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## ORIGINAL ARTICLE

# Intermittent hypoxia, brain glyoxalase-1 and glutathione reductase-1, and anxiety-like behavior in mice

Alicia Carissimi,[1] Denis Martinez,[2,3,4] Lenise J. Kim,[5] Cintia Z. Fiori,[2,3] Luciana R. Vieira,[4] Darlan P. Rosa,[4,6] Gabriel N. Pires[5]

[1]*Programa de Pós-Graduação em Psiquiatria e Ciências do Comportamento, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.* [2]*Programa de Pós-Graduação em Cardiologia e Ciências Cardiovasculares, UFRGS, Porto Alegre, RS, Brazil.* [3]*Divisão de Cardiologia, Hospital de Clínicas de Porto Alegre (HCPA), UFRGS, Porto Alegre, RS, Brazil.* [4]*Programa de Pós-Graduação em Medicina, Ciências Médicas, UFRGS, Porto Alegre, RS, Brazil.* [5]*Departamento de Psicobiologia, Universidade Federal de São Paulo (UNIFESP), São Paulo, SP, Brazil.* [6]*Faculdade Cenecista de Bento Gonçalves (CNEC), Bento Gonçalves, RS, Brazil.*

**Objective:** Sleep apnea has been associated with anxiety, but the mechanisms of the sleep apnea-anxiety relationship are unresolved. Sleep apnea causes oxidative stress, which might enhance anxiety-like behavior in rodents. To clarify the apnea-anxiety connection, we tested the effect of intermittent hypoxia, a model of sleep apnea, on the anxiety behavior of mice.
**Methods:** The rodents were exposed daily to 480 one-minute cycles of intermittent hypoxia to a nadir of 7±1% inspiratory oxygen fraction or to a sham procedure with room air. After 7 days, the mice from both groups were placed in an elevated plus maze and were video recorded for 10 min to allow analysis of latency, frequency, and duration in open and closed arms. Glyoxalase-1 (Glo1) and glutathione reductase-1 (GR1) were measured in the cerebral cortex, hippocampus, and striatum by Western blotting.
**Results:** Compared to controls, the intermittent hypoxia group displayed less anxiety-like behavior, perceived by a statistically significant increase in the number of entries and total time spent in open arms. A higher expression of GR1 in the cortex was also observed.
**Conclusion:** The lack of a clear anxiety response as an outcome of intermittent hypoxia exposure suggests the existence of additional layers in the anxiety mechanism in sleep apnea, possibly represented by sleepiness and irreversible neuronal damage.

**Keywords:** Sleep; biological markers; molecular biology; stress; anxiety

## Introduction

Obstructive sleep apnea (OSA) is often associated with psychiatric and neurobehavioral symptoms, including depression and anxiety.[1-4] Indeed, the presence of mood disorders or cognitive dysfunction is a diagnostic criterion for OSA according to the International Classification of Sleep Disorders.[5] The prevalence of anxiety disorders among OSA patients varies from 11 to 70%, and a remarkable overlap of anxiety and OSA symptoms is frequently observed.[6] This wide range in the prevalence of anxiety among OSA patients could be a result of different definitions and methods of assessment.

The main possible mechanism for exacerbating anxiety in sleep apnea is the recognized association of apnea with sympathetic hyperactivity.[7,8] This could enhance "fight or flight" mechanisms, leading to anxiety-like behavior.[9] However, mechanisms for the emergence of apnea-induced anxiety behavior are still unclear. Besides sympathetic hyperactivity, a possible pathway to explain the concomitance of anxiety and sleep apnea could be the response to oxidative stress triggered by intermittent hypoxia in OSA patients. Intermittent hypoxia is a well-established cause of oxidative stress,[10,11] which in turn is associated with neuronal cell loss in rodents[11-13] and with alterations similar to those observed in patients with residual sleepiness after effective OSA treatment.[14,15] This interplay between intermittent hypoxia and oxidative stress might explain part of the neurobehavioral phenotype observed in OSA patients.

