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Effects of carbon dioxide exposure on early brain development in rats

M Kiray[1], AR Sisman[2], UM Camsari[3], M Evren[4], A Dayi[1], B Baykara[5], I Aksu[1], M Ates[6], Nazan Uysal[1]

[1]*Dokuz Eylul University, School of Medicine, Department of Physiology, Division of Behavioral Physiology,* [2]*Dokuz Eylul University, School of Medicine, Department of Biochemistry, Izmir,Turkey,* [3]*Mayo Clinic, Department of Neuroscience, Jacksonville, Florida,* [4]*Ege University, Natural and Applied Sciences, Department of Biotechnology, Bornova, Izmir,* [5]*Dokuz Eylul University, College of Physical Therapy and Rehabilitation, and* [6]*Dokuz Eylul University, School of Medicine, Vocational School of Health Services, Pharmacology, Balcova, Izmir, Turkey*

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Abstract

The developing brain is vulnerable to environmental factors. We investigated the effects of air that contained 0.05, 0.1 and 0.3% CO_2 on the hippocampus, prefrontal cortex (PFC) and amygdala. We focused on the circuitry involved in the neurobiology of anxiety, spatial learning, memory, and on insulin-like growth factor-1 (IGF-1), which is known to play a role in early brain development in rats. Spatial learning and memory were impaired by exposure to 0.3% CO_2 air, while exposure to 0.1 and 0.3% CO_2 air elevated blood corticosterone levels, intensified anxiety behavior, increased superoxide dismutase (SOD) enzyme activity and MDA levels in hippocampus and PFC; glutathione peroxidase (GPx) enzyme activity decreased in the PFC with no associated change in the hippocampus. IGF-1 levels were decreased in the blood, PFC and hippocampus by exposure to both 0.1 and 0.3% CO_2. In addition, apoptosis was increased, while cell numbers were decreased in the CA1 regions of hippocampus and PFC after 0.3% CO_2 air exposure in adolescent rats. A positive correlation was found between the blood IGF-1 level and apoptosis in the PFC. We found that chronic exposure to 0.3% CO_2 air decreased IGF-1 levels in the serum, hippocampus and PFC, and increased oxidative stress. These findings were associated with increased anxiety behavior, and impaired memory and learning.

Key words: air quality, amygdala, carbon dioxide, hippocampus, insulin-like growth factor-1, IGF-1, learning, memory, prefrontal cortex

Carbon dioxide (CO_2) is a normal component of air at a concentration of 0.03%. It is an end product of metabolism of all living organisms and humans are the main source of CO_2 in indoor environments. It is known that poor indoor air quality has adverse effects on health (American Society of Heating Refrigerating and Air-Conditioning Engineers (ASHRAE) 2004, Stellman 1998, Center for Disease Control and Prevention (CