



Activation of Astrocytes in the Persistence of Post-hypoxic Respiratory Augmentation

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Acute hypoxia increases ventilation. After cessation of hypoxia loading, ventilation decreases but remains above the pre-exposure baseline level for a time. However, the mechanism of this post-hypoxic persistent respiratory augmentation (PHRA), which is a short-term potentiation of breathing, has not been elucidated. We aimed to test the hypothesis that astrocytes are involved in PHRA. To this end, we investigated hypoxic ventilatory responses by whole-body plethysmography in unanesthetized adult mice. The animals breathed room air, hypoxic gas mixture (7% O₂, 93% N₂) for 2 min, and again room air for 10 min before and after i.p. administration of low (100 mg/kg) and high (300 mg/kg) doses of arundic acid (AA), an astrocyte inhibitor. AA suppressed PHRA, with the high dose decreasing ventilation below the pre-hypoxic level. Further, we investigated the role of the astrocytic TRPA1 channel, a putative ventilatory hypoxia sensor, in PHRA using astrocyte-specific *Trpa1* knockout (*asTrpa1*^{-/-}) and floxed *Trpa1* (*Trpa1*^{fl/fl}) mice. In both *Trpa1*^{fl/fl} and *asTrpa1*^{-/-} mice, PHRA was noticeable, indicating that the astrocyte TRPA1 channel was not directly involved in PHRA. Taken together, these results indicate that astrocytes mediate the PHRA by mechanisms other than TRPA1 channels that are engaged in hypoxia sensing.

Keywords: astrocyte, hypoxia, post-hypoxic respiratory augmentation, plasticity, short-term potentiation, respiratory control, arundic acid, TRPA1

INTRODUCTION

Acute hypoxia increases ventilation. After brief hypoxic exposure, a switchback to room air is accompanied by a ventilatory fall-off in the recovery phase, but ventilation remains above the pre-hypoxic baseline for a time. Post-hypoxic persistent respiratory augmentation (PHRA) is a form of neural plasticity, which is defined as a change in the neural control system based

on the memory-like experience (Mitchell and Johnson, 2003). The poststimulus overshoot in ventilatory activity may even go above the stimulus level as is evident in the acute hypoxic ventilatory response (HVR) to static exercise, with the mechanism ascribed to the interaction with the cardiovascular brain control or rapid release of the volitional hypothalamic control over sustained muscle tension (Pokorski et al., 1990). Neural plasticity is essential for stabilizing respiratory control, but the underlying mechanisms are not yet well known (Eldridge and Millhorn, 1986; Dahan et al., 1995; Eldridge, 1996; Powell et al., 1998).

There are plastic interactions in relay circuits of hypoxic stimulus between peripheral chemoreceptors, among which carotid body chemoreceptors are most engaged in creating the HVR, and brain respiratory control pathways (Pamenter and Powell, 2016). The multipronged complexity of PHRA is highlighted by increased carotid chemoreceptor sensitivity due to the withdrawal of the central efferent activity component running down the sinus nerve to the carotid body (Lahiri et al., 1983). That feature has been unraveled in adaptive plasticity to chronic hypoxia but is plausibly also present in repeat acute hypoxic episodes characteristic of sleep apnea syndrome, the disease that distinctly affects brain function and increases chemoreflex sensitivity (Prabhakar, 2016).

Limited understanding of peripheral and central underliers of respiratory plasticity spurred novel lines of research, one of which is the role of transient receptor potential ankyrin 1 (TRPA1) channel. These channels participate in shaping the acute HVR (Pokorski et al., 2014). However, the channels have never been verified in carotid chemoreceptor cells and their effects on the HVR are mediated by mechanisms other than the carotid body (Pokorski et al., 2014). It has been shown that TRPA1 is localized in the chemosensitive parafacial respiratory group (pFRG/RTN) astrocytes in which hypoxia-induced TRPA1 activation facilitates exocytosis of ATP-containing vesicles (Uchiyama et al., 2020). On the basis of these findings, TRPA1 channels in astrocytes have been proposed as an oxygen sensor for respiratory control (Uchiyama et al., 2020). The proposition is in line with studies that show the role of astrocytes in brain synaptic plasticity (De Pittà et al., 2016; Schiera et al., 2020). Astrocytes are also influential for various aspects of respiratory control, including rhythm generation (Okada et al., 2012; Sheikhabahaei et al., 2018) and hypoxic and hypercapnic ventilatory responses (Gourine et al., 2010; Funk et al., 2015; Pokorski et al., 2016; Beltrán-Castillo et al., 2017; Gourine and Funk, 2017; Funk and Gourine, 2018; Sheikhabahaei et al., 2018; Guyenet et al., 2019). It has been reported that astrocytes can detect hypoxia (Tadmouri et al., 2014; Angelova et al., 2015; Fukushi et al., 2016; Onimaru et al., 2021). Therefore, we aimed to test the hypothesis that astrocytes are involved in PHRA and define the role of astrocytes, notably through TRPA1 channels, in the PHRA phenomenon. We used arundic acid (AA) as a pharmacological tool to inhibit astrocytic function in wild-type mice. We also used astrocyte *Trpa1* knockout mice to investigate the role of astrocytic TRPA1 channels in PHRA. We found that the presence of active astrocytes is indispensable for the expression of PHRA, but their action is mediated by mechanisms other than TRPA1 channels.

MATERIALS AND METHODS

Animal Welfare

All animal experiments were performed with the approval of the Ethics Committee for Animal Experiments of the Murayama Medical Center in Tokyo and complied with the Guidelines for Care and Use of Laboratory Animals released by the National Research Council of the National Academies (8th edition, revised 2011) and with the Guiding Principles for Care and Use of Animals of the Physiological Society of Japan. A total of 34 mice (including the mice in experiments for **Supplementary Figures 1, 2**) were used in the experiments. All efforts were made to minimize the number of animals used.

