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Augmented Chemosensitivity in Black-Eyed White *Mitf*$^{mi\text{-}bw}$ Mice, Lacking Melanocytes

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Microphthalmia-associated transcription factor (Mitf) is responsible for differentiation of melanocytes, and a recessive *Mitf* mutant, black-eyed white (bw) mouse, is characterized by the lack of melanocytes in the skin and inner ear. To search for the hitherto unknown roles of melanocytes, we analysed the ventilatory responses of unanaesthetized bw mice by whole body plethysmography. During air breathing, bw mice showed lower breathing frequency and larger tidal volume, compared with age-matched wild-type mice, although there was no difference in the minute ventilation. Importantly, bw mice present normal haematocrit values and red blood cell counts. We next measured the immediate ventilatory responses to acute hypoxia (10% O_2) and to hyperoxic hypercapnia (10% CO_2). Hypoxic and hypercapnic ventilatory responses represent the functions of the chemoreceptors in the carotid body and the brainstem, respectively. The bw mice retain the peripheral hypoxic and central hypercapnic sensing functions, but exhibited augmented ventilatory responses to both hypoxia and hypercapnia. Unexpectedly, RT-PCR analysis has shown the expression of melanocyte-specific Mitf mRNA in the brain of bw mice, suggesting the presence of leptomeningeal melanocytes. These findings suggest a functional link between skin melanocytes and the central respiratory controller that generates respiratory rhythm and pattern.

Key words: brain, chemosensing, heart, hypoxia, hypercapnia, lung, microphthalmia-associated transcription factor, melanocyte, ventilation.

Abbreviations: bw, black-eyed white; CCHS, congenital central hypoventilation syndrome; Mitf, microphthalmia-associated transcription factor.

INTRODUCTION

The immediate ventilatory responses to hypoxia and hypercapnia are essential for survival. Hypoxia stimulates the peripheral chemoreceptors, which in turn leads to a rapid increase in ventilation (*1*). Glomus cells in the carotid body, which are of neural crest-origin, serve as a principal peripheral chemoreceptor (*2*). On the other hand, CO_2 sensing mainly depends on chemoreceptors in the brainstem (*3, 4*). The central respiratory controller, which regulates autonomic ventilation, is derived at least in part from the neural crest, as judged by the molecular basis of congenital central hypoventilation syndrome (CCHS) (*5–7*). CCHS is defined as the failure of automatic control of breathing, which is characterized by the marginal ventilatory sensitivity to hypoxia and hypercapnia during sleep. CCHS is sometimes associated with mutations of the gene coding for the receptor tyrosine kinase RET (Rearranged during transfection) (*5–7*). Some patients with CCHS also manifest Hirschsprung's disease (*6*). These results indicate that the neural crest-derived cells play an important role in the ventilatory responses to chemical loadings.

Microphthalmia-associated transcription factor (Mitf) plays an essential role in development and survival of neural crest-derived melanocytes and optic cup-derived retinal pigment epithelium (RPE) (*8–10*). The mutations of the human MITF gene are associated with some cases of Waardenburg syndrome type 2, a dominantly inherited auditoty–pigmentary syndrome, which is caused by the lack of melanocytes in the skin and inner ears (*11–14*). Likewise, mutations in the murine *Mitf* gene cause hypopigmentation and hearing impairment (*8, 9*). MITF/Mitf consists of many isoforms with distinct amino-termini in humans and mice, such as MITF/Mitf-M, MITF/Mitf-A and MITF/Mitf-H (*15–19*; reviewed in *20*), but shares the entire carboxyl-terminal region, including a basic helix-loop-helix-leucine zipper structure. Among multiple Mitf isoforms, Mitf-M is essential for melanocyte development, as judged by the molecular lesion of black-eyed white *Mitf*$^{mi\text{-}bw}$ (bw) mice, in which the insertion of an L1 retrotransposable element in the intron 3 between exon 3 and exon 4 of the Mitf gene leads to complete repression of Mitf-M mRNA expression in melanocytes (*21*). However, other Mitf isoforms, such as Mitf-A and Mitf-H, are expressed in the bw mouse

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skin, RPE (*21*), and testis (*22*). Accordingly, the bw mice exhibit a unique black-eyed white phenotype: complete white coat color and deafness due to the lack of the skin and inner ear melanocytes, associated with normally pigmented RPE (*21, 23*).

Multiple environmental and hormonal signals converge on melanocytes (*20, 24*). However, little is known about the physiological consequences of the lack of melanocytes, except for auditory-pigmentary abnormality. In order to search for the hitherto unknown roles of melanocytes or a genetic factor that may influence the hypoxic and/or hypercapnic ventilatory responses, we examined the respiratory function and ventilatory responses of bw mice by whole body plethysmography (*25, 26*).

EXPERIMENTAL PROCEDUERS

Mice—The original *Mitf*$^{mi\text{-}bw}$ (bw) mouse was maintained on a C3H background at Tohoku University, Graduate School of Life Sciences, as detailed previously (*21, 23*). C3H/He mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed based on the institutionally approved protocols of Tohoku University School of Medicine. Male mice (8–12 weeks) were maintained under 12 h light/12 h dark cycle at 20°C and allowed free access to standard mice food and water. Blood samples were drawn from the retro-orbital plexus of unanaesthetized mice (*27*) for measuring red blood cell counts and haematocrit with a heparinized capillary tube (Drummond Scientific Co., Broomall, PA) (*28*).

Measurement of Ventilation—Male mice (8–12 weeks) used were bw ($n=9$) and age-matched C3H/He mice ($n=13$). A freely moving mouse with body weight of about 25 g was permitted to acclimate in the chamber at least 30 min before ventilation was measured by the barometric methods of pressure type plethysmography (*25–27*). The respiratory frequency (f) and tidal volume (TV) were obtained, as detailed previously (*27, 29*). Minute ventilation (VE) was calculated from $f \times TV$. TV and VE were normalized with body weight (g). The mean values from the two trials were accepted as individual data. The O_2 fractional concentration (FO_2), the CO_2 concentration (FCO_2) in the 700 ml Plexiglass chamber, and pressure changes were measured with a gas analyzer (Model 1H21A; NEC Sanei, Tokyo, Japan). For hypoxic ventilatory responses, N_2 gas was mixed with background room air to maintain the chamber FO_2 at 10%. For ventilatory responses to hypercapnia, the FCO_2 in the chamber was adjusted to 10% using a gas mixture of 40%CO_2 and 60%O_2 in N_2. Under such a hyperoxic hypercapnic condition, the contributions of peripheral hypoxic chemoreceptors were minimized. The ventilatory responses to hypoxia or hypercapnia were measured for 15–20 s, with an interval of at least 30 min and in random order. The response of each respiratory variable was assessed as value during hypoxia or hypercapnia minus corresponding basal value during room air breathing (*25*). Data are presented as mean ± SEM. Comparison between two groups was by an unpaired Student's t-test. Differences between mean values were considered significant when $P<0.05$.

RT-PCR Analysis of Mitf Isoforms—Total RNA was prepared using TRI REAGENT™ (Sigma-Aldrich Japan, Tokyo, Japan) from the skin of bw and heterozygous littermates at 4.5 days of age (*30*). Total RNAs were also prepared from the brain, heart and lung of bw and its heterozygous male littermates at 7 months of age, and from melan-a immortalized mouse melanocytes (*31*) and B16 mouse melanoma cells (*30, 32*). RT-PCR for Mitf-A, Mitf-H and Mitf-M mRNA was performed with a forward primer located in each exon 1 (5′-CAC AGT TGG AGT TAA GAG TGA GCA TAG CC-3′ for Mitf-A; 5′-TTA GAT TTG AGA TGC TCA TCC CCT GCT CCT-3′ for Mitf-H; and 5′-TAC AGA AAG TAG AGG GAG GAC TAA G for Mitf-M, respectively), and a common reverse primer located in exon 2 (5′-CCT GGT GCC TCT GAG CTT GCT GTA TGT GGT AC-3′) (*30, 33*). The predicted sizes of the amplified fragments for Mitf-A, Mitf-H and Mitf-M are 344 bp, 357 bp and 167 bp, respectively. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was performed with a forward primer (5′-ACC ACA GTC CAT GCC ATC AC-3′) and a reverse primer (5′-TCC ACC ACC CTG TTG CTG TA-3′). The PCR conditions were 42 cycles of 96°C for 20 s, 60°C for 20 s and 72°C for 1 min, except for Mitf-M. The PCR conditions for Mitf-M were 42 cycles of 96°C for 20 s, 55°C for 20 s and 72°C for 30 s.

RESULTS

Room Air Breathing Patterns of bw Mice—The bw mice appear to be in good physical conditions, despite the lack of melanocytes in the skin, the choroid of the eye, and the inner ear (*21*). In fact, there is no difference in the body weight between wild-type mice and bw mice (Table 1). Moreover, haematocrit and red blood cell counts are 49.44 ± 0.76% and $9.50 \pm 0.23 \times 10^{12}$/l, respectively, in bw mice ($n=5$), the values of which are indistinguishable from 48.32 ± 0.29% and $9.47 \pm 0.46 \times 1012$/l in age-matched wild-type mice ($n=5$). Thus, bw mice are free of anaemia and polycythaemia.

To search for hitherto unknown abnormality in bw mice, we next measured the basal breathing patterns of freely moving mice (Table 1). Unanaesthetized mice were permitted to acclimate in the chamber for at least 30 min before ventilation was measured by plethysmography. The parameters measured under room air breathing were breathing frequency, TV and VE. During air breathing, there was no difference in the VE, which reflects overall ventilation, between the age-matched wild-type mice and bw mice. However, to achieve the similar VE, bw mice showed the significantly lower breathing frequency and significantly larger TV than did wild-type mice. In other words, bw mice showed slow and deep breathing patterns, compared with the wild-type mice with rapid and shallow breathing.

Enhanced Ventilatory Responses to Hypoxia and Hypercapnia in bw Mice—We next analysed the immediate ventilatory responses to acute hypoxia (10% O_2) and to acute hyperoxic hypercapnia (10% CO_2) in freely moving mice by whole-body plethysmography (Table 1 and Fig. 1). We measured the initial phase of ventilatory

Table 1. **Respiratory variables during room air breathing, and hypoxic and hypercapnic ventilatory responses of wild-type and bw mice.**

Genotype	*n*	Body weight (g)		VE (ml/min/g)	*f* (min)	TV (μl/g)
Wild-type	13	25.0 ± 0.6	Room air	2.57 ± 0.07	222.9 ± 9.2	11.7 ± 0.5
			10%O_2	4.30 ± 0.16	260.7 ± 6.2	16.5 ± 0.4
			10%CO_2	7.05 ± 0.26	307.8 ± 3.9	22.9 ± 0.8
bw	9	23.6 ± 0.9	Room air	2.50 ± 0.10	162.2 ± 10.2*	15.7 ± 0.8*
			10%O_2	5.84 ± 0.49*	292.4 ± 22.9	20.0 ± 0.6*
			10%CO_2	8.87 ± 0.32*	305.5 ± 12.5	29.4 ± 1.5*

VE, VE ($f \times$ TV); *f*, respiratory frequency and TV, tidal volume. Values shown are means ± SEM. *Significant difference ($P < 0.05$) was determined for each dependent variable.

responses to acute hypoxia, which were detected within 20 s after challenge. It is important to measure the immediate ventilatory responses to acute hypoxia to exclude the secondary effects caused by complicated compensatory responses. Hypoxic ventilatory responses reflect the function of the carotid body, which senses reduced arterial oxygen tension to enhance ventilation. Hypercapnic ventilatory responses are mainly achieved through the central chemoreceptor and respiratory controller in the brainstem (*3, 4*).

In response to hypoxia or hypercapnia, the bw mice increased the breathing frequency and the TV, thereby leading to a significant increase in the VE (Table 1). However, there were no significant differences in the maximum breathing frequency achieved in response to hypoxia or hypercapnia between the control and bw mice (Table 1), although the relative increase in the breathing frequency was greater in bw mice than in wild-type mice (Fig. 1). Likewise, the TV was significantly larger in bw mice during hypoxic and hypercapnic conditions, compared with wild-type mice (Table 1), but the relative increase in the TV was similar in bw and wild-type mice (Fig. 1). Accordingly, the increase in the VE was significantly higher in response to acute hypoxia or hypercapnia in bw mice. These results suggest that the bw mice retain the functions of the oxygen-chemosensors in the carotid body and the central chemoreceptor for hypercapnic chemoreception. However, the augmented chemosensitivity to both hypoxia and hypercapnia suggests a functional alteration in the central respiratory controller, which governs ventilation.

Expression of Mitf Isoform mRNAs in the Brain, Heart and Lung—As a first step to explore the molecular basis for the augmented ventilatory responses in bw mice, we attempted to localize the neurons that express Mitf in the brain by immunohistochemistry, but the trials were unsuccessful probably due to the low expression levels of Mitf. Northern blot analysis of total RNA detected faint broad signals, representing mRNAs of Mitf isoforms, in the brain, heart and lung of bw and its heterozygous littermates (data not shown). Consequently, we performed RT-PCR to identify Mitf isoform mRNAs expressed in the brain of bw mice. As expected, Mitf-M mRNA was undetectable in the bw mouse skin, but detected in the heterozygous mouse skin and in mouse melanocyte and melanoma cell lines (Fig. 2A). These results confirm the validity of the RT-PCR analysis. Unexpectedly, Mitf-M mRNA is expressed in the brain of bw and its heterozygous mice, although its expression is undetectable in the heart and lung (Fig. 2B). In contrast, Mitf-A and Mitf-H mRNAs are expressed in the brain, heart and lung of bw and its heterozygous mice (Fig. 2B). These results indicate that there are Mitf-M expressing cells in the bw brain, which probably include leptomeningeal melanocytes (*34*).

DISCUSSION

Various biochemical processes in the brain are responsible for generation of respiratory rhythm and respiratory pattern (*4*), and are influenced by the changes in O_2 tension and CO_2 tension. The bw mice show the lower breathing frequency and the larger TV than the wild-type C3H mice during air breathing, thereby maintaining the overall ventilation (VE) similar to that of wild-type mice. The lower breathing frequency indicates that inspiratory time and/or expiratory time are prolonged in bw mice. Moreover, bw mice show the lowest breathing frequency and the largest TV during air breathing, compared with other mouse strains analysed by the same method, including A/J, AKR/N, BALB/c, C57BL/6, DBA/2, NZW, SWR/J and 129Sv (*29*). Thus, the breathing patterns of bw mice are unique among those mouse strains and may reflect an alteration in the central chemosensitivity that determines the breathing frequency and the respiratory depth (Fig. 3). In addition, bw mice show augmented chemosensitivity to both hypoxia and hypercapnia. Taken together, these results suggest that bw mice are altered in chemosensing at the central respiratory controller, including the nucleus tractus solitarius, which represents the first synapse site of the cardiopulmonary reflex afferents (*25, 26, 35*).

Incidentally, C3H mouse strain has been classified as hypercapnic low responsive by other investigators (*36*), which is consistent in part with our recent report, showing the lower TV in C3H mice during hypercapnia (*29*). In contrast, bw mice on the C3H background showed the largest TV under hypercapnia among the nine strains examined: A/J, AKR/N, BALB/c, C3H, C57BL/6, DBA/2, NZW, SWR/J and 129Sv (*29*). Five of these mouse strains are albino, while other four are pigmented, C3H, C57BL/6, DBA/2 and 129Sv. Thus, a loss of tyrosinase enzyme activity (*37*) or lack of melanin production is unlikely to cause the altered chemosensing observed in bw mice. Moreover, we have

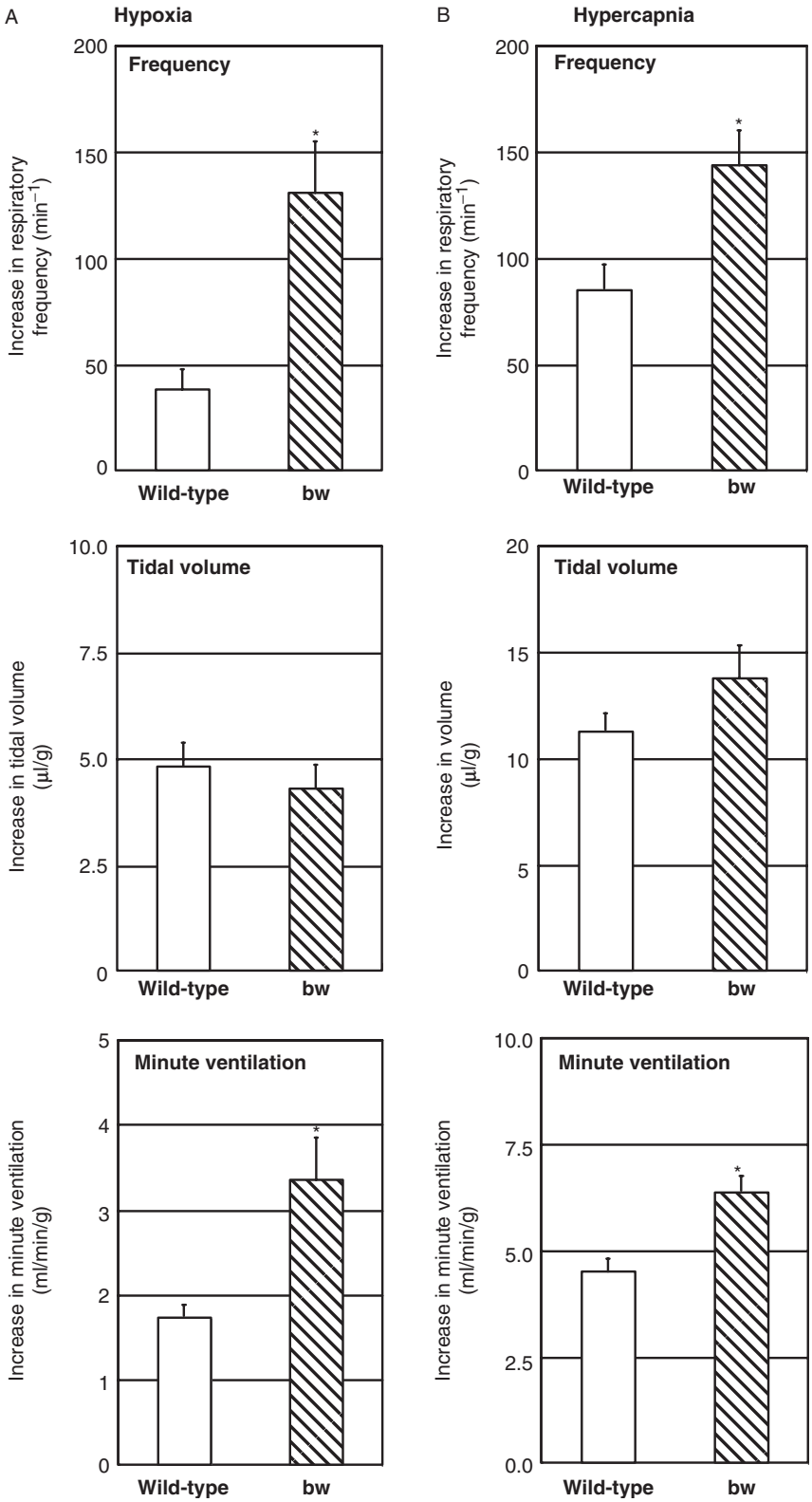

Fig. 1. **Immediate ventilatory responses in bw mice.** Ventilatory responses were measured in unanaesthetized and unrestrained mice by whole body plethysmography. Shown are (A) ventilatory responses to hypoxia (10% O_2) and (B) ventilatory responses to hypercapnia (10% CO_2): the increase in respiratory frequency (top), TV (middle) and VE (bottom). Wild-type C3H mice ($n = 13$) and bw mice ($n = 9$). Error bars indicate mean values and SEM for the indicated ventilatory parameters (*$P < 0.05$).

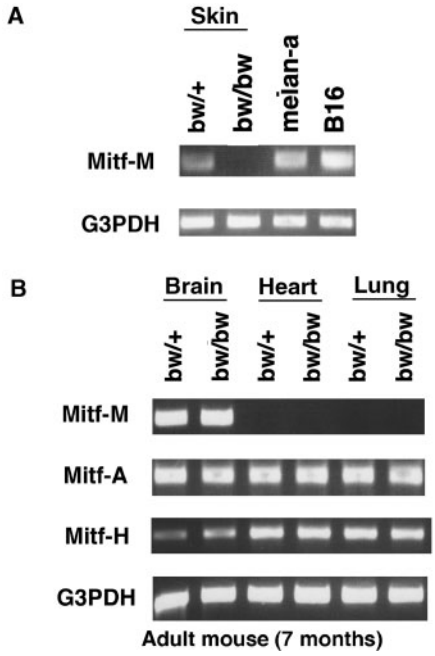

Fig. 2. **RT-PCR analysis of Mitf isoform mRNAs in bw mouse.** (A) Expression profile of Mitf-M mRNA in the skin of bw and its heterozygous littermates. Total RNA was prepared from the skin at postnatal 4.5 days (P4.5), melan-a immortalized melanocytes and B16 melanoma cells, shown at the top of the lanes. Mitf-M cDNA was amplified with an isoform-specific forward primer. The amplified DNA segments were visualized with ultraviolet transilluminator. Bottom panels show the expression of G3PDH mRNA as an internal control. Mitf-M mRNA expression is undetectable in the bw mouse skin. (B) Expression profiles of Mitf isoform mRNAs in the brain, heart and lung of bw and its heterozygous littermates at 7 months of age. Each Mitf cDNA was amplified with an isoform-specific forward primer. Other conditions were the same as described earlier.

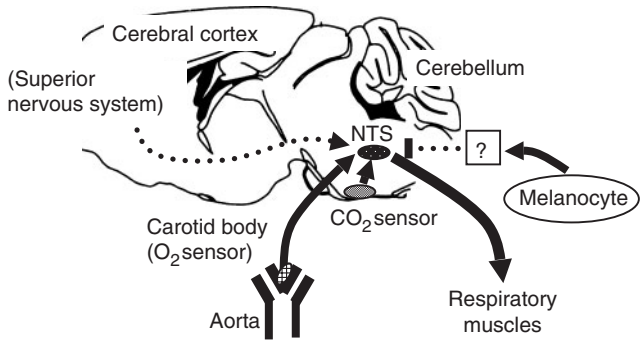

Fig. 3. **A proposed role of melanocytes in the regulation of the central respiratory controller.** Shown is a lateral view of the mouse brain. Hypoxia stimulates the peripheral chemoreceptor, glomus cells in the carotid body, which in turn leads to a rapid increase in ventilation through the nucleus tractus solitarius (NTS) in the medulla. NTS represents an essential centre for the augmentation of ventilation during hypoxia and hypercapnia (*4, 25, 26*). Hypercapnia stimulates ventilation through the central chemoreceptors in the ventrolateral medullary area (CO_2 sensor). Melanocytes, which are located in the skin and/or inner ear, secrete a hitherto unidentified factor, which may influence the function of the NTS or the superior control centre. Thus, bw mice exhibit altered function of the respiratory controller at the NTS or its higher control centre. The carotid body is shown at the bifurcation of the aorta Ø.

shown the expression of Mitf-M mRNA in the brain of bw mice. It is, therefore, conceivable that melanocytes may be involved in the modulation of the function of central respiratory pattern generator.

In the central nervous system, melanocytes are distributed on the leptomeninges of the entire human brain (*34*). Leptomeningeal pigment cells were found over the ventrolateral surfaces of the medulla oblongata. The bw mouse carries the insertion of an L1 retrotransposable element in the intron between exon 3 and exon 4 of the Mitf gene (*21*), which has been considered to cause the loss or the reduced expression of Mitf-M in melanoblasts during fetal development. In this context, the M promoter of the Mitf gene, encoding Mitf-M, represents the most downstream promoter (*11, 38*), and may be most susceptible to the transcriptional repression caused by the insertion of the L1 element (*21*). The dosage-sensitive role of Mitf-M may account for the lack of skin and inner ear melanocytes in bw mice (*39, 40*). The present study, however, suggests that differentiation or survival of leptomeningeal melanocytes might be less sensitive to the threshold level of Mitf-M, compared with the skin and inner ear melanocytes. Alternatively, the L1 insertion may not reduce the transcription from the M promoter in the leptomeningeal melanocytes; namely, the L1 element may have exerted different effects on transcription from the M promoter in the brain.

The target genes of Mitf include melanogenesis genes (*15, 16, 41*). By the cDNA microarray analysis with bw skin RNA, we have recently identified lipocalin-type prostaglandin D-synthase (L-PGDS) as a new melanocyte marker (*30*). L-PGDS catalyses the isomerization of prostaglandin (PG) H_2 to produce PGD_2 and also functions as a secreted protein abundantly present in cerebrospinal fluid (*42*). It is therefore tempting to speculate that L-PGDS or its product PGD_2, derived from melanocytes in the skin, inner ear and/or the choroid of the eye, might be involved in the regulation of ventilatory responses.

It is also noteworthy that acutely administered morphine decreased ventilatory responses to hypoxia and hypercapnia in normal subjects (*43*), which is in contrast to the augmented ventilatory responses to hypoxia and hypercapnia observed in the bw mice. Likewise, patients with heroin addiction, who were treated with methadone, a long-acting μ-opioid agonist, showed blunted hypercapnic ventilatory response but augmented hypoxic ventilatory response; namely, these patients have impaired central chemosensitivity but enhanced peripheral chemosensitivity (*44*). These studies in humans suggest the inhibitory role of opioids in the hypercapnic ventilatory response. Moreover, human melanocytes express β-endorphin, an endogenous opioid, which is generated from proopiomelanocortin (45; for review, 20). It is, therefore, conceivable that the reduction of the melanocyte-derived opioids may account for the augmented ventilatory response to hypercapnia in the bw mice.

The present study contains a limitation, which remains to be investigated. To confirm the contribution of the melanocytes to the ventilatory responses, we need to analyse other melanocyte-deficient mice with relatively good health. However, to the best of our knowledge, there are no available mutant mice with the black-eyed white phenotype, which present no haematological abnormality.

In the present study, we have shown that bw mice exhibit the exaggerated ventilatory responses to hypoxia and hypercapnia, which may be due to the altered function of the central respiratory controller in the brainstem. We therefore suggest that certain factors derived from melanocytes may influence the generation of respiratory rhythm and respiratory pattern.

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