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Post-natal hypoxic activity of the central respiratory command is improved in transgenic mice overexpressing Epo in the brain

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ABSTRACT

Previous studies indicated that erythropoietin modulates central respiratory command in mice. Specifically, a one-hour incubation of the brainstems with erythropoietin attenuates hypoxia-induced central respiratory depression. Here, using transgenic mice constitutively overexpressing erythropoietin specifically in the brain (Tg21), we investigated the effect of chronic erythropoietin stimulation on central respiratory command activity during post-natal development. In vitro brainstem-spinal cord preparations from mice at 0 (P0) or 3 days of age (P3) were used to record the fictive inspiratory activity from the C4 ventral root. Our results show that erythropoietin already stimulates the hypoxic burst frequency at P0, and at P3, erythropoietin effectively stimulates the hypoxic burst frequency and amplitude. Because the maturation of the central respiratory command in mice is characterized by a decrease in the burst frequency with age, our results also suggest that erythropoietin accelerates the maturation of the newborn respiratory network and its response to hypoxia.

1. Introduction

Erythropoietin (Epo) is a cytokine that is expressed in the central nervous system (CNS). It exerts a neuroprotective role, which was recently reviewed (Alnaeeli et al., 2012), by promoting cell survival following several types of brain injuries during adulthood (Xu et al., 2012; Yu et al., 2013) and infancy (Fan et al., 2013; Schober et al., 2014). Moreover, under physiological conditions, studies by our research group have shown that Epo modulates central respiratory command. During adulthood, transgenic mice overexpressing Epo specifically in the brain (Tg21) exhibit an increased hypoxic respiratory frequency (Soliz et al., 2005), most likely by regulating catecholaminergic synthesis in the pons and the medulla oblongata (Soliz et al., 2005). However, at early ages, the effect of Epo in newborn respiratory control has only recently been explored. The acute effect of Epo on central respiratory command was investigated in brainstem-spinal cord preparations (*en bloc* technique) (Khemiri et al., 2012). The hypoxia-related frequency depression in these preparations was attenuated by acute stimulation with Epo in a time- and dose-dependent manner, and this attenuation was reversed by antagonizing Epo signaling. Remarkably, these results are in line with clinical observations showing that chronic subcutaneous treatment with Epo (300 IU/kg dose three times per week) reduces the need for mechanical ventilation and oxygen supplementation in premature neonates (Tempera et al., 2011). Because respiratory improvement is not observed during blood transfusion (Kasat et al., 2011), these results suggest that chronic central Epo elevation impacts the maturation of the respiratory network during post-natal development. Indeed, aside from its neuroprotective role, Epo is also implicated in neural development. Epo and its receptor (EpoR) are expressed in the embryonic brain of mice (Yu et al., 2002), and the genetic deletion of Epo or EpoR leads to the increased apoptosis of neural cells and a decreased number and defective migration of neural progenitor cells. Moreover, Epo promotes increased proliferation of primary cultured neural cells from Epo or EpoR mutant mice, as well as cultured human NT2 neuronal cells, when exposed to hypoxic conditions (Yu et al., 2002). According to these findings, we hypothesized that chronic Epo stimulation of central respiratory command protects the brainstem of neonatal mice during hypoxia and accelerates the maturation of the central respiratory system during early post-natal development, the age at which the establishment of neural control of respiratory function occurs (van Vonderen et al., 2014). The absence of enhanced erythropoiesis renders our transgenic Tg21 strain a

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suitable model to address this hypothesis. Indeed, Tg21 mice show a fourfold increase of Epo specifically in the brain (Wiessner et al., 2001), which allow to study the chronic Epo stimulation on the central respiratory centers without peripheral Epo effect, e.g. erythropoiesis. *En bloc* electrophysiology was performed to obtain recordings of isolated brainstems from neonatal mice. This technique, which is frequently used in the respiratory research field, facilitates the isolated examination of central respiratory command under defined experimental conditions and without the influence of peripheral afferents. Central respiratory command consists of a complex network involving several structures, such as the pons (e.g., the parabrachial/Kölliker-Fuse complex) and the medulla (e.g., pre-Bötzinger complex, retrotrapezoid nucleus/parafacial respiratory group, and nucleus tractus solitarius) (Feldman et al., 2013). The rhythm generated by the central respiratory command in the absence of peripheral input is composed of individual slow bursts that are linked to the inspiration signal. Isolated brainstem-spinal cord preparations are easily recordable in mice from P0 to P4, but recordings become challenging at older ages (Viemari et al., 2003). Our results show that chronic Epo overexpression inhibits hypoxia-induced frequency depression. Moreover, because the maturation of isolated brainstem-spinal cord preparations in mice is characterized by a lower burst frequency with age (Viemari et al., 2003), our results suggest that chronic Epo overexpression accelerates the maturation of central respiratory command at birth.

2. Material and methods

2.1. Animals

In this study, we used mutant mice overexpressing Epo specifically in the brain (referred to henceforth as Tg21) and corresponding wild type non-Epo-overexpressing mice (referred to as control), which are Tg21 mice back-crossed with C57BL/6 mice for at least 8 to 10 generations. Tg21 and control male and female mice at post-natal day 0 (P0) and 3 (P3) from at least three litters for each group were used to perform the experiments. The Tg21 mouse strain was obtained as a generous gift from Dr. Max Gassmann of the University of Zurich in Switzerland. A detailed description of this strain is available in the literature (Ruschitzka et al., 2000; Wiessner et al., 2001). The animal experiments were approved by the Laval University Animal Ethics Committee (protocol #12-119-1) and were performed in accordance with the standards and guidance of the Canadian Council on Animal Care and EU Directive 2010/63/EU.

2.2. Electrophysiological recordings

Electrophysiological recordings were performed on isolated brainstems. Neonatal mice were cryoanesthetized via total immersion in ice for 4–5 min (Danneman and Mandrell, 1997). Two types of preparations were used: medulla oblongata-spinal cord preparations with and without the pons (total number of animals: P0, $n = 44$; P3, $n = 45$), as previously described (Khemiri et al., 2012; Viemari et al., 2003). Transection to remove the pons was performed on the anterior inferior cerebellar artery (Ruangkittisakul et al., 2007). The preparation was superfused in a recording chamber with artificial cerebrospinal fluid (aCSF: 129 mM NaCl, 3.35 mM KCl, 1.26 mM $CaCl_2$, 1.15 mM $MgCl_2$, 21.0 mM $NaHCO_3$, 0.58 mM NaH_2PO_4, and 30.0 mM glucose; final pH value in gassed aCSF of 7.4) bubbled with carbogen (95% O_2, 5% CO_2). The temperature was maintained at $26 \pm 1\,^{\circ}C$ (Temperature Controller TC-324B, Warner Instruments, Hamden, CT, USA). Inspiratory-related activity in phrenic motoneuron axons from the C4 ventral root was recorded using a suction electrode (model 573000; A-M Systems, Everett, WA, USA). The signal was amplified (gain = 10,000) and filtered (low threshold, 10 Hz; high threshold, 5 kHz) using a differential AC amplifier (model 1700; A-M Systems, Everett, WA, USA). The raw signals were integrated using a moving averager (model MA-821; CWE, Ardmore, PA, USA) and were recorded in a digitized form using a data acquisition system (model DI-720; Dataq Instruments, Akron, OH, USA). The sampling rate of the analog to digital conversion of the raw signal was 2.5 kHz. The respiratory motor output produced by the preparations was recorded under baseline conditions for 40 min to determine the viability of the preparations. The hypoxia protocol was only applied to the medulla oblongata-spinal cord preparations (P0: control, $n = 9$; Tg21, $n = 14$; P3: control, $n = 11$; Tg21, $n = 20$) and consisted of baseline recording followed by 10 min of hypoxia, induced by converting the superfusion solution from aCSF bubbled with carbogen to aCSF bubbled with 95% N_2 and 5% CO_2. Finally, the post-hypoxic recovery activity was recorded for 10 min by superfusing the preparations with aCSF bubbled with carbogen. Comparisons of the recordings from male and female newborn mice displayed no clear differences, so the data were pooled.

2.3. Statistical analysis

The frequency was defined as the number of bursts per minute. The amplitude was calculated as the difference between the background noise and the peak of the burst and was expressed in mV. The burst duration was calculated as the duration from the beginning to the end of the burst and was expressed in seconds. Finally, the burst area represented the area under the curve of the integrated signal. The burst duration and area were calculated using the LabChart software (ADInstruments, Colorado Springs, CO, USA). The frequency, amplitude, burst duration and burst area under normoxic (baseline), hypoxic and post-hypoxic recovery conditions were calculated as the average of the last 5 min of the recordings for each condition. Baselines from different genotypes were compared using one-way ANOVA (i.e. for the factor genotype, control and Tg21 were compared). Hypoxia and post-hypoxic recovery were expressed as a percentage of the baseline level for the corresponding recording. The results were expressed as the means ± SEM. Statistical analyses were conducted by adjusting baselines to a "100" arbitrary value, and then the baseline-normalized values of hypoxia and post-hypoxic recovery were compared to "100" by performing two-way repeated measures ANOVA using the Prism software (Graphpad, La Jolla, CA, USA) followed by multiple comparisons analysis using Fisher's exact test. For these analyses, the factors are condition (i.e. hypoxia and post-hypoxic recovery) and genotype (i.e. control and Tg21). The threshold of significance was fixed at 0.05.

3. Results

3.1. The pons displays inhibitory activity in Tg21 brainstem-spinal cord preparations

The ability of Tg21 and control brainstem-spinal cord preparations to burst was evaluated for 40 min under normoxic conditions, facilitating the comparison of the rhythm evolution over a period that corresponds to the entire experimental protocol (i.e., baseline, hypoxia and post-hypoxic recovery) in both types of preparations at P0 and P3. These measurements were repeated to confirm the stability of the preparations. When the pons was included in Tg21 (Fig. 1A) or control (Fig. 1C) medulla oblongata-spinal cord preparations, the signal rapidly disappeared due to pons-mediated inhibition. However, when the pons was removed, the rhythm frequency and amplitude in Tg21 (Fig. 1B) and control

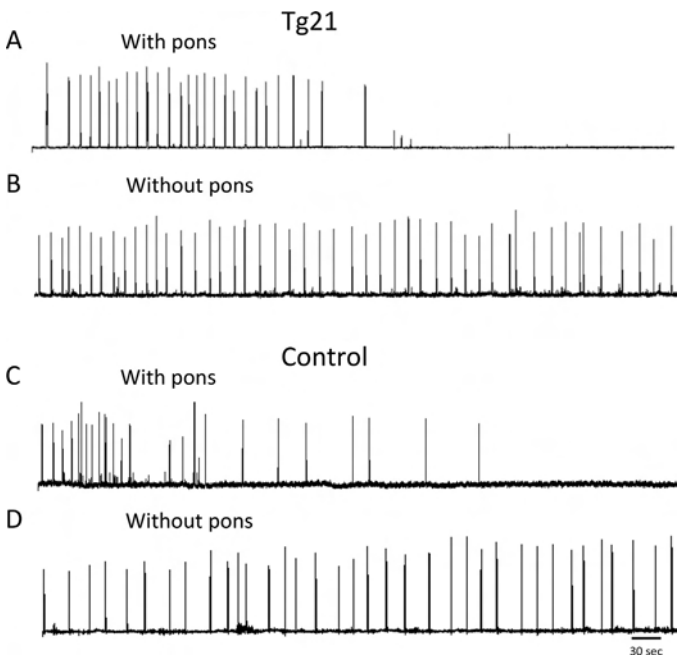

Fig. 1. The pons displays inhibitory activity in Tg21 brainstem-spinal cord preparations. (A) Representative integrated signal of fictive inspiratory activity recorded from the C4 ventral root of preparations containing the pons from P3 Tg21 mice under normoxic conditions. (B) Representative integrated signal of fictive inspiratory activity recorded from the C4 ventral root of preparations lacking the pons from Tg21 mice under normoxic conditions. (C) Representative integrated signal of fictive inspiratory activity recorded from the C4 ventral root of preparations containing the pons from P3 control mice under normoxic conditions. (D) Representative integrated signal of fictive inspiratory activity recorded from the C4 ventral root of preparations lacking the pons from control mice under normoxic conditions. The preparations containing the pons stopped releasing bursts after a few minutes. The preparations lacking the pons did not stop releasing bursts after a few minutes ($n = 45$).

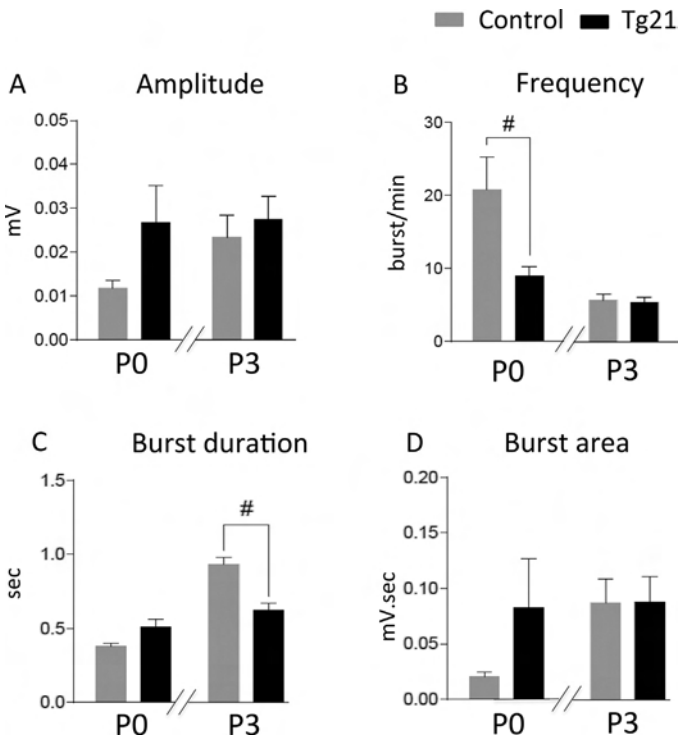

Fig. 2. The baseline parameters of P0 Tg21 preparations are similar to those of P3 preparations. (A) Basal burst amplitude (expressed in mV). (B) Basal burst frequency (expressed in bursts/min). (C) Basal burst duration (expressed in seconds). (D) Basal burst area (expressed in mV s). P0: control, $n = 9$; Tg21, $n = 14$; P3: control, $n = 11$; Tg21, $n = 20$. #Significantly different compared with the age- and condition-matched control.

(Fig. 1D) medulla oblongata-spinal cord preparations were normal throughout the recording period. No pattern difference was detected between the medulla oblongata-spinal cord preparations with and without the pons (burst area: $p = 0.1867$; burst duration: $p = 0.2373$). The same observations were made on P0 and P3 brainstem-spinal cord preparations. In subsequent experiments, medulla oblongata-spinal cord preparations without the pons were used to perform the hypoxic challenge, and these preparations are henceforth referred to as brainstem-spinal cord preparations.

3.2. Compared with the controls, the Tg21 brainstem-spinal cord preparations at P0 displayed lower basal frequency

The burst activity from control and Tg21 mouse brainstem-spinal cord preparations was recorded to determine whether chronic Epo stimulation modulates the inspiratory motor output at P0. The basal burst amplitude was not significantly different between the control and Tg21 mice ($p = 0.1759$; Fig. 2A). The basal frequency was higher in the preparations from the control mice than it was in those from the Tg21 mice (Fig. 2B). There was no difference in the burst duration ($p = 0.1438$; Fig. 2C). The burst area was not significantly different between the two genotypes ($p = 0.2107$; Fig. 2D); however, the burst area tended to be higher in the Tg21 preparations than in the control preparations.

3.3. Compared with the controls, the Tg21 brainstem-spinal cord preparations at P3 displayed a shorter basal burst duration

The amplitude, frequency and burst area were not different between the control and Tg21 preparations under normoxic conditions at P3 (Fig. 2A–C). However, the burst duration was significantly shorter in the Tg21 preparations than in the control preparations (Fig. 2D).

3.4. Compared with the controls, the Tg21 brainstem-spinal cord preparations at P0 were less strongly affected by hypoxia

Compared with the control preparations, the Tg21 brainstem-spinal cord preparations maintained the output rhythm during hypoxia (Fig. 3A; represented recordings are provided to illustrate the hypoxic response, regardless of the concordance with the baseline mean frequency). More precisely, compared with the baseline burst amplitude, the hypoxia and post-hypoxic recovery burst amplitude were significantly enhanced in the control preparations but were not altered in the Tg21 preparations (Fig. 3B). Moreover, during post-hypoxic recovery, normalized burst amplitude is significantly lower in Tg21 preparations compared with control (Fig. 3B). As reported previously (Viemari et al., 2003), compared with baseline, the frequency during hypoxia in the control preparations was significantly decreased, whereas the frequency during hypoxia in the Tg21 preparations did not decrease (Fig. 3C; Tg21 P0 baseline versus hypoxia: $p = 0.4402$; Tg21 P0 baseline versus hypoxic recovery: $p = 0.7323$). During hypoxia and post-hypoxic recovery, the burst duration was enhanced in the Tg21 group compared with baseline but not with control (Fig. 3D). During hypoxia and post-hypoxic recovery, the burst area was significantly increased compared to baseline but no significant difference between the control and Tg21 brainstem-spinal cord preparations occurs (Fig. 3E).

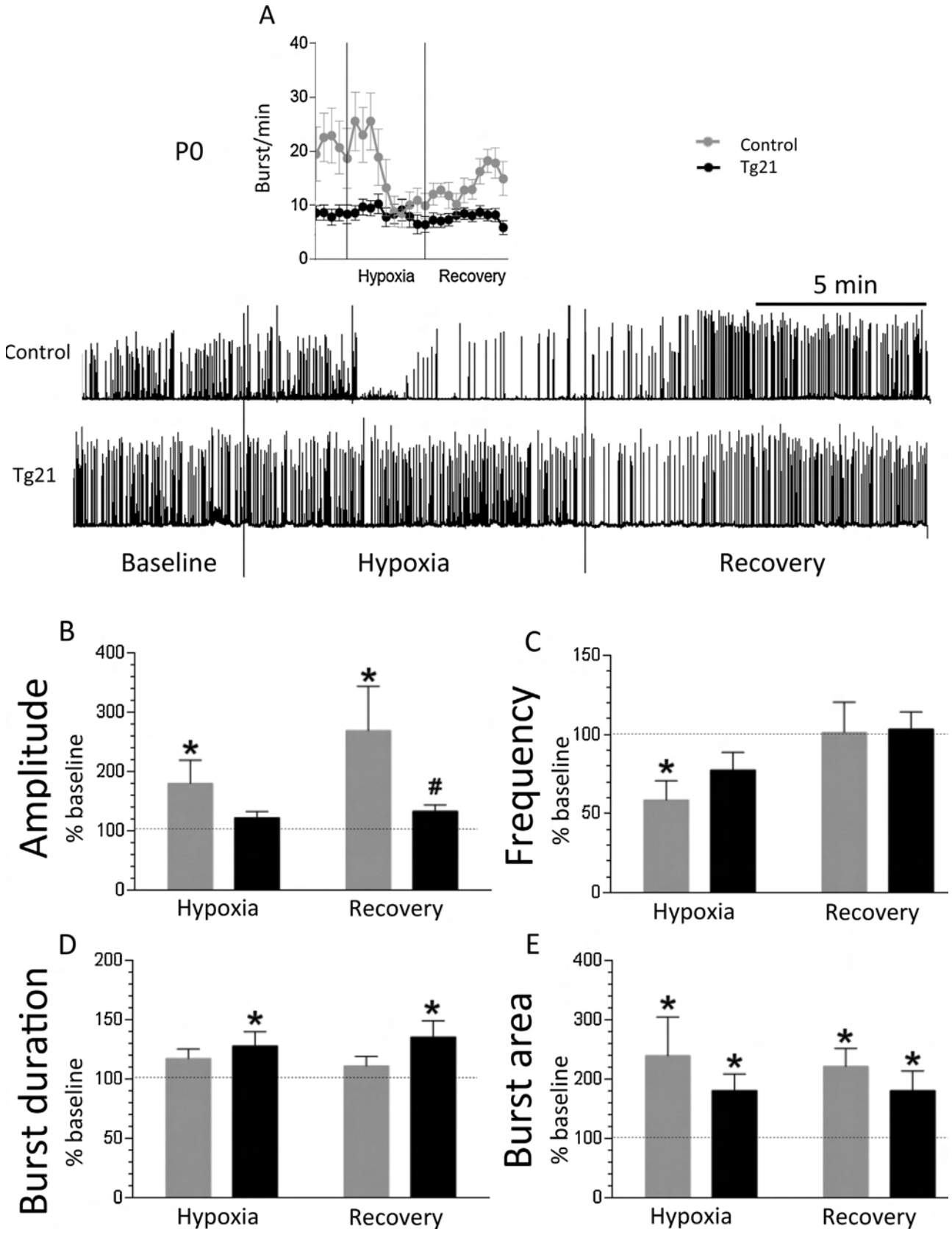

Fig. 3. P0 Tg21 brainstem-spinal cord preparations do not display hypoxia-induced frequency depression. (A) Minute-by-minute frequency recordings of P0 preparations. The graph shows the mean burst frequency over time, and the traces illustrate full recordings of control (upper trace) or Tg21 (lower trace) preparations. (B) The burst amplitude of the P0 preparations (expressed as % of baseline). (C) The burst frequency of the P0 preparations (expressed as % of baseline). (D) The burst duration of the P0 preparations (expressed as % of baseline). (E) The burst area of the P0 preparations (expressed as % of baseline). Control, $n = 9$; Tg21, $n = 14$. *Significantly different compared with the baseline level of the same group. #Significantly different compared with the age- and condition-matched control.

3.5. The hypoxic response at P3 is higher in the Tg21 brainstem-spinal cord preparations than in the control preparations

Compared with the controls, the Tg21 brainstem-spinal cord preparations maintained the output rhythm during hypoxia (Fig. 4A). During hypoxia, the amplitude was enhanced, and the frequency did not change in the Tg21 preparations compared with the baseline levels (Fig. 4B and C; Tg21 P3 baseline frequency versus hypoxia: $p = 0.8084$; Tg21 P3 baseline frequency versus hypoxic recovery: $p = 0.5159$). Alternatively, in the control preparations, the amplitude was not altered but the frequency was decreased compared with the baseline levels (Fig. 4B and C). Burst frequency during hypoxia was significantly higher in Tg21 preparations than in control preparations (Fig. 4C). During post-hypoxic recovery, the Tg21 preparations displayed higher burst amplitude compared with the control preparations (Fig. 4B), and the control preparations maintained a lower frequency compared with the baseline levels (Fig. 4C). Moreover, compared with baseline, the burst duration is enhanced in both control and Tg21 brainstem-spinal cord preparations upon hypoxic and post-hypoxic conditions. In addition, compared with control preparations, the post-hypoxic recovery burst duration was longer in the Tg21 preparations (Fig. 4D). During hypoxia, the burst area was not significantly altered in control preparations (Fig. 4E), but during post hypoxic recovery, the burst area was significantly increased compared with the baseline level (Fig. 4E) in both genotypes.

4. Discussion

In vitro recordings of isolated brainstems from control and Tg21 mice were used to examine the influence of endogenous Epo overexpression in the CNS on normoxic and hypoxic fictive ventilation at P0 and P3. The primary findings of the present study are that chronic central Epo overexpression in mice stimulates central breathing activity during hypoxia at early post-natal ages. Furthermore, our results suggest that chronic central Epo overexpression accelerates the maturation of the brainstem respiratory network, and this effect was detected at birth.

4.1. Corresponding wild type mice are the appropriate control for Tg21 Epo-overexpressing mice

Although the C57BL/6 strain was originally used to generate the transgenic Tg21 mouse line, this strain is not a suitable control for the present investigations. Instead, proper control animals were generated by backcrossing Tg21 animals with C57BL/6 animals (Wiessner et al., 2001). Differences between generated control animals and wild type background animals are common in the literature, and it is generally accepted that these differences are due to incomplete recovery of the original genetic background and breeding or housing practices (Editor, Nature Neuroscience, 2009). The burst frequency of brainstem-spinal cord preparations in previous studies was lower than that of control brainstem-spinal cord preparations in the present study. This difference could be explained by differences between generated control and wild type animals and by differences between the mouse strains and ages (P0 to P2, pooled group of different strains in Viemari et al. (2003); C57BL/6 P4 in Khemiri et al. (2012)). Significant differences between wild type mouse strains are now well characterized (Arata et al., 2010; Campen et al., 2005; O'Neill and Gu, 2013), as is the age-dependent evolution of hypoxic responses (Arata et al., 2010). The frequency of C57BL/6 brainstem-spinal cord preparations lacking the pons decreased between P0 and P2, as did their variability (Arata et al., 2010). Therefore, it is plausible that the higher burst frequency in our study is partially due to the early age and specific strain of the mice. Alternatively, the pattern of neural respiratory activity, which shifts from altered frequency to altered amplitude depending on the incubation time or the Epo dose (Khemiri et al., 2012), may also explain the differences between the response amplitudes between the control and Tg21 brainstem-spinal cord preparations according to their age.

4.2. The pons remains inhibitory despite chronic Epo stimulation

Aside from the cells that generate the respiratory rhythm, several groups of brainstem neurons are involved in the regulation of breathing (Spyer, 2009). The pons contains major clusters of catecholaminergic neurons that are involved in the regulation of breathing (Hilaire et al., 2004). These groups include A6 neurons in the locus coeruleus and A5 neurons in the ventrolateral pons. The A5 nucleus exerts an inhibitory effect on central respiratory command, whereas the A6 nucleus facilitates rhythm generation (Hilaire et al., 2004). Indeed, the inhibition of A5 neurons via noradrenaline application increases the respiratory frequency (Hilaire and Duron, 1999). These results are in line with a previous study performed using adult Tg21 mice, which revealed that Epo overexpression increases the noradrenaline levels in the A5 pontine respiratory nucleus and that Tg21 adult mice exhibit a higher breathing frequency than control mice under severely hypoxic conditions (Soliz et al., 2005). Compared with rats, the pons-mediated inhibition detected in the brainstem-spinal cord preparations from mice is especially strong. Keeping in mind that adult Tg21 mice express higher levels of noradrenaline in A5 neurons, we hypothesized that pons-mediated inhibition is attenuated at early post-natal ages. However, similar to the controls, the pons-mediated inhibitory effect is present in Tg21 brainstem-spinal cord preparations containing the pons, and we found no differences in the burst pattern between the presence and absence of the pons. Several reasons might explain this finding, such as a lower expression of EpoR in A5 neurons or insufficient Epo-mediated up-regulation of noradrenaline in newborn Tg21 brainstems.

4.3. Does the effect of Epo overexpression on central respiratory command begin at a post-natal age?

It is important to determine when Epo overexpression begins modulating neural respiratory control during brainstem development. Indeed, the effect of Epo on central respiratory command at birth and early post-natal ages should depend on the duration of overexpression, leading to the question of when Epo overexpression begins in the Tg21 mouse brain. Epo and EpoR are extensively expressed in the mouse (Knabe et al., 2004) and human (Juul et al., 1998) brain of fetuses, but the effect of Epo on neural respiratory control has only been investigated at post-natal ages. Whether Epo overexpression is already present at fetal ages in Tg21 mice is unknown. Given that the overexpression of the transgene is driven by the platelet-derived growth factor (PDGF) promoter and that PDGF expression in the brain is crucial during early gestation (Winkler et al., 2010), Epo overexpression could begin early in fetal development. As such, the differences in the responses to hypoxia already detected between the control and Tg21 brainstem-spinal cord preparations at P0 suggests an early effect of Epo overexpression on the modulation of neural respiratory control.

4.4. Acute versus chronic Epo stimulation of the hypoxic fictive response

The acute Epo stimulation of brainstem-spinal cord preparations attenuated hypoxia-induced depression but did not influence the basal parameters (Khemiri et al., 2012). In contrast to the results

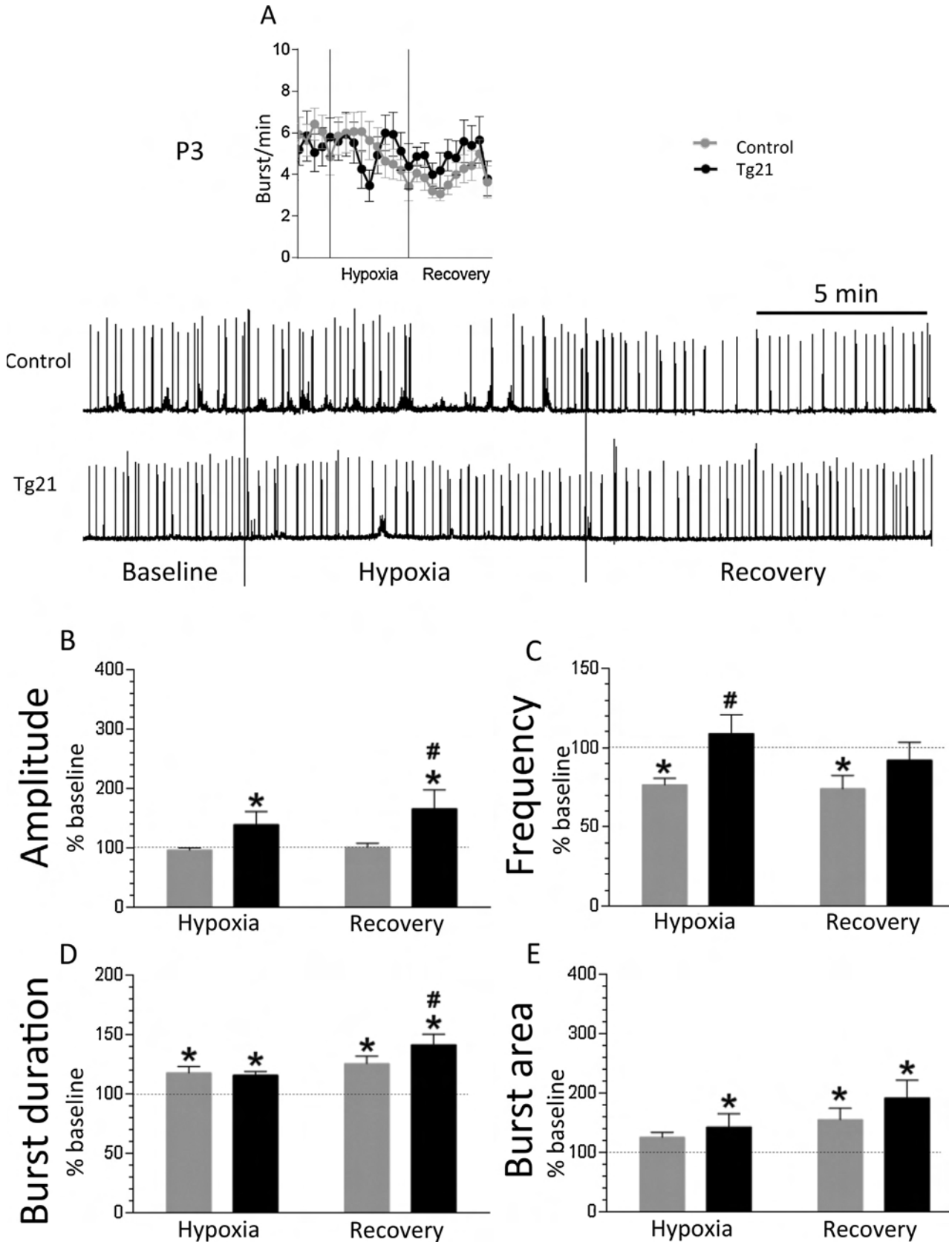

Fig. 4. P3 Tg21 brainstem-spinal cord preparations display hypoxia-induced amplitude enhancement but no hypoxia-induced frequency depression. (A) Minute-by-minute frequency recordings of P3 preparations. The graph shows the mean burst frequency over time, and the traces illustrate full recordings of control (upper trace) or Tg21 (lower trace) preparations. (B) The burst amplitude of the P3 preparations (expressed as % of baseline). (C) The burst frequency of the P3 preparations (expressed as % of baseline). (D) The burst duration of the P3 preparations (expressed as % of baseline). (E) The burst area of the P3 preparations (expressed as % of baseline). Control, $n = 11$; Tg21, $n = 20$. *Significantly different compared with the baseline level of the same group. #Significantly different compared with the age- and condition-matched control.

of Khemiri et al., we detected an effect of Epo on the basal parameters, indicating that chronic Epo stimulation in Tg21 brainstems acts on central respiratory command differently than acute stimulation on C57BL/6 brainstems. In both studies, the frequency in control brainstem-spinal cord preparations was decreased during hypoxia (Khemiri et al., 2012; Viemari et al., 2003), whereas the frequency in Tg21 brainstem-spinal cord preparations remained stable during hypoxia. Indeed, in the present study, hypoxia-induced frequency depression fully disappeared rather than merely being attenuated; indicating that chronic Epo stimulation in the brainstem exerts a stronger effect than acute (1 h) Epo stimulation (25 U). This result might be explained by the chronic stimulation of Epo (acute versus chronic), or by a different local Epo concentration, which is unknown in both cases (chronic overexpression and acute incubation) in these regions.

4.5. Chronic Epo stimulation affects central respiratory command differentially according to age

An interaction between Epo and estradiol has been previously characterized to modulate the hypoxic ventilatory response in adult female mice overexpressing Epo, in which the hypoxic ventilatory response is enhanced compared with that of males (Gassmann et al., 2010; Soliz et al., 2009). In the kidneys, the expression of Epo is decreased by estradiol (Mukundan et al., 2004). Alternatively, the expression of HIF, the transcription factor responsible for Epo synthesis, is increased by sexual hormones in the placenta (Pringle et al., 2010). Furthermore, the estradiol and testosterone levels are elevated in the brain during late gestation and immediately after birth, returning to lower levels hours after birth in rats (Rhoda et al., 1984) and mice (Corbier et al., 1992). The effect of chronic Epo stimulation on the basal parameters was detected only at P0, which may be due to the interaction of Epo with these sexual hormones. Moreover, this interaction between Epo and sexual hormones, if effective in the gestational context, may persist during the first hours after birth and modify the effect of Epo on central respiratory command.

We also hypothesize that the distinct effects of Epo according to age are due to differences in catecholamine expression during development (Roux et al., 2003) or to a potential age-dependent change of EpoR expression, which may also be linked to sexual hormones.

4.6. Does Epo accelerate the maturation of the central network that controls respiration?

Maturation of central respiratory command in rats is characterized by increased burst frequency, and decreased apnea frequency in brainstem-spinal cord preparations (Fournier et al., 2013). However, in mouse brainstem-spinal cord preparations, maturation is defined by a lower burst frequency and a longer burst duration (Viemari et al., 2003). These parameters were analyzed to determine whether Epo impacts the maturation of the central respiratory command. Interestingly, during baseline recording, the Tg21 preparations already displayed a decreased frequency of activity at P0, which remained decreased at P3. Moreover, the baseline burst area for the control P0 brainstems tended to be lower than the other groups, whereas the baseline burst amplitude tended to be higher. At these ages, neither we nor Viemari et al. (2003) reported any change in the burst duration. Thus, the brainstem-spinal cord preparations from Tg21 P0 mice displayed the same pattern as the preparations from P3 mice. Supporting this result, there are reports that Epo improves brain development in cultured rat embryos exposed to hypoxic conditions by stimulating vascular endothelial growth factor synthesis (Torun et al., 2014). Moreover, Epo promotes the differentiation and/or maturation of oligodendrocytes and enhances the proliferation of astrocytes in cultured glia (Sugawa et al., 2002). Brain-derived Epo is also required for normal brain development: when Epo or EpoR is lacking, severe embryonic neurogenesis defects occur (Alnaeeli et al., 2012). Together, these findings support the hypothesis that Epo improves the maturation of the central control command at P0. However, additional experiments are necessary to prove this hypothesis. Indeed, chronic Epo stimulation could induce some modifications of the pattern output that resemble maturation but are not truly molecular or cellular neural system maturation.

5. Conclusions

Constitutive overexpression of Epo in the CNS allows the brainstem to maintain the respiratory rhythm under hypoxic conditions. Moreover, in Tg21 brainstem-spinal cord preparations, fictive breathing is differentially affected according to age: the pattern output of the brainstem-spinal cord preparations appears to be more mature at P0, and the response to hypoxia is stronger at P3. Chronic stimulation with Epo in the brain already modulates the fine-tuning of the central respiratory network and improves hypoxic fictive ventilation at the first post-natal day in mice. The present results are of major interest for determining the neonatal impact of Epo on the brain because they demonstrate, for the first time, the effect of chronic Epo stimulation on the neonatal respiratory system. Moreover, these data are in line with the clinical observations reported by Tempera et al. (2011), who noted that chronic subcutaneous treatment with Epo (300 U/kg; 3 times/week) reduced the need for mechanical ventilation and O_2 supplementation in preterm neonates. These results are of high interest for the improvement of treatments for newborn respiratory disorders, as Epo may reduce the need for mechanical and heavy respiratory support. In humans, the immaturity of the respiratory network is linked to several brainstem-related respiratory pathologies, such as apnea of prematurity, infant distress respiratory syndrome and sudden infant death syndrome (Carroll and Agarwal, 2010). As such, the present results are of therapeutic interest.

Conflict of interest

The authors have no actual or potential conflicts of interest to disclose.

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