Experiments With Arundic Acid

We used unanesthetized adult male C57BL/6 mice aged 24.0 ± 3.0 weeks (mean \pm SE). It should be the same weeks, weighing 29.6 ± 0.7 g ($n=9$). The respiratory flow was measured noninvasively using an “open flow” whole-body plethysmograph (PLY 310, EMMS, Bordon, United Kingdom) consisting of recording (volume of 530 ml) and reference chambers as previously described (Oyamada et al., 2008; Pokorski et al., 2014; Fukushi et al., 2016, 2020). Briefly, the chambers were placed inside a transparent acrylic box (size $20 \times 20 \times 20$ cm). Each mouse was placed in the pre-calibrated recording chamber. The chamber temperature was maintained at 25°C throughout. The air in the recording chamber was suctioned with a constant flow generator (MV-6005VP, E.M.P-Japan, Tokyo, Japan), with a flow rate of 250 ml/min. To calculate the airflow, the pressure difference between the recording and reference chambers was measured with a differential pressure transducer (TPF100, EMMS) connected to an amplifier (AIU060, Information & Display Systems, Bordon, United Kingdom) and was bandpass filtered at 0.1–20 Hz. We calculated tidal volume (V_T ; $\mu\text{l/g b.w.}$) for each breath by integrating the airflow whose changes are proportional to those in the chamber pressure (Lundblad et al., 2002). We counted the number of breaths and obtained respiratory rate (RR; breaths/min). Minute ventilation (V_E ; ml/g/min) was calculated as $V_T \times \text{RR}$ for each minute. The V_E during hypoxia was calculated as a 2-min average and during the recovery phase as an average of the first 5 min (Recovery 1) and second 5 min (Recovery 2). The O_2 concentration in the chamber was monitored with an O_2 analyzer incorporating a polarographic sensor (Respina IH 26, San-ei, Tokyo, Japan) and was adjusted by controlling the mixing of N_2 and air blown into the acrylic box. The pressure and O_2 concentration data were simultaneously digitized at a 400 Hz sampling rate with an A/D converter (PowerLab4/26) and stored in a PC with LabChart7 software. The signal processing was performed using MATLAB 2020a (MathWorks, Natick, MA).

To evaluate the HVR, mice breathed room air, then a hypoxic gas mixture (7% O_2 , 93% N_2 for 2 min), and room air again before and after i.p. administration of AA. The experimental protocol consisted of three repeats of hypoxic challenges. First, dimethyl sulfoxide (DMSO), a vehicle for AA diluted in saline, was injected and the mouse was placed into the chamber to acclimatize in room air for 60 min. Then, after recording

normoxic baseline data for 1 min, N₂ gas was blown into the acrylic box. The chamber O₂ concentration rapidly declined to 7%, which was maintained for 2 min and followed by a switchback to room air. The measurement for the recovery continued for 10 min. This protocol was repeated after injections of two doses of AA solubilized in a mixture of DMSO and saline (1:4:5 v/v) at 30-min intervals. Thus, injections were made in the following sequence (1) vehicle – 0.45 ml/kg DMSO, (2) AA – 100 mg/kg, and (3) AA – 200 mg/kg (cumulative AA dose of 300 mg/kg). Although DMSO alone can affect the brain function when the dose is high, a total dose of DMSO used in the present experiment did not exceed 2.0 g/kg, which is much below the 3.5 g/kg, a dose that starts affecting respiration (Takeda et al., 2016). The total volume of saline used in the experiment was 2.24 ml/kg, which is much below the 10 ml/kg reported to affect respiration in mice (Receno et al., 2018). The dosing of AA was chosen according to previous studies using this agent in *in-vivo* rodents (Higashino et al., 2009; Fukushi et al., 2016, 2020). Any apparent movement and sniffing artifacts interfering with breathing patterns were discarded off-line from the recording traces during the final data elaboration. The mean values of V_E were submitted to a two-factor within-subject analysis of variance (ANOVA), with three pharmacological conditions: DMSO vehicle and the two doses of AA, and four air phases (Baseline room air, Hypoxia, Recovery 1, and Recovery 2). The same statistical tests were performed for RR and V_T as for V_E. A Greenhouse–Geisser adjustment was used to correct for violations of sphericity whenever necessary. Then, to quantitatively evaluate the magnitude of PHRA, we calculated the difference in V_E between the post-hypoxic recovery and pre-hypoxic baseline levels. This difference was divided by the difference in V_E between the hypoxic loading and pre-hypoxic levels to normalize for the degree of hypoxic ventilatory augmentation. The calculation provided the parameter $\Delta V_{E\text{Recovery}}/\Delta V_{E\text{Hypoxia}}$ to compare the PHRA magnitude among three drug conditions (without AA and with low and high doses of AA) in the post-hypoxic Recovery 1 and Recovery 2 phases. Statistical differences were assessed with a paired t-test. Bonferroni correction was performed for the multiple comparisons.

Experiments Using Astrocyte-Specific *Trpa1* Knockout Mice

We examined the role of astrocyte TRPA1 channels in HVR and PHRA using astrocyte-specific *Trpa1* knockout mice (as*Trpa1*^{-/-}). To generate the as*Trpa1*^{-/-}, two lines of mice were crossed: a transgenic mouse GFAP-Cre (mGFAP-Cre) and a recombinant *Trpa1* floxed (*Trpa1*^{fl/fl}) mouse (Gregorian et al., 2009; Zappia et al., 2017; Uchiyama et al., 2020). We conducted 7% hypoxia loading experiments in as*Trpa1*^{-/-} mice (seven males and five females, aged 21.8 ± 0.4 weeks, weighing 25.8 ± 1.1 g) and *Trpa1*^{fl/fl} mice (two males and four females, aged 22.7 ± 1.3 weeks, weighing 24.2 ± 0.9 g) according to the same protocol and measurement methods as outlined above for the AA experiments. The mean values of V_E, V_T, and RR were submitted to two-way ANOVA with two TRPA1 conditions (as*Trpa1*^{-/-} and *Trpa1*^{fl/fl}) as between-factor and with four air phases (Baseline room air, Hypoxia, Recovery 1, and Recovery 2)

as within-factor. A Greenhouse–Geisser adjustment was used to correct for violations of sphericity. We calculated the $\Delta V_{E\text{Recovery}}/\Delta V_{E\text{Hypoxia}}$ to compare the PHRA magnitude between the two TRPA1 conditions (as*Trpa1*^{-/-} and *Trpa1*^{fl/fl}) in the post-hypoxic Recovery 1 and Recovery 2 phases using the Welch test. The Bonferroni correction was used for multiple comparisons in *post hoc* tests. A *p* < 0.05 defined statistically significant differences. The analysis was performed using SPSS 24.0 (IBM, Armonk, NY).