Oxidative stress has been proposed as a common factor for anxiety and other long-term consequences and co-morbidities of OSA, such as hypertension and insulin resistance.[16] Among all oxidative stress mediators, an association between anxiety-related phenotype and overexpression of two genes, glyoxalase-1 (Glo1) and glutathione reductase-1 (GR1), was originally described by Hovatta et al. in rats.[17] GR is the chief redox buffer in most cells, being critical for the balance of oxidative processes and resistance to oxidative stress,[18] while Glo1 is an important enzyme for the neutralization of cytotoxic byproducts of glycolysis.[19] Despite their relevance, Hovatta et al.'s[17] findings were, at least in part, contradicted by subsequent results.[20-22] Animals treated with L-buthionine-(S,R)-sulfoximine (BSO) for 7 days to produce oxidative

Correspondence: Alicia Carissimi, Laboratório de Cronobiologia e Sono, Hospital de Clínicas Porto Alegre (HCPA), Rua Ramiro Barcelos, 2350, CEP 90035-003, Porto Alegre, RS, Brazil.
E-mail: alicia.ufrgs@gmail.com
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stress showed lower expression of Glo1 and glutathione than controls.[23] After 7 days of BSO, the time in open arms was lower, indicating higher anxiety; lower levels of glutathione were also observed in the hippocampus and amygdala. Rats receiving the antioxidant tempol, used to counteract the effect of BSO, behaved similarly to controls in anxiety tests.[24] This finding suggests that these proteins, involved in oxidative stress regulation, are potential anxiety-like behavior mediators.[17,25]

We hypothesized that intermittent hypoxia may be associated with reduced Glo1 and GR1 expression and with higher anxiety-like behavior. The present study aimed to investigate anxiety mechanisms that may involve oxidative stress and alterations in Glo1 and GR1 levels in mice submitted to 7 days of intermittent hypoxia.

## Methods

### *Animals*

Two groups of two-month-old male Balb/c mice were maintained in an isolated room under controlled environmental conditions (22±1 °C; 12:12-h light-dark cycle) and received food and water *ad libitum*. These groups, 19 mice in total, were subjected to intermittent hypoxia or sham hypoxia for 7 days. All experimental procedures were approved by the institutional ethics committee (protocol 09-482). The experimental protocols were designed in accordance with the Guide for the Care and Use of Laboratory Animals.[26]

### *Intermittent hypoxia system*

The intermittent hypoxia system utilized in the present study has been previously described elsewhere.[27,28] In brief, 13 mice were exposed to a protocol of intermittent hypoxia for 7 days, 8 h/day (09:00 a.m. to 05:00 p.m.) in the light phase, totaling 480 cycles of hypoxia/reoxygenation per day. The mice were submitted to cycles of 30 s of hypoxia induced by insufflation in a sealed cage with a gas mixture (92% nitrogen and 8% $CO_2$) that reduced the inspired oxygen fraction from 21 to 7±1% and increased the $CO_2$ concentration to 5±1%, which was followed by 30 s of reoxygenation when room air was again insufflated into the cages. An automated system controlled the gas cycling. Intermittent hypoxia was conducted for 7 consecutive days. Six control animals were submitted to sham intermittent hypoxia, being housed in similar cages and conditions as the intermittent hypoxia group, except for the insufflated gas mixture being room air.

### *Anxiety assessment*

Anxiety behavior was assessed after 7 days of intermittent hypoxia using the elevated plus-maze test.[29,30] All behavioral tests were performed between 09:00 a.m. and noon. To habituate the mice to the testing room conditions, they were moved there 30 min prior to the test. The apparatus utilized for the test was elevated 50 cm from the floor and consisted of two open arms (25 x 5 x 0.5 cm) and two closed arms (25 x 5 x 16 cm). The mice were placed at the center of the platform (5 x 5 x 0.5 cm) with their nose directed towards a closed arm. The animals were allowed to explore the apparatus for 10 min while their behavior was recorded by a video camera. Posterior video analyses were performed semi-manually, with the assistance of data-counting software. Entrances into any space in the apparatus (open arms, closed arms, or center) were only counted when the animal placed all the four paws in it. Latency, frequency, and duration in the open and closed arms were analyzed. The total frequency (TF) of entries was calculated by adding the frequencies in the open and closed arms.

### *Molecular analyses*

After 7 days of intermittent hypoxia exposure and anxiety assessment, the animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The mice were euthanized by cervical dislocation, and the brains were immediately removed. The hippocampus, cerebral cortex, and striatum were separated and rapidly frozen in liquid nitrogen. These three brain regions were selected because they are involved in the regulation of anxiety and fear.[17] The samples were stored at -80 °C until the moment of analysis. Glo1 and GR1 (Santa Cruz Biotechnology, Santa Cruz, USA) levels were determined by Western blotting in the cerebral cortex, hippocampus, and striatum. The protein bands were visualized by ethidium bromide (0.5 μg.mL-1) (Vilber Lourmat apparatus, Marne-la-Vallée, France).The images were captured using an L-Pix Chemi photo digitizer (Loccus Biotecnologia, Cotia, Brazil) for subsequent editing and analysis with a digitizer (Loccus Biotecnologia).

### *Statistical analysis*

All statistical analyses were conducted using SPSS version 18. The results from elevated plus-maze test and the protein levels in the hippocampus, cerebral cortex, and striatum were expressed as median and interquartile range. The comparisons between the two groups were performed using Mann-Whitney *U* tests. Differences with $p < 0.05$ for alpha error were considered significant.

## Results

### *Body weight*

The average baseline body weights in the hypoxia and control groups were 23.2 g (22.0-24.9) and 23.5 g (22.9-25.2), respectively, with no significant difference observed. The body weights were stable throughout the experimental period in both groups.

### *Elevated plus-maze test*

Mice exposed to intermittent hypoxia scored lower on the anxiety behavior indices than the control group (Table 1). Differences in behavior were indicated by higher frequencies of open-arm entries and by longer duration and higher frequency of head-dipping, which indicate anxiolysis and increased risk-taking behavior. There were no

**Table 1** Effect of intermittent hypoxia on elevated plus-maze parameters

| | Control (n=6) | Intermittent hypoxia (n=13) | p-value |
|---|---|---|---|
| Total entries (frequency) | 4.00 (1.00-13.00) | 11.50 (6.25-18.00) | 0.11 |
| Open entries (frequency) | 0.00 (0.00-2.00) | 3.00 (1.25-6.50) | **0.04** |
| Closed entries (frequency) | 4.00 (1.00-10.00) | 9.00 (4.00-11.00) | 0.20 |
| Time in open arms (s) | 0.00 (0.00-10.00) | 23.00 (2.00-70.75) | 0.09 |
| Time in closed arms (min) | 4.00 (2.40-5.00) | 2.90 (2.10-3.70) | 0.20 |
| Stretching (frequency) | 11.00 (6.00-13.00) | 7.50 (3.50-11.75) | 0.19 |
| Stretching (duration, s) | 32.00 (15.00-72.00) | 27.00 (6.00-51.00) | 0.37 |
| Head-dipping (frequency) | 0.00 (0.00-5.00) | 13.00 (2.75-19.25) | **0.03** |
| Head-dipping (duration, s) | 0.00 (0.00-6.00) | 14.50 (5.00-25.00) | **0.02** |

Data presented as median and 25-75% quartiles.
p-values from the Mann-Whitney *U* test.
Bold font indicates statistical significance.

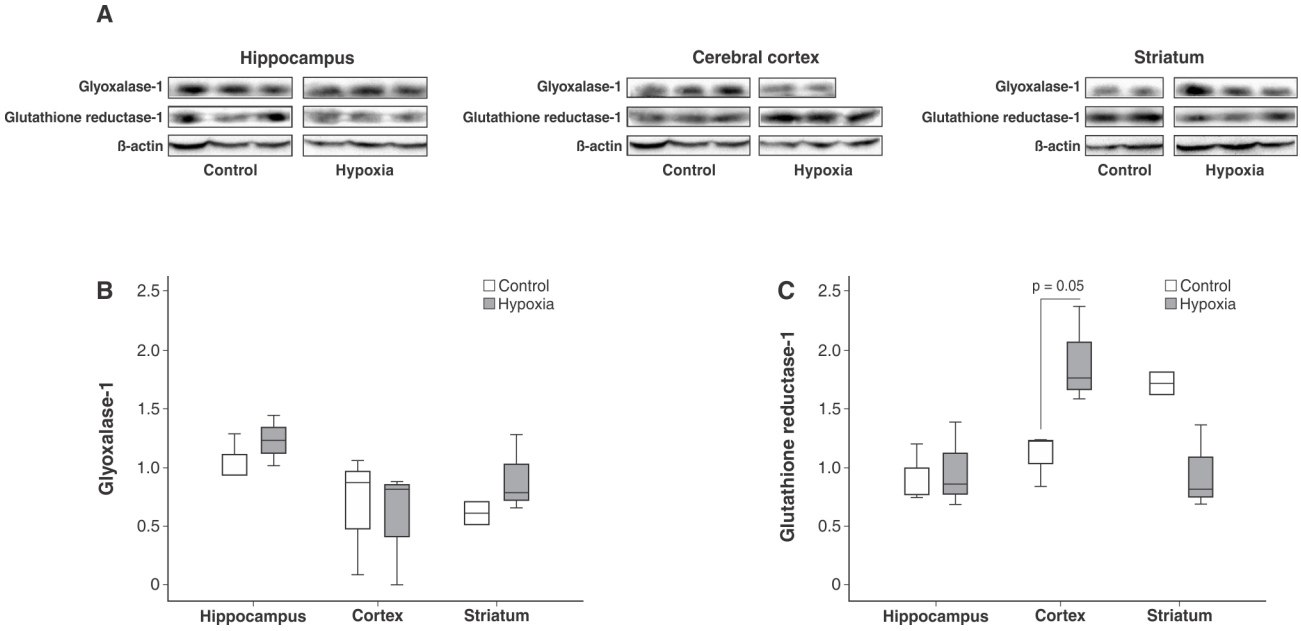


**Figure 1** The molecular findings for glutathione reductase-1 and glyoxalase-1 levels. A) Western blots from three nervous system areas showing the expression of glyoxalase-1, glutathione reductase-1, and the structural protein beta-actin used as a control between groups. B) Glyoxalase-1 levels in the hippocampus, cerebral cortex, and striatum were similar between the hypoxia (n=13) and control groups (n=6); C) Glutathione reductase-1 levels were 42% higher in the cerebral cortex of animals exposed to intermittent hypoxia.

significant differences in total entries, frequency of closed arm entry, time spent in open and closed arms, or stretching duration and frequency.

*Western blot analysis*

The GR1 level was 43% higher in the cerebral cortex (p = 0.05), 8% higher in the hippocampus (p = 0.51), and 52% lower in the striatum (p = 0.13) of the intermittent hypoxia group than the control group. The Glo1 level was 6% lower in the cerebral cortex (p = 0.56), 32% higher in the hippocampus (p = 0.51), and 28% higher in the striatum (p = 0.08) of the intermittent hypoxia than the control group (Figure 1).

## Discussion

Our results demonstrated decreased anxiety-like behavior, which is contrary to previous hypotheses. The plus-maze parameters that reached significance in the intermittent hypoxia group also indicate compatibility with increased impulsivity, risk-taking, or mania-like behavior. Regarding the enzymes analyzed, unlike previous assumptions, no significant results were observed for GR1 or Glo1 expression in the analyzed areas. The only borderline difference was in GR1 expression level in the cerebral cortex, which was also unexpected. These results suggest the existence of additional factors in the relationship between enzyme expression and anxiety, and the results should be interpreted in light of the peculiarities of different intermittent hypoxia models.

The elevated plus maze is based on an approach-avoidance paradigm built on the opposition of two natural rodent behaviors: the propensity to explore the environment (mainly the open arms, denoting anxiolysis) and the propensity to remain in a safe environment (the closed arms, denoting anxiogenesis). Sleep restriction leads to increased anxiety as part of a complex phenotype,

including inattention, impulsivity, and mania-like behavior. In this method, anxiety and impulsivity/mania are opposite and mutually exclusive, since the former is associated with increased time in the closed arms, while the latter corresponds to increased time in the open arms. Thus, in our experiment, reduced anxiety could represent a mania-like state induced by intermittent hypoxia, as a primary or secondary consequence of brain damage.

Similar experiments evaluating the effects of intermittent hypoxia on anxiety-like behavior in an elevated plus maze have already been performed. Zhu et al.[31] submitted adult rats to an intermittent hypoxia model, rather than sleep apnea, using hypobaric exposure for 4 h per day, with an oxygen pressure equivalent to an inspiratory fraction of 12% oxygen. After 14 days, an antidepressant-like effect and reduced anxiety were observed after a chronic mild stress paradigm. Furthermore, Leconte et al.[32] showed that repeated hypoxic episodes to an 8% $F_IO_2$ nadir, applied for 1 h and repeated three times per week for 6 weeks, decreased anxiety-like behavior in the plus-maze and light/dark transition tests. A similar nadir of inspired $O_2$ was employed in our study in an intermittent hypoxia system, and the frequency of open arm entries in the elevated plus-maze test was similar in our results. Finally, Perry et al.[33] demonstrated that intermittent hypoxia to a 10% $F_IO_2$ nadir for 3 days does not change anxiety behavior in the elevated plus-maze test in comparison with controls or with animals exposed to paradoxical sleep deprivation. It should be pointed out that all these experiments have slightly different hypoxia protocols. Thus, ours is the only study design utilizing a 7-day intermittent hypoxia protocol that mimics an apnea-hypopnea index of 60/h with a nadir of inspired $O_2$ of 7%. Despite the dissimilar intermittent hypoxia protocols used in these studies, the observed anxiolysis agrees with our results. These data attest that, contrary to our hypothesis, intermittent hypoxia might have no effect on or lead to decreased anxiety in rodents.

