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Short communication

PI3K and $MEK_{1/2}$ molecular pathways are involved in the erythropoietin-mediated regulation of the central respiratory command

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ABSTRACT

Erythropoietin stimulation modulates the central respiratory command in newborn mice. Specifically, the central respiratory depression induced by hypoxia is attenuated by acute (1 h) or abolished by chronic erythropoietin stimulation. However, the underlying mechanisms remain unknown. As MEK and PI3K pathways are commonly involved in Epo-mediated effects of neuroprotection and erythropoiesis, we investigated here the implication of PI3K and $MEK_{1/2}$ in the Epo-mediated regulation of the central respiratory command. To this end, *in vitro* brainstem–spinal cord preparations from 3 days old transgenic (Tg21; constitutively overexpressing erythropoietin in the brain specifically) and control mice were used. Our results show that blockade of PI3K or $MEK_{1/2}$ stimulates normoxic bursts frequency in Tg21 preparations and abolish hypoxia-induced frequency depression in control preparations. These results show that $MEK_{1/2}$ and PI3K pathways are involved in the Epo-mediated regulation of the central respiratory command. Moreover, this is the first demonstration that $MEK_{1/2}$ and PI3K are involved in the brainstem central respiratory command.

1. Introduction

Erythropoietin (Epo) is a cytokine well known for its erythropoietic function. Epo is also synthesized by different organs, particularly the brain. Cerebral Epo is synthetized by both astrocytes (80%) and neurons (20%) and has important neuroprotective effects against hypoxia/ischemia insults. Cerebral Epo protects from focal cerebral ischemia by dual activation of extracellular-signal regulated kinase (ERK) and kinase protein B (Akt) pathways (Kilic et al., 2005). In addition, Epo induces phrenic motor facilitation via $ERK_{1/2}$ and Akt (Dale et al., 2012) in adult rats. Indeed, blockade of $ERK_{1/2}$ and Akt before Epo injection abolished Epo-induced phrenic motor facilitation, demonstrating that $ERK_{1/2}$ and Akt are both required for Epo-induced phrenic nerve facilitation in the spinal cord.

Cerebral Epo is also able to modulate the central control of breathing in the brainstem during childhood (Soliz, 2013). In brainstem–spinal cord preparations, acute hypoxia induces a rhythm depression, which is reversed during post-hypoxic recovery (Viemari et al., 2003): rhythm frequency is highly reduced from the first minutes until the end of the hypoxia exposure. In our laboratory, we recently demonstrated that Epo modulates this hypoxic-induced depression in the medulla oblongata. Indeed, the central burst frequency depression induced by hypoxia is attenuated by acute (1 h) (Khemiri et al., 2012) and abolished by chronic erythropoietin stimulation in brainstem–spinal cord preparations (Caravagna et al., 2014). However, the underlying mechanisms remain unknown.

Keeping in mind the previous research of molecular mechanisms implicated in Epo neuroprotection and phrenic motor facilitation, we hypothesized that Epo effect on the central respiratory command at neonatal ages may also be mediated by MEK–ERK and PI3K–Akt pathways. To test this hypothesis, we performed electrophysiological recordings on isolated preparations (en bloc technique) of transgenic (Tg21; constitutively overexpressing erythropoietin in the brain specifically) and control mice of post-natal (P) day 3. Preparations were incubated with increasing

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doses of U0126 ($MEK_{1/2}$ inhibitor) or LY294002 (PI3K inhibitor) for 1 h, and then exposed to a hypoxic challenge. Our results show that inhibition of $MEK_{1/2}$ or PI3K enhances normoxic burst frequency in Tg21 preparations, and inhibits hypoxic-induced frequency depression in control preparations. Inhibition of $MEK_{1/2}$ or PI3K does not modify hypoxic frequency in Tg21 preparations. Thus, $MEK_{1/2}$ and PI3K are involved in Epo action on the central respiratory command.

2. Materials and methods

2.1. Animals

Tg21 mice line was initially generated by Epo cDNA injection in B6C3F1 oocytes and crossing the resulting mice with C57BL/6 mice. Tg21 and line-specific control male and female mice at post-natal day 3 (LY294002 group: control $n = 10$; Tg21 $n = 20$; U0126 group: control $n = 14$; Tg21 $n = 21$) from at least three litters in each group were used to perform the experiments. The Tg21 mouse line, that overexpresses Epo in the brain, was obtained as a generous gift from Professor Max Gasmann, University of Zurich, Switzerland, and has already been used in our team (Caravagna et al., 2014). Animal experiments were approved by 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.

2.2. Electrophysiological recordings

Electrophysiological recordings were performed on isolated medulla oblongata and spinal cord, as previously described (rostral section at the level of the anterior inferior coronary artery) (Khemiri et al., 2012). The preparations were recorded during 20 min, as pre-incubation baseline. The preparations were then placed 1 h in an incubation chamber perfused with an antagonist of PI3K–Akt pathway (LY294002, Cell Signaling, Cat# 9901) or an antagonist of MEK–ERK pathways (U0126, Cell Signaling, Cat# 9903) at different concentrations (0.1 μM, 1 μM and 100 μM) diluted in aCSF-, followed by 20 min of post-incubation baseline recording, 10 min of hypoxia, obtained by switching the superfusion solution from aCSF bubbled with carbogen to aCSF bubbled with 95% N_2 and 5% CO_2 (the partial dioxygen pressure in the recording chamber was around 20 mmHg). Finally, post-hypoxic recovery activity was recorded for 10 min by superfusing preparations with aCSF bubbled with carbogen. The drug effect could be attenuated by the post-incubation baseline delay, but since this time was already present in the dose-dependent protocol (Fig. 1) we consider the drug dose used in the following experiment is sufficient to produce an effect after being "rinsed" during the post-incubation baseline.

2.3. Statistical analysis

The baselines frequency and amplitude of the integrated discharges at the C4 root were calculated as the average of the last 5 min of the baseline recordings. Frequency is expressed in burst/minute, amplitude (data not shown) is expressed in mV. For each preparation and condition, only the last 5 min of post-incubation baseline, hypoxia and post-hypoxic recovery were considered. The results are expressed as mean ± SD. Statistical analyses were conducted by performing two-way repeated ANOVAs on Prism software, with multiple comparisons by Fisher's test. The threshold of significance was fixed at 0.05.

3. Results

3.1. U0126 and LY294002 in "en bloc" preparations is effective at 1 μM

Initially, a dose-dependent effect was investigated to obtain the effective concentrations of U0126 and LY294002 to be used in preparations from Tg21 P3 mice. LY294002 induced enhancement of burst frequency only at 1 μM during post-incubation baseline, hypoxia and post-hypoxic recovery ($p < 0.0001$; $p < 0.0001$; $p = 0.0073$ respectively; Fig. 1). No burst amplitude modification was observed (data not shown). U0126 significantly increased burst frequency only at 1 μM (during post-incubation baseline, $p = 0.025$; and during post-hypoxic recovery, $p = 0.0456$) and 100 μM (during post-incubation baseline, $p < 0.0001$; during hypoxia, $p = 0.0001$; during post-hypoxic recovery, $p = 0.0006$) (Fig. 1). At 0.1 μM, no significant effect was observed (post-incubation baseline, $p = 0.7982$; hypoxia, $p = 0.9086$; post-hypoxic recovery, $p = 0.6087$), and at 100 μM tonic discharges appear and did not allow burst discrimination versus ectopic discharges. No burst amplitude modification was observed (data not shown). Accordingly to these results, the concentration of 1 μM was used for both drugs during this study.

3.2. Normoxic burst frequency is not modified in control but is enhanced in Tg21 preparations by U0126 and LY294002

Burst frequency was quantified before and after drug incubation under normoxic condition to visualize whether these drugs modify the central respiratory command under normoxic conditions. In control preparations, no modification was observed in burst frequency (U0126, $p = 0.905$; LY294002, $p = 0.1357$; Fig. 2.B) or amplitude (data not show). In Tg21 preparations, burst amplitude was not modified (data not show) while burst frequency increased compared with pre-incubation baseline (U0126, $p = 0.0034$; LY294002, $p < 0.0001$; Fig. 2.B). There is no significant difference between Tg21 and control preparation normoxic bursts frequency (U0126, $p = 0.0543$; LY294002, $p = 0.2351$).

3.3. U0126 or LY294002 abolish hypoxic-induced frequency depression in control preparations

We performed hypoxic challenge on preparations after drug incubation and post-incubation baseline. Control preparations did not show the usual hypoxia-induced burst frequency depression (compared with post-incubation baseline, U0126, $p = 0.7905$; LY294002, $p = 0.8843$). There is no hypoxia-induced depression in Tg21 preparations after drugs incubation, as previously demonstrated without drug incubation (Caravagna et al., 2014). Tg21 preparations incubated with LY294002 show higher hypoxic burst frequency compared with pre-incubation baseline ($p = 0.0001$), while Tg21 preparations incubated with U0126 display hypoxic burst frequency not significantly different from pre-incubation baseline ($p = 0.0924$). Burst frequency of Tg21 preparations was significantly higher than burst frequency of control preparations during post-incubation baseline after incubation with U0126 ($p = 0.0183$) but not LY294002 ($p = 0.1372$). Burst frequency of Tg21 preparations remains higher than pre-incubation during post-hypoxic recovery (U0126, $p = 0.0093$; LY294002, $p = 0.0134$).

4. Discussion

We performed electrophysiological recordings on isolated medullary and spinal cord preparations after incubation with

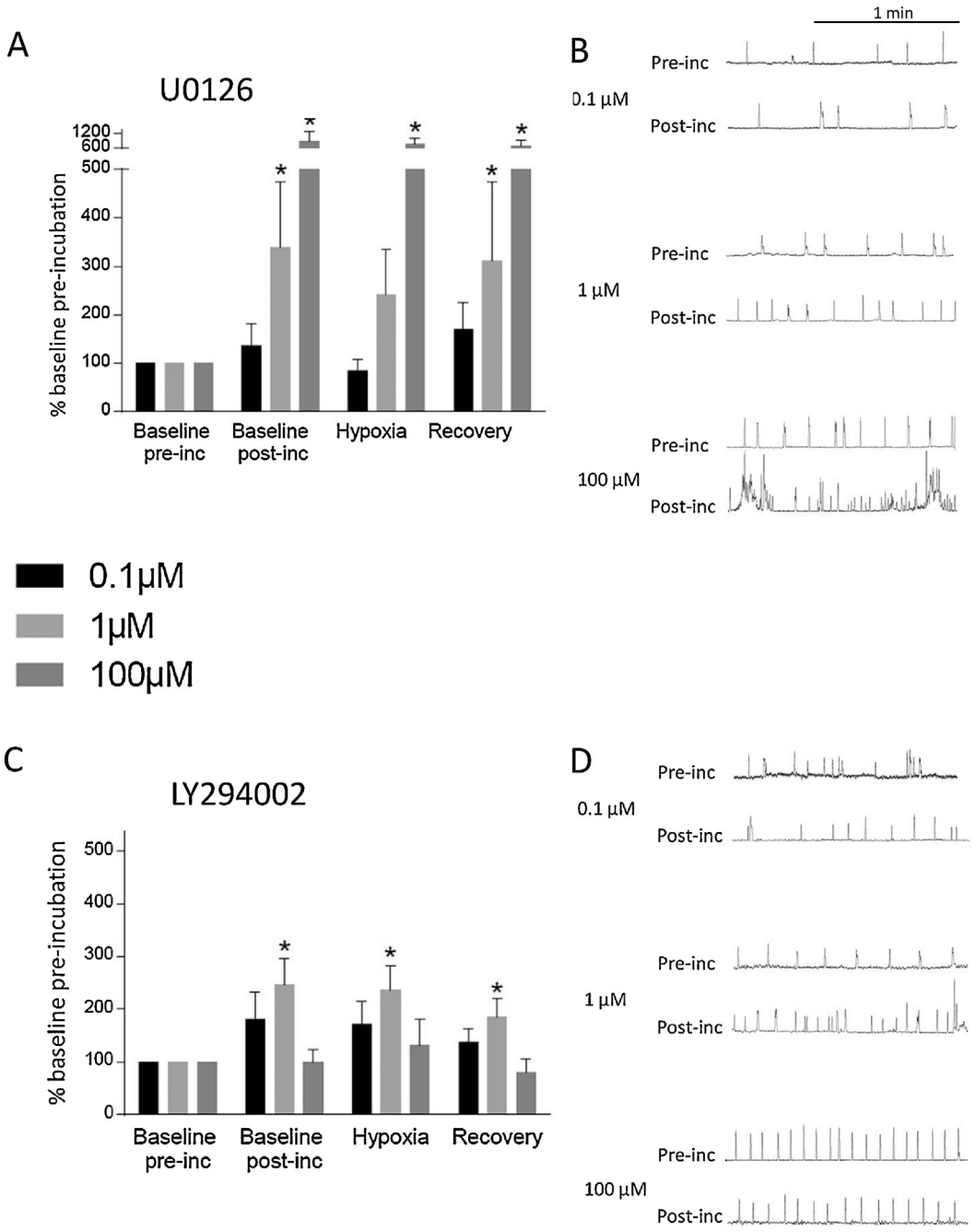

Fig. 1. 1 µM is the right dose to apply of U0126 and LY294002 on preparations. (A) Baseline-corrected of preparations from Tg21 mice bursts frequency after 1 h incubation in 0.1 µM, 1 µM or 100 µM of U0126, expressed in %. (B) Examples of recordings from the C4 ventral root during normoxia before and after U0126 incubation at different doses. Note that 100 µM induces irregular bursts that cannot be associated with respiratory rhythm. (C) Baseline-corrected of preparations from Tg21 mice bursts frequency after 1 h incubation in 0.1 µM, 1 µM or 100 µM of LY294002, expressed in %. (D) Examples of recordings from the C4 ventral root during normoxia before and after LY294002 incubation at different doses. *Different from baseline.

$MEK_{1/2}$ or PI3K inhibitors, on Tg21 and control mice. Blockade of $MEK_{1/2}$ or PI3K abolishes the usual hypoxia-induced depression in control medulla oblongata. In Tg21 medulla oblongata, blockade of $MEK_{1/2}$ or PI3K increases normoxic burst frequency during post-incubation baseline and post-hypoxic recovery. This study shows that $MEK_{1/2}$ and PI3K pathways are involved in the basal normoxic activity, the response to hypoxia and the post-hypoxic recovery of preparations, which is the first demonstration of $MEK_{1/2}$ and PI3K implication in the central respiratory command in the medulla oblongata.

4.1. Tg21 preparations display lower frequency than C57BL/6 preparations

Literature reported activity of P3 mice medullary–spinal cord preparations baseline frequency is around 10 bursts/min, while our medullary–spinal cord preparations, both Tg21 and control, display a baseline frequency around 3 bursts/min. As previously discussed (Caravagna et al., 2014), differences between generated animals and wild type background animals are common in the literature, and it is generally accepted that these differences are due to

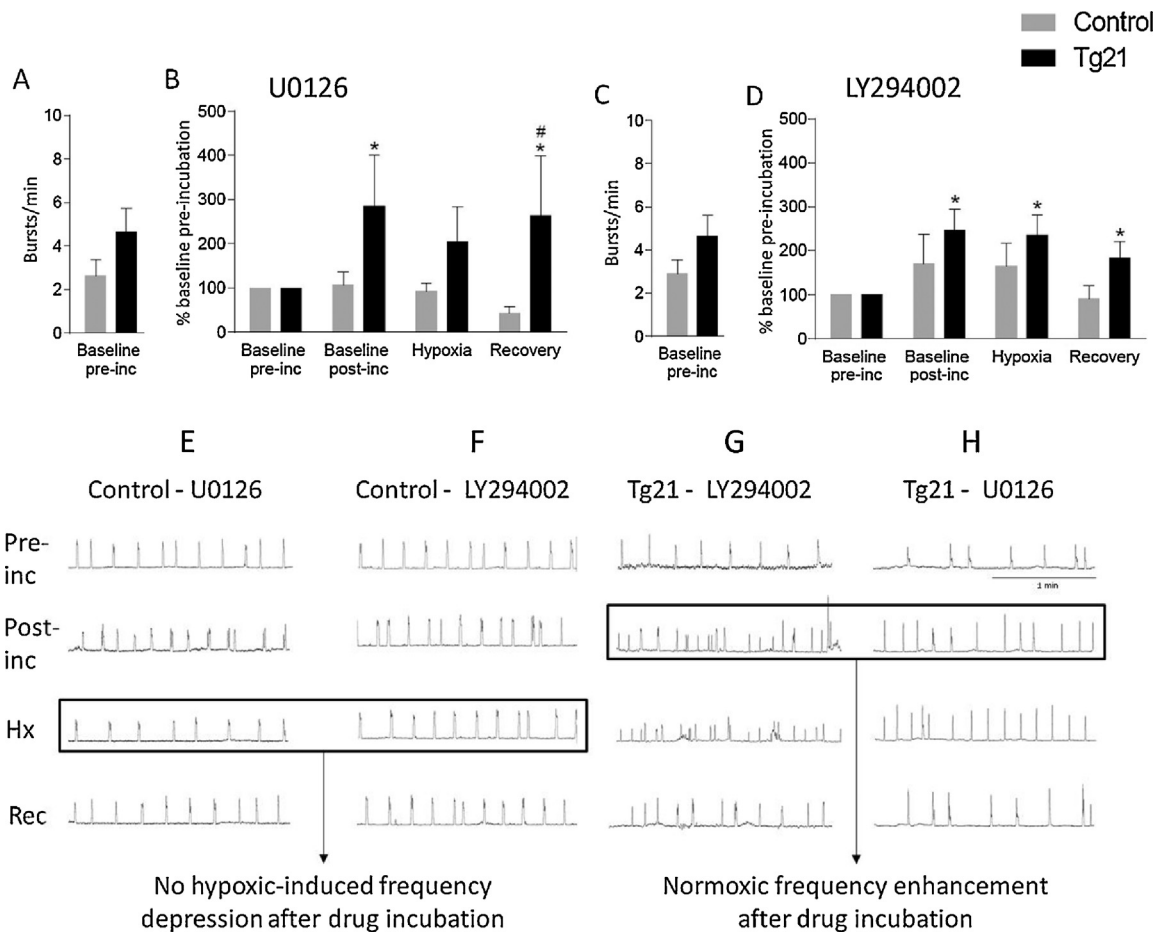

Fig. 2. Antagonization of PI3K-Ak and MEK–ERK pathways in preparations of Tg21 and control animals stimulates bursts frequency. (A) Basal bursts frequency of Tg21 and control preparations, expressed in burst/min. (B) Burst frequency of Tg21 and control preparations after 1 μM U0126 incubation, expressed in %. (C) Basal bursts frequency of Tg21 and control preparations, expressed in burst/min. (D) Burst frequency of Tg21 and control preparations after 1 μM LY294002 incubation, expressed in %. (E–H) Examples of respiratory output recorded from the C4 ventral root of preparations from P3 control (E and F) and Tg21 (G and H) mice before and after incubation with LY294002 (F and G) and U0126 (E and H). *Different from baseline, same genotype. #Genotype difference, same condition.

incomplete recovery of the original genetic background and breeding or housing practices. Thus, the Tg21 mice line may display some particular characteristics. To avoid inappropriate conclusions, we used the proper control to Tg21 mice, which have been generated and housed in the exact same conditions than Tg21 mice.

4.2. $MEK_{1/2}$ and PI3K are implicated in basal activity of Tg21 medulla oblongata and in the response to hypoxia of control medulla oblongata

We first hypothesized that Epo inhibition of burst frequency hypoxic-depression in Tg21 preparations would be mediated by $MEK_{1/2}$ or PI3K. However, it appears that inhibition of $MEK_{1/2}$ or PI3K does not restore control phenotype on Tg21 preparations, but mimic chronic Epo overexpression effect on control preparations. Indeed, the usual hypoxic-induced frequency depression does not appear as previously noticed (Caravagna et al., 2014; Khemiri et al., 2012). Thus, $MEK_{1/2}$ and PI3K are necessary to the hypoxic-induced frequency depression to appear. This data can be interpreted in different ways. Epo may not activate $MEK_{1/2}$ or PI3K in the medulla oblongata respiratory context. In this case, repressive respiratory modulators, such as opioids, endorphins or various anesthetics, could recruit $MEK_{1/2}$ and PI3K under hypoxic conditions, and Epo should act via others pathways to stimulate the rhythm frequency, perhaps JAK-STAT pathway. Otherwise, it is also possible that Epo activates $MEK_{1/2}$ and PI3K pathways in respiratory groups involved in the hypoxia-induced depression, such as the RTN/pFRG (Gourine et al., 2010). Moreover, the RTN/pFRG chemosensitivity mechanisms involve astrocytes, which are also cells producing Epo. Finally, Epo could activate $MEK_{1/2}$ or PI3K pathways in different respiratory groups, such as the parapyramidal area, the resulting effect on the C4 respiratory output of this Epo activation being an increased frequency.

As discussed below, a potential compensation between these two pathways is conceivable. Nevertheless the present data allows us to conclude that $MEK_{1/2}$ or PI3K alone is not sufficient to support Epo stimulation of hypoxic activity, and is implicated in the central respiratory command process. Finally, it has to be kept in mind that these results can only be interpreted in the context of medullary–spinal cord preparations and do not represent the response of full *in vivo* brainstem. This is the first step in the decipheration of Epo action in the central respiratory command.

4.3. Potential compensation of PI3K–Akt and MEK–ERK pathways

An important point is that PI3K–Akt and MEK–ERK pathways could compensate for each other during the inhibition of $MEK_{1/2}$ or PI3K. When blocking $MEK_{1/2}$ or PI3K, a usual cross-inhibition between the two pathways could be released, effectively activating the other pathway. Indeed, this action has already been

demonstrated in different contexts, e.g. expression of epidermal growth factor via Akt pathway is enhanced in presence of MEK inhibitors (Mendoza et al., 2011).

However, MEK–ERK and PI3K–Akt pathways have much more complicated cross-talk. As such, it is known for example that PI3K activity is necessary for Epo-induced MEK–ERK activation depending on the Epo concentration and interactions with other growth factors (Schmidt et al., 2004). Thus, it cannot be sure if PI3K is directly or indirectly necessary for Epo action, because it is required to activate ERK.

Moreover, PI3K inhibitor LY294002 blocks the activation of ERK in fluoxetine-treated NSCs (Huang et al., 2013). However, a detailed study of direct LY294002 interactions with various proteins (including PI3K family members) revealed no interactions between LY294002 and MEK or ERK (study detailed in Huang et al., 2013). A detailed review of the known cross-talk between MEK–ERK and PI3K–Akt pathways is available in the discussion of (Huang et al., 2013).

In expected, the study of MEK–ERK and PI3K–Akt pathways is complex. Still, a cross-inhibition could be present in the central respiratory command, and explain why the blockade of one pathway mimics Epo stimulation: the other pathway, still activated by endogenous Epo, is up-regulated and induces the Tg21 phenotype in control mice, while it accentuates the Epo stimulation in Tg21 mice during normoxia.

4.4. Potential drugs effect on the phrenic motor neurons

Dale et al. (2012) demonstrated that the phrenic motor facilitation is dependent of Epo, MEK and PI3K. This leads to the question of the location of overexpressed-Epo and drugs effect in our preparations. Because there is no phrenic motor facilitation in Tg21 preparations during normoxic pre-incubation baseline, because phrenic motor facilitation is about amplitude and not frequency, and because Dale et al. demonstrated that LY294002 and U0126 inhibit Epo-induced phrenic motor facilitation, we believe that the observed drugs effect in Tg21 preparations cannot be related to phrenic motor facilitation. Nevertheless, in accordance with the effect on the medulla oblongata respiratory groups, a potential drugs effect on the phrenic motor neurons cannot be excluded in this study. This potential effect does not cast doubt on the major finding of this study, which is that $MEK_{1/2}$ or PI3K are necessary for the hypoxia-induced depression to appear.

5. Conclusion

In the present study, two different pathways, PI3K–Akt and MEK–ERK, have been tested in the medulla oblongata central respiratory command because of the known link with Epo in neural and non-neural cell types. Inhibition of both these pathways resulted in the inhibition of hypoxic-induced frequency depression in control brainstem–spinal cord preparations and the enhancement of normoxic central respiratory command activity in Tg21 brainstem–spinal cord preparations. This data shows an interaction between Epo and MEK and PI3K in the medulla oblongata: when Epo is overexpressed, the inhibition of MEK or PI3K produces a stimulatory effect during in normoxic conditions. This data should be very helpful to further investigators to understand the central respiratory command mechanisms.

Conflict of interest

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

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