RESULTS

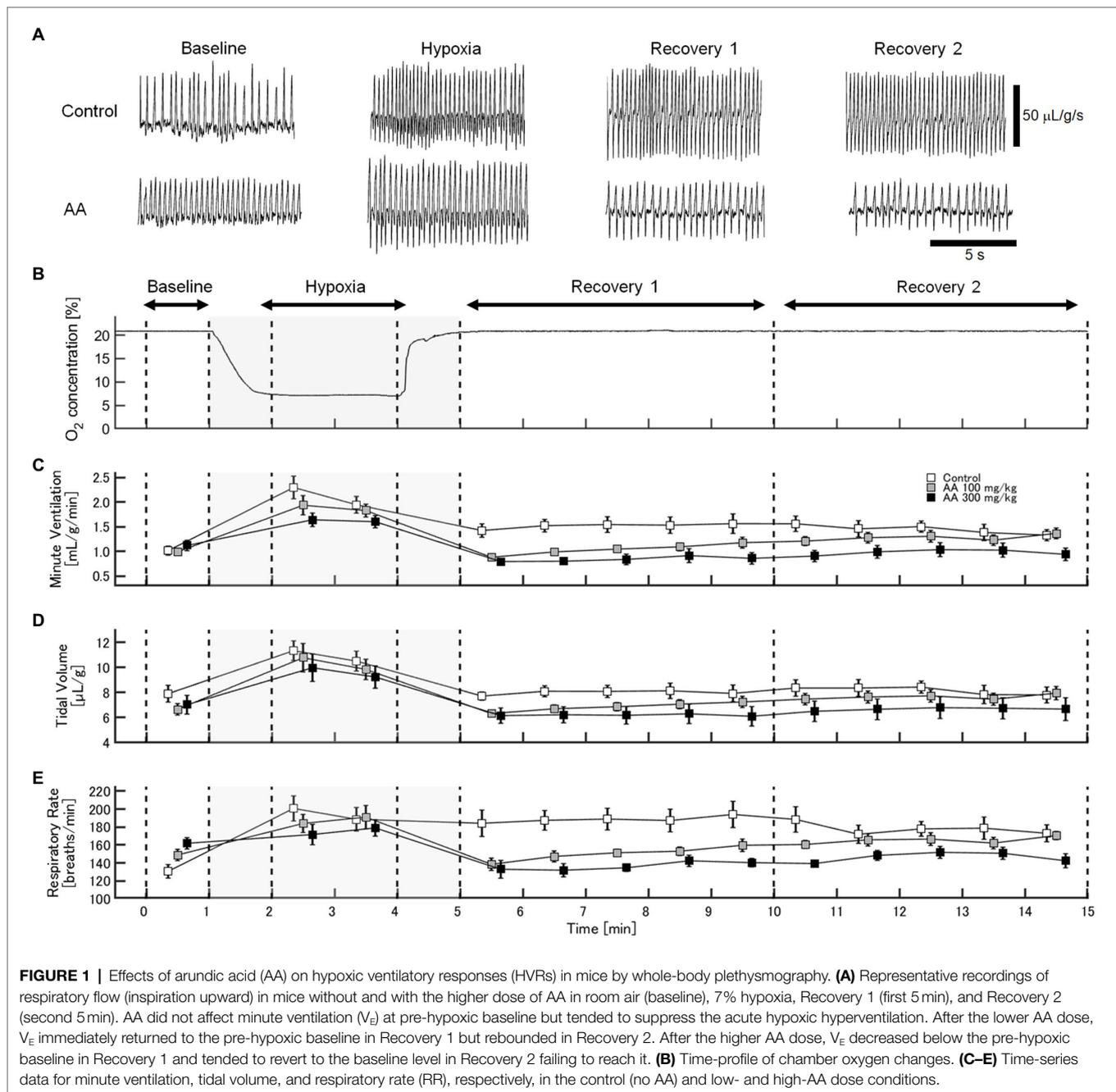
Effects of Arundic Acid on HVRs

The exemplary recordings of V_E profiles in the two AA conditions vs. the control condition with no AA across the baseline room air, hypoxia, and Recovery 1 and 2 phases are shown in **Figure 1**. There was a significant interaction between pharmacological conditions × ventilatory phases [*F*(6, 42) = 8.08, *p* < 0.001]. On average, AA failed to affect V_E, despite some increases in RR after the higher dose of AA in room air. While V_E increased during hypoxia on the background of AA, there were differences in the post-hypoxia recovery course. In the control condition, V_E decreased from the hypoxic hyperventilation level but remained higher than the pre-hypoxic baseline level in both recovery phases. In the low-dose AA condition, V_E immediately returned to the pre-hypoxic baseline level during Recovery 1 but increased again above it during Recovery 2. In the high-dose AA condition, V_E decreased significantly below the pre-hypoxic baseline level during Recovery 1 and then tended to revert to the baseline level in Recovery 2 failing to reach it. The time courses of V_E, V_T, and RR as outlined in the example shown above are summarized in **Figure 2**. **Figure 3A** shows that the $\Delta V_{E\text{Recovery}}/\Delta V_{E\text{Hypoxia}}$, assessing the PHRA magnitude, was significantly smaller in Recovery 1 between control (no AA) and 100 mg/kg AA (*p* < 0.01) or 300 mg/kg AA (*p* < 0.001), and between 100 and 300 mg/kg AA (*p* < 0.05). The differences between control (no AA) and 300 mg/kg AA and between 100 and 300 mg/kg AA distinctly persisted in Recovery 2 (**Figure 3B**). Thus, blockade of astrocyte activation significantly attenuated PHRA; the effect was greatly potentiated at the higher AA dose.

HVRs in as*Trpa1*^{-/-} Mice

V_E profiles in as*Trpa1*^{-/-} and *Trpa1*^{fl/fl} mice are shown in **Figures 4A,B**. There was a significant main effect of the ventilatory response phases [*F*(3, 48) = 85.011, *p* < 0.001] but not between the TRPA1 conditions [*F*(1, 16) = 1.843, *p* = 0.193]. In both as*Trpa1*^{-/-} and *Trpa1*^{fl/fl} mice, V_E increased during hypoxia when compared to the pre-hypoxic baseline level (*p* < 0.001) and then decreased in Recovery 1. However, V_E stayed above the baseline level throughout the recovery phases in both as*Trpa1*^{-/-} and *Trpa1*^{fl/fl} mice. Both V_T and RR components drove ventilatory changes throughout the hypoxic course in both as*Trpa1*^{-/-} and *Trpa1*^{fl/fl} mice (**Figures 4C,D**).

Although there was no significant interaction of TRPA1 conditions × ventilatory phases [*F*(3, 48) = 2.352, *p* = 0.084], we performed a between-TRPA1 comparison in each phase.



V_E tended to be smaller in *Trpa1^{fl/fl}* than *asTrpa1^{-/-}* mice during hypoxia, but the difference was not significant ($p=0.158$). V_E became significantly smaller in *asTrpa1^{-/-}* mice during Recovery 1 ($p=0.034$), but the PHRA phenomenon remained noticeable in both *asTrpa1^{-/-}* and *Trpa1^{fl/fl}* mice (Figure 4B). On average, $\Delta V_{E\text{Recovery}}/\Delta V_{E\text{Hypoxia}}$ percentage values denoting PHRA magnitude were little different between *asTrpa1^{-/-}* and *Trpa1^{fl/fl}* in post-hypoxic Recovery 1 and 2 phases (Figures 5A,B, respectively). Although we did not conduct statistical analysis because the number of *Trpa1^{fl/fl}* mice was small, there seems to be a gender difference; $\Delta V_{E\text{Recovery1}}/\Delta V_{E\text{Hypoxia}}$ values in male and female *Trpa1^{fl/fl}*, and male and female *asTrpa1^{-/-}* mice were 52, 61,

67, and 16%, respectively. $\Delta V_{E\text{Recovery2}}/\Delta V_{E\text{Hypoxia}}$ values in these mice were 38, 36, 51, and 40%, respectively.

DISCUSSION

This study investigated the role of astrocytes in the PHRA, representing short-term potentiation of respiration. The findings show that astrocytes mediate PHRA. Pharmacological blockade of astrocyte activation by AA inhibited PHRA. The knockout *asTrpa1^{-/-}* mice showed less increase in ventilation in response to hypoxia than *Trpa1^{fl/fl}* mice. However, the magnitude of PHRA

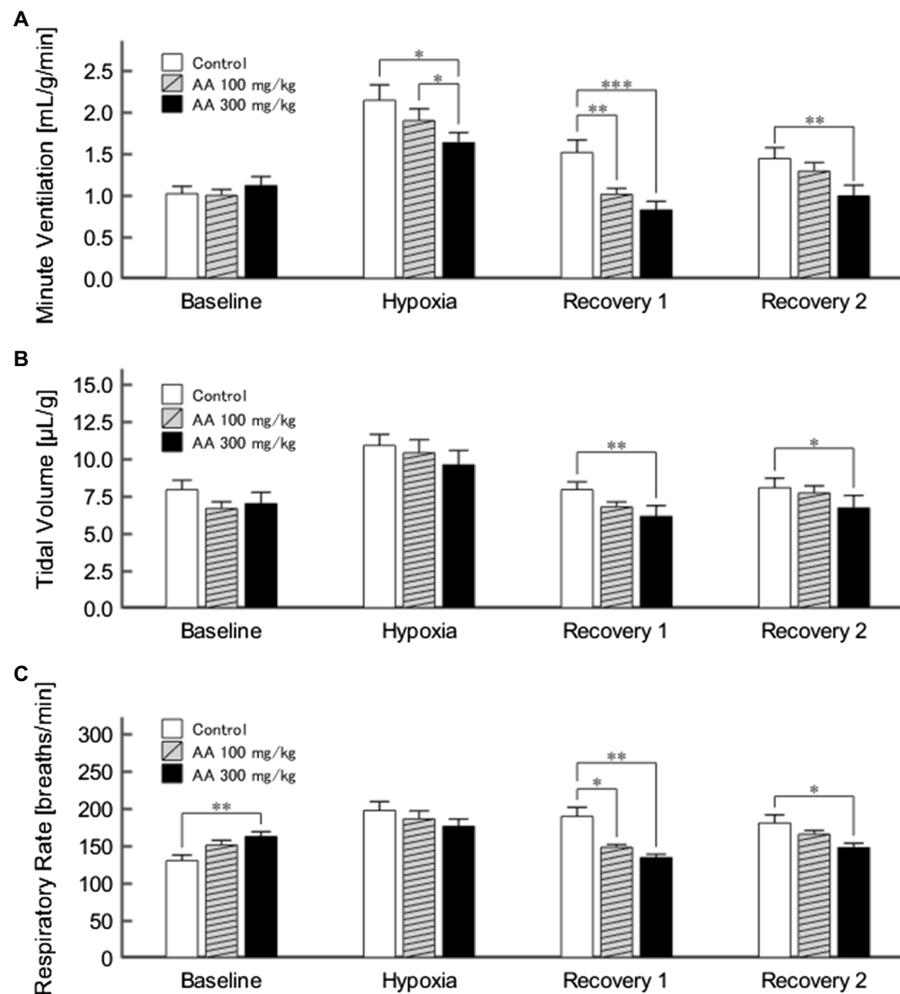
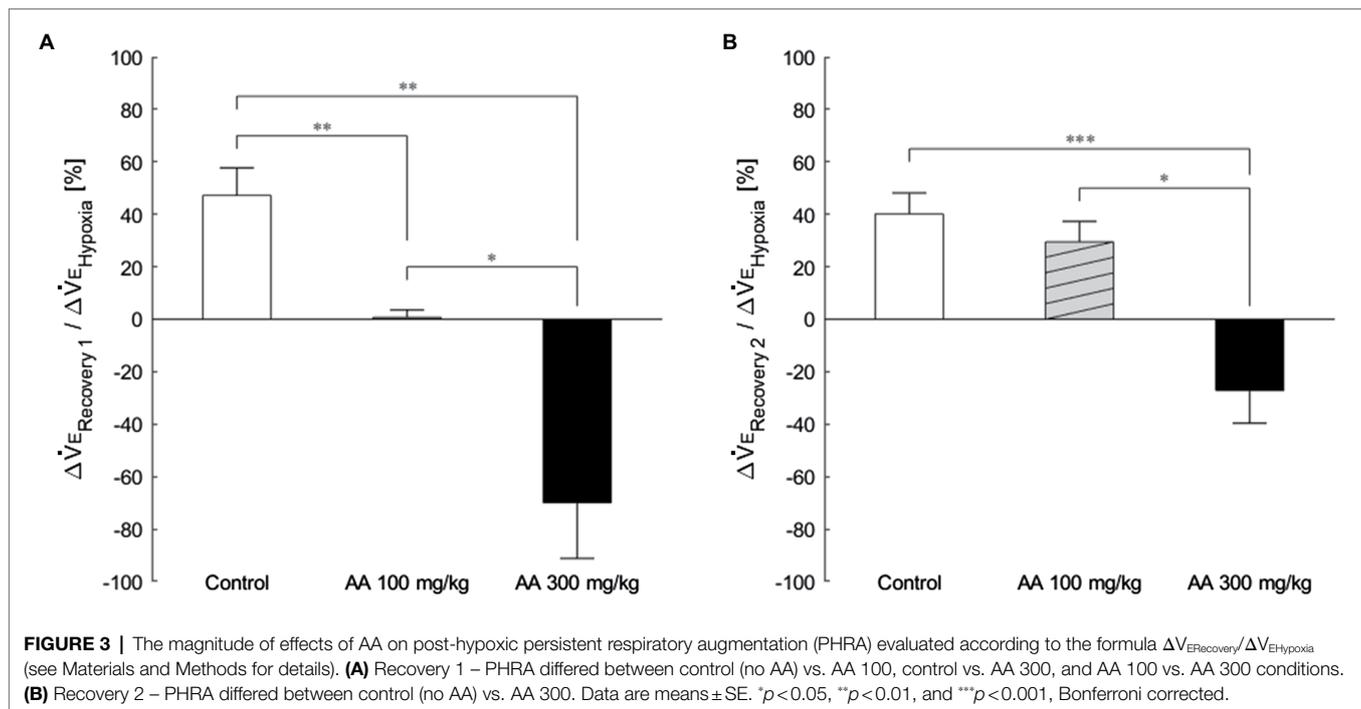


FIGURE 2 | (A) Minute ventilation (V_E) profiles ($n=9$) in the control (no AA) and low- and high-AA dose conditions across the successive ventilatory phases. V_E differed significantly in the following pairwise comparisons: Control vs. AA 100 and AA 100 vs. AA 300 in hypoxia (both $p<0.05$); Control vs. AA 100 ($p<0.01$) and Control vs. AA 300 ($p<0.001$) in Recovery 1; and Control vs. AA 300 ($p<0.01$) in Recovery 2. **(B)** The time-course of tidal volume (V_T). There were main effects on V_T of the AA condition ($F_{2,16}=6.596$, $p<0.01$) and oxygen concentration ($F_{3,24}=89.579$, $\epsilon_{GG}=0.424$, $p<0.001$), but no interaction between the two ($F_{6,48}=1.205$, $\epsilon_{GG}=0.522$, $p=0.329$). V_T differed significantly in the following comparisons: Control vs. AA 300 in Recovery 1 and Control vs. AA 300 in Recovery 2 (both $p<0.01$). **(C)** Time-course of RR. There was a significant interaction between control (no AA) and two AA conditions \times HVR phases ($F_{6,42}=12.208$, $p<0.001$). RR differed significantly in the following comparisons: Control vs. AA 300 at baseline ($p<0.01$); Control vs. AA 100 ($p<0.05$) and Control vs. AA 300 ($p<0.01$) in Recovery 1; and Control vs. AA 300 ($p<0.05$) in Recovery 2. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$, Bonferroni corrected.

was not attenuated in *asTrpa1^{-/-}* when compared to *Trpa1^{fl/fl}* mice. Our findings demonstrate the putative role of the astrocyte TRPA1 channels in hypoxia sensing, which confirms the recent findings by Uchiyama et al. (2020). We expanded the role of astrocytes to the mediation of PHRA as well. However, TRPA1 detects mild hypoxia (13%) more closely than severe hypoxia (7%; Takahashi et al., 2011; Pokorski et al., 2014). This suggests that PHRA is more likely to occur under conditions of severe hypoxia. Astrocyte-related action on the short-term PHRA occurs through yet unsettled mechanisms other than TRPA1 channels. The involvement of astrocyte TRPA1 channels has been reported in the hippocampal long-term potentiation in mice (Shigetomi et al., 2013). The contribution of these channels may vary depending on the type of brain plasticity.

In the present study, we used AA, as an inhibitory modulator of astrocyte function. AA inhibits the inflammatory response of astrocytes by reducing GFAP and S100 protein synthesis, increasing the expression of the astroglial glutamate transporter GLAST and releases the glutamate receptor antagonist kynurenic acid from astrocytes (Tateishi et al., 2002; Mori et al., 2004; Asano et al., 2005; Wajima et al., 2013; Yamamura et al., 2013; Yanagisawa et al., 2015). We have previously reported that AA delays the occurrence of seizures and prevents respiratory arrest in severe hypoxia (Fukushi et al., 2020).

