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Chronic overexpression of cerebral Epo improves the ventilatory response to acute hypoxia during the postnatal development

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ABSTRACT

Clinicians observed that the treatment of premature human newborns for anemia with erythropoietin (Epo) also improved their respiratory autonomy. This observation is in line with our previous *in vitro* studies showing that acute and chronic Epo stimulation enhances fictive breathing of brainstem-spinal cord preparations of postnatal day 3-4 mice during hypoxia. Furthermore, we recently reported that the antagonization of the cerebral Epo (by using the soluble Epo receptor; sEpoR) significantly reduced the basal ventilation and the hypoxic ventilatory response of 10 days old mice. In this study, we used transgenic (Tg21) mice to investigate the effect of the chronic cerebral Epo overexpression on the modulation of the normoxic and hypoxic ventilatory drive during the post-natal development. Ventilation was evaluated by whole body plethysmography at postnatal ages 3 (P3), 7 (P7), 15 (P15) and 21 (P21). In addition Epo quantification was performed by RIA and mRNA EpoR was evaluated by qRT-PCR. Our results showed that compared to control animals the chronic Epo overexpression stimulates the hypoxic (but not the normoxic) ventilation assessed as $\dot{V}_E / \dot{V}_{O_2}$ at the ages of P3 and P21. More interestingly, we observed that at P7 and P15 the chronic Epo stimulation of ventilation was attenuated by the down regulation of the Epo receptor in brainstem areas. We conclude that Epo, by stimulating ventilation in brainstem areas crucially helps tolerating physiological (e.g., high altitude) and/or pathological (e.g., respiratory disorders, prematurity, etc.) oxygen deprivation at postnatal ages.

236 words

INTRODUCTION

By activating erythropoiesis in bone marrow and augmenting the oxygen caring capacity of the arterial blood, erythropoietin (Epo) is known as a central molecule regulating tissue oxygenation (Jelkmann 2007). However, apart from the peripheral erythropoietic system, our research demonstrated that Epo is also a crucial factor regulating the neural control of ventilation by interacting with the respiratory network located in the brainstem (Soliz, et al. 2005). In our last study we showed that the intracisternal administration of soluble Epo receptor (sEpoR, a competitive antagonist of Epo) in the brainstem of newborn and adult C57/Bl6 mice significantly decreases the minute ventilation and the hypoxic ventilatory response. Accordingly, we concluded that endogenous cerebral Epo is a potent respiratory stimulant (Ballot, et al. 2015). Furthermore, recent data obtained on the *in vitro* preparation of the isolated brainstem spinal cord of newborn mice showed that Epo reduces the classical hypoxic depression of the fictive breathing frequency (Khemiri, et al. 2011). Moreover, the hypoxic depression was blunted when using brainstem spinal cord preparations of transgenic (Tg21) mice constitutively overexpressing Epo in the brain (Caravagna, et al. 2014; Caravagna and Soliz 2015).

Considering that respiratory disorders in newborns (especially on those born prematurely) are major causes of morbidity, our findings are of clinical relevance. In fact, subcutaneous treatment of premature neonates (gestational age < 30 weeks) with Epo (300 U/kg/dose, 3 times/week) has been recently reported to improve both erythropoiesis (higher hemoglobin, hematocrit and reticulocytes) and ventilatory function (reduced need of assisted ventilation and O_2 supplementation) (Tempera, et al. 2011). Although a better hematological status may help to stabilize respiration, the remarkable improvement of ventilatory function had not been reported in studies where only blood transfusions were used (Kasat, et al. 2011). Keeping in mind that Epo is able to cross the blood-brain barrier (Banks, et al. 2004; Ehrenreich, et al. 2002; Juul 2004; Statler, et al. 2007), the findings of our research perfectly explain the output of this clinical investigation.

In the present study we wanted to use our transgenic Tg21 mice to ask how the chronic overexpression of cerebral Epo modulates the ventilatory response to acute hypoxia during the postnatal development. Our results show that the chronic overexpression of Epo in brainstem regions stimulates the hypoxic ventilatory response at P3 and P21, but not in P7 and P15 mice. However, at P7 and P15 the expression of the mRNA for the Epo receptor (EpoR) was significantly decreased compared to control mice. We conclude that chronic cerebral overexpression of Epo increases the hypoxic ventilatory response during the postnatal development. These results support the evidence that Epo may be used in clinic to treat the neural-related respiratory disorders in newborns, including the inefficient ventilation observed in preterm babies.

MATERIAL AND METHODS

Animals

The transgenic Tg21 mouse line that overexpresses Epo in the brain was a generous gift of Professor Max Gassmann, University of Zurich, Switzerland. Detailed description of the Tg21 strain was reported previously (Ruschitzka, et al. 2000; Wiessner, et al. 2001). In brief, the Tg21 were created from the C57BL/6 mouse line. In our laboratory, Tg21 heterozygotes mice were backcrossed with C57Bl/6 mice for more than six generations to obtain the corresponding control mice used in this study. For the experimentation only Tg21 homozygotes were used. As comparisons of the recordings from male and female newborn mice displayed no differences the data was pooled. Animal experiments were approved by the Laval University Animal Ethics Committee (protocol #12-119-1) and carried out in accordance with the standards and guidance of the Canadian Council on Animal Care.

Ventilatory recordings by Plethysmography

Tg21 and control male and female mice at post-natal day 3 (control n=20; Tg21 n=21), 7 (control n=13; Tg21 n=39), post-natal day 15 (control n=11; Tg21 n=16) and post-natal day 21 (control n=23; Tg21 n=22) from at least three litters in each group were used to perform the plethysmography experiments. All genotyping was performed after analysis of the plethymography recordings, which explains the different numbers of animals in each group. A whole-body flow-through plethysmograph (EMKA Technologies, France) was used to record respiration in non-restrained and un-anaesthetized animals, as adapted from published protocols (Blanchi, et al. 2003; Kilic, et al. 2005). The body weights of mice were measured routinely before the plethysmography protocol. Mice were placed in a chamber continuously supplied with airflow at 0.05 (P3) 0.12 – 0.13 (P7) or 0.20 – 0.25 (P15 and P21) L/min using flow restrictors. Temperature inside the chamber was maintained at 32°C (P3), 30°C (P7) or 28°C (P15 and P21) with a temperature control loop (TCAT-2 – Physytemp, Clifton, NJ, USA). The percent of O_2 was measured in the in-flowing and out-flowing air with a two channel O_2 analyzer (S-3AII, AEI technologies, Pittsburg, PA, USA), CO_2 and pH_2O were measured in the out-flowing air (CD-3A, AEI technologies and RH 300, Sable Systems International, Las Vegas, NV, USA). Calibration of the plethysmography chamber was performed by injection of 0.5 mL of air, and the signal was amplified and recorded using computer respiratory acquisition software (IOX data acquisition and analysis, EMKA Technologies, France). As soon as the animal was familiarized with the new environment (approximately 30-45 minutes), basal ventilation was recorded at 21% O_2 for 5 minutes. Respiratory recordings at 10% O_2 were performed for 15 min. Then, the oxygen concentration in the chamber was further reduced to 6% and recordings were performed for 15 min. Finally, recovery at 21% O_2 was recorded for 15 min. Body temperature was measured before and after the hypoxia protocol using an oral thermocouple (Fluke Corporation, USA).

Flow tracings from the plethysmograph were integrated using the IOX software and used to calculate the following parameters. Frequency (Fr) was calculated as the number of respiratory cycles (inspiration and expiration) per minute. Tidal volume (V_T) was calculated using the standard equation described for whole body plethysmography (Bartlett and Tenney 1970a), as the amplitude of the integrated signal corresponding to inspiration and corrected for atmospheric and water pressures as well as temperature. Ventilation ($\dot{V}_E$) was calculated as tidal volume multiplied by frequency. Steady state O_2 consumption was measured at P3, P7 and P21 for the last 5 minutes of each condition. O_2 consumption was reported in the P3, P7 and P21 mice. The portion of the calibrated flow trace corresponding to inspiration was integrated by the software, and the corresponding volume was corrected by using the standard equation described for whole body plethysmography (Bartlett and Tenney 1970b). $\dot{V}_{O_2}$ was calculated as follow:

$$\dot{V}_{O_2} = Flowi(FiO2 - (\frac{FeO2(1 - FiO2 - FiCO2 - FiH2O)}{1 - FeO2 - FeCO2 - FeH2O})$$

where $Flow_i$ is the flow rate of gas measured in the inflowing line, *Fi* and *Fe* are the fractions of the corresponding gas measured in the inflowing (F_i) and outflowing (F_e) lines respectively. This equation allows the correction for the changes of gas composition in the inflowing and outflowing gas lines due to the activity of the animal, and the day-to-day variability of CO_2 and H_2O in ambient air (Lighton and Halsey 2011). Since in our previous study the most important effect of Epo on hypoxic ventilatory response in newborn mice was assessed by the ratio of minute ventilation ($\dot{V}_E$) to O_2 consumption ($\dot{V}_E / \dot{V}_{O_2}$) (Ballot, et al. 2015), this value was reported in this work.

Radioimmunoassay

Control and Tg21 male and female mice at P1 (control n=4; Tg21 n=4), P4 (control n=4; Tg21 n=4), P7 (control n=4; Tg21 n=4), P15 (control n=3; Tg21 n=3) and P21 (control n=4; Tg21 n=4) were used to quantify Epo in brainstem. According to previously published protocols (Bavis, et al. 2007; Kilic, et al. 2005), Epo levels in the brain were quantified using an 125I-Epo-based radio-immunoassay (RIA) (Amersham, Zurich, Switzerland), The lower detection limit of our RIA was 4 U l–1, and the intrassay/interassay variances were <2% and < 6%, respectively.

qRT-PCR

mRNA was isolated and purified (ReliaPrep RNA Miniprep System, Promega) from control and Tg21 brainstem tissues at P3, P7, P14 and P21. The yield and purity of mRNA was determined using spectrophotometry (Nanodrop 2000, Thermo Scientific). The yield of total mRNA obtained was determined

at 260 nm; the purity was estimated by the relative absorbance at 260 nm and 280 nm (A260/A280), which in all cases was superior to 2.0. Efficiency of RNA extraction was monitored with the Agilent 2100 Bioanalyzer (Agilent Technologies) giving a RIN score over 9.4, indicating high quality RNA extraction by the RNA extraction Promega Kit.

We performed two-step RT-PCR. For reverse transcription (first step) we used 1µg of RNA with 1µg Oligo dT_{15} (Promega) and nuclease-free water to a final volume of 10 µl. PCR tubes were incubated 5 minutes at 70°C and then RT buffer (1X), dNTP (1mM), RNAasin inhibitor (40U, Promega) and reverse transcriptase (200 U) were added to the PCR tubes to a final volume of 20 µl. The thermal cycle program consisted of 5 minutes at 25°C (annealing), 1 hour at 42°C (transcription) and 15 minutes at 70°C (inactivation). qPCR (second step) experiments were performed on a 7500 fast real time PCR system (Applied Biosystems, Foster City, CA, USA) using Select Master mix SYBR Green.

Murine EpoR forward (5'ACAAGGGHTAACTTCCAQGCTGTG3') and reverse (5'GATCCTCAGGGAAGGAGCTG3') primers were designed using Primer 3.0. Software. Oligo properties were calculated using Oligo Analyzer 3.1 (IDT) Software. Each assay was performed in duplicate using 2µL of cDNA (0.5µg/µL), appropriate forward and reverse primer (1µM) and fast SYBR green master mix (A&B Systems). The thermal cycle protocol included one step of 50°C during 2 minutes and one step of 95°C during 10 minutes for denaturation and 40 cycles of 95°C during 0:15 minutes and 60°C during 0:34 minutes for amplification.

Amplification efficiency of target and reference genes was between 90 and 110%. Threshold cycle (Ct) was set at 20% of the fluorescent amplification plot. Ct value is inversely related to the starting amount of cDNA assuming 100% efficiency, which is the case. Post-amplification melting-curve analysis ensured reaction specificity.

Relative EpoR mRNA expression analysis was done by normalizing values to the housekeeping gene murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5' AATGTGTCCGTCGTGGATCTG – 3'; reverse: 5'CATACTTGGCAGGTTTCTCCAG-3'). DCt (Ct_{EpoR} - Ct_{GAPDH}) was calculated for each sample and DCt was compared between WT and Tg21 for each age group.

Statistical analysis

Baseline values were calculated as the mean of the last 5 min of baseline recording. Hypoxic and post-hypoxic values were calculated every 5 minutes under hypoxic and post-hypoxic recovery conditions. Baseline Hypoxic and post-hypoxic data from different groups were compared using one-way ANOVA for

repeated measures with genotype as the grouping variable and time as the repeated variable using the Prism software (Graphpad, La Jolla, CA, USA) followed by multiple comparisons analysis using Fisher's exact test. The results were expressed as the means ± SEM. For Epo and EpoR mRNA quantification, the analyses were performed using Prism software (from Graphpad, La Jolla, CA, USA). The reported values are the means ± SEM. Data were analyzed using two-way ANOVA followed by a *post hoc* Fisher's test. Differences were considered significant when $p<0.05$. The data of radioimmunoassay and the q-RT-PCR tests were analyzed using one-way ANOVA followed by a *post hoc* Fisher's test.

RESULTS

Transgenic Tg21 mice overexpress Epo in the brainstem at postnatal ages

While the overexpression of Epo in the brain of Tg21 animals was shown at adult ages (Ruschitzka, et al. 2000), it was not yet demonstrated if Epo overexpression in Tg21 mice was already present at postnatal ages. To answer this question Epo levels in the brainstem were evaluated by performing RIA assays. Compared to control brainstem samples, Epo was significantly higher in transgenic brainstems at all tested ages (P1, P4, P7, P14, P21; $p<0.05$). Moreover, while the Epo overexpression in Tg21 brainstems remains stable during the postnatal development, Epo levels in the brainstem of control animals significantly decrease after the postnatal day 4 (Fig. 1a).

EpoR mRNA expression is decreased in the brainstem of Tg21 mice at P7 and P15

As the overexpression of a protein may induce a negative feedback loop of its receptor, we wanted to determine whether the expression level of the Epo receptor (EpoR) in the brainstem of transgenic animals remained unchanged during the development. As such, the EpoR mRNA expression was evaluated by qRT-PCR assay. Our results showed no significant differences of EpoR mRNA expression between transgenic and control brainstem samples at P3, but a significant reduction of EpoR mRNA expression in Tg21 mice at P7 and P15. This difference however disappeared at P21 (Figure 1b). This data suggest that a compensatory balance of Epo stimulation by decreasing the expression of its receptor occurs at the ages of P7 and P15. Nevertheless, later on, the level of EpoR mRNA is restored when the animals reach the age of P21.

Tg21 mice showed increased hypoxic ventilatory response at P3.

Ventilation ($\dot{V}_E$), respiratory frequency (Fr) and tidal volume (VT) were evaluated during normoxia (21%), hypoxia (at 10% and 6%; 15m each), and post hypoxic recovery (21%; 15 min). Compared to Ve in control animals, $\dot{V}_E$ in transgenic mice was significantly higher at all the tested conditions (Fig. 2a). The increased Ve was due to an elevated VT (Fig. 2c) rather than Fr (Fig. 2b). While the metabolic rate ($\dot{V}_{O_2}$) was similar between control and transgenic mice at all conditions (Fig. 2e), $\dot{V}_E / \dot{V}_{O_2}$ at 10% and 6% hypoxia was significantly augmented in transgenic mice compared to control animals (Fig. 2e). No differences of $\dot{V}_E / \dot{V}_{O_2}$ were found at basal minute ventilation, neither after post hypoxic recovery (Fig. 2e).

Despite decreased expression of EpoR mRNA in the brainstem at P7 and P15, HVR appears to be preserved in transgenic mice.

At postnatal day 7, minute ventilation, hypoxic ventilatory response (at 10%, but not at 6% O_2), and post-hypoxic recovery (after the first 5 min only) were significantly lower in transgenic mice compared to control animals (Fig. 3a). This decrease was mainly due to significant decreased tidal volume (Fig. 3c), rather than a reduction of Fr (except after 5 min of post hypoxic condition - Fig. 3b). Oxygen consumption at this age is similar in transgenic and control animals (Fig. 3d). More important, when $\dot{V}_E$ was normalized by the $\dot{V}_{O_2}$, no difference were observed between the animal groups (Fig. 3e). Keeping in mind that at P7 transgenic mice show a reduced amount of mRNA EpoR in the brainstem, this result suggests that the chronic Epo overexpression may partially compensate for this decreased receptor expression..

At postnatal day 15, transgenic mice still show decreased level of mRNA EpoR in the brainstem, and no differences in $\dot{V}_E$, Vt and Fr at all tested conditions were observed between Tg21 and control mice (Fig. 4).

Tg21 mice showed increased hypoxic ventilatory response at P21.

At P21, transgenic and control mice showed similar basal ventilation, HVR at 10% O_2, and post-hypoxic recovery. However, HVR at 6%O_2 was higher in Tg21 mice than in control animals (Fig 5a). Regarding the ventilatory pattern, while no differences were observed in Fr at all tested conditions (Fig 5b), VT was significantly higher in transgenic mice than in control animals under hypoxic and post-hypoxic conditions (Fig 5c). Moreover, while non-significant differences were observed in $\dot{V}_{O_2}$ (Fig 5d), $\dot{V}_E / \dot{V}_{O_2}$ at hypoxic and post-hypoxic conditions was higher in transgenic mice compared to control animals (Fig 5e). These results are in line with the reestablishment of a normal mRNA EpoR expression observed in transgenic mice at this age.

DISCUSION

In the present study we used a transgenic mouse strain (Tg21) that overexpresses Epo in the brain only to determine *in vivo* whether the chronic Epo overexpression modulates basal ventilation, the hypoxic ventilatory response (HVR) and the post-hypoxic recovery during the postnatal development. Our results clearly indicate that Epo stimulates ventilation at the ages of P3 and P21. Moreover, at postnatal ages of P7 and P15, despite significant decreased expression of EpoR mRNA in the brainstem, the chronic overexpression of Epo in the brainstem stimulated the hypoxic ventilation in transgenic mice at similar levels than in control animals. We conclude that Epo is a key factor in the regulation of the central respiratory network that should protect against harmful impacts of extreme hypoxia during the postnatal development.

Epo stimulate the fictive breathing in *in vitro* preparation

We previously investigated the impact of cerebral Epo in the neuronal control of ventilation. To do so, *in vitro* preparations of brainstem-spinal cord were obtained from mice at postnatal days P3-P4, and the hypoxic fictive breathing was recorded by electrophysiology. When this preparation is exposed to hypoxia, the activity registered at the C4 ventral nerve decreases (Viemari, et al. 2003) evoking the hypoxic ventilatory decline or "*roll-off*" observed in vivo (Powell, et al. 2000). Our experiments showed that the incubation with 25 U of Epo for 1h was enough to attenuate the hypoxic-mediated decrease of the central respiratory command. Moreover, we observed that the incubation with the soluble erythropoietin receptor (sEpoR, a competitive antagonist of Epo) dramatically decreases the neural hypoxic respiratory activity (Khemiri, et al. 2011). In next step experiments we wanted to investigate the effect of chronic Epo stimulation in the activity of the central respiratory command. To this aim, *in vitro* brainstem-spinal cord preparations of transgenic mice overexpressing Epo in brain only (Tg21) were used. In comparison to acutely incubated preparations (for 1h), the chronic Epo stimulation blunted the hypoxic-mediated depression of the central respiratory command (Caravagna, et al. 2014). These results showed for the first time that Epo is involved in the neural network controlling the hypoxic brainstem circuitry in newborn mice. Despite the merit of this research, the en bloc electrophysiological technique has important limitations. First, *in vivo*, the respiratory frequency at early ages in mice is about 200 breaths/min. This frequency drastically falls down in *in vitro* preparations to about 8-10 burst/min. As such, the extrapolation from *in vitro* to *in vivo* experiences is far to be direct. On the other hand, with age the perfusion of the "en bloc" brainstem becomes harder. As such, the en bloc technique can be performed in animals younger than postnatal day 4 only. Clearly, the small window in which this technique can be used does not allow the evaluation of respiration during the whole duration of the postnatal development.

Epo stimulates the ventilation in *in vivo* preparations

In order to evaluate the impact of Epo in the neural control of ventilation during the frame that correspond to the postnatal development we performed studies *in vivo* by measuring the ventilation (and the hypoxic ventilatory response) on mice by using the whole body plethysmography technique. The advantage of this method is that it allows recording the ventilatory drive in not anesthetized and not restrained animals. As such, we recently reported that at early ages Epo is a potent respiratory stimulant. This investigation was performed by injecting the soluble Epo receptor (sEpoR, a competitive antagonist of Epo) in the brainstem of mice through the cisterna magna (intrasisternal injection; ICI). Interestingly, the sEpoR was able to reduce the basal ventilation and the hypoxic ventilatory response of 10 days old pups. As such, we concluded that Epo is a crucial respiratory stimulant of the neural respiratory network during development (Ballot, et al. 2015). In the present study, we used transgenic mice (Tg21) to determine how the chronic overexpression of cerebral Epo modulates the ventilatory response to acute hypoxia during the postnatal development. In addition, here we evaluated the ventilation in mice at several developmental ages (P3, P7, P15 and P21). Coherently with our previous *in vitro* and *in vivo* reports, our results showed that Epo stimulates the hypoxic ventilatory response at postnatal day P3.

In following, we observed that at P7 and P15 postnatal days the mRNA expression of the Epo receptor (EpoR) was down regulated. As a reliable antibody for EpoR is not yet available (Elliott, et al. 2006), this result was not confirmed by the evaluation of the protein expression. It was interesting however to observe that despite the decreased amount of EpoR in the brainstem areas, transgenic mice were able to show similar hypoxic ventilatory response than control animals. The data obtained in this study are however limited to explain whether the down regulation of EpoR at these ages appear as a negative feedback of the Epo overexpression, or whether the neural Epo system needs to moderate the Epo stimulation at ages when the neural respiratory system is actively completing its development and maturation.

The neural network controlling the ventilatory output in mice reaches the maturity around P15-P21 days old (Bissonnette and Knopp 2001). At P21, our results show that while Epo is still overexpressed in the transgenic brainstem, the level of EpoR (mRNA) returns to control values. Under this condition, the hypoxic ventilatory response, as well as the post-hypoxic ventilatory recovery of Tg21 mice is higher than control animals. This result is in line with the ventilatory responses to hypoxia obtained in adult Tg21 mice (Soliz, et al. 2005). Thus suggesting that once the respiratory network is mature, the chronic neural Epo overexpression stimulates ventilation under hypoxia.

The cerebral Epo overexpression does not modify the basal ventilation

One important difference observed between the work in which the sEpoR was administrated (Ballot, et al. 2015) and the present study is that sEpoR was able to significantly decrease the basal ventilation (normalized by the oxygen consumption) while the Epo overexpression did not. Comparable outcomes were obtained in Tg21 adult mice that showed similar basal ventilation and hypoxic ventilatory response to 10% O_2, but increased HVR at 6%O_2 (Soliz, et al. 2005). These findings suggest that upon conditions of normoxia or moderate hypoxia the endogenous expression of Epo in wild type animals should be enough to saturate its receptor (EpoR), but not under conditions of extreme hypoxia. In addition, we observed in adult Tg21 mice that Epo in hypoxic, but not in mormoxic conditions modulates the synthesis of catecholamines, which are potent modulators of ventilation in hypoxia (Soliz, et al. 2005). At difference to adulthood, we observed here that Tg21 mice at P21 show increased ventilation at moderate (10 %O_2), and extreme (6 %O_2) hypoxia, and after 15 min of post-hypoxic recovery. This suggests that the neural respiratory command is more sensible to Epo at early ages than in adults.

No sex-specific effect of Epo in Tg21 mice

Other important difference with the work published by Ballot et al (Ballot, et al. 2015), is that in contrast to the effect of the antagonist (the sEpoR), in the present study not sex dimorphic results were evidenced. While sEpoR was able to significantly decrease ventilation in newborn male and female mice, this decrease was due to decreased VT in males, but due to reduced VT and Fr in females (Ballot, et al. 2015). As mentioned in material and method section, in this study the results of male and female animals were pooled because no sex-related differences were observed. Similarly, while sex-dimorphic results were observed when adult wild type mice were treated with sEpoR (Ballot, et al. 2015), the absence of a ventilatory sex dimorphism was previously reported in adult Tg21 mice in normoxia and in acute hypoxia (Gassmann, et al. 2009). This interesting result suggests that the endogenous synthesis of Epo in the brain should interact with neurosteroids, but that the overexpression of Epo apparently fails to do so. Keeping in mind the fact that the morbidity and mortality related to respiratory disorders in newborns and during infancy are higher in males than in females (Mage and Donner 2006), these sex-specific effects of endogenous *vs* exogenous Epo are intriguing and deserve more investigation.

In concussion, our results show that the chronic Epo overexpression in brain only produces a significant augmentation of the hypoxic ventilatory response at very early neonatal age (P3) and once the maturation of the respiratory network is completed (at P21). Moreover, we showed that at P7 and P15 the neural stimulation of Epo in the brainstem becomes less evident, most probably due to the down regulation of the EpoR. These results may have significant implications for clinicians by helping to understand the mechanism underlying systemic therapy with Epo injections as part of the observed effects might be due to a central action of Epo, as suggested by the present work. Moreover, the present report will provide a

rationale for exploring novel research avenues to improve our understanding of the physiology and pathophysiology of neonatal respiratory disorders related to neural dysfunction and/or immaturity.

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Figure Legends

Figure 1. Epo protein and mRNA EpoR expression in brainstems of Tg21 and control mice. a) Epo concentration was measured by RIA and expressed in mU/mg of protein. Compared to control animals, transgenic mice show higher Epo in the brainstem during development. b) mRNA EpoR was evaluated by qRT-PCR and normalized to GAPDH and DCt (Ct_{EpoR} - Ct_{GAPDH}) was calculated. mRNA EpoR was significantly lower in transgenic vs control animals at P7 and P14. *p<0.05 transgenic *vs.* control at the same age.

Figure 2. Tg21 mice showed increased hypoxic $\dot{V}_E/\dot{V}_{O_2}$ at P3. a,b and c) Hypoxia was achieved in two steps, from 21% to 10% O2, and from 10% to 6% O2 (black triangles in the right direction). Post hypoxic recovery was achieved in one step, from 6% to 21% O2 (black triangles in the left direction). $\dot{V}_E$, Fr and VT were measured in Tg21 and control mice during 15 min at each hypoxic and recovery conditions. The bars represent the average of the last 5min at hypoxic and recovery conditions of the $\dot{V}_{O_2}$ (d), and $\dot{V}_E/\dot{V}_{O_2}$ (e). *p<0.05 transgenic *vs.* control at the same time and condition.

Figure 3. Tg21 and control mice showed similar hypoxic $\dot{V}_E/\dot{V}_{O_2}$ at P7. a,b and c) Hypoxia was achieved in two steps, from 21% to 10% O2, and from 10% to 6% O2 (black triangles in the right direction). Post hypoxic recovery was achieved in one step, from 6% to 21% O2 (black triangles in the left direction). $\dot{V}_E$, Fr and VT were measured in Tg21 and control mice during 15 min at each hypoxic and recovery conditions. The bars represent the average of the last 5min at hypoxic and recovery conditions of the $\dot{V}_{O_2}$ (d), and $\dot{V}_E/\dot{V}_{O_2}$ (e). *p<0.05 transgenic *vs.* control at the same time and condition.

Figure 4. Tg21 and control mice showed similar hypoxic ventilatory parameters at P15. a,b and c) Hypoxia was achieved in two steps, from 21% to 10% O2, and from 10% to 6% O2 (black triangles in the right direction). Post hypoxic recovery was achieved in one step, from 6% to 21% O2 (black triangles in the left direction). $\dot{V}_E$, Fr and VT were measured in Tg21 and control mice during 15 min at each hypoxic and recovery conditions.

Figure 5. Tg21 mice showed increased hypoxic $\dot{V}_E/\dot{V}_{O_2}$ at P21. a,b and c) Hypoxia was achieved in two steps, from 21% to 10% O2, and from 10% to 6% O2 (black triangles in the right direction). Post hypoxic recovery was achieved in one step, from 6% to 21% O2 (black triangles in the left direction). $\dot{V}_E$, Fr and VT were measured in Tg21 and control mice during 15 min at each hypoxic and recovery

conditions. The bars represent the average of the last 5min at hypoxic and recovery conditions of the $\dot{V}_{O_2}$ (d), and $\dot{V}_E / \dot{V}_{O_2}$ (e). *p<0.05 transgenic *vs.* control at the same time and condition.

Figure 1

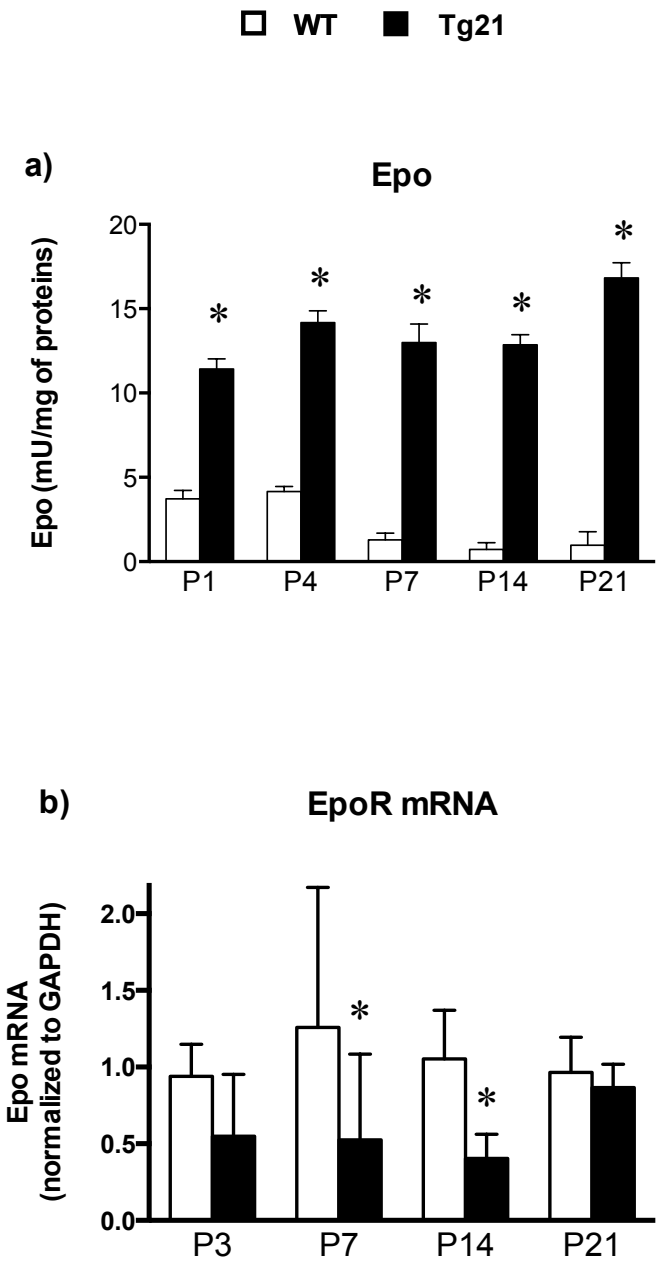

WT
Tg21
a)
Epo
Epo (mU/mg of proteins)
20
15
10
5
0
P1
P4
P7
P14
P21
b)
EpoR mRNA
Epo mRNA (normalized to GAPDH)
2.0
1.5
1.0
0.5
0.0
P3
P7
P14
P21

Figure 2

postnatal day 3

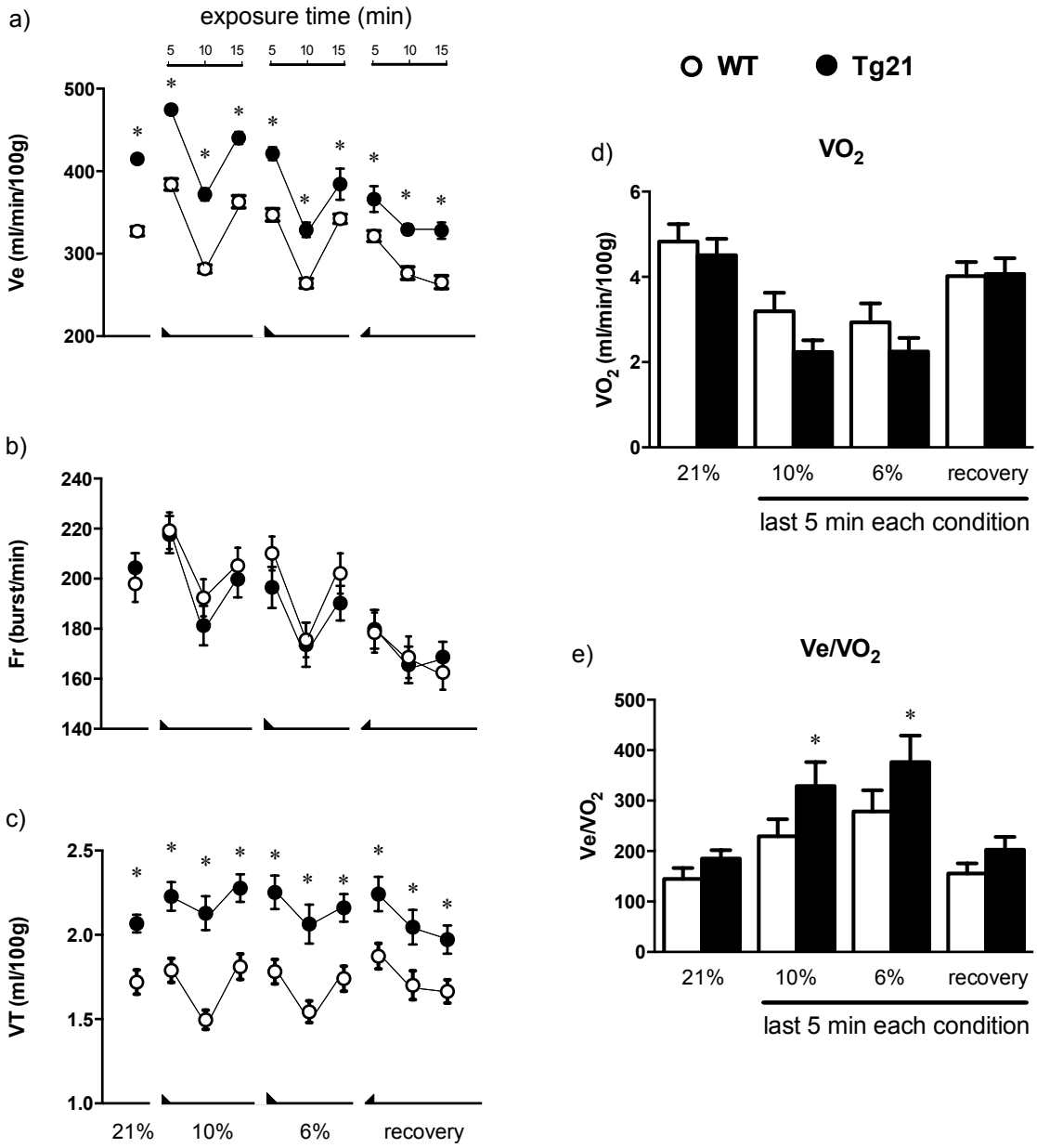


Figure 3

postnatal day 7

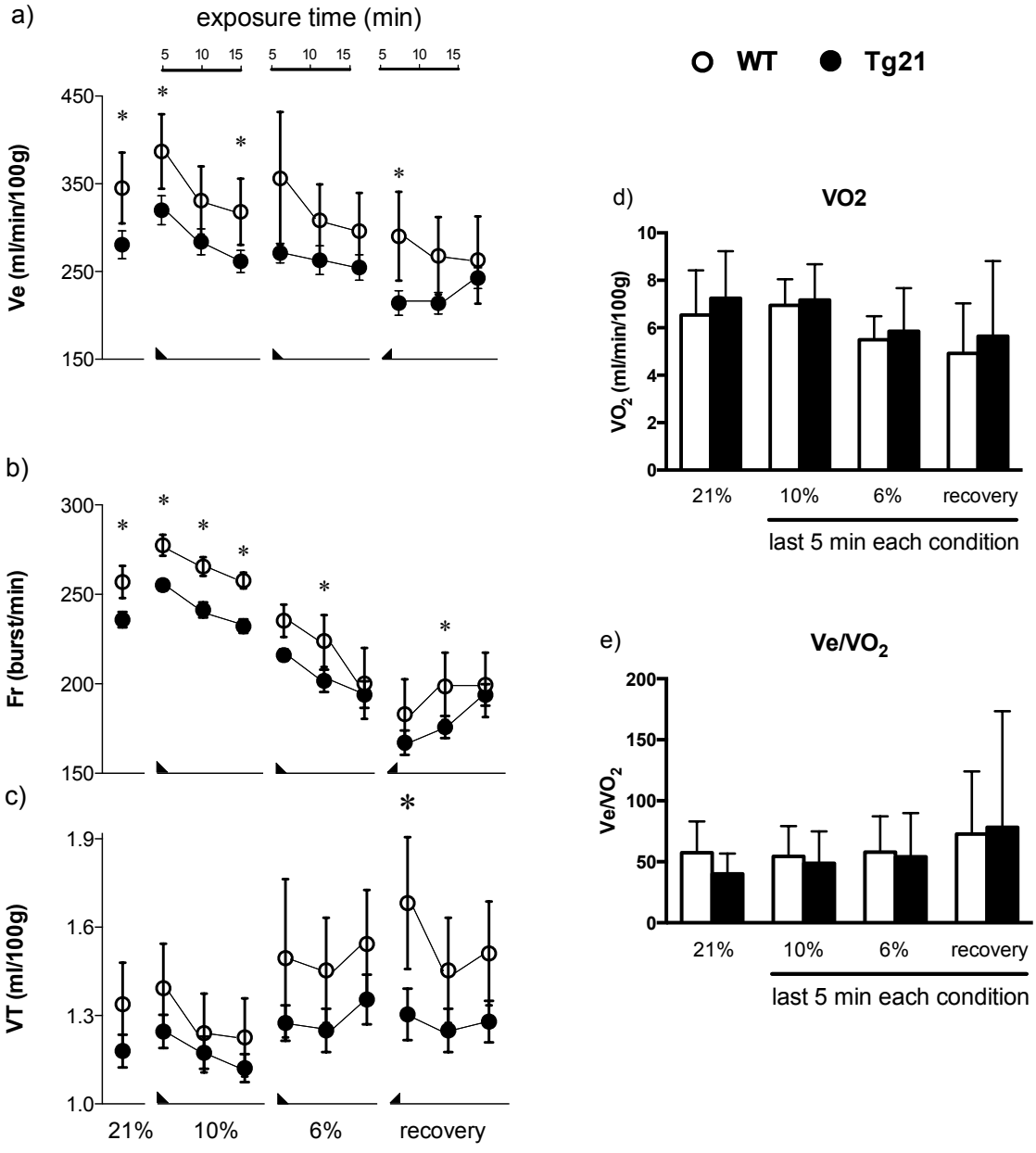


Figure 4 **postnatal day 15**

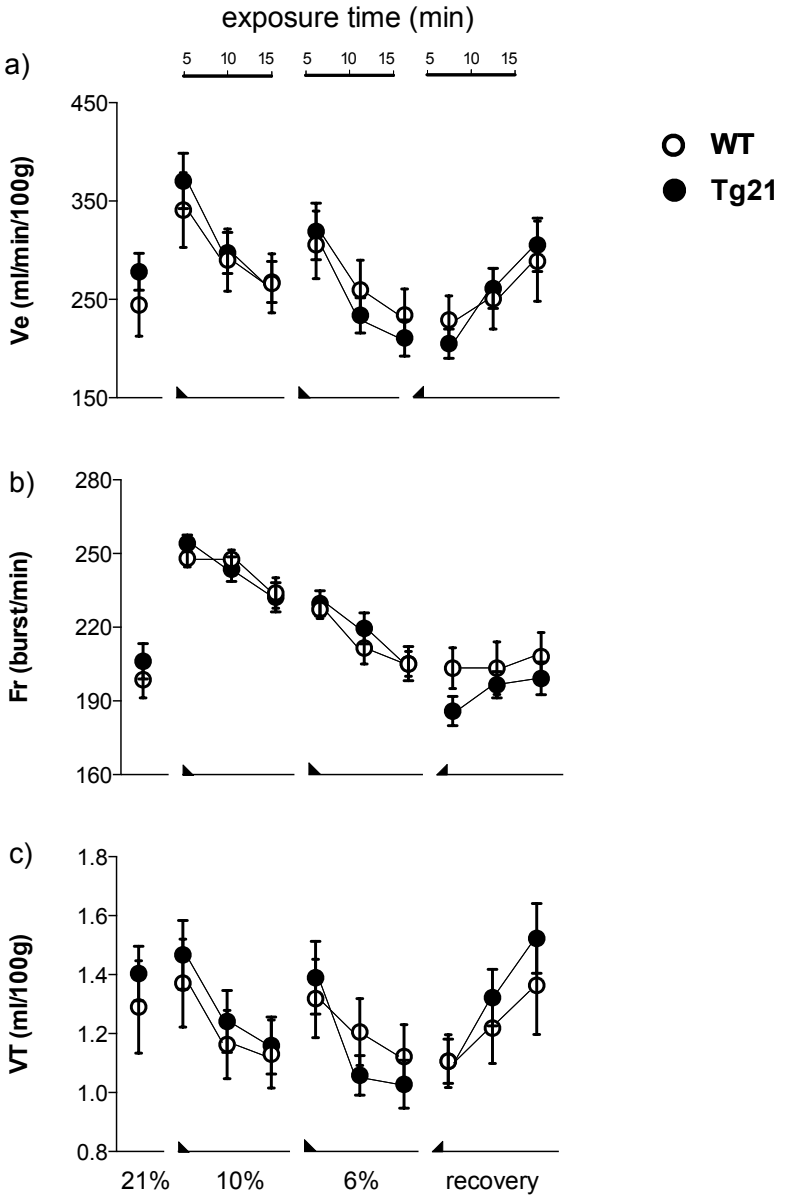
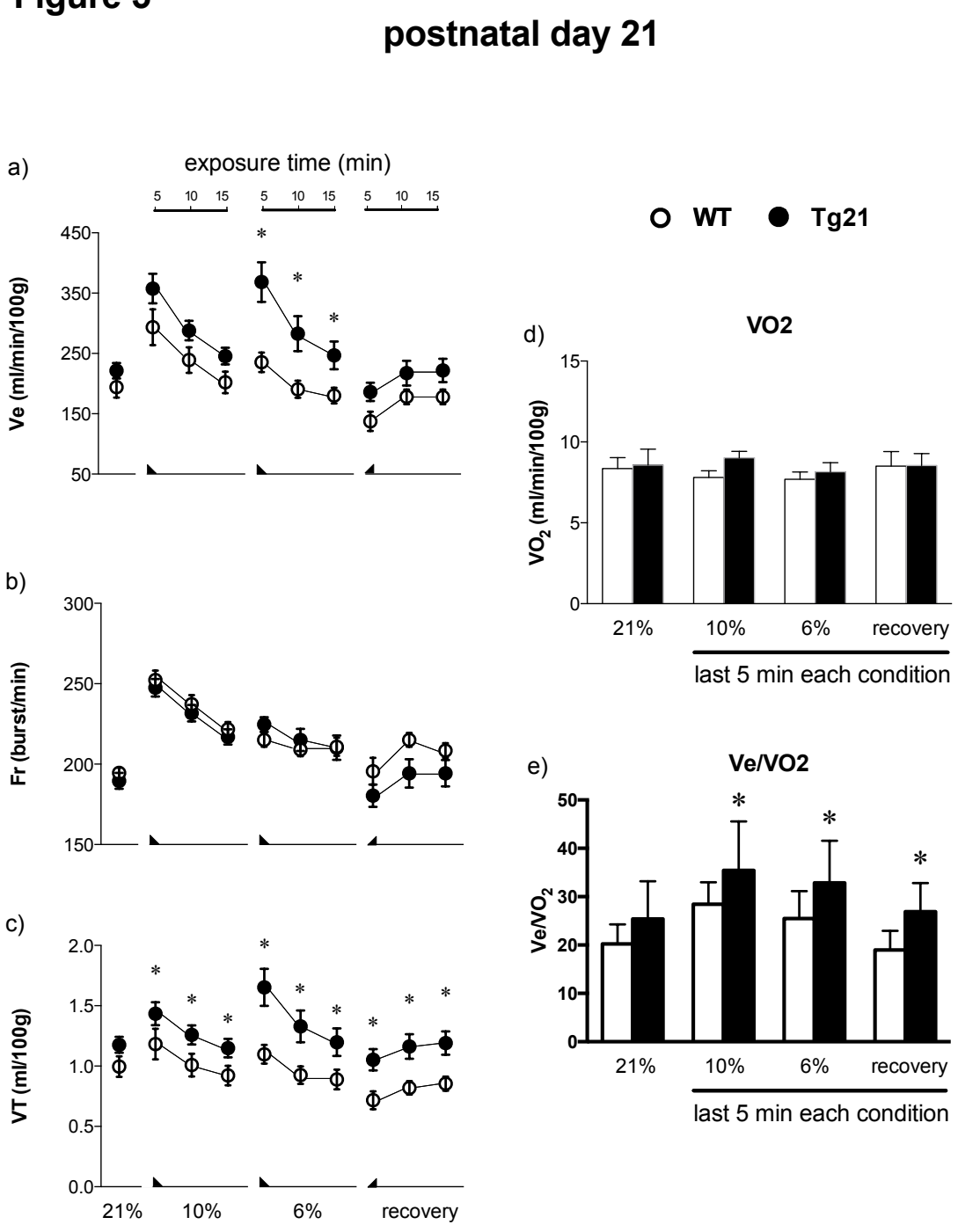


Figure 5


postnatal day 21
a)
exposure time (min)
5 10 15 5 10 15 5 10 15
Ve (ml/min/100g)
450
350
250
150
50
b)
Fr (burst/min)
300
250
200
150
c)
VT (ml/100g)
2.0
1.5
1.0
0.5
0.0
21%
10%
6%
recovery
WT
Tg21
d)
VO2
VO2 (ml/min/100g)
15
10
5
0
21%
10%
6%
recovery
last 5 min each condition
e)
Ve/VO2
Ve/VO2
50
40
30
20
10
0
21%
10%
6%
recovery
last 5 min each condition