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Acute hypoxia exposure following prenatal stress impairs hippocampus and novelty-seeking behavior in adolescent rats

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Abstract

Objectives: The present study aimed to investigate the effects of acute hypoxia exposure following prenatal stress on the novelty-seeking behavior and hippocampus of adolescent rats.

Methods: The offspring were divided into prenatal stress (PS) and non-stress (NS) groups. Both groups were exposed to hypoxia on postnatal day 10 (P10) while control groups were undisturbed. Novel object recognition task was performed in each group. Next, brains were collected to examine hippocampus via immunohistochemical and biochemical studies on postnatal day 35 (P35).

Results: PS decreased novelty discrimination and synaptophysin (SYN) expressions in both CA1 and CA3 of the hypoxia group prominently ($p < 0.05$). Nestin-expressing cells were reduced while vascular endothelial growth factor (VEGF) expression was enhanced in the subgranular zone (SGZ) of PS-hypoxia group ($p < 0.05$). VEGF enhancement triggered angiogenesis in the CA1 and CA3 significantly ($p < 0.05$). PS also increased thiobarbituric acid reactive substances (TBARS) levels in the hypoxia group as a result of oxidative stress ($p < 0.05$).

Conclusion: These findings demonstrated that PS exacerbates neurodevelopmental deficits in the hippocampus of acute hypoxia-induced offspring in adolescence.

KEYWORDS
hippocampus, hypoxia, novelty-seeking, prenatal stress

1 | INTRODUCTION

Early life stress usually causes neurodevelopmental, psychiatric, cardiovascular, and intestinal problems in long term (Charil et al., 2010; Weinstock, 2008). Stress during pregnancy affects fetal development by increasing the activity of the hypothalamic–pituitary–adrenal (HPA) (O'donnell et al., 2009). Dysregulation of the HPA axis causes glucocorticoids to be transferred through placenta and accumulate in the developing brain (Huizink et al., 2000). Prenatal stress (PS) especially affects the hippocampus which is crucial for emotional behaviors, learning, and memory (Weinstock, 2008). Therefore, PS may lead to long-term behavioral and cognitive problems

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by changing synaptic transmission, neurogenesis, neuronal morphology, and migration in the hippocampus (Fujioka et al., 2006; Martínez-Téllez et al., 2009; Mulder et al., 2002; Stevens et al., 2013). However, the effects of PS depend on the species in the experiment, duration and intensity of the stress, and when during pregnancy occurred (Weinstock, 2008).

Hypoxic encephalopathy is the most common brain injury in early life and one of the reasons for neurodevelopmental disorders (Huseynova et al., 2017). PS affects the ventilatory response to hypoxia (Golubeva et al., 2015), and it increases hypoxia-inducible factors (Jašarević et al., 2021). Furthermore, Palma-Gudiel et al. revealed that prenatal adverse environment decrease methylation of EP300 gene, which is neuroprotective against hypoxic conditions (Palma-Gudiel et al., 2019). Several studies indicate that acute hypoxia increases oxidative stress markers such as thiobarbituric acid reactive substances (TBARS) (Coimbra-Costa et al., 2017; Irarrázaval et al., 2017; McGinnis et al., 2014). Myeloperoxidase (MPO) that is released from neutrophils reacts with H_2O_2 that can also cause oxidative stress and plays role in pathogenesis of many neurodevelopmental disorders (Klebanoff, 1999). The antioxidant enzymes such as catalase (CAT) provide protection to the brain; however, oxidative stress may remain afterward, and reactive oxygen species may cause unavoidable damage in the long term (Coimbra-Costa et al., 2017). Furthermore, hypoxia activates voltage-gated $Ca2^+$ and K^+ channels as well as AMPA receptors, causing synaptic excitotoxicity (Bickler & Donohoe, 2002; Mukandala et al., 2016; Sanchez et al., 2001). It also upgrades vascular endothelial growth factor (VEGF), which is crucial for angiogenesis, the formation of new blood vessels in the brain (Brantley-Sieders & Chen, 2004; Croll & Wiegand, 2001).

The subgranular zone (SGZ) is significant for brain development since it contains nestin-expressing neural progenitor cells (Amaral et al., 2007). Limited hippocampal neurogenesis in SGZ contributes to the formation of granular neurons in dentate gyrus (DG), which is an essential region for the synaptic network of novel object recognition (Kitamura et al., 2009; Luo et al., 2010). However, considerable studies suggest that the perirhinal cortex plays a critical role rather than hippocampus in novel object discrimination (Kumaran & Maguire, 2007). Therefore, in this study, we investigated whether the hippocampus involves in novelty-seeking behavior after the effects of PS and acute hypoxia exposure.