The present finding of counteracting the PHRA by AA indicates that astrocytes are influential in shaping respiratory neural plasticity. Hypoxia activates the carotid body, and the information is relayed *via* the carotid sinus nerve to the



medullary solitary tract nucleus, emanating to other respiratory regions in the brainstem and spinal cord (Guyenet, 2014). Astrocytes around the excited neurons are activated *via* neurotransmitters spilled from neurons. Once activated, they release gliotransmitters that in turn activate respiratory neurons responsible for the sustenance of respiratory potentiation. Of note, the hitherto mechanistic studies on respiratory neural plasticity have been explicitly focused on neurons but not on glial cells. The present study is the first to demonstrate that astrocytes mediate the neural plasticity of respiration.

The short-term potentiation of brain excitability, leading to the continuation of respiratory augmentation after the stimulus cessation, referred to as neural plasticity, has been previously reported (Eldridge, 1973, 1976; Tawadrous and Eldridge, 1974; Eldridge and Gill-Kumar, 1980; Wagner and Eldridge, 1991). The mechanisms of respiratory plasticity are also present in the spinal cord (Feldman et al., 2003; Mitchell and Johnson, 2003; Fuller and Mitchell, 2017). One of the most extensively investigated phenomena in this context is the phrenic long-term facilitation following acute intermittent hypoxia. Regarding the cellular mechanism of facilitation, the Q and S signaling cascades in the phrenic motor nucleus have been proposed, induced by activation of metabotropic receptors coupled to Gq and Gs proteins, respectively, interacting *via* crosstalk inhibition. The serotonin-dependent Q pathway dominates in the phrenic facilitation during mild-to-moderate hypoxia. In contrast, the S pathway is serotonin-independent and dominates during severe hypoxia (Devinney et al., 2013; Fuller and Mitchell, 2017).

Recent studies have revealed an active role of astrocytes in brain plasticity related to other than respiratory functions, with a notable reference to hippocampal memory (Magistretti, 2006; Ota et al., 2013; Croft et al., 2015; Sims et al., 2015;

De Pittà et al., 2016). Astrocytes secrete synapse-modulating gliotransmitters such as glutamate, ATP, D-serine, and GABA (Jourdain et al., 2007; Henneberger et al., 2010; Takata et al., 2011; Kang et al., 2013; Shigetomi et al., 2013; Verkhatsky et al., 2016; Zorec et al., 2018; Santello et al., 2019). The regulation of postsynaptic glutamate receptors, particularly α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, is dependent on ATP released from astrocytes. The elevation in astrocytic Ca^{2+} , occurring slowly in the order of seconds, stimulates glutamate release which activates astrocytic metabotropic glutamate receptors (Agulhon et al., 2012; Navarrete et al., 2012). The classical form of neural plasticity also depends on N-methyl-D-aspartate (NMDA) receptors and Ca^{2+} -dependent slow release of D-serine from astrocytes (Henneberger et al., 2010). Further, astrocytes express a variety of receptors such as acetylcholine, ATP, GABA, and endocannabinoids (Porter and McCarthy, 1997; Haydon, 2001; Charles et al., 2003).

There are an increasing number of studies referring to the functional role of astrocytes in respiratory control other than respiratory plasticity. Astrocytes in the brainstem are sensitive to hypoxia and involved in HVR (Tadmouri et al., 2014; Angelova et al., 2015; Marina et al., 2015; Fukushi et al., 2016; Pokorski et al., 2016; Rajani et al., 2018; Uchiyama et al., 2020). Astrocytes in the ventral respiratory network, including the pre-Bötzinger complex, release ATP, which increases respiratory activity during hypoxia, putatively counteracting the depressive effects of hypoxia (Gourine et al., 2005; Marina et al., 2016a; Gourine and Funk, 2017; Funk and Gourine, 2018; Rajani et al., 2018). ATP acts *via* P2Y₁ receptors in the pre-Bötzinger complex to increase the respiratory burst rate with increases in intracellular Ca^{2+} and glutamate release (Lorier et al., 2007; Huxtable et al., 2010). Astrocytes also are strongly

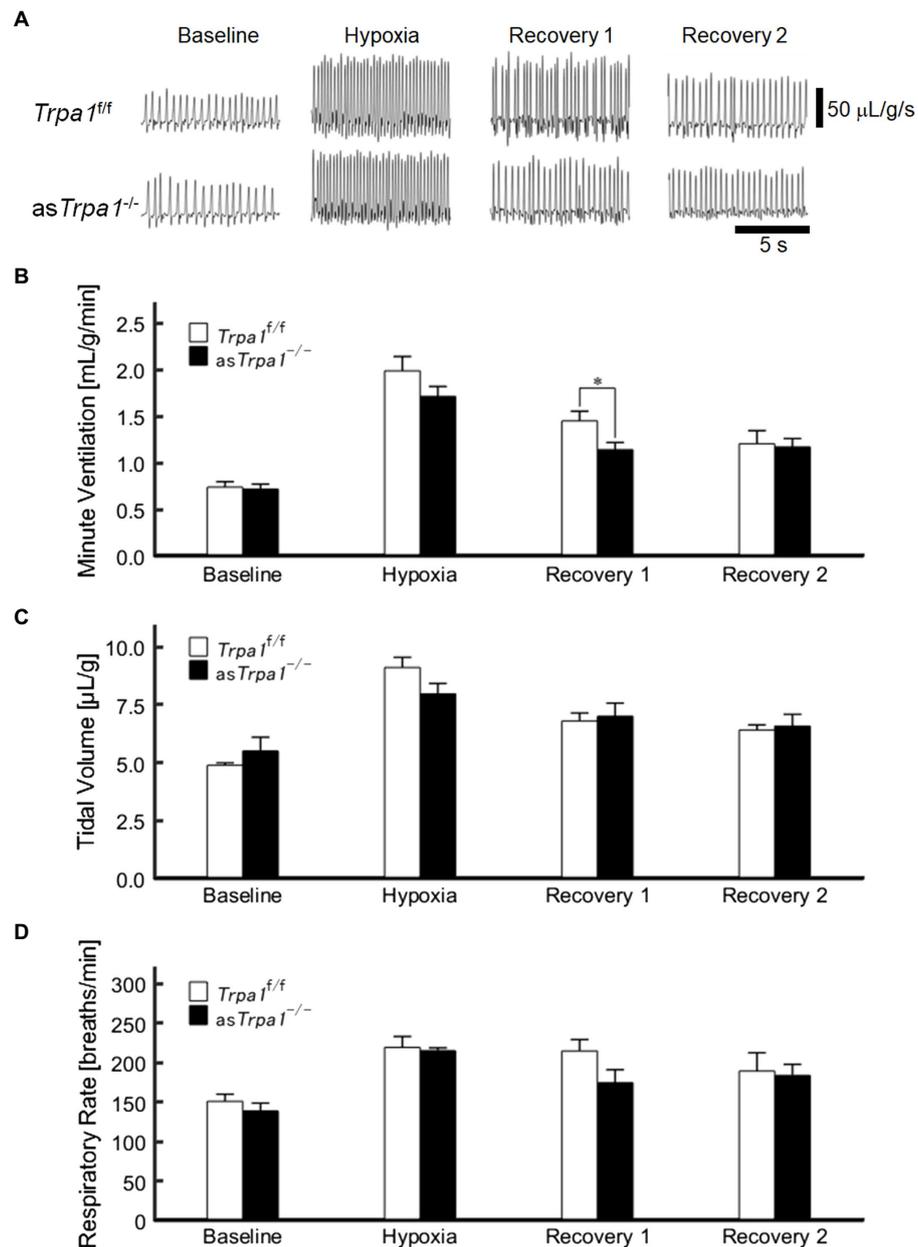
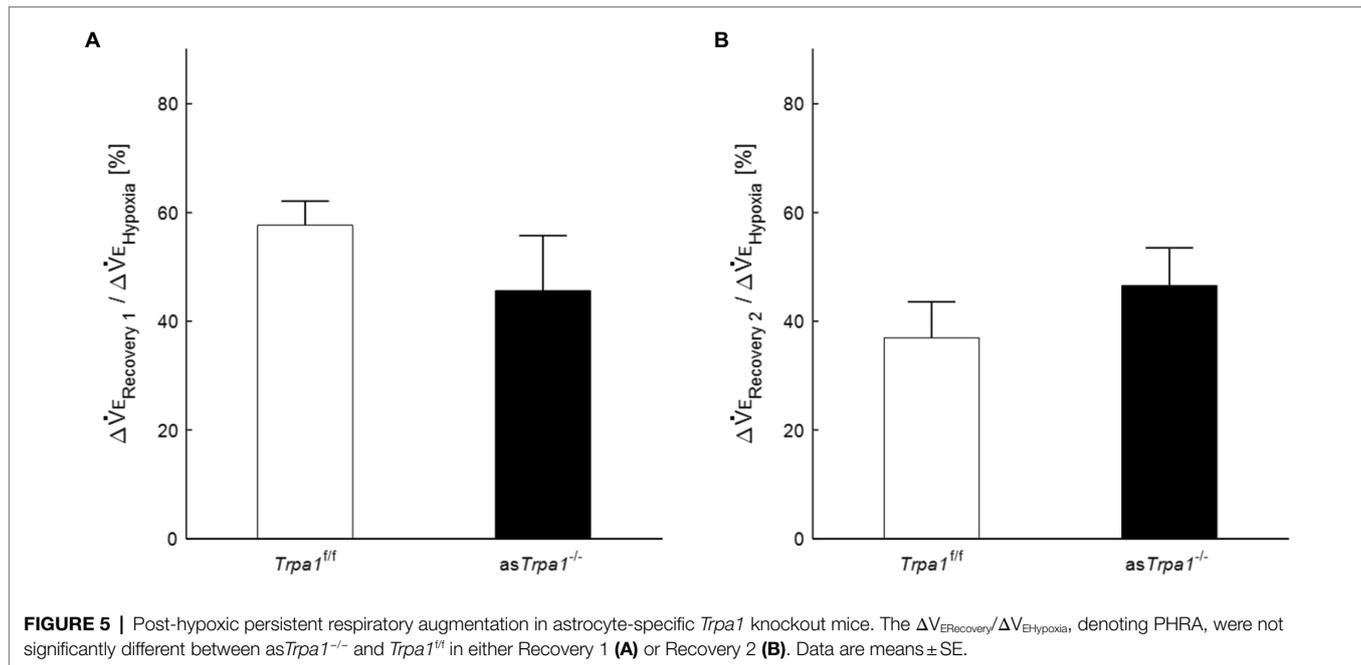


FIGURE 4 | Hypoxic ventilatory responses in *asTrpa1^{-/-}* and *Trpa1^{fl/fl}* mice. **(A)** Representative recordings of respiratory flow (inspiration upward) in room air (baseline), 7% hypoxia, Recovery 1 (first 5 min), and Recovery 2 (second 5 min). In both *asTrpa1^{-/-}* and *Trpa1^{fl/fl}* mice, minute ventilation (V_E) increased during hypoxia and then decreased in Recovery 1, remaining significantly elevated over the baseline level throughout both recovery phases. In Recovery 1, V_E was smaller in *asTrpa1^{-/-}* than *Trpa1^{fl/fl}* mice **(B)** Minute ventilation (V_E) in *Trpa1^{fl/fl}* ($n=12$) and *asTrpa1^{-/-}* ($n=6$) mice in successive ventilatory phases. Of note, V_E was significantly smaller in *asTrpa1^{-/-}* than *Trpa1^{fl/fl}* in Recovery 1 ($p=0.034$). **(C)** Tidal volume (V_T) in successive ventilatory phases. There was a significant interaction between transient receptor potential ankyrin 1 (TRPA1) conditions (*asTrpa1^{-/-}* and *Trpa1^{fl/fl}*) \times ventilatory response phases ($F_{3,48}$ in *asTrpa1^{-/-}* and *Trpa1^{fl/fl}* mice = 3.318, $\epsilon_{GG}=0.658$, $p<0.05$) but not between TRPA1 condition and V_T in any of the ventilatory phases. Pairwise comparisons in *asTrpa1^{-/-}*: Baseline vs. Hypoxia ($p<0.001$), Baseline vs. Recovery 1 and Baseline vs. Recovery 2 (both $p<0.01$), and Hypoxia vs. Recovery 2 ($p<0.05$) and in *Trpa1^{fl/fl}* Baseline vs. Hypoxia ($p<0.001$), Baseline vs. Recovery 1, Baseline vs. Recovery 2, Hypoxia vs. Recovery 1, and Hypoxia vs. Recovery 2 (all $p<0.01$). **(D)** RR in successive ventilatory phases. There was a significant main effect on RR of the ventilatory response phases ($F_{3,48}=17.967$, $p<0.001$) but no significant interaction between RR and TRPA1 conditions ($F_{3,48}=1.396$, $p=0.08$). There were no significant pairwise differences between ventilatory response phases in either *asTrpa1^{-/-}* or *Trpa1^{fl/fl}*. Data are means \pm SE. * $p<0.05$; Bonferroni corrected.

involved in the central control of sympathetic activity and cardiovascular function, including systemic hypertension (Marina et al., 2016b), which are enhanced by acute and particularly

repeat hypoxia episodes sensed by carotid chemoreceptors (Prabhakar et al., 2015). There is a biological plausibility that medullary astrocytes, respiratory neurons, and peripheral



chemosensing intertwine with each other in shaping PHRA. Alternative study designs are needed to further explore this issue.

In the present study, AA failed to affect V_E , although RR was increased in mice receiving a high dose of AA in room air. This phenomenon suggests that AA can affect breathing, i.e., inhibition of astrocyte activation may alter breathing patterns. In line with this notion, we showed that HVR was attenuated by a high dose of AA. However, AA blunted PHRA much more, suggesting that PHRA is activity-dependent plasticity.

There may be a concern over the time-dependent stability of minute ventilation on the background of a high dose of AA. Our additional investigation revealed that minute ventilation was stable over 240 min in this condition (Supplementary Figure 1). Likewise, another set of control investigations showed that hypoxia loadings repeated three times provide close reproducibility (Supplementary Figure 2).

One potential limitation of this study could be a lack of the animal's temperature control. In the classical "closed chamber" whole-body plethysmography, tidal volume is calculated by measuring the chamber pressure based on the combined gas law stating that the ratio of the product of gas pressure and volume to the absolute gas temperature is equal to a constant (Drorbaugh and Fenn, 1955). The chamber pressure is recorded while the chamber is sealed, and the body temperature weighs in on the result (Mayer et al., 2014; Rourke et al., 2016; Baby et al., 2018). In practice, however, the body temperature changes are so small during the hypoxic challenges of a couple of minutes that they are usually neglected for the sake of simplicity (Onodera et al., 1997). In the present study, we adopted the "open flow" plethysmography in which the chamber gas is continuously suctioned at a constant flow rate during the continuous recording. We calculated the tidal volume by integrating the airflow whose changes are proportional to those in the chamber pressure (Lundblad et al., 2002). In this case,

tidal volume is expressed at ambient temperature (25°C), which obviates the need for taking the animal's body temperature. Another limitation of this study was that we failed to examine metabolic rate in knock-out mice or its potential alterations by AA, which could influence respiration. Metabolic aspects require further exploration using alternative study designs.

The ultimate purpose of this research was to refer to the mechanism of post-hypoxic short-term respiratory plasticity in unanesthetized humans, which is essential to get insights into the pathophysiology of and preventive measures for periodic breathing, e.g., sleep apnea. This purpose stemmed from the studies showing that PHRA is involved in the mitigation of periodic breathing in sleep apnea (Georgopoulos et al., 1992; Mahamed and Mitchell, 2007; Mateika and Syed, 2013; Mateika and Komnenov, 2017) and heart failure (Ahmed et al., 1994); the notion supported in a computer simulation study (Eldridge, 1996). Our results showed that astrocytes, but not the astrocytic TRPA1 channel, were involved in the development of PHRA, suggesting that the TRPA1 is engaged in shaping HVR but not PHRA. The TRPA1 channel likely plays a (patho)physiological role in acute hypoxic conditions such as an attack of bronchial asthma (Shen et al., 2012). In diseases with periodic breathing such as sleep apnea, astrocytes may contribute to its prevention by exerting PHRA. Additionally, the observation that $\Delta V_{E, \text{Recovery1}} / \Delta V_{E, \text{Hypoxia}}$ tended to be smaller in the female *asTrpa1*^{-/-} mice raises the implication of a greater role of astrocytic TRPA1 in the female gender, which requires further exploration.

In conclusion, we have provided novel aspects of PHRA's role linking it to astrocyte activation and suggesting that this tandem arrangement contributes to respiratory stability and potentially might be influential in the prevention of periodic breathing. However, caution should be exercised in the translation of animal findings to human settings before further exploratory research. We conclude that astrocytes mediate the post-hypoxic

persisting respiratory augmentation by mechanisms other than the hitherto recognized role of TRPA1 channels in hypoxia sensing.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee for Animal Experiments of the Murayama Medical Center.

AUTHOR CONTRIBUTIONS

IF conceived and designed the study, performed the animal experiments, analyzed the data, and drafted the manuscript.

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KT performed the statistical analysis and drafted the manuscript. MP edited and revised the manuscript. YK, MY, and YH participated in the design of the study. AN and YM provided the animals and revised the manuscript. HO supervised the experiments and revised the manuscript. YO conceived and designed the study, analyzed the data, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2021.757731/full#supplementary-material>

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