



## Effect of postnatal intermittent hypoxia on locomotor activity and neuronal development in rats tested in early adulthood

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### Abstract

The present study evaluated the effects of postnatal intermittent hypoxia on locomotor activity and neuronal cell survival in early adulthood rats. During a critical period of brain development on postnatal day (PD) 7-11, male rat pups were exposed to intermittent hypoxia and randomly assigned to three experimental groups: (1) intermittent hypoxia, (2) normoxia, and (3) control (unhandled). One and a half months later on PD56, a behavioral test was conducted, and cell survival was estimated in the hilus, dental gyrus, and CA1 and CA3 subfields of the hippocampus, nucleus accumbens shell and core, dorsal and ventral striatum, and prefrontal cortex. Our results showed that intermittent hypoxia produced hyperactivity that correlated well with psychomotor agitation observed in patients with schizophrenia. Moreover, post-hypoxic rats exhibited a reduction of the number of neurons in the hilar region of the hippocampus and dorsal striatum, structures that have been neuropathologically associated with schizophrenia. These findings suggest that intermittent hypoxia can modify the pattern of locomotor activity and selectively affect neurons in rats tested in early adulthood.

**Keywords:** hypoxia, neurodevelopment, hyperactivity, cell survival, schizophrenia.

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### Introduction

Schizophrenia is a complex neuropsychiatric disease that has genetic, environmental, and developmental aspects. The neurodevelopmental hypothesis of schizophrenia proposes that lesions caused by birth complications, infections, toxin exposure, or maternal stress *in utero* or during the postnatal period disturb the establishment of fundamental cerebral structural and functional aspects, rendering the brain vulnerable to adverse effects during puberty that precipitate the disease in young adulthood (Sandager-Nielsen, Andersen, Sager, Werge, & Scheel-Kruger, 2004; Schwabe, Klein, & Koch, 2006). A large number of epidemiological studies

have demonstrated that perinatal complications, often involving fetal or neonatal hypoxia, are associated with an increased risk for the development of schizophrenia (McNeil, 1995; Cannon, 1997; Cantor-Graae, Ismail, & McNeil, 2000; Vaillancourt, & Boksa, 2000).

The slow process of rodent brain maturation enhances the vulnerability of neuronal survival. Some functional components do not fully mature until postnatal day (PD) 15-20 (Golan & Huleihel, 2006). Exposure to intermittent hypoxia (IH) during this period was associated with marked increases in the number of apoptotic cells in the CA1 area of the hippocampus and cortex (Gozal, Daniel, & Dohanich, 2001a; Gozal, Row, Schurr, & Gozal, 2001b) and changes in the dopaminergic system (Decker, Hue, Caudle, Miller, Keating, & Rye, 2003; Decker, Jones, Solomon, Keating, & Rye, 2005). These alterations were related to cognitive deficits and increased motor activity (Gozal et al., 2001a; Row, Kheirandish, Neville, & Gozal, 2002; Decker et al., 2005), both of which are considered clinical symptoms of schizophrenia.

Based on the assumption that the clinical features of schizophrenia typically appear in adolescence and early adulthood (Juarez, Silva-Gomez, Peralta, & Flores, 2003), the aim of the present study was to investigate whether IH during a critical period of brain development (PD7-11) alters the pattern of locomotor activity and induces neuronal loss in early adulthood (PD56) in rats.

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## Methods

### Subjects

Male and female Wistar rats were mated in the animal facility of the Universidade Federal de São Paulo, São Paulo campus and allowed to deliver normally. Delivery times were recorded. The litters were culled to eight pups. PD1 was defined either as the day of delivery or as the next day if the pups were born after than 6:00 PM. Only males were used in this study, but female pups were allowed to stay in the litter until they were weaned. All of the rats were weaned at 4 weeks of age, and male rats were subsequently housed socially with two to three animals from the same litter that belonged to the same experimental group under a 12 h/12 h light/dark cycle (lights on at 7:00 AM) at  $23 \pm 1^\circ\text{C}$  and  $55 \pm 5\%$  humidity with food and water available *ad libitum* throughout the study. The experiments occurred between 9:00 AM and 5:00 PM. All of the experiments were performed in compliance with the recommendations of the Brazilian Society for Neuroscience and Behavior, which are based on the United States National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985) and were approved by the ethics committee of the Universidade Federal de São Paulo (207/07). All efforts were made to minimize the number of animals used and their suffering.

### Intermittent hypoxia protocol

Intermittent hypoxia was induced in a custom-built chamber (30 × 20 × 20 in; Oxycycler model A44X0, Biospherix, Redfield, NY, USA) connected to a supply of O<sub>2</sub> and N<sub>2</sub> gas. Sensors measured O<sub>2</sub> concentration, CO<sub>2</sub> concentration (<0.01%), humidity (40-50%), and temperature (22-24°C). The inflow of O<sub>2</sub> and N<sub>2</sub> into the chamber was controlled by a computer programmed to produce cycles of 2 min of room air and 2 min of 10% O<sub>2</sub>. The IH profile consisted of alternating room air and 10% O<sub>2</sub> every 2 min, whereas treatment of the corresponding controls (i.e., the normoxic group) consisted of flushing room air into the chamber every 2 min (Gozal et al., 2001a; Perry, D'Almeida, Souza, Schoorlemmer, Columbari, & Tufik, 2007).

On PD7, male rat pups were randomly assigned to three experimental groups as described by Decker et al. (2005): (1) intermittent hypoxia-inducing chamber, (2) normoxic chamber, and (3) control (unhandled). Once inside their respective chambers, the rat pups were exposed to either IH or normoxia during the subsequent 2 h. The pups were then removed from the chambers and returned to their dams for 45 min. The pups were then returned to their respective chambers and again exposed to IH or normoxia for another 2 h. Upon completion of the second 2-h session, all of the pups were returned to their dams and left undisturbed until the following morning. During the next 4 days (PD8-11), the pups that were designated as hypoxic and normoxic were removed from their dams and placed in the hypoxia and normoxia

chambers. During this period, hypoxic pups received 6 h of IH per day. They were exposed to 2 h of IH followed by a 45 min feeding and grooming session that was repeated three times each day. Normoxic pups were exposed to room air, and the non-handled control pups were left undisturbed with their dams. The rats were subjected to this schedule during the light period (8:00 AM-5:00 PM). At the conclusion of the protocol on PD11, all of the pups were returned to their dams where they remained undisturbed until weaning on PD21. The rats were subsequently housed socially with two to three animals from the same litter that belonged to the same experimental group. They were tested for locomotor activity on PD56.

### Locomotor activity protocol

The openfield was a circular enclosure (arena) made of Plexiglas (60 cm diameter, 50 cm height) with a white acrylic floor divided into 12 equal sections (13.3 × 15.0 cm rectangles; Insight Equipment, Ribeirão Preto, SP, Brazil).

On PD56, the rats were tested in the open field to assess locomotor activity. The animals were individually placed in the center of the arena and allowed a 10 min period of free exploration, during which the number of crossings (i.e., the number of floor sections crossed with all four paws) was recorded. The behavior of the animals was recorded by a video camera (Safety View) positioned 60 cm above the arena, with the signal relayed to a video monitor in another room via a closed circuit. Testing was conducted under dim light (16 lux) to avoid the possible aversive effect of bright light. After each trial, the apparatus was cleaned with a 10% ethanol solution.

### Histological analyses: Nissl staining

Upon completion of the locomotor activity test, the rats were deeply anaesthetized with urethane and intracardially perfused with 100 ml phosphate-buffered saline (PBS) and 400 ml of 4% paraformaldehyde. Coronal brain sections (35 μm thick) were made with a cryostat between 0.98 and -3.28 mm relative to bregma according to the stereotaxic coordinates of a rat brain atlas (Paxinos & Watson, 2007). The sections were selected (12 per animal) by targeting the areas and subareas of the hippocampus (hilus, granular layer of the dentate gyrus, and CA1 and CA3 areas), nucleus accumbens shell and core, dorsal and ventral striatum, and prefrontal cortex (M2 and Cg1). The selected sections were Nissl-stained with Cresyl violet (0.2% Cresyl violet acetate in acetate buffer, pH 4.0), dehydrated with alcohol and xylene, and cover slipped.

The slides were examined using a light microscope (Nikon 80i), and images were captured and digitized using a Nikon ACT-1 v.2 system. Cell damage in the three selected subregions was estimated by counting the absolute number of surviving intact cells in the Nissl-stained sections by two observers who were blind to

the experimental conditions of the animals. The cells were manually counted under a microscope at 40× magnification in 10 random, non-overlapping fields for all of the animals in the three groups (control, normoxia, and hypoxia). Cells with somas < 3 μm were considered glial or necrotic cells and excluded. The value attributed to each animal represents the mean value for all of the images taken from that animal.

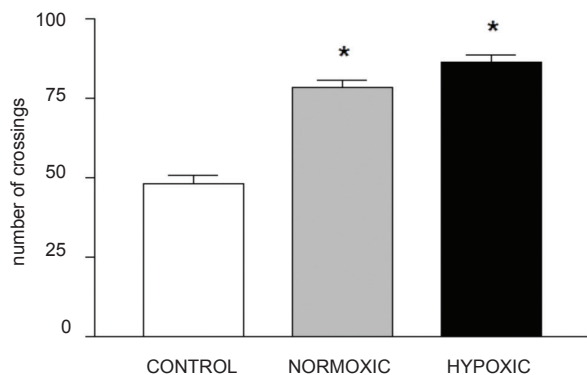
### Statistical analysis

One-factor (treatment) analysis of variance (ANOVA) was performed for the number of crossings and cell counts using Prism 4.0 software (GraphPad, San Diego, CA, USA), followed by the Tukey *post hoc* test. The alpha level was set at 0.05 for all of the statistical analyses.

## Results

### Behavioral results

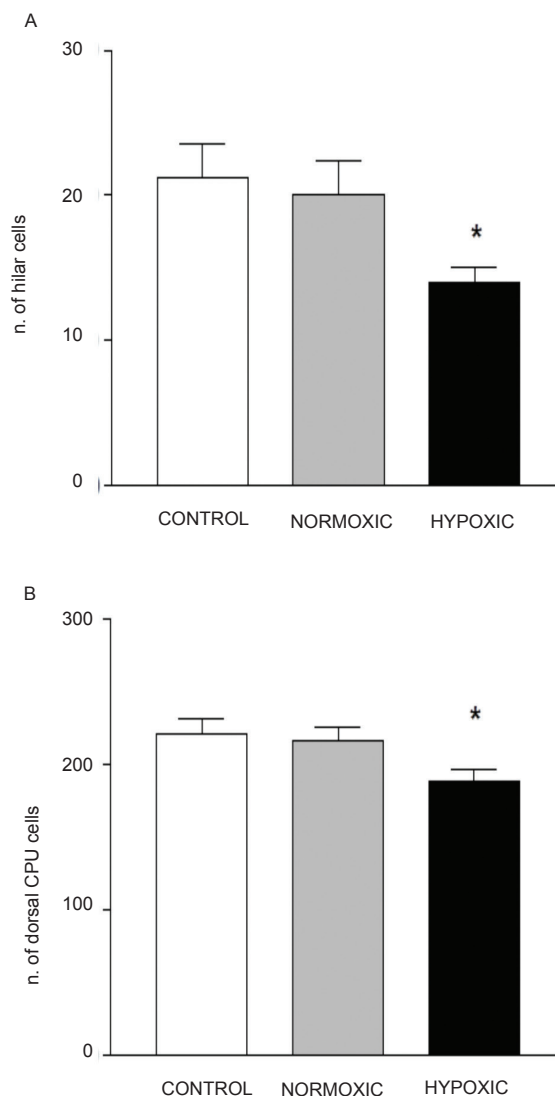
One-way ANOVA revealed a significant effect of treatment on the number of crossings in the open field test ( $F_{2,28} = 9.16$ ,  $p < 0.05$ ; Figure 1). The *posthoc* analysis showed that both the hypoxia and normoxia groups exhibited an increase in the number of crossings compared with controls.



**Figure 1.** Mean + SEM number of crossings in the open field test in male Wistar rats in the control group ( $n = 9$ ), normoxic group ( $n = 11$ ), and hypoxic group ( $n = 11$ ). \* $p < 0.05$  (one-way ANOVA followed by Tukey test).

### Neuronal cell damage

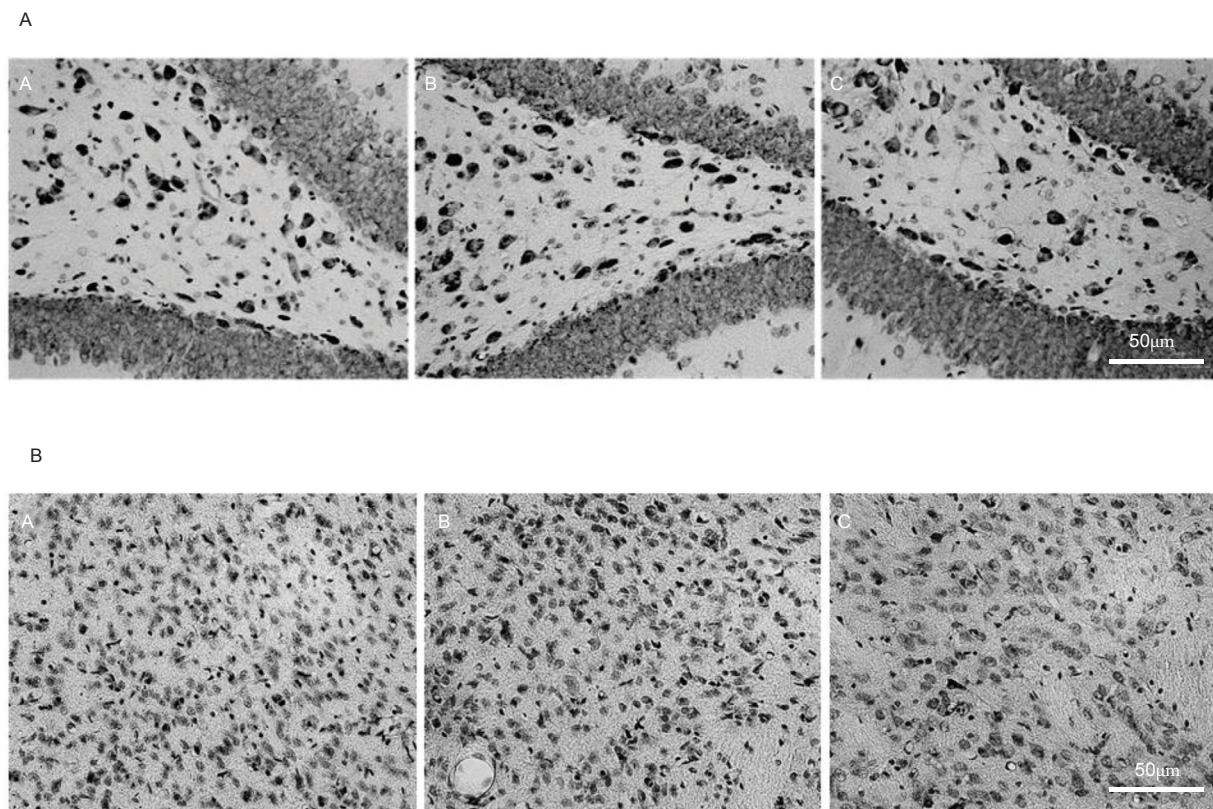
Histological analyses were performed in Nissl-stained sections to estimate neuronal death caused by IH by counting the number of intact cells in the studied brain regions. One-way ANOVA revealed a significant decrease in the number of intact cells in the hilus ( $F_{2,31} = 5.4$ ,  $p < 0.05$ ; Figures 2A and 3A) and dorsal striatum ( $F_{2,50} = 3.6$ ,  $p < 0.05$ ; Figures 2B and 3B) in the hypoxia group compared with the control and normoxia groups, indicating cell loss. No significant cell loss was observed in the CA1 area ( $F_{2,31} = 0.37$ ,  $p > 0.05$ ), CA3 area ( $F_{2,31} = 0.20$ ,  $p > 0.05$ ), prefrontal cortex ( $F_{2,90} = 2.3$ ,  $p > 0.05$ ), or nucleus accumbens ( $F_{2,33} = 0.04$ ,  $p > 0.05$ ) in these hypoxic rats.



**Figure 2.** Cell survival estimated by quantifying the number of intact Nissl-stained cells. A significant reduction of the number of Nissl-stained was observed in the hilus of the hippocampal (A) and dorsal striatum (B) in the hypoxic group compared with the control group ( $n = 9$ ); normoxic, ( $n = 11$ ); hypoxic, ( $n = 11$ );  $p < 0.05$ ; (one-way ANOVA followed by Tukey test).

## Discussion

Schizophrenia results from a cumulative interaction between numerous risk factors, some of which are neurodevelopmental. According to the neurodevelopmental hypothesis of schizophrenia, brain damage that occurs early in life increases the vulnerability of young adults to adverse events, thereby increasing the probability of clinical symptoms in adulthood (Jacob & Beckmann, 1986, 1994; McNeil, Cantor-Graae, & Ismail, 2000). An interaction between early neurodevelopmental disturbances and periadolescent brain maturation appears to be necessary to trigger the onset of full-blown psychotic behavior, which typically emerges during adolescence or early adulthood (Decker et al., 2003; Sandager-Nielsen et al., 2004; Meyer & Feldon, 2010).



**Figure 3.** Photomicrograph of standard Nissl staining that shows the hilus of the hippocampus (A) and dorsal striatum (B) in the three groups: (A) control, (B) normoxic, and (C) hypoxic. Notice the reduction of the number of cells in the hilus and dorsal striatum in the hypoxic group. Magnification = 40 $\times$  (A) and 20 $\times$  (B). Scale bar = 50  $\mu$ m.

The behavioral data obtained in the present study indicate that IH in rat pups during early developmental phases (PD7-11) led to an increase in locomotor activity when these animals were tested in early adulthood (PD56) compared with controls (Figure 1). Hyperactivity is thought to model some of the positive symptoms of schizophrenia and correlate well with psychomotor agitation observed in schizophrenia patients (Kilts, 2001). Our results are consistent with Decker et al. (2003, 2005), who observed an increase in the basal levels of locomotor activity in 42-day-old rats exposed to IH between PD7 and PD11. Other studies that used different protocols of hypoxia (Row et al., 2002) or anoxia (Dell'Anna, Calzolari, Molinari, Luvone, & Calimici, 1991) also reported an increase in locomotor activity in adult animals exposed to these procedures early in life.

Interestingly, we observed an increase in the number of crossings in the open field in normoxic rats during adulthood compared with controls (Figure 1). One possible explanation for development hyperactivity in this group is the effect of stress associated with early maternal deprivation. Several animal studies have addressed the outcome of maternal deprivation and found long-term changes in brain neurochemistry and behavioral tasks (i.e., the open field, elevated plus maze, and forced swim test), suggesting increased anxiety,

hyperactivity, and behavioral despair (George, Bordner, Elwafi, & Siemen, 2010; Anand & Scalzo, 2000; Hofer, 1980).

The hippocampus is a brain structure that has been traditionally associated with the neuropathology of schizophrenia. We found that post-hypoxic rats showed a reduction of the number of neurons restricted to the hilar region of the hippocampus (Figures 2A and 3A). However, the changes in the number of hilar cells reported in this study were quite subtle and therefore difficult to compare directly with the effects of gross disruption of hippocampal circuitry. Some studies suggested that beyond the age of the animals tested in the present study, strain differences and the severity of hypoxia can be critical factors in determining the pattern of brain injury and extent to which neurological function will be affected, thus influencing the behavioral parameters evaluated in the present study. Moreover, the intensity and site of hippocampal damage might be related to the age at which the animals were tested (Simonová, Sterbová, Brozek, Komarék, & Syková, 2003; Leonardo, Eakin, Ajmo, & Gottschall, 2008). In the present study, the animals were exposed to a moderate level of hypoxia (10% O<sub>2</sub>) on PD7-11 and evaluated on PD56. Perhaps a more intense hypoxic insult (e.g., 5% O<sub>2</sub>; Polotsky et al., 2006) could lead to more severe neuronal damage compared with the present

protocol. Indeed, Gozalet et al. (2001a,b) showed that hypoxia induced neuronal death selectively in the CA1 region and cortex without modifying other hippocampal regions. Other studies also suggested that hypoxia induces selective neuronal death in the hippocampus (Feng, Zhao, Gurkoff, Van, Shahlaie, & Lyeth, 2012), especially in the hilus (Schwartz, Massarweh, Vinters, & Wasterlain, 1992).

Our findings demonstrated that hypoxia induced neuronal damage in the dorsal striatum (Figures 2B and 3B). The striatum is one of the main regions that is damaged after neonatal hypoxia in rats (Rice, Vannucci, & Brierley, 1981; Galvin & Oorschot, 2003) and humans (Volpe, 2001). These data are consistent with other studies that reported neuronal loss in the striatum at different ages (Leonardo et al., 2008, Galvin & Oorschot, 2003). During the critical period of brain development, hypoxia can damage the functional integrity of the dopaminergic system, thus influencing relevant behavioral and neurochemical alterations (Decker et al., 2003, 2005). Row et al. (2002) verified that newborn rats (PD10) that were subjected to hypoxia exhibited an increase in locomotor activity when they reached 30 days of age.

Striatal dopaminergic dysfunction is commonly observed in schizophrenia and could be related to the dysfunction of dopamine-glutamate interactions. Usun, Eybrard, Meyer, & Louilot (2013) showed that ketamine (20 mg/kg, i.c.) increased the release of dopamine in the dorsal striatum and increased locomotor activity in freely moving adult rats that had their prefrontal cortex inactivated by a microinjection of tetrodotoxin on PD8. The rats were monitored using *in vivo* voltammetry in the dorsal striatum. These data suggest that transient inactivation of the prefrontal cortex during early development results in greater behavioral and striatal dopaminergic reactivity to ketamine in adulthood.

Our results also showed that the nucleus accumbens core and shell and prefrontal cortex were not damaged after IH on PD7-11. Neurons in the hippocampus and dorsal striatum are less resistant to injury and die before neurons in the nucleus accumbens and prefrontal cortex during the critical developmental period evaluated in the present study (Golan & Huleihel, 2006). Moreover, the intensity and duration of the hypoxic insult might influence the severity of damage (Ten, Bradley-Moore, Gingrich, Stark, & Pinsky, 2003). Moreover, other significant aspects, such as the unmyelinated state of the immature brain with a greater volume of the extracellular space, immaturity of the blood-brain barrier, cerebral blood flow during the first weeks of life, different glucose utilization, and immaturity of enzymes and receptors, are important for the cerebral response to injury and may affect the evolution of the injury (for review, see Hagberg, Bona, Gilland, & Puka-Sundvall, 1997).

In conclusion, IH insult and maternal deprivation in rat pups on PD7-11 produced hyperactivity on PD56, which correlated well with psychomotor agitation

observed in patients with schizophrenia. Post-hypoxic rats also exhibited a reduction of the number of neurons in the hilar region of the hippocampus and dorsal striatum, structures that have been traditionally associated with the neuropathology of schizophrenia. The nucleus accumbens and prefrontal cortex were not affected by IH. Several factors, such as the concentration of O<sub>2</sub>, duration of exposure to hypoxia, and age of the animals at the time of testing, can influence the response to hypoxia.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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