Considering the effects of PS and hypoxia on brain development, we questioned the effects of acute hypoxia following PS on hippocampus in adolescence. In this study, we measured TBARS, MPO, and CAT levels to evaluate oxidant and antioxidant balance. Regarding neurogenesis, angiogenesis, and synaptic transmission, we examined nestin, VEGF, and synaptophysin (SYN) expressions in hippocampus. This study, thus, exhibits supportive and distinctive evidence to show the effects of PS and neonatal acute hypoxia on hippocampus and novelty-seeking behavior in adolescence.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental design

Adult female Wistar albino rats (200–400 g) were obtained from Ege University, Laboratory of Animal Research and Application Center, and housed with ad libitum food and water under 20-25Co, 12L/12D laboratory conditions. The female rats ($n = 6$/each group) were housed with the male rats overnight following the determination of estrous cycle via vaginal smear. The next day, vaginal plugs were observed and presumed as the first day of pregnancy. The offspring ($n = 11$/each group) stayed in their home cage until weaning and separated by their genders on postnatal day 21. The experimental groups were set as (1) PS-hypoxia, (2) PS-control, (3) NS-hypoxia, and (4) NS-control. This study was carried out after the approval by the Institutional Animal Care and Ethical Committee of Ege University (2017-082).

2.2 | Prenatal stress procedure

The pregnant rats were housed individually and restrained in a plexiglass transparent cylinder (19 cm × 6 cm) three times/day for 45 min under two bulbs (100 W) (Edwards et al., 2002). The restraint stress was induced between embryonic days 12.5–17 due to the hippocampus begins to form on embryonic day 12 in rats (Amaral et al., 2007). The NS group of pregnant dams was not disturbed.

2.3 | Hypoxia exposure

Forty-four different litters (20 male/24 female) were exposed to 100% CO_2 in an airtight chamber for 5 min on P10, and re-oxygenation was induced with 100% O_2 for 5 min gradually (Okur et al., 1995). Freezing, hyperventilation, head shaking, and tachycardia were observed for approximately 1 h. Control group was taken to the experiment room without any application. After the

experimental process, all groups were taken to their home cages until weaning.

2.4 | Novel object recognition task

Novel object recognition (NOR) task was described by Ennaceur and Delacour (Ennaceur & Delacour, 1988). Animal learns the novel object without any penalty or reward and encodes the information depending on exploration and novelty-seeking behavior. On the habituation day (P33), the animals ($n = 10$/each group) were placed into a 60 × 60 × 40 box for 5 min and expected to explore the box. The next day, two familiar objects (two green toy carousels with and without horses) were placed on different quadrants of the box symmetrically, and the rats discovered the objects for 10 min. On the test day, the familiar object was replaced with a different shaped, sized, and colored object (a heart-shaped toy). The animals were placed into the box and expected to explore familiar and novel objects for 10 min. The experiment was carried out during their active phase in a room with dim light daytime. All sessions were recorded, the time spent nearby the novel object was calculated by two different stopwatches, and the mean was considered. The box and the objects were cleaned with 70% alcohol between sessions to avoid odor factor. The novelty detection time was calculated as when the animal approached the object less than 2 cm, sniffed, pawed, and self-cleaned alongside the object (Lueptow, 2017).

2.5 | Tissue preparation and immunohistochemistry

The animals ($n = 6$) in each group were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) on P35 following behavioral assessment. Intra-cardiac perfusion was administered with 4% formaldehyde in 0.1-M phosphate-buffered saline (PBS). Brains were collected and washed in 0.1-M cacodylate buffer and stored in 4% formaldehyde in 0.1-M PBS for 3 days, respectively. The next day, brain slices at the level of the dorsal hippocampus (plate 21 and 23) were prepared according to the stereotaxic atlas of Paxinos and Watson (Paxinos & Watson, 2006). The slices embedded into the paraffin and sectioned (5 μm) for immunostainings. The sections were incubated with anti-nestin (1:100, Bioss Antibodies, China), anti-VEGF (1:100, Sigma Aldrich), and monoclonal anti-synaptophysin (1:100, Sigma Aldrich) primary antibodies. The following day, the sections were washed and incubated with anti-mouse IgG (1:200, Sigma Aldrich) for 40 min and DAB staining applied.

2.6 | Image analysis

Synaptophysin (SYN) expression in CA1 and CA3, nestin, and VEGF positive cells in the SGZ (cells/mm^2) were examined under a light microscope and microphotographs were captured (Olympus BX-51, light microscope, Olympus C-5050 digital camera). Image J (https://imagej.nih.gov/ij) and cellSens (Olympus Corporation, Japan) were used for image analysis. For this analysis, the hippocampus of each animal was randomly chosen for unbiased counting, and the averages of 10 images (magnification ×40) from different sections were examined by two researchers to ensure objectivity (Turgut et al., 2006). Further, angiogenesis was measured as counting VEGF-positive vessels on CA1 and CA3 (vessels/mm^2). Only clear lumen and tubular structures were counted for 10 images from different sections/each animal by two researchers, and the mean was considered (Zand et al., 2005).

2.7 | Biochemistry

The brains ($n = 5$) were collected without intra-cardiac perfusion, and hippocampus was dissected and homogenized in phosphate buffer (0.5 M, pH: 7.0), (1/10: w/v). Homogenates were centrifuged for 5 min at 700×g at 4°C. Supernatants were collected immediately to determine myeloperoxidase (MPO), catalase (CAT), and thiobarbituric acid reactive substances (TBARS) levels. Hydrogen peroxide degradation was recorded at 240 nm by spectrophotometry, and CAT activity was assessed (Aebi, 1984). MPO, an enzyme present in granulocytic and monocytic cells, was calculated by following these stages: Homogenates were centrifuged at 10,000 rpm for 15 min. Pellets were re-homogenized in 0.5% HETAB (hexadecyltrimethylammonium bromide) in PBS (50 mM, pH: 6.0) and centrifuged 10,000 rpm for 10 min. Supernatants were added to a reactive solution containing 0.5-M o-dianisidine. Hydrogen peroxide solution (20 mM) was added, and absorbance was recorded at 492 nm with a microplate reader for 3 min with 15-s intervals. Finally, MPO activity was calculated using the standard curve (Grisham et al., 1986). Furthermore, homogenates were incubated with TBARS solution (0.12-M TBA in 15% TCA and 1% HCl) for 30 min at 95°C, and the TBARS levels were calculated using 1,1,3,3-tetramethoxypropane standard curve (Sözmen et al., 2001).

2.8 | Data analysis

One-way ANOVA and post hoc Bonferroni tests were performed in normal distributed and homogenous data according to Shapiro–Wilk test. Independent *t* test was used to compare hypoxia and control groups between PS and NS in normality circumstances. Kruskal–Wallis and Mann–Whitney non-parametric tests were used in the absence of normal distribution. Pearson correlation was used to understand whether the results of NOR are correlated with immunohistological parameters. The significance was considered as $p < 0.05$, and data are shown as mean ± standard error of the mean (S.E.M.). IBM SPSS Statistics 22.0 was used for statistical analysis.

3 | RESULTS

3.1 | Novel object recognition (NOR)

The discrimination index (DI) was calculated as $DI = (new\ object/total\ exploration) \times 100$. ANOVA results of the DI demonstrated a significant difference between groups ($F_{(3,40)} = 3.38$, $p = 0.028$). Post hoc test revealed that PS decreased novelty-seeking in the hypoxia group (41.58 ± 4.75) compared with controls of both NS (75.87 ± 6.71, $p = 0.044$) and PS (74.88 ± 7.62, $p = 0.05$). Similarly, independent *t* test results showed the negative effects of PS on the novelty discrimination of hypoxia group compared with NS-hypoxia (71.85 ± 12.28, $p = 0.041$) (Figure 1). There was no statistical difference between control and hypoxia in the NS group.

3.2 | Synaptophysin expressions in CA1 and CA3

Synaptophysin (SYN) immunoreactivity was measured in CA1 and CA3 regions of hippocampus. Neuron loss and disorganized pyramidal layers were observed in both regions of PS-hypoxia group (Figure 2a,b). Moreover, the axonal sprouting was aberrant in PS-hypoxia group caused by increased angiogenesis presumably. ANOVA results of the SYN expression in the CA1 ($F_{(3,24)} = 3.99$, $p = 0.35$) and CA3 ($F_{(3,24)} = 14.2$, $p = 0.001$) exhibited significant difference between groups. The only significant difference in CA1 was between PS-hypoxia (30.68 ± 2.82) and PS-control group (43.59 ± 2.27, $p = 0.033$). Similarly, PS decreased SYN expression in the CA3 of hypoxia group (25.26 ± 2.5) compared with all groups significantly ($p < 0.05$) (Figure 2c,d).

3.3 | VEGF and nestin expressions in SGZ

SGZ was detected from the hippocampal sections under light microscope. According to ANOVA results, there is a significant difference between groups for nestin-expressing cells in the SGZ ($F_{(3,24)} = 4.481$, $p = 0.025$). PS decreased nestin-positive cells in the hypoxia group (22.08 ± 2.70) compared with NS-hypoxia (32.98 ± 1.68, $p = 0.05$) and NS-control (33.68 ± 3.68, $p = 0.038$). There was also an enlargement between the granular zone and hilus in the PS-hypoxia group as evidence of developmental deficits (Figure 3a).

ANOVA results ($F_{(3,24)} = 6.061$, $p = 0.009$) and post hoc revealed that VEGF expression in the PS-hypoxia

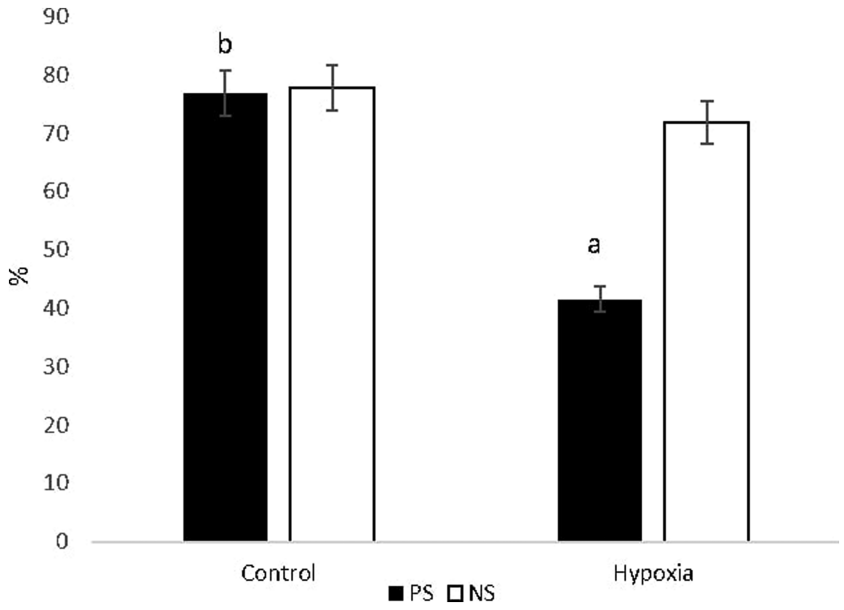

FIGURE 1 The discrimination index (DI) of novelty-seeking behavior. PS decreased novelty discrimination in hypoxia group significantly ($n = 10$/each group). pa < 0.05 versus NS-control and NS-hypoxia, pb < 0.05 versus PS-hypoxia

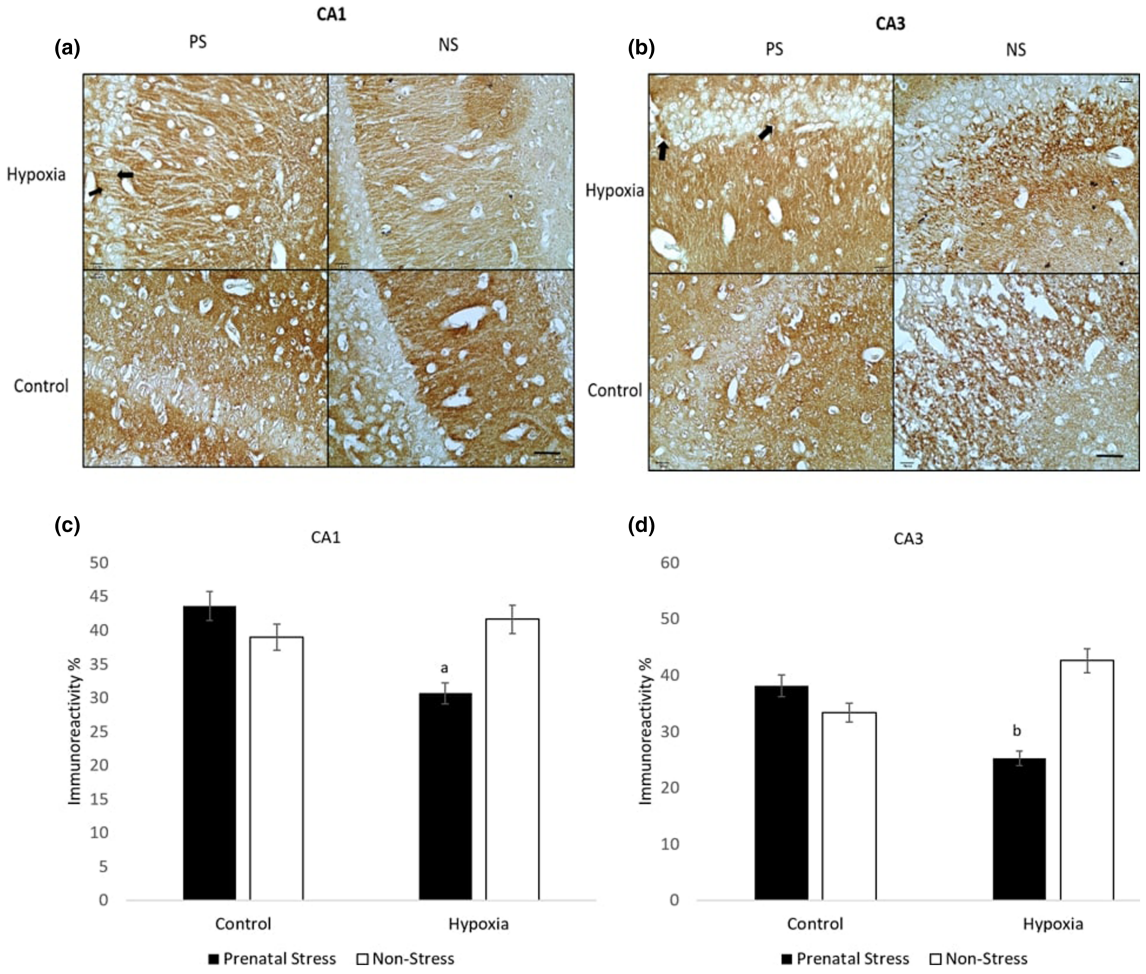

FIGURE 2 Synaptophysin expressions in CA1 and CA3. The arrows indicate the neuronal loss and disordered pyramidal layers, causing decreased synaptophysin immunoreactivity in the PS-hypoxia group (a, b). *p*a < 0.05 versus PS-control (c). *p*b < 0.05 versus all groups (d) ($n = 6$/each group, scale bar = 20 μm, magnification = ×40)

group (32.04 ± 2.184) was higher than other groups ($p < 0.05$). Furthermore, VEGF-positive cells were quite prominent in the PS-hypoxia group regarding increased angiogenesis (Figure 3b). The quantitive results of immunoreactivity in the SGZ are shown in Figure 3c,d following image analysis.

3.4 | Measurement of angiogenesis

Angiogenesis in the CA1 and CA3 areas was measured on the microphotographs of VEGF immunostaining at ×40 magnification, and results are shown in Figure 4. There was a significant difference between groups both in CA1 ($F_{(3,24)} = 8.510$, $p = 0.001$) and CA3 ($F_{(3,24)} = 4.011$, $p = 0.022$). PS increased VEGF-positive tubular structures in the CA1 of hypoxia group (86.86 ± 8.20) compared with NS-hypoxia (55.01 ± 2.04, $p = 0.039$) and NS-control (47.37 ± 5.85, $p = 0.015$) significantly. There was no significant difference in CA3 of hypoxia groups ($p > 0.05$). However, PS increased angiogenesis in the CA3 of control group (40.71 ± 4.48) compared with NS-control (34.67 ± 0.87, $p = 0.017$) significantly.

3.5 | TBARS, MPO, and CAT levels

The biochemical results revealed that there was a significant difference between groups for TBARS ($F_{(3,20)} = 4.080$, $p = 0.028$) and CAT levels ($F_{(3,20)} = 11.15$, $p = 0.001$). PS increased TBARS levels in the hypoxia group (3.43 ± 0.61) compared with NS-control significantly (1.04 ± 0.30, $p = 0.025$). Furthermore, the highest level of the CAT levels was in NS-control compared with other groups (1.99 ± 0.45, $p = 0.016$) (Figure 5). There was no significant difference between groups for MPO levels ($p > 0.05$).

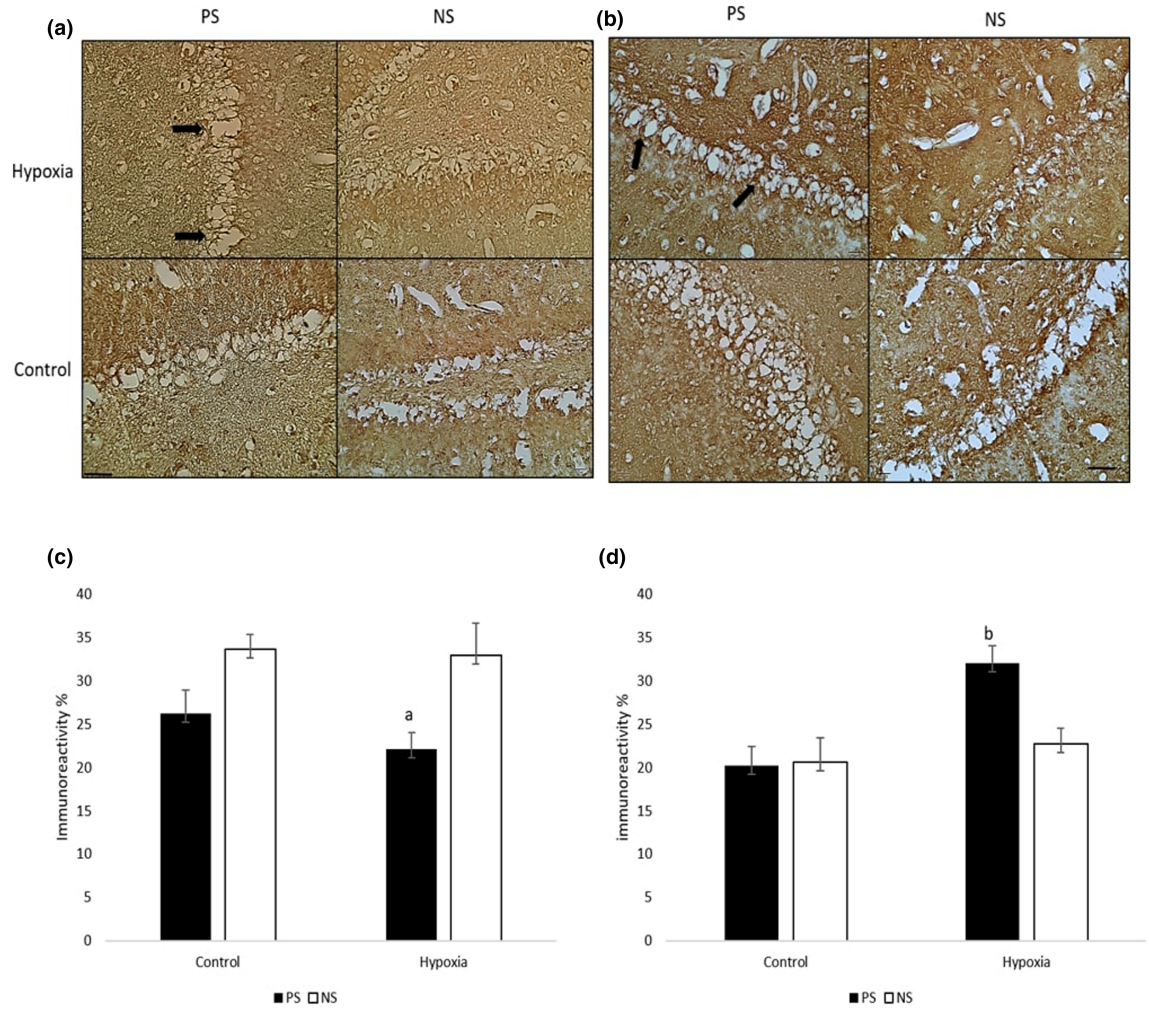

FIGURE 3 Nestin and VEGF expressions in SGZ. The large cavities between nestin-expressing cells were observed in the SGZ of PS-hypoxia as shown by arrows (a). Increased VEGF-positive cells were seen in the PS-hypoxia group (b). PS decreased nestin-positive cells in hypoxia group. pa = 0.05 versus NS-hypoxia (c). PS increased VEGF expression in hypoxia group pb < 0.05 versus other groups (d) (n = 6/each group, scale bar = 20 μm, magnification = ×40)

4 | DISCUSSION

Environmental stress during pregnancy may cause neurodevelopmental and cognitive deficits in later life. In this study, we investigated whether PS exacerbates the damage of neonatal acute hypoxia exposure in the hippocampus of adolescent offspring. Furthermore, we analyzed the novelty-seeking behavior of adolescent rats in each group to understand the behavioral alterations caused by PS and hypoxia exposure.

Rodents exhibit natural curiosity and exploration of novel environments or unknown objects (Douglas et al., 2003; Peters et al., 2007). This complex behavior is related to stress responsiveness, maternal care, age, and anxiety levels (Redolat et al., 2009). Therefore, it involves many pathways including hippocampus, perirhinal, and entorhinal cortex (Barker & Warburton, 2011; Broadbent et al., 2010; Olarte-Sánchez et al., 2015). In our previous study, PS caused neuronal loss in CA1 and decreased novel object recognition in adolescent rats with early acute-pentylenetetrazole-kindling (Çelik et al., 2021). Similarly, the current data showed that PS decreased novelty-seeking behavior correlated positively with synaptophysin immunoreactivity in CA1 (r = 0.538, p < 0.05) and CA3 (r = 0.665, p < 0.05) of hypoxia group (Table 1).

Several studies indicated that adolescent rats in stressful conditions engage in more active, exploratory, risk-taking, and novelty-seeking behaviors (Douglas et al., 2003; Shumake et al., 2005; Toledo & Sandi, 2011). Other studies suggest that PS affects stress responsiveness and does not alter anxiety levels as well as novelty-seeking behavior (Clinton et al., 2008; Pastor et al., 2018). Furthermore, mild hypoxia exposure leads

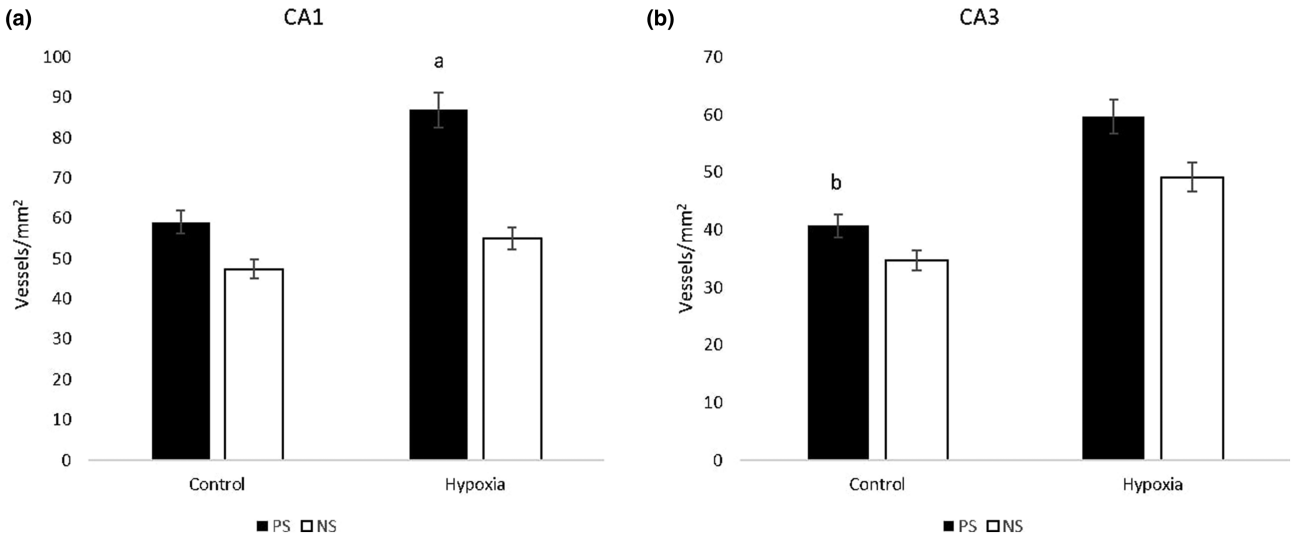

FIGURE 4 Quantitive measurement of angiogenesis. PS increased angiogenesis in CA1 of hypoxia group and CA3 of control group. pa < 0.05 versus NS-hypoxia and NS-control (a). pb < 0.05 versus NS-control (b) ($n = 6$/each group)

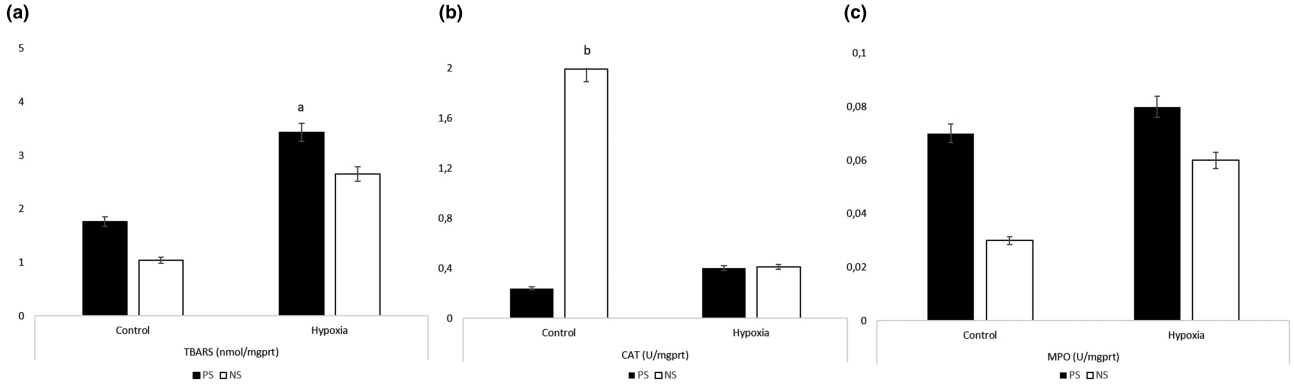

FIGURE 5 Results of biochemical parameters. (TBARS: thiobarbituric acid reactive substances, CAT: catalase, MPO: myeloperoxidase) ($n = 5$/each group) pa < 0.05 versus NS-control, pb < 0.05 versus other groups

to increased risk-taking and novel object recognition (Gozal et al., 2017; Pighin et al., 2020). Contrary, human studies showed the negative effects of acute hypoxia on cognition (McMorris et al., 2017). Our results emphasize the primary role of CA3 region in novelty-seeking behavior linked with the SYN expressions. In this paradigm, disruption of synaptic transmission between CA1 and CA3 led to decreased novelty-seeking in PS-hypoxia group.

CA3 also receives inputs via Mossy fibers from DG formed by SGZ in prenatal and postnatal brain development. Therefore, we also focused on SGZ area of the hippocampus that has a potential contribution to the synaptic network between CA3 and DG. We examined VEGF and nestin-expressing progenitor cells in the SGZ. Immunohistochemical studies demonstrated that PS reduced nestin-expressing precursor cells significantly. Previously, it has been reported that nestin deficiency is associated with object recognition deficits (Wilhelmsson et al., 2019). The positive correlation between nestin and DI ($r = 0.544$, $p < 0.05$) demonstrated that the lack of nestin-expressing cells in the SGZ worsen synaptic transmission and novel object discrimination in the PS-hypoxia group. However, other specific markers are required to detect those progenitor cells in SGZ. Furthermore, we predicted that hypoxia would increase angiogenesis in CA1&CA3 regions associated with the increase of VEGF in SGZ. PS increased VEGF and angiogenesis even more in hypoxia-induced adolescent offspring. Accordingly, previous studies established that acute stress increases neurogenesis associated with VEGF expression and PS has angiogenic effects (Neigh et al., 2017; Uysal et al., 2012). PS also increased angiogenesis in the CA3 region of controls. These results generally refer to the repair and ameliorative functions of VEGF on the damage of PS and hypoxia.

TABLE 1 Correlations between DI and immunohistological parameters

			SYN CA1	SYN CA3	Nestin	VEGF	DI
PS-hypoxia	SYN CA1	Pearson correlation	1	0.680**	0.106	−0.627*	0.538*
		Sig. (two-tailed)		0.004	0.696	0.009	0.032
		N	6	6	6	6	6
	SYN CA3	Pearson correlation	0.680**	1	0.356	−0.378	0.665**
		Sig. (two-tailed)	0.004		0.176	0.149	0.005
		N	6	6	6	6	6
	Nestin	Pearson correlation	0.106	0.356	1	−0.472	0.544*
		Sig. (two-tailed)	0.696	0.176		0.065	0.029
		N	6	6	6	6	6
	VEGF	Pearson correlation	−0.627*	−0.378	−0.472	1	−0.620
		Sig. (two-tailed)	0.009	0.149	0.065		0.010
		N	6	6	6	6	6
	DI	Pearson correlation	0.538*	0.665**	0.544*	−0.620	1
		Sig. (two-tailed)	0.032	0.005	0.029	0.010	
		N	6	6	6	6	6

*Correlation is significant at the 0.05 level (two-tailed).
**Correlation is significant at the 0.01 level (two-tailed).

TBARS, CAT, and MPO levels were investigated to comprehend the effects of PS and hypoxia on hippocampal oxidant and antioxidant balance. It has been reported that both PS and hypoxia increase TBARS, which is an end-product of lipid peroxidation (Coimbra-Costa et al., 2017; Qulu et al., 2016). Another study revealed that hippocampal TBARS level was decreased over time after hypoxia exposure (Vetrovoy et al., 2017). In our previous study, we found that PS increases TBARS and MPO levels in adolescent rats with early pentylenetetrazole-kindling (Çelik et al., 2021). Similarly, these results showed that PS increases TBARS levels in the hypoxia group compared to NS-hypoxia. Pimentel and colleagues found that hypoxia-ischemia increased MPO levels in hippocampus regarding inflammation (Pimentel et al., 2011). However, our results demonstrated no significance for MPO levels in the hippocampus, neither in PS nor in hypoxia groups Furthermore, several studies revealed that PS increases CAT activity in primary cortical neurons (Luft et al., 2021) and hippocampus (Fatima et al., 2019). Contrary, Ahlbom and colleagues indicated that CAT activity was decreased both in neonatal and adolescent offspring (Ahlbom et al., 2000). In addition to these controversial results, our results showed no difference between groups despite decreased CAT levels. Consequently, increased lipid peroxidation and insignificant CAT levels suggest that PS causes developmental disruptions in the hippocampus by changing the oxidant and antioxidant balance.

5 | CONCLUSION

This study demonstrated that acute hypoxia exposure following PS impacted hippocampus development and novelty behavior of adolescent offspring. Oxidative stress markers and angiogenesis were increased, and synaptic transmission in CA1 and CA3 and neural progenitors in SGZ were disrupted. Therefore, this study provides multidisciplinary and unique evidence for the profound effects of PS and early acute hypoxia on hippocampus development. Nevertheless, investigating gender differences, underlying mechanisms of PS and hypoxia in the adult hippocampus, as well as other regions of the brain, are limitations of this study.

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AUTHOR CONTRIBUTIONS

KÇ: Conceptualization and design, methodology, validation, data analysis, investigation, and writing, editing, and revising the original draft. PB: Conceptualization, investigation, data curation, and review and editing. GG:

Investigation, data curation, and review and editing. BD: Investigation, data curation, and review and editing. EYS: Methodology, validation, writing, review and editing, and supervision. MB: Conceptualization and design, methodology, validation, investigation, writing and editing the original draft, supervision, and project management.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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