferents play an essential role in the expression of sleep apnea (Smith et al., 2003). Despite considerable normal physiology, the C57BL6 mouse is of major interest to sleep-apnea researchers because it displays disordered breathing (irregular breaths and irregular breathing patterns including apneas, sighs, sniffs) during sleep and wakefulness and markedly disordered breathing upon return to room-air after exposure to HX gas challenges (Han et al., 2000, 2001, 2002; Tagaito et al., 2001; Yamauchi et al., 2008a,b,c, 2010). Post-HX breathing in sleep-apnea patients is associated with episodes of glottal closures (Dempsey et al., 2010; Strohl et al., 2012). The finding that the working heart-brainstem preparation from C57BL6 mice displays spontaneous central apneas associated with active laryngeal closure (Stettner et al., 2008) further supports the concepts that disordered breathing in conscious unrestrained C57BL6 mice include obstruction of the upper airway, and that this strain is a true model of sleep apnea (Stettner et al., 2008).

The genetic bases for sleep disordered breathing in mouse strains have received extensive investigation (Tankersley et al., 1994, 1998, 2000, Tankersley, 2000, 2001, 2003; Han and Strohl, 2000; Han et al., 2001, 2002; Tagaito et al., 2001; Schneider et al., 2003; Strohl, 2003; Tankersley and Broman, 2004; Balbir et al., 2006; Yamauchi et al., 2008b; Gillombardo et al., 2012) as have the neurochemical processes (Tankersley et al., 2002b; Price et al., 2003; Groeben et al., 2005; Yamauchi et al., 2007, 2008a, 2012; Moore et al., 2012, 2014), and structural features of respiratory structures such as the carotid bodies (Yamaguchi et al., 2003, 2006; Chai et al., 2011).

Despite intensive investigation of post-HX breathing, the possibility that disordered breathing in C57BL6 mice increases upon return to room-air following exposures to HC challenges and especially more physiological/pathophysiological relevant H-H challenges (see Dempsey et al., 2010) has received minimal attention (see Yamauchi et al., 2007, 2008c). Analyses of disordered breathing patterns following return to room-air after HX, HC and H-H challenges will allow for examination of the dynamic interplay of HX and HC on the occurrence of apneas, sighs and irregular breathing per se. Moreover, the issue as to whether the actual level of the frequency of breathing ($\mathbf{f_R}$) is associated with or is essential to the expression of disordered breathing upon return to room-air after HX, HC or H-H challenges is not settled (Han et al., 2001, 2002; Tagaito et al., 2001; Yamauchi et al., 2008a,b, 2010). The analysis of breathing patterns is a complicated process that certain groups have mastered (e.g., Tankersley et al., 1994, 1998, 2000, Tankersley, 2000, 2001, 2003; Han and Strohl, 2000; Han et al., 2001, 2002; Tagaito et al., 2001; Schneider et al., 2003; Strohl, 2003; Tankersley and Broman, 2004; Balbir et al., 2006; Yamauchi et al., 2008b; Gillombardo et al., 2012). One less intense (and less informative) method to examine disordered breathing is the Rejection Index (Rinx) feature in whole body plethysmography systems including the Buxco system recently acquired by Data Sciences International (St. Paul, MN). As will be described, the Buxco system allows the investigator to set key

ventilatory parameters to include or reject irregular breathing patterns, apneas, sighs and sniffs. The number of rejected breaths in each recording epoch (e.g., 15 sec) is counted and presented as the Rinx. To our knowledge, the applicability of this method of detecting disordered breathing has not been examined previously. In strict terms, Rinx does not give information about individual breathing events (e.g., apneas, sniffing) but rather is an index of the time that is spent in non-eupneic breathing rather than an actual measurement of the events that define disordered breathing. As such, will use the term disordered breathing only when events such as apneas and sighs were actually determined (including the post-HX phase in C57BL6 mice of the present study). We will use the term "non-eupneic breathing" when referring to our findings pertaining to Rinx that were not accompanied by actual calculations of individual events.

The aims of this study were to determine whether (1) an increase in the level of non-eupneic breathing occurs upon return to room-air after HC or H-H challenges as well as HX challenge, (b) Rinx is applicable for the detection of non-eupneic breathing at rest, during HX-challenge and upon return to room-air in conscious C57BL6 mice, and (c) the level of f_R is a determinant of Rinx before and during the HX and post-HX phases in C57BL6 mice, Swiss-Webster mice, and in B6AF1 mice which are derived from a C57BL6 dam and an A/J sire. The data from the three strains is presented because they demonstrate that Rinx and thereby non-eupneic breathing is not simply a function of the level of f_R. It should be noted that unlike C57BL6 mice, the B6AF1 and A/J mouse show little spontaneous or post-HX-associated disordered breathing (Han et al., 2002, Yamauchi et al., 2008a,b) and we serendipitously determined that Swiss Webster mice displayed substantial post-HX elevations in f_R that were accompanied by minimal increases in Rinx.

2. Methods

2.1. Mice

All studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996. The protocols were approved by the Animal Care and Use Committees of the University of Virginia, and Case Western Reserve University. Adult male C57BL6 mice and B6AF1 mice were from Jackson Laboratories (Bar Harbor, ME). Male Swiss-Webster mice were from Harlan (Indianapolis, IN). The mice were exposed to the protocols when they were approximately 90 days of age to eliminate development influences on the results. It should be noted that corrections for body weights are not necessary for the parameters under investigation in this study, namely f_R and Rinx (although volume parameters were computed to determine Rinx, each mouse served as its own control).

2.2. Whole-body plethysmography

Ventilatory parameters were recorded in conscious mice by whole body plethysmography (PLY3223; Data Sciences International, St. Paul, MN) as described previously (Palmer et al., 2013a,b, 2014; Gaston et al., 2014). The parameters were f_R; tidal volume (V_T) , minute ventilation (V_E), inspiratory time (T_I), expiratory time (T_E) and peak inspiratory (PIF) and peak expiratory (PEF) flows. The provided software (Fine Pointe, BUXCO) constantly

corrected digitized values for changes in chamber temperature and humidity. Pressure changes associated with the respiratory waveform were converted to volumes (i.e., V_T, PIF and PEF) using the algorithm of Epstein and colleagues (Epstein and Epstein, 1978; Epstein et al., 1980). More specifically, factoring in chamber temperature and humidity, the cycle analyzers filtered the acquired signals, and algorithms (Fine Pointe, BUXCO) generated an array of box flow data that identified a waveform segment as an acceptable breath. From that data vector, the minimum and maximum values were determined. The flows at this point were box-flow signals. From this array, the minimum and maximum box flow values were determined and then multiplied by the compensation factor provided by the selected algorithm (Epstein and Epstein, 1978; Epstein et al., 1980), thus producing V_T, PIF and PEF that are used to determine accepted and rejected waveforms. In all protocols described below, the conscious unrestrained mice were placed in the plethysmography chambers and allowed at least 60 min to acclimatize before exposure to the gas challenges.

2.3 Protocols for gas challenges

2.3.1. Study 1—Conscious unrestrained C57BL6 mice (n=16) were exposed to a hypoxic (10% O_2, 90% N_2) challenge for 5 min after which time they were re-exposed to room air for 15 min.

2.3.2. Study 2—Conscious unrestrained C57BL6 mice were exposed to either a hypoxic (10% O_2, 90% N_2; n= 16 mice), hypercapnic (5% CO_2, 21% O_2, 74% N_2, n=16) or hypoxic-hypercapnic challenge (5% CO_2, 10% O_2, 85% N_2, n=16) for 15 min after which time they were re-exposed to room air for 15 min.

2.3.3. Study 3—Conscious unrestrained C57BL6 mice, Swiss Webster mice and B6AF1 mice (n=16 mice per group) were exposed to a hypoxic (10% O_2, 90% N_2; n= 16 mice) challenge for 15 min after which time they were re-exposed to room air for 15 min.

2.4. Rejection Index

The Buxco system includes a Rinx algorithm that was set to reject breaths that did not reflect normal tidal volume breathing and as such typically rejected (a) abnormal breaths (abnormal balance of inspiratory and expiratory volumes), apneas, sighs, post-sighs, sniffs and waveforms that most likely arose from activities such as grooming the face and hands, and rearing. For each accepted breath, the soft-ware determined the number of rejected breaths since the last accepted breath and calculated the percentage of breaths accepted. This percentage of accepted breaths is then aver-aged over each epoch (e.g. 15 s) to arrive at the value reported asRinx. In the present studies, minimum V_t was set at 0.05 ml, mini-mum T_i was set at 0.04 s, maximum T_e was set at 0.5 s, the ratio of exspiratory volume/inspiratory volume (volume balance) was set to the range of 60% to 140%. Values were rejected when they fell outside the above criteria and when (a) T_i was greater than two times T_e, (b) PIF could not be distinguished from PEF, (c) expiratory flow at 50% V_t, time to maximum expiratory flow, relaxation time or conditioning coefficient could not be computed. Using in-house soft-ware programs, analyses of the waveforms over 5 min periods before and 5–10 min after return to room-air following HX challenge was performed on 16 C57BL6 mice (88 ± 2 days of age) to compare the values with Rinx values generated by the Buxco soft-ware

for these mice. One mouse was tested at a time in order to allow monitoring of behaviors (see below).