The absence of an increase in anxiety levels might be explained by confounders such as sleep restriction or the effect of sleepiness on anxiety. It should be pointed out that the intermittent hypoxia protocol in rodents leads to sleep restriction, which is less distressing than total sleep deprivation. A recent meta-analysis of clinical studies demonstrated that sleep deprivation has significant anxiogenic potential in humans, although no such effect was observed with sleep restriction.[34] In addition, the increase in anxiety level is proportional to the duration of sleep loss. Considering that we employed a period of intermittent hypoxia shorter than that of similar studies, this might have affected the results. Furthermore, rodent studies evaluating the relationship between sleep and anxiety have failed to reproduce the sleep deprivation-induced anxiety observed in humans.[35-37] While sleep deprivation increases anxiety in humans,[34] a meta-analysis of 49 rodent studies[37] found reduced anxiety-like behavior in both mice and rats, with no species-specific effects. The discrepancy between our results and those of the meta-analysis of preclinical studies might be due to use of the elevated plus-maze, which despite being the gold-standard technique for anxiety-like behavior in rodents, has led to most of the conflicting data in this field of study. These comparisons indicate that there might be additional factors modulating the relationship between sleep deprivation and hypoxia.

Furthermore, the possible reduction in anxiety might be directly related to intermittent hypoxia, which acts over and above simple sleep restriction. It is well-documented that intermittent hypoxia causes oxidative damage to neurons.[13] Higher locomotion to stimulate anxiolysis might occur as a direct result of brain damage, rather than as an effect of sleep restriction. Intermittent hypoxia might lead to impulsivity or mania-like events, just as classically observed in REM sleep deprivation.[38,39] In this case, the increased locomotion and apparent anxiolysis might be a direct effect of sleep deprivation or a brain damage-induced transient maniac state.

Sleepiness may be acting as a confounder in this context, partially explaining the observed anxiolysis. In sleepy women scoring over 10 on the Epworth sleepiness scale, the frequency of depression is higher than in non-sleepy women, although the frequency of anxiety is similar.[40] Rodents exposed to intermittent hypoxia increase sleep time by more than 3 h per day, indicating sleepiness. Even 2 weeks after the discontinuation of intermittent hypoxia exposure, the total sleep time remains higher than normal.[14] This finding suggests that sleepiness attenuates anxiety-like behavior.

Regarding oxidative stress effects, in a review, Bouayed et al.[25] observed a link between oxidative stress and high-anxiety behaviors, but no causal relationship. Oxidative stress produced by the intermittent hypoxia model may increase the expression of antioxidant enzymes.[17,21,41] A study by Ntalapascha et al.[42] found increased oxidative burden, possibly via the protein oxidation pathway, in OSA patients without comorbidities. The antioxidant enzymes Glo1 and GR1 are significantly lower in the hippocampus of rats submitted to social stress.[43] These enzymes have been described as overexpressed in the cerebral cortex of animals with high anxiety-related behavior.[17] The expression of these enzymes is increased after 24-h sleep deprivation.[44] This effect is corroborated by our findings and may be explained by an initial antioxidant response elicited to face the immediate rise in oxidative stress. Differently from injected substances that remain circulating for prolonged periods, intermittent hypoxia is promptly reversible as soon as the procedure is interrupted. Instead of consuming antioxidant enzymes, intermittent hypoxia may lead to their overexpression and higher tissue levels immediately after ceasing the experiment. In the present study, we observed a 43% higher level of GR1 in the cerebral cortex, indicating that this compensatory mechanism may be causing the apparent inverse relationship between the enzyme and anxiety.

The limited number of anxiety-related oxidative stress markers assessed and the problems entailed in anxiety-like behavior assessment in rodents are the present study's main limitations. The small sample size and time of outcome assessment might also have impacted the results, although previous data have suggested that there is no circadian effect on the elevated plus maze.[45] The use of intermittent hypoxia mimics only part of the features of OSA. The pattern of hypercapnia induced by $CO_2$

insufflation in the cage is different from that observed in humans, and the hypoxic events remain present even during waking periods. Moreover, the duration of the hypoxic period might have been short, especially considering that OSA is chronic disease, usually diagnosed years after the onset of symptoms. However, articles about the short-term effects of Continuous Positive Airway Pressure withdrawal provide interesting insights about the effects of being under intermittent hypoxic conditions for short periods, impacting both behavioral performance and oxidative stress-related enzymatic expression.[46,47]

Further investigation with longer intermittent hypoxia exposure and a greater range of molecular and behavioral evaluations is necessary to ensure the validity of the present results. In conclusion, mice submitted to intermittent hypoxia show no increase in anxiety behavior. The mechanism for this change may involve the anxiety-modulating effects of antioxidants like GR1.

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## Disclosure

DM is co-owner of a sleep medicine clinic. GNP is currently an employee of Springer Nature, although this position has no relation with the present article or any of this author's academic activities. The other authors report no conflicts of interest.

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