2.6. Activity Scores

The Activity Scores during the 5 min periods before and 5-10 min after return to room-air following HX challenge were performed on the same 16 C57BL6 mice (88 ± 2 days of age) used to determine non-eupneic breathing events (see above). Initial analyses in which we simultaneously monitored behaviors of mice in the plethysmography chambers and the accompanying respiratory wave-forms, we determined that non-eupneic breathing was most marked during body and/or paw grooming, and progressively less intense during moving and rearing, moving or head movements. Accordingly, the observers (2 per mouse) were asked to note the presence of these activities during each 15 sec epoch. Each mouse was also simultaneously filmed with a VIXIA HF R500 HD Flash Memory Camcorder (Canon Model: 9176B001s) so that (1) the behaviors displayed by each mouse could be scored by another two observers blinded as to (1) which of the 16 mice was being viewed, and (2) whether the recording was from the pre- or post-HX phases. The recorded behaviors were then given a score based on our initial findings regarding the increasing magnitude and duration of effects of each behavior on the plethysmography wave-form. The scores ascribed to each behavior were, head movements (score 1), moving (score 2), moving and rearing (score 3), and episodes of body and/or paw grooming (score 4). The four sets of results from each mouse per epoch were averaged.

2.6. Statistics

All data are presented as mean ± SEM. To determine the total responses (cumulative %changes from pre-hypoxia values) during gas challenge and return to room air for each mouse, we summed the values recorded before and during the challenge and those upon return to room air. We then determined the cumulative response by the formula, total response (%change) = {[(sum of values during hypoxic challenge or return to room air) − (sum of values before hypoxic challenge)]/sum of values before hypoxic challenge} × 100. We then determined the mean and SEM of the group data. All data were analyzed by one-way or two-way ANOVA followed by Student's modified t-test with Bonferroni corrections for multiple comparisons between means (Palmer et al., 2013a,b).

3. Results

3.1. Relationship between Rinx and disordered breathing

The total duration of spontaneous pauses (apneas), post-sighs, irregular breaths and sniffing determined by our in-house analyses was increased during the post-HX period (i.e., return to room-air after HX challenge) whereas the time due to movement artifacts was not different between the two periods (**Table 1**). The total times of disordered breathing before and during the post-HX period were consistent with the Rinx values determined by the plethysmography software (see last two rows of **Table 1**).

3.2. Hypoxic challenge in C57BL6 mice

Typical examples of resting f_R (values were those not rejected by the Buxco soft-ware), Rinx and Activity Score of a conscious C57BL6 mouse before, during and after a 5 min exposure to the HX gas (10% O_2, 90% N_2) challenge are shown in the left hand columns of **Figure 1**. Prior to HX, resting f_R was stable at about 170 breaths/min and Rinx was stable at about 15%. Within some 15 sec recording periods, the mouse displayed episodes of head movements (score 1), moving (score 2), moving and rearing (score 3), or body and/or paw grooming (score 4). This activity was not overtly influential on Rinx suggesting that the majority of rejected breaths were not due to movement or grooming which can elicit an artifactual effect on breathing waveforms. Consistent with established findings, exposure of the C57BL6 mice to HX challenge elicited an initial increase in f_R that waned substantially during the challenge, a phenomenon known as “roll-off” (Palmer et al., 2013a,b; Gaston et al., 2014). Rinx initially increased during the HX challenge but subsided substantially well before roll-off in f_R was prominent. Again, the sporadic increases in Activity Score were not obviously related to Rinx. Upon return to room-air, there was an abrupt increase in f_R and Rinx that was accompanied by sporadic increases in Activity Score. Rinx remained elevated during many time periods in which Activity Score was zero.

A summary of findings with 16 male C57BL6 mice is shown in the right-hand panels of **Figure 1**. The major conclusions regarding Rinx were: (1) Rinx occurs about 15% of time in conscious unrestrained C57BL6 mice, (2) Rinx increases during the initial exposure to hypoxic gas but declines rapidly to baseline values while f_R is still elevated, (3) Rinx is markedly elevated following return to room-air, and (4) the steady state Rinx and the changes during and following HX challenge were not obviously related to Activity Score.

3.3. Comparison of hypoxic, hypercapnic or hypoxic-hypercapnic challenges in C57BL6 mice

The summaries of f_R, Rinx and Rinx/f_R values of conscious C57BL6 mice recorded before, during and following a 5 min exposure to a (1) HX challenge (10% O_2, 90% N_2), (2) HC challenge (5% CO_2, 21% O_2, 74% N_2) or (3) H-H challenge (10% O_2, 5%CO_2, 85% N_2) are shown in the left-hand panels of **Figure 2**. Resting values prior to gas challenges were similar to one another (**Table 2**). HX challenge elicited initial increases in f_R and Rinx that were subject to roll-off (roll off was more rapid and complete for Rinx). HC and H-H challenges elicited sustained increases in f_R accompanied by an initial increase and then sustained decreases in Rinx (**Figure 2**, **Table 2**). The return to room-air after the HX, HC and H-H challenges were associated with sudden increases in f_R that gradually declined toward pre-challenge values and abrupt and sustained increases in Rinx. The maximal changes at key points are summarized in the right-hand panels of **Figure 2**. The HX, HC and H-H challenges elicited robust and similar maximal increases in f_R (column Gas – max). There was substantial roll-off during the HX challenge (column GAS - 5 min) but minimal roll-off during the HC and H-H challenges. The maximal increases in f_R that occurred upon return to room-air were similar following exposure to the HX, HC or H-H challenges (column RA - max). The return to pre-challenge levels over 15 min (column RA - 15 min) was substantial for each group but less pronounced in the HX than the HC and H-H groups.

The HX, HC and H-H challenges elicited robust maximal increases in Rinx that were slightly less in the HC group (column Gas - max). Rinx gradually returned to pre-gas levels during the HX challenge whereas Rinx values fell below zero in the HC group (column Gas - 5 min). The fall in the H-H group was less than in the HC group. The maximal increases in Rinx upon return to room-air were similar following exposure to the HX or H-H challenges but smaller in the HC group (column RA – max). The return to pre-challenge levels (column RA - 15 min) was substantial for HX group, more so for the HC group but less in the H-H group. As a result of these changes, the maximal increases in $Rinx/f_R$ were greater in the H-H group than the HX or HC groups (column Gas - max). $Rinx/f_R$ subsequently fell during the HX, HC and H-H challenges with the falls being greater in the HC and H-H groups (Gas - 5 min). The maximal increases in $Rinx/f_R$ that occurred upon return to room-air were similar following exposure to the HX or H-H challenges but substantially smaller in the HC group (column RA – max). The return to pre-challenge levels (column RA - 15 min) was substantial for the HX group, more so for the HC group but substantially less in the H-H group.

The total changes in f_R, Rinx and $Rinx/f_R$ during the gas challenges and upon return to room-air are summarized in **Figure 3**. The total changes in f_R were similar in the HX, HC and H-H groups over the first min (top panel: Gas - min 1). Because of the pronounced roll-off, the total response over the 5 min HX challenge was smaller than in the HC and H-H groups (Middle panel: Gas – 5 min). The initial total increase in Rinx was smaller in the HC than the HX or H-H groups (top panel: Gas - min 1) whereas there was an increase in total Rinx during the entire HX challenge (middle panel: Gas – 5 min), a fall in total Rinx during HC challenge but no overall change in total Rinx during H-H challenge. Taken together, there were small initial increases in total $Rinx/f_R$ in the HX and H-H groups but a decrease in the HC group (top panel: Gas - min 1). There was a small decrease in total $Rinx/f_R$ during the entire HX challenge but substantial decreases in total $Rinx/f_R$ during the entire HC and H-H challenges. The decrease in the H-H group was less than that in the HC group. The total increase in f_R following return to room-air was greater in the HX group than in the HC or H-H groups (Bottom panel: Room-air). The total increase in Rinx was greatest in the H-H group and lowest in the HC group with the value in the HX group being between the HC and H-H groups. The increase in total $Rinx/f_R$ was higher in the H-H group than either the HX or HC groups.

3.4. Hypoxic gas challenges and return to room-air in C57BL6, Swiss Webster and B6AF1 mice

Summary statistics for the C57BL6, Swiss-Webster and B6AF1 mice are provided in **Table 3**. The mice were of equivalent age. The body weights of the Swiss-Webster mice were greater than the C57BL6 or B6AF1 mice. Resting f_R was higher in B6AF1 mice than C57BL6 or Swiss Webster mice. Rinx was higher in C57BL6 mice than in Swiss-Webster or B6AF1 mice, which were similar to one another. $Rinx/f_R$ was higher in C57BL6 mice than in Swiss-Webster mice, which in turn was higher than in B6AF1 mice. The changes in f_R, Rinx and $Rinx/f_R$ during hypoxic challenge (10% O_2, 90% N_2) in C57BL6 and Swiss Webster mice (left-hand panels) and C57CL6 and B6AF1 mice (right hand panels) are summarized in **Figure 4**. Both groups of C57BL6 mice showed initial increases in f_R upon

HX challenge that displayed substantial roll-off. Both groups of C57BL6 mice showed a transient increase in Rinx during the challenge but minimal changes thereafter. Both groups of C57BL6 mice showed sustained decreases in Rinx/f_R during HX challenge. The Swiss-Webster mice displayed an increase in f_R that did not roll-off substantially. There were small increases in Rinx and Rinx/f_R during initiation of the HX challenge in the Swiss Webster mice that were followed by gradual declines thereafter. The B6AF1 mice also displayed an increase in f_R with minimal roll-off during the HX challenge. Both Rinx and Rinx/f_R fell substantially during the HX challenge.

The changes in f_R, Rinx and Rinx/f_R upon return to room-air in following the hypoxic challenges in C57BL6 and Swiss Webster mice and C57BL6 and B6AF1 mice are also summarized in **Figure 4**. Both groups of C57BL6 mice showed initial increases in f_R upon return to room-air that were sustained for at least 15 min. Both groups of C57BL6 mice also displayed somewhat gradually occurring (compared to the rate of increase in f_R) increases in Rinx and Rinx/f_R that remained elevated for over 10 min. Upon return to room-air, the Swiss-Webster mice displayed simple time-dependent decreases in f_R toward baseline levels whereas Rinx and Rinx/f_R fell virtually to zero. Upon return to room-air, the B6AF1 mice maintained the high level of f_R that was reached during the HX challenge whereas Rinx and Rinx/f_R merely returned toward baseline values.

The percent changes in f_R, Rinx and Rinx/f_R (maximal responses and those at 15 min) that occurred upon exposure to the HX challenge and upon return to room-air in the C57BL6 and Swiss-Webster study are shown in the left-hand panels of **Figure 5**. The maximal increases in f_R (column “Max”) elicited by the HX challenge was greater in C57BL6 than in Swiss-Webster mice. However, roll-off was greater in C57BL6 than Swiss-Webster mice (column 15 min, under Hypoxic Challenge). The maximal increases in Rinx were greater in C57BL6 mice than in Swiss-Webster mice whereas there was a minor decrease in Rinx in Swiss-Webster but not C57BL6 mice at 15 min. The maximal changes in Rinx/f_R upon exposure to the HX challenge were similar in both groups whereas the fall in Rinx at 15 min was greater in the Swiss-Webster than the C57BL6 mice. Upon return to room-air, f_R, Rinx and Rinx/f_R values all increased in C57BL6 mice and remained elevated at 15 min (left-hand panels). In the Swiss-Webster mice, f_R rose initially and then subsided by 15 min whereas there was an immediate and sustained fall in Rinx and Rinx/f_R.

The percent changes in f_R, Rinx and Rinx/f_R (maximal responses and those at 15 min) that occurred upon exposure to the HX challenge and upon return to room-air in the C57BL6 and B6AF1 study are shown in the right-hand panels of **Figure 5**. The maximal increases in f_R elicited by the HX challenge was greater in C57BL6 mice than in B6AF1 mice whereas roll-off was greater in C57BL6 mice. Rinx initially rose upon exposure to the HX challenge in C57BL6 mice but had declined at 15 min. In contrast, Rinx fell initially in the B6AF1 mice but normalized by 15 min. The maximal decreases in Rinx/f_R and those recorded at 15 min of the HX challenge were greater in B6AF1 mice than in C57BL6 mice. Upon return to room-air, f_R, Rinx and Rinx/f_R values all increased in C57BL6 mice and except for Rinx/f_R remained elevated at 15 min (right-hand panels). The increases in f_R, Rinx and Rinx/f_R in the B6AF1 mice were smaller than those in C57BL6 mice.

The total percent changes in f_R, Rinx and Rinx/f_R that occurred during the HX challenge and upon return to room-air in the C57BL6 and Swiss-Webster study are shown in the left-hand panels of **Figure 6**. During the HX challenge, the total increases in f_R were greater in Swiss Webster than in C57BL6 mice, the total increase in Rinx was slightly greater in Swiss-Webster than in C57BL6 mice, whereas the total decline in Rinx/f_R were similar in both groups. Upon return to room-air, C57BL6 mice displayed increases in f_R, Rinx and Rinx/f_R. In contrast, Swiss-Webster mice displayed minor total increases in f_R that were accompanied by substantial falls in total Rinx and Rinx/f_R.

The total percent changes in f_R, Rinx and Rinx/f_R that occurred during the HX challenge and upon return to room-air in the C57BL6 and B6AF1 study are shown in the right-hand panels of **Figure 6**. During the HX challenge, the total increases in f_R were greater in B6AF1 mice than in C57BL6 mice. The total change in Rinx was not significant in C57BL6 mice whereas there was a fall in B6AF1 mice. As a result, there was a greater fall in Rinx/f_R in B6AF1 mice than in C57BL6 mice during the HX challenge. Upon return to room-air, the C57BL6 mice displayed substantial increases in f_R, Rinx and Rinx/f_R. The increases in f_R were smaller in the B6AF1 mice than in the C57BL6 mice and the B6AF1 mice displayed minor total changes in Rinx and Rinx/f_R.

4. Discussion

4.1. Rinx and its relationship to disordered breathing

The present study provides compelling evidence that Rinx may be a valuable parameter for investigating the genetic and mechanistic processes underlying disordered breathing in mice. In addition, these studies pave the way for using Rinx as a marker for the efficacy of drugs on disordered breathing. In this study, the Buxco parameter settings used to define disordered breathing (Rinx) were set to pick up any abnormal breaths as well as apneas/pauses, sighs and sniffs, and irregular breaths due to abrupt movement. Indeed, these settings gave Rinx values for the percent time spent in disordered breathing at rest and post-HX that were very similar to those determined by manual analyses. With respect to these analyses, it is evident that the post-HX period in C57BL6 mice was associated with substantial increases in the total time associated with spontaneous pauses (apneas), post sighs and sniffing, and a marked increase in irregular breathing (unbalanced inspiratory/expiratory volumes). Since movement artifacts were minimal at rest and upon return to room-air, it is evident that the occurrence of this markedly disordered breathing is a unique feature of the physiological/pathophysiological make-up of the C57BL6 mouse.

It should be noted that the manual analyses of the C57BL6 mice were performed between 5 and 10 min post-HX, a period of enhanced and relatively stable Rinx recorded in these mice. The question of the relationships between f_R and Rinx in the first few min of post-HX as a function of the duration of the HX challenge will be discussed below. It should also be noted that the input variables for assessing Rinx (e.g., V_T, T_I, T_E, balance between inspiratory and expiratory volumes, and minimum box pressure change) can be modified to selectively reject a particular event such as a sniff thereby allowing more detailed analyses of the disordered breathing events.

4.2. Ventilatory responses during and following gas challenges in C57BL6 mice

The changes in f_R during the 5 and 15 min HX challenges in conscious C57BL6 mice (increases followed by roll-off) and upon return to room-air (substantial increase from roll-off levels for at least 10 min) are consistent with our previous findings as to the effects of 15 min HX challenges and post-HX phase in conscious C57BL6 mice (Palmer et al., 2013a,b; Gaston et al., 2014). Although the pattern of changes of f_R and Rinx (initial increases followed by roll-off) were similar during the HX challenge, it was evident that the degree of Rinx per level of f_R (Rinx/fr) fell during HX challenge, but, not as markedly as during HC or H-H challenge (see **Figure 2**). It is therefore apparent that HX does not have the impact on disordered breathing elicited by HC or H-H. Since the degree of post-HX changes in f_R were similar after the 5 and 15 min HX challenges, it is apparent that the mechanisms responsible for the post-HX responses are recruited rapidly in these mice. However, the duration of HX exposure dramatically influenced the time to appearance of Rinx in the post-HX phase. More specifically, f_R and Rinx rose immediately following return to room-air after the 5 min HX challenge (**Figure 2**). In contrast, whereas f_R rose immediately after return to room-air following the 15 min HX challenge, Rinx actually fell initially for 1-2 min before gradually rising with significant increases in Rinx not occurring for 3-4 min (**Figure 4**). Extrapolating to the generation of disordered breathing in animals and humans, it is possible that more brief episodes of apnea destabilize breathing more effectively than longer apneas, which suggests that neurochemical processes recruited to defend against ventilatory instability upon cessation of the apnea take a definite amount of time to become effective. It should be noted that return to room air after even more brief (1 min) episodes of HX is associated with pronounced ventilatory instability in C57BL6 mice (Yamauchi et al., 2007, 2008c).

In contrast to HX challenge, the HC challenge elicited an increase in f_R that was not subject to roll-off and which was accompanied by robust decreases in Rinx and Rinx/f_R. As such, it is evident that hypercapnia elicits dramatic effects on disordered breathing in C57BL6 mice, most probably via actions within the carotid bodies and brain. Nonetheless, the return to room-air following the HC challenge also elicited a pronounced increase in f_R that was associated with an equally pronounced increase in Rinx. These findings raise the possibility that hypercapnia and resultant generation of H^+ ions via the carbonic anhydrase system plays a vital role in the etiology of disordered breathing. Moreover, evidence that inhibition of carbonic anhydrase markedly diminishes disordered breathing in C57BL6 mice upon return to room-air after 1 min HX or HC challenges (Yamauchi et al., 2007, 2008c) strongly suggests that H^+ ions and acid-sensing ion channels (ASICs), which exist within the carotid bodies (Lu et al., 2013; Tan et al., 2014) and central neurons involved in ventilatory control (Huda et al., 2012; Song et al., 2012), play a ubiquitous role in disordered breathing. Indeed, HX stimulates the activity of the H^+ ion extruding Na^+-H^+ exchanger (NHE) in neocortical (Jørgensen et al., 1999), and CA1 hippocampal (Yao et al., 2001) neurons from the mouse, which in turn could activate ASICs during HX challenge, and depending on the rapidity of change in extracellular H^+-ion concentrations, the return to room-air phase. Indeed, in rat CA1 hippocampal neurons, stimulation of NHE only occurred after the neurons were returned to normoxia (Diarra et al., 1999; Sheldon and Church, 2002). Moreover, although acute HX challenge (5-10 min induration) had little effect on steady-state pH_i of rat

hippocampal astrocytes, HX stimulated HCO_3^--independent acid extrusion (NHE activity) and inhibited HCO_3^--dependent acid extrusion (Bevensee and Boron, 2008). Moreover, intermittent HX also increases the expression of ASICS in neurons of trapezoid body and lateral paragigantocellular nuclei in the rat brain (Cao et al., 2009). Of direct relevance to the present study is evidence that HX can decrease intracellular pH of carotid body glomus cells and most likely extracellular extrusion of H^+-ions (Pang and Eyzaguirre, 1993), which would allow for activation of glomus cell ASICs (Lu et al., 2013; Tan et al., 2014). It is also relevant to our H-H findings in particular, that HX augments H^+-ion induced activation of transient receptor potential vanilloid receptors (TRPV1) in rat dorsal root ganglia (Henrich and Buckler, 2009). In light of existing evidence at the time, Yamauchi et al (2007) postulated that unstable breathing is caused by carbonic anhydrase (H^+ ion-dependent) pathways to or within the central respiratory controller, which encourages reentry into apnea and unstable breathing. The more recently acquired data discussed above strongly support this postulation.

A key observation of the present study was that exposure to H-H challenge elicited an increase in f_R that was reminiscent of that elicited by HC challenge rather than HX challenge (i.e., no roll-off during H-H or HC challenge) and a decrease in Rinx that was observed for the HC but not the HX challenge. These findings support a wealth of evidence that HC activates neurochemical processes that dominate those elicited by the HX challenge (see Campen et al., 2004, 2005; Dempsey et al., 2010). These interactions may be in the brain rather than the carotid bodies since hypoxia augments the effects of hypercapnia on glomus cell activity and *vice versa* (Dasso et al., 2000; Roy et al., 2000). However, similar to HX and HC challenges, the return to room-air after H-H challenge elicited robust increases in Rinx and Rinx/f_R, which at 15 min was substantially greater than for the post-HX or post-HC phases. It appears that HX and HC play a *synergistic* role in the expression of disordered breathing, which may involve the above mentioned synergisms between HX and HC on carotid body glomus cell/chemoafferent activity. However the possibility of direct brain involvement is suggested by evidence that the gain of brain CO_2/H^+ chemoreceptors in dogs is critically dependent on carotid body afferent activity and that brain-carotid body interaction results in hyper-additive ventilatory responses to central HC (Blain et al., 2010).

With respect to the return to room-air, we found that post-HX tachypnea (1) is markedly diminished in C57BL6 mice in which the carotid sinus nerves were transected several days before-hand (Gaston et al., 2014), (2) is markedly diminished in C57BL6 mice in which the cysteine at the 93 position in the β-chain of hemoglobin in red blood cells was converted into an alanine, thereby preventing HX-induced generation of S-nitrosothiols (Gaston et al., 2014), and (3) markedly augmented in C57BL6 mice null in S-nitrosoglutathione reductase, an enzyme playing a major role in the catalysis of S-nitrosoglutathione and overall S-nitrosylation status of functional proteins (see Palmer et al., 2014). Taken together, these findings raise the possibility that HX generates blood S-nitrosothiols, which in turn activate carotid body glomus cells and/or chemoafferent terminals to elicit the post-HX ventilatory response. This may involve the ability of S-nitrosothiols to activate ASICs on glomus cells since S-nitroso-N-acetylpenicillamine has been shown to potentiate H^+-gated currents in

dorsal root ganglion neurons and H^+-gated currents in CHO cells expressing ASIC sub-units, most probably via S-nitrosylation events (Cadiou et al., 2007). This possibility is supported by earlier evidence that HX challenge may involve direct activation of carotid body chemoafferents via HX-sensitive proteins or external chemical influences (Sun and Reis, 1994; Roy et al., 2000). We are currently determining whether the generation of circulating S-nitrosothiols is responsible for the post-HX increases in disordered breathing (Rinx) using the mouse models described above.

4.3. Comparisons between C57BL6, Swiss-Webster and B6AF1 mice

C57BL6 mice have been used extensively to study the effects of HX, HC and H-H gas challenges on ventilatory function (see Palmer et al., 2013a,b, 2014; Gaston et al., 2014) and disordered breathing (Han et al., 2001, 2002; Tagaito et al., 2001; Schneider et al., 2003; Yamauchi et al., 2007, 2008a, 2012; Moore et al., 2012, 2014). Although Swiss-Webster mice have been used in studies concerned with the effects of 2 min episodes of HX and HC challenges on ventilatory parameters (Schenkler et al., 2002) and the effects of 5 min episodes of hypoxia-induced gasping (Jacobi and Thach, 1989), no studies have addressed the relationships between ventilatory performance and level of disordered breathing at rest, during HX or HC challenges, and upon return to room-air. Our findings comparing the three mouse strains was that resting Rinx was not necessarily a function of the magnitude of resting f_R since Swiss-Webster (and in particular B6AF1 mice) had higher f_R at rest than C57BL6 mice but substantially lower Rinx values than the C57BL6 mice. This would suggest that resting Rinx is a function of intrinsic mechanisms regulating breathing patterns rather than the level of f_R per se. A key finding of the present study was that Swiss-Webster mice did not display of roll-off during HX challenge and displayed a gradual subsidence of f_R upon return to room-air, which were in stark contrast to the substantial roll-off and pronounced post-HX tachypnea observed in C57BL6 mice. Other than initial short-lived increases, Rinx did not change markedly during the HX challenge in Swiss-Webster mice (as in C57BL6 mice). It could be expected that Rinx would fall during the HX challenge due to enhanced tidal volume breathing, which would diminish the relative occurrence of disordered breathing including apneas. However, as with C57BL6 mice, this did not happen in Swiss-Webster mice, which suggests that neural pathways recruited by HX are not sufficient to overcome the mechanisms responsible for disordered breathing in these mice. Whereas C57BL6 mice displayed pronounced increases in f_R, Rinx and Rinx/f_R upon return to room-air after the HX challenge, f_R gradually declined in the Swiss-Webster mice and Rinx and Rinx/f_R gradually fell to virtually zero values during the post-HX phase. These dramatic differences in post-HX changes in f_R and levels of disordered breathing between C57BL6 and Swiss-Webster mice certainly reinforce extensive knowledge that genetic factors play a vital role in determining ventilatory patterns (Han and Strohl, 2000; Han et al., 2001, 2002; Groeben et al., 2005; Gillombardo et al., 2012).

The B6AF1 mouse (off-spring of a C57BL6 dam, A/J sire) more closely behaves like the A/J mouse than the C57BL6 mouse in that it shows minimal disordered breathing at rest and a minimal expression of disordered breathing during the post-HX phase (Han et al., 2002; Yamauchi et al., 2008a,b). As such, the issue that was addressed in the B6AF1 mice used in this study was whether Rinx and Rinx/f_R values at rest and during the post-HX phase would

be consistent with direct measurements of disordered breathing (Han et al., 2002; Yamauchi et al., 2008a,b). First, it should be noted that the B6AF1 mice had higher resting f_R but lower resting Rinx and Rinx/f_R values than C57BL6 mice. Second, unlike C57BL6 mice, f_R did not show roll-off during the HX challenge and moreover, Rinx and Rinx/f_R values fell dramatically at the onset of the HX challenge, which probably reflects a dominance of normal tidal volume breathing (e.g., matching of inspiratory and expiratory volumes), before recovering toward pre-HX values during the later period of the challenge (the Rinx/f_R values of the C57BL6 mice also showed this trend). Taken together, it is evident that B6AF1 mice behave more like A/J mice than C57BL6 mice during HX challenge. The data from the B6AF1 mice clearly demonstrate that HX can elicit a substantial decrease in disordered breathing in a mouse strain, with the reason(s) for the slow recovery of Rinx and Rinx/f_R during the challenge indicative of adaptive mechanisms taking place.

A vital set of findings pertained to the differences between C57BL6 mice and B6AF1 mice during the post-HX phase. More specifically, the B6AF1 mice displayed substantial post-HX elevations in f_R values that were similar qualitatively and quantitatively to the C57BL6 mice. However, in contrast to the C57BL6 mice, the B6AF1 mice displayed only minor increases in Rinx and Rinx/f_R values merely retuned to pre-HX values. These findings demonstrate that post-HX elevations in breathing are not necessarily associated with disordered breathing (elevated Rinx) and that extend previous evidence that the B6AF1 mouse behaves like the A/J mouse rather than the C57BL6 mouse with respect to post-HX breathing patterns (Han et al., 2002; Yamauchi et al., 2008a,b). The mechanisms responsible for the differences in expression of disordered breathing in C57BL6, A/J and B6AF1 in the post-HX phase may be multifactorial, however there is evidence that the genetic mechanisms that produce strain differences in ventilatory function do not associate with carotid body structure or tyrosine hydroxylase morphology and that the A/J chromosome 1 contributes little to the morphology of the C57BL6 carotid body (Chai et al., 2011).

4.4. Perspectives

The present study provides evidence that Rinx and Rinx/f_R are reliable analytic tools, which detect breathing patterning and disordered breathing in mice placed in whole-body plethysmography chambers. The parameters are reproducible within a strain and distinguish breathing differences among strains; and may be valuable for detection of non-eupneic breathing during the various stages of the awake-sleep cycle. The use of Rinx and Rinx/f_R may be considered as a first-level phenotyping for breathing at rest, during a gas challenge and upon return to room-air, and would complement the detailed analyses of waveforms as elegantly performed by several groups (Dick and Coles, 2000; Tankersley et al., 2000, Strohl, 2003; Balbir et al., 2006). Moreover, use of Rinx and Rinx/f_R may be valuable in pharmacological studies aimed at uncovering the mechanisms of disordered breathing, in assessment of gene-by-drug interactions, and in studies designed to establish the efficacy of drugs on respiratory disorders such as sleep apnea.

4.5. Study Limitations

The major limitations of Rinx as a stand-alone parameter are that (1) the individual events that make up a disordered breathing pattern such as apneas are not identified, and (2)

without concomitant recording of behaviors, Rinx cannot be a true index of disordered breathing since tachypnea and sniffing for example have a close temporal association with outward behaviors such as grooming and motor activity in rats (see Kabir et al., 2010) and most probably mice. Nonetheless, our studies in C57BL6 mice clearly demonstrate that Rinx and individual breathing events such as apneas during the post-hypoxic phase are not overtly related to behavioral activity and indeed there are many epochs during which Rinx and the expression of disordered breathing events are high even though the mice are not displaying any outward behaviors. Since Rinx can only be equated to disordered breathing if concomitant measures of individual breathing events and behaviors are recorded we used the term non-eupneic breathing rather than disordered breathing on the basis that behavioral contributions to Rinx did not change during the post-hypoxia period (see Table 1). Nonetheless, once an initial finding of an elevated Rinx is established then more detailed analyses of individual breathing events and behaviors must be performed to establish that the increase in Rinx was indeed indicative of greater disordered breathing.

In any experiment, it is pertinent to question whether a change in Rinx reflects the effects of a particular challenge on peripheral or central respiratory homeostatic mechanisms, altered behavioral/locomotor patterns, or effects on the level of arousal/anxiety, knowing for example that hypoxic (Roth et al., 2002; Kumar and Goyal, 2008a,b; Ninot, 2011) and hypercapnic (Battaglia and Ogliari, 2005; Johnson et al., 2012; Battaglia et al., 2014) challenges are anxiogenic stimuli in humans and mice. As mentioned above, our studies show that the marked increase in disordered breathing events (e.g., apneas, sighs) during the post-hypoxic phase are not obviously related to behavioral activity at specific times during this period but it is certainly possible that anxiety elicited during the hypoxic and/or hypercapnic challenges are intimately involved in the expression of breathing pattern upon return to room-air in C57BL6 mice, whereas this may not be the case for Swiss-Webster or B6AF1 mice which show little disordered breathing during the post-hypoxia phase. Moreover, the finding that the number of sighs and %sniffing is a reflection of anxiety states in rats (Carnevali et al., 2013) is no doubt relevant to our studies which show a substantial increase in the number of sighs and %sniffing during the post-hypoxia phase in C57BL6 mice.

During the HX challenge, it would be expected that the vital ventilatory response consisting of more rapid and deeper breathing would dominate non-eupneic breathing events (i.e., diminish behaviors such as sniffing and sighing) via the increase in chemical drive designed to maintain PO_2. Moreover, anxiety-related breathing events initiated during the HX challenge would be un-masked during the post-HX phase and strongly influence breathing. This anxiety-related (emotional) component of post-HX breathing may be unique to C57BL6 mice since there was little evidence of non-eupneic breathing during the post-HX phase in Swiss-Webster or more strikingly in B6AF1 mice, which displayed robust post-HX increases in f_R but minimal changes in Rinx (see **Figure 4**). However, even though the HX challenge diminished Rinx in B6AF1 mice it did not do so in C57BL6 or Swiss-Webster mice. As such, the ability of HX to mask non-eupneic breathing may not only be absent in the latter two strains but also have little effect on post-HX enhancement of non-eupneic

breathing because C57BL6 mice did whereas the Swiss-Webster did not display this phenomenon.

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Highlights

- C57BL6 mice display marked non-eupneic breathing upon cessation of a hypoxic challenge.
- They also showed non-eupneic breathing after hypercapnic or hypoxic-hypercapnic challenges.
- B6AF1 and Swiss-Webster mice displayed reduced non-eupneic breathing post-hypoxia.
- Post-hypoxic non-eupneic breathing is independent of resting breathing frequency.

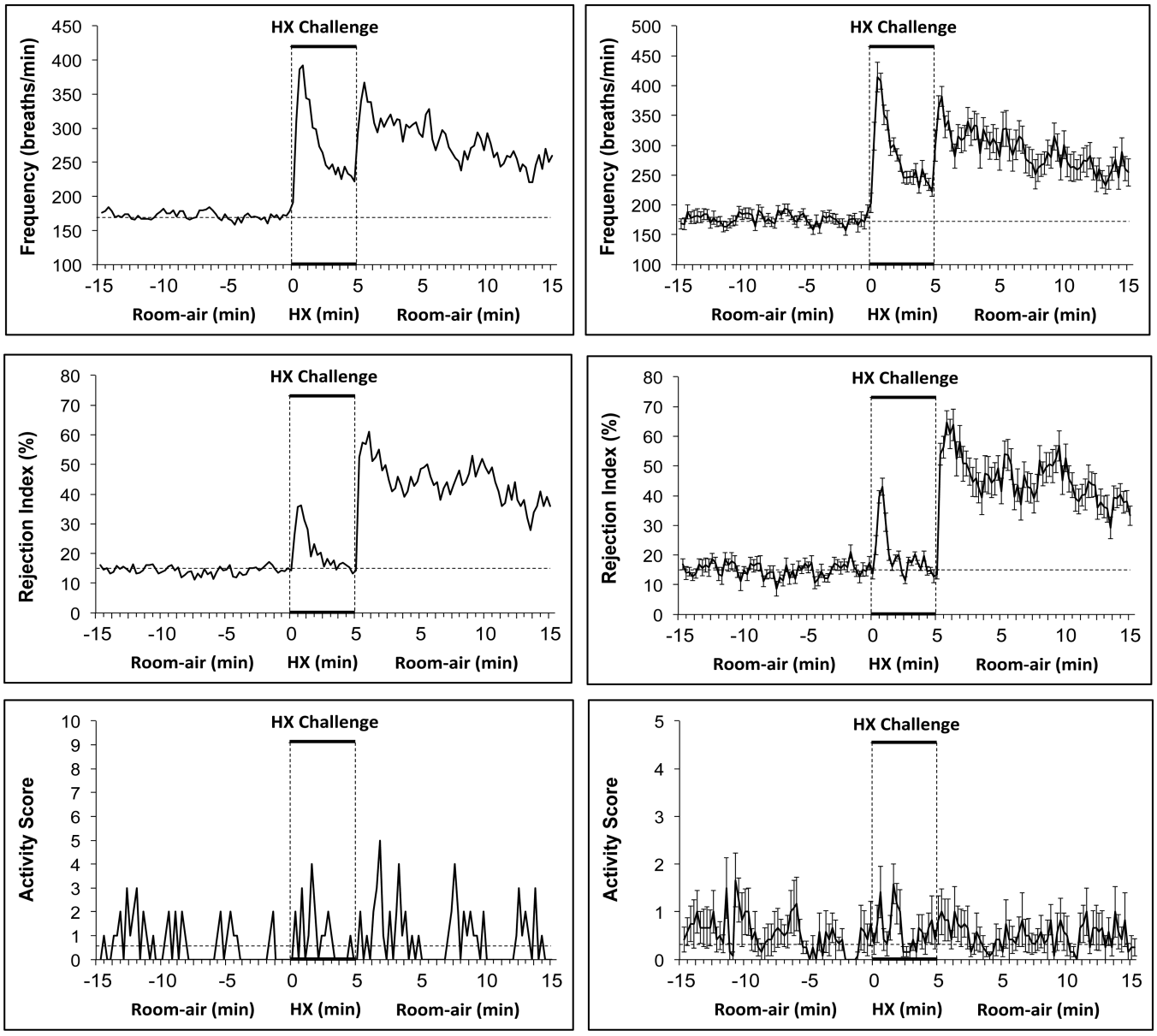

Figure 1.

Typical examples (left-hand panels) and summary graphs (right-hand panels) of frequency of breathing, Rejection Index (0% = no disordered breathing, 100% = completely disordered breathing) and Activity Score (see Methods for details) of conscious C57BL6 mice (n=16) before, during a 5 min challenge with a hypoxic (HX) gas mixture (10% O_2, 90% N_2), and following return to room-air. The data in the right-hand panels are presented as mean ± SEM.

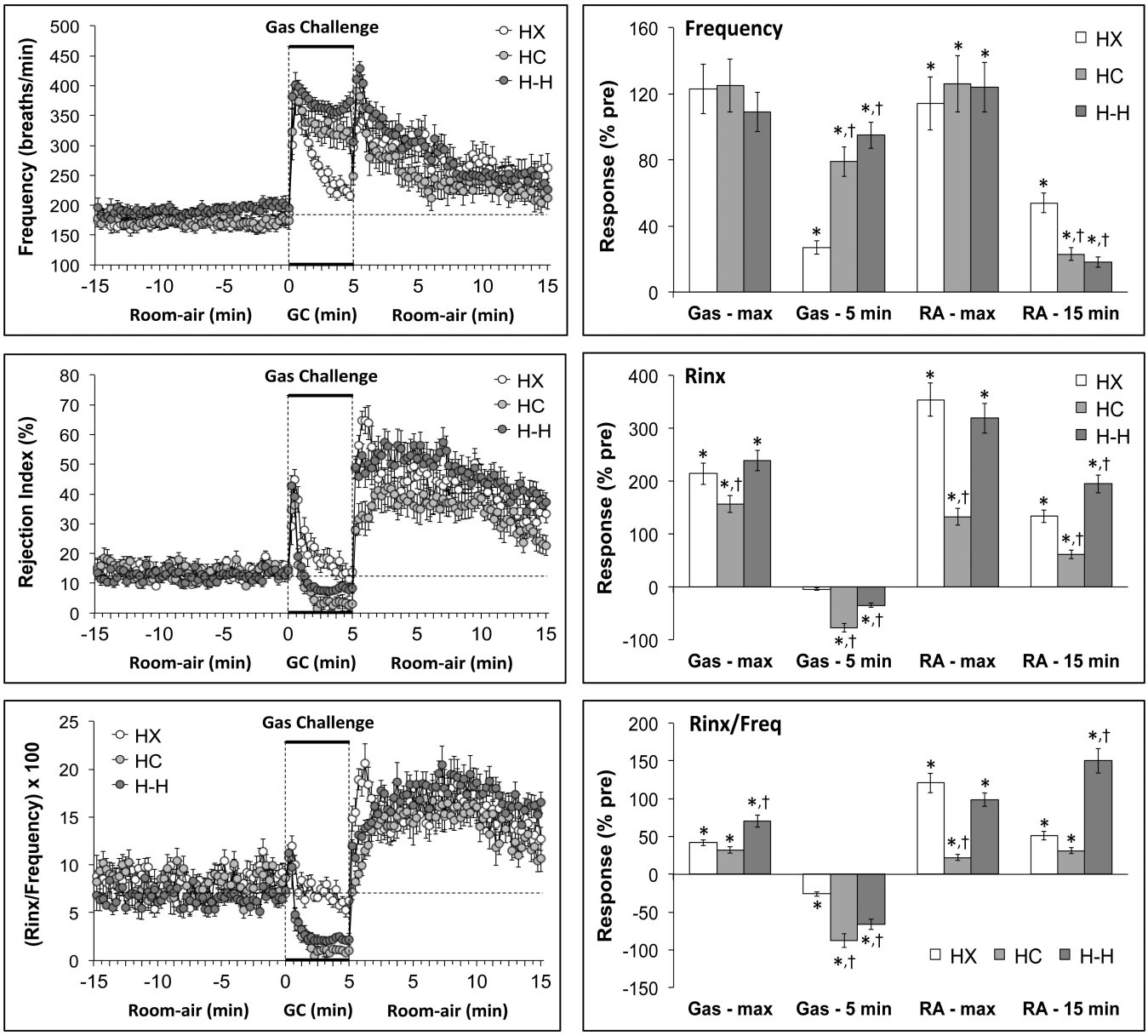

Figure 2.

Left-hand panels. Changes in frequency of breathing, Rejection Index and Rejection Index/frequency (Rinx/frequency) in conscious C57BL6 mice before, during a 5 min challenge with a hypoxic (HX) gas mixture (10% O_2, 90% N_2), a hypercapnic (HC) mixture (5% CO_2, 21% O_2, 74% N_2), or a hypoxic-hypercapnic (H-H) mixture (5% CO_2, 10% O_2, 85% N_2), and following return to room-air. **Right-hand panels.** Maximal responses recorded during the hypoxic challenge and upon return to room-air (HX – max and RA - max, respectively). The values at the end of the hypoxic challenge and the return to room-air (HX – 5 min and RA - 15 min, respectively) are also shown. The data are presented as mean ± SEM. There were 16 mice in each group. *$P < 0.05$, significant response. †$P < 0.05$, HC or HH *versus* HX.

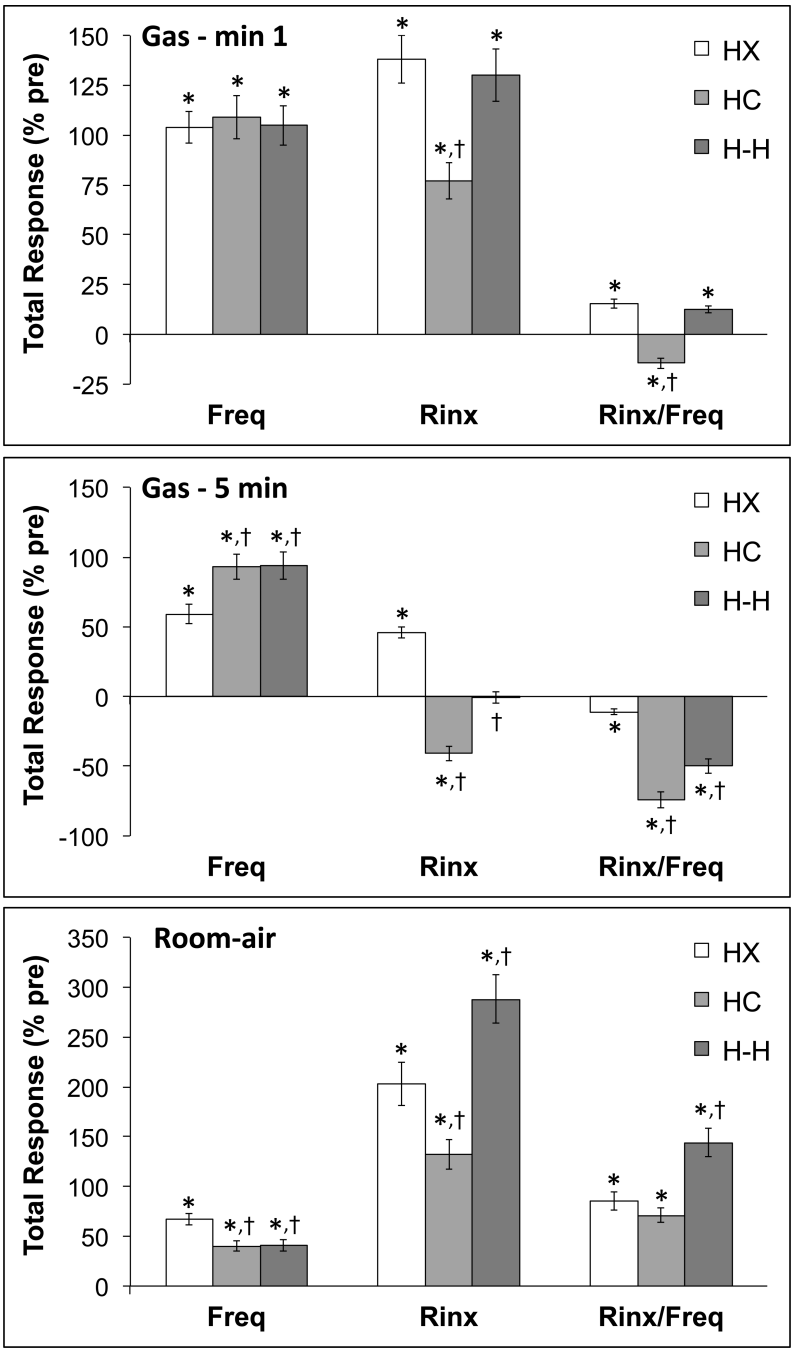

Figure 3.

Total changes in frequency of breathing (Freq), Rejection Index (Rinx) and Rejection Index/frequency (Rinx/freq) in C57BL6 mice before, during the first minute (Gas – 1 min) and for the entire 5 min (Gas – 5 min) challenge with a hypoxic (HX) gas mixture (10% O_2, 90% N_2), a hypercapnic (HC) mixture (5% CO_2, 21% O_2, 74% N_2), or a hypoxic-hypercapnic (H-H) mixture (5% CO_2, 10% O_2, 85% N_2), and upon return to room-air (15 min total). The data are mean ± SEM. There were 16 mice in each group. *$P < 0.05$, significant response. †$P < 0.05$, HC or HH *versus* HX.

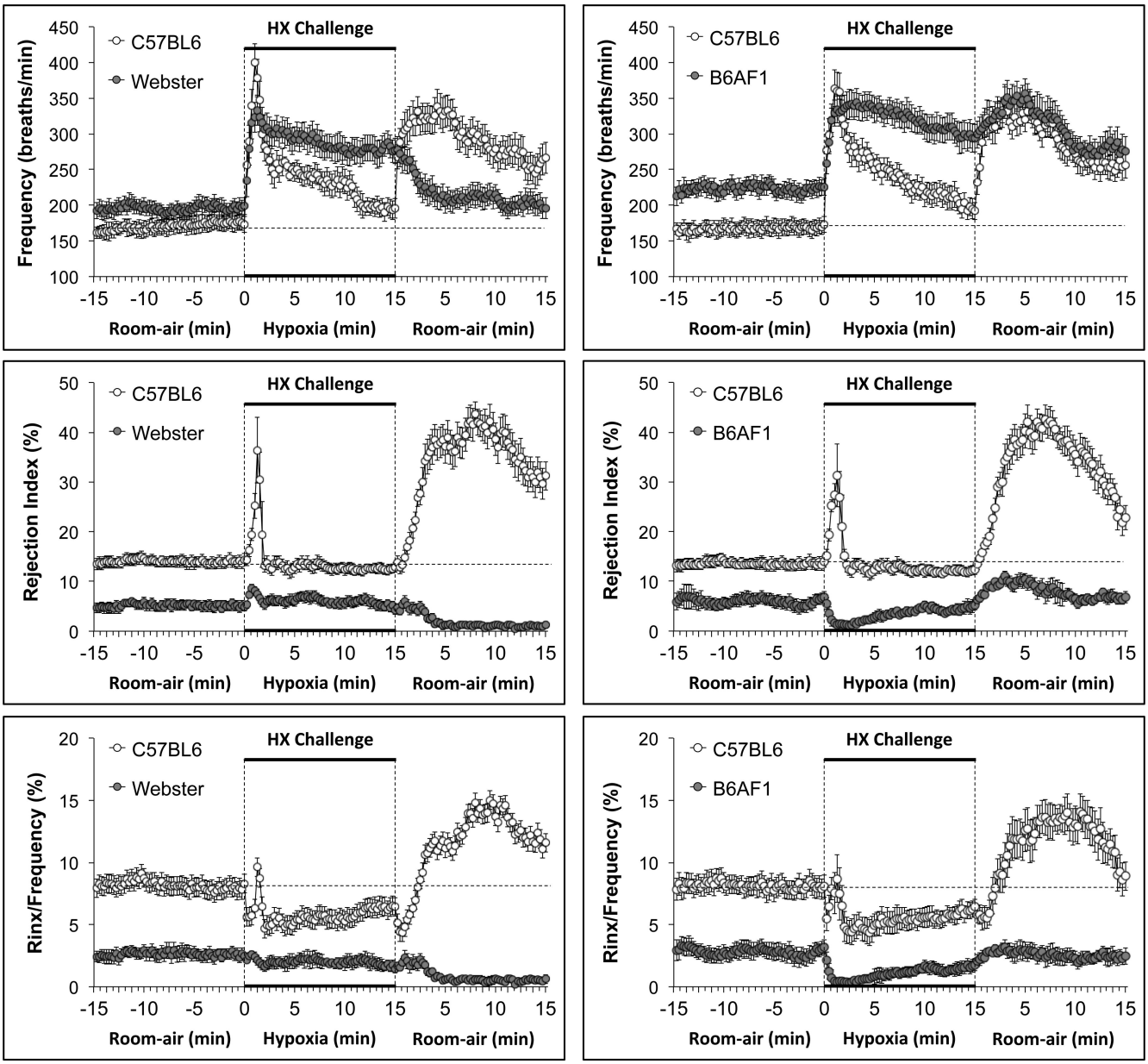

Figure 4.

Left-hand panels. Changes in the frequency of breathing, Rejection Index (0% = no disordered breathing, 100% = completely disordered breathing) and Rejection Index/frequency (Rinx/frequency) in conscious C57BL6 mice and Swiss-Webster (Webster) mice before, during a 15 min challenge with a hypoxic (HX) gas mixture (10% O_2, 90% N_2), and following return to room-air. **Right-hand panels.** Changes in frequency, Rejection Index and Rejection Index/Frequency in conscious C57BL6 mice and B6AF1 mice before, during a 15 min challenge with a hypoxic (HX) gas mixture (10% O_2, 90% N_2), and following return to room-air. There were 16 mice in each of the above groups. The data in all panels are presented as mean ± SEM.

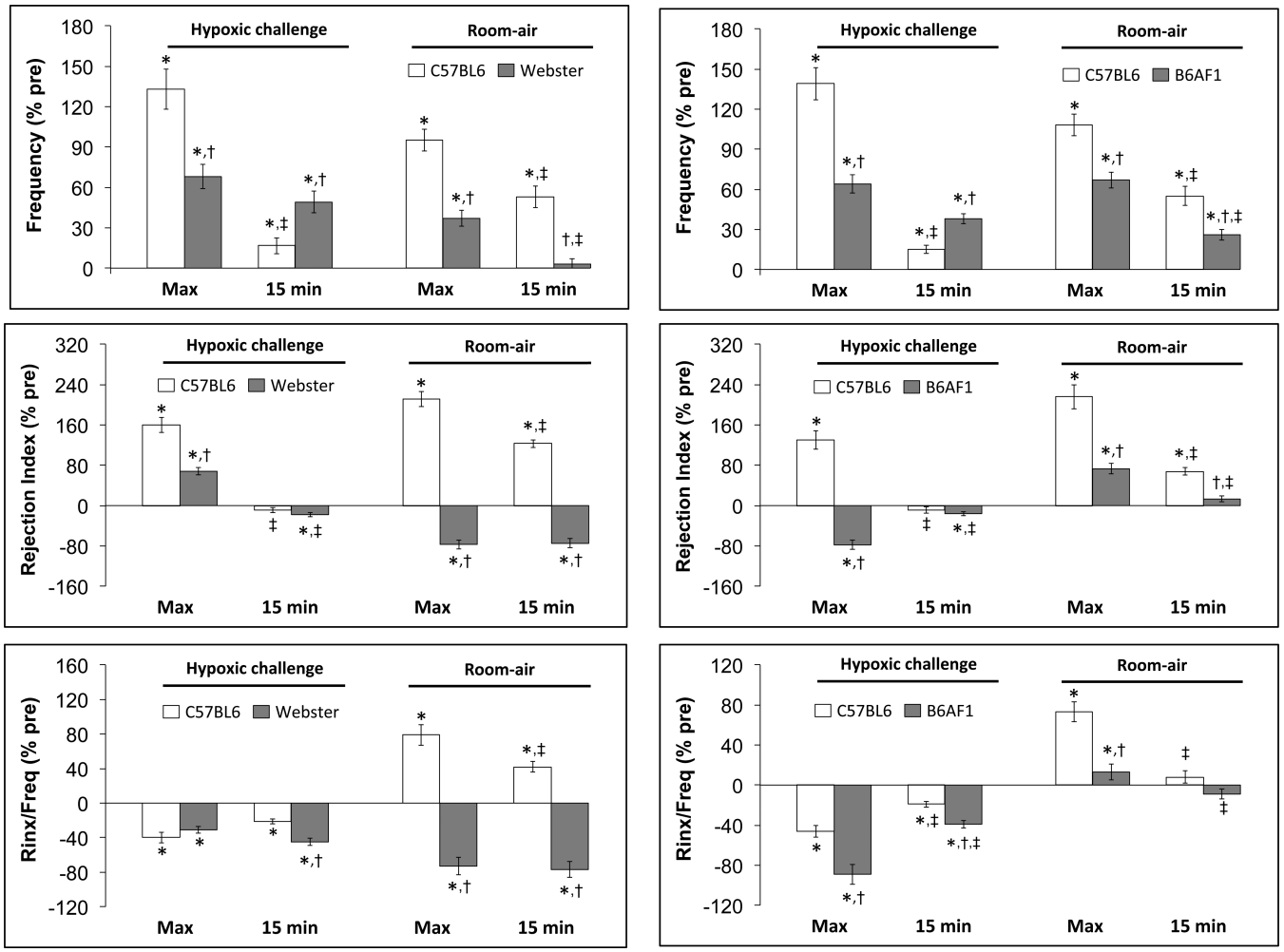

Figure 5.

Left-hand panels. Maximal changes in frequency of breathing, Rejection Index (0% = no disordered breathing, 100% = completely disordered breathing) and Rejection Index/frequency (Rinx/freq) in conscious C57BL6 and Swiss-Webster (Webster) mice recorded during a hypoxic challenge (10% O_2, 90% N_2) and upon return to room-air (HX – max and RA - max, respectively). The values at the end of the hypoxic challenge and the return to room-air (HX – 15 min and RA - 15 min, respectively) are also shown. **Right-hand panels.** Maximal changes in frequency of breathing, Rejection Index and Rejection Index/Frequency (Rinx/freq) in conscious C57BL6 and B6AF1 mice recorded during a hypoxic challenge (10% O_2, 90% N_2) and upon return to room-air (HX – max and RA - max, respectively). The values at the end of the hypoxic challenge and the return to room-air (HX – 15 min and RA - 15 min, respectively) are also shown. The data in all panels are presented as mean ± SEM. There were 16 mice in each group. *$P < 0.05$, significant response. $^{\dagger}P < 0.05$, Swiss-Webster or B6AF1 *versus* C57BL6. $^{\ddagger}P < 0.05$, 15 min values *versus* Max values.

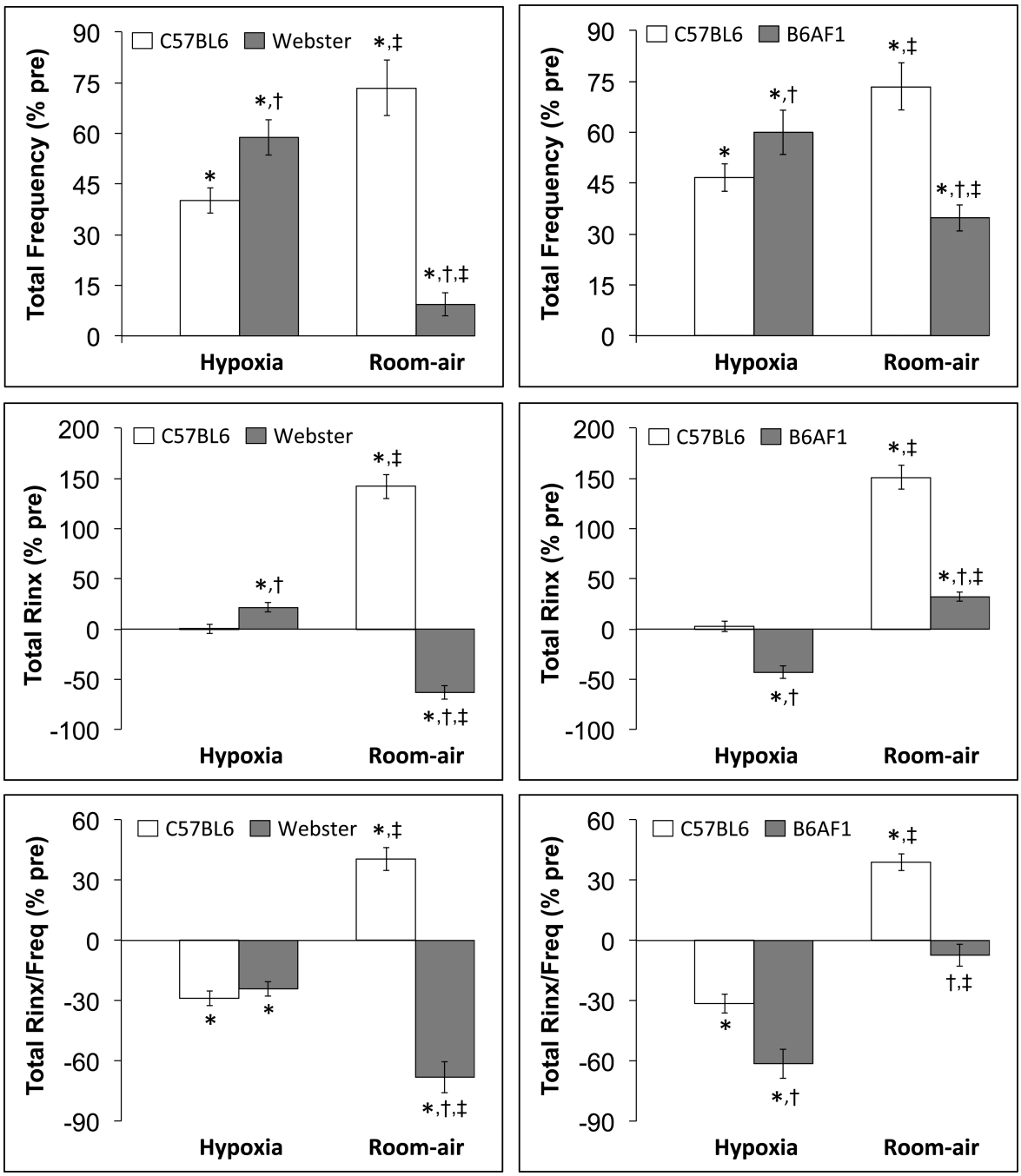

Figure 6.

Left-hand panels. Total changes in frequency of breathing, Rejection Index (Total Rinx) and Rejection Index/Frequency (Total Rinx/freq) in conscious C57BL6 and Swiss-Webster (Webster) mice recorded during a hypoxic challenge (10% O_2, 90% N_2) and upon return to room-air (HX – max and RA - max, respectively). **Right-hand panels.** Total changes in frequency of breathing, Rejection Index (Total Rinx) and Rejection Index/Frequency (Total Rinx/freq) in conscious C57BL6 and B6AF1 mice recorded during a hypoxic challenge (10% O_2, 90% N_2) and upon return to room-air (HX – max and RA - max, respectively).

The data in all panels are presented as mean ± SEM. There were 16 mice in each group. *$P < 0.05$, significant response. †$P < 0.05$, Swiss-Webster or

Table 1

Breathing events before and after return to room-air following exposure to a hypoxic challenge

Phase	Number	Pre	Post-HX
Spontaneous pause	Number/min	1.82 ± 0.18	5.22 ± 0.65*
	Duration	0.99 ± 0.12	1.62 ± 0.18*
	Total, sec	1.76 ± 0.27	8.36 ± 1.9*
Post-sigh without apnea	Number/min	0.36 ± 0.04	1.53 ± 0.22*
	Duration	0.68 ± 0.73	0.89 ± 0.12*
	Total, sec	0.25 ± 0.36	1.37 ± 0.24*
Post-sigh - type 1	Number/min	0.25 ± 0.03	1.04 ± 0.18*
	Duration	0.63 ± 0.08	0.69 ± 0.08*
	Total, sec	0.16 ± 0.03	0.72 ± 0.11*
Post-sigh - type 2	Number/min	0.17 ± 0.02	0.83 ± 0.11*
	Duration	0.65 ± 0.09	0.71 ± 0.12*
	Total, sec	0.11 ± 0.02	0.59 ± 0.08*
	Total per min	2.28 ± 0.39	10.97 ± 2.3*
	%Time per min	3.8 ± 0.5	18.3 ± 3.3*
Irregular breathing	Total, sec	2.69 ± 0.32	7.46 ± 0.93*
Sniffing	Total, sec	2.44 ± 0.26	6.23 ± 0.83*
Movement artifacts	Total, sec	1.11 ± 0.21	1.27 ± 0.15
	Total per min	6.24 ± 0.83	14.96 ± 2.33*
	%Time per min	10.4 ± 2.2	24.9 ± 3.8*
	Overall Total, min	8.52 ± 1.12	25.93 ± 4.43
	%Time per min	14.1 ± 2.3	43.2 ± 6.6*
	Rinx, %	13.5 ± 1.8	41.9 ± 5.2*

The data are presented as mean ± SEM. HX, hypoxic gas challenge. There were 16 male C57BL6 mice in the group.

* $P < 0.05$, post-HX values *versus* Pre values.

Table 2

Values at baseline (Pre) and during exposure to gas challenge and return to room-air

Parameter	Variables	HX	HC	H-H
Frequency (breaths/min)	Pre	170 ± 9	173 ± 11	192 ± 11
	GC - max	380 ±14*	388 ± 19*	401 ± 21*
	GC - 5 min	216 ± 8*	308 ± 23	373 ± 16
	RA - max	364 ± 17*	391 ± 20*	428 ± 22*
	RA - 15 min	262 ± 24*	212 ± 18*	226 ± 19
Rejection Index (%)	Pre	14 ± 2	14 ± 2	13 ± 2
	GC - max	45 ± 3*	36 ± 4*	43 ± 3
	GC - 5 min	14 ± 2	3 ± 1*	8 ± 2*
	RA - max	65 ± 6*	33 ± 5*	53 ± 4*
	RA - 15 min	33 ± 3*	23 ± 3*	37 ± 4*
(Rejection Index/Frequency) × 100	Pre	8 ± 1	8 ± 1	7 ± 1
	GC - max	12 ± 1*	11 ± 1*	11 ± 1*
	GC - 5 min	6 ± 1*	1 ± 0*	2 ± 1
	RA - max	18 ± 2*	10 ± 2	13 ± 2*
	RA - 15 min	13 ± 2*	11 ± 2	17 ± 1*

The data are presented as mean ± SEM. GC, gas challenge. RA, return to room-air. HX, hypoxic-gas challenge. HC, hypercapnic gas challenge. H-H, hypoxic-hypercapnic gas challenge. There were 16 male C57BL6 mice in each gas challenge (mice only received one of the challenges).

* $P < 0.05$, significantly different from Pre values.

Table 3

Parameters relevant to the Swiss-Webster and B6AF1 studies

	Swiss-Webster Study		B6AF1 study	
Parameter	**C57BL6**	**Webster**	**C57BL6**	**B6AF1**
N	16	16	16	16
Age (days)	88 ± 1	85 ± 2	88 ± 1	86 ± 1
Weight (g)	28.2 ± 0.6	36.4 ± 0.8*	28.1 ± 0.5	29.6 ± 0.6†
Frequency (breaths/min)	172 ± 11	196 ± 13	168 ± 12	213 ± 12*
Rejection Index (%)	14.2 ± 1.3	5.1 ± 0.9*	13.6 ± 1.2	6.0 ± 1.3*
(Rejection Index/Frequency) × 100	8.1 ± 0.8	5.0 ± 0.6*	8.4 ± 0.9	2.8 ± 0.6*,†

The data are presented as mean ± SEM. There were 16 male C57BL6 mice in each group.

*$P < 0.05$, significantly different from appropriate C57BL6 group.

†$P < 0.05$, B6AF1 *versus* Swiss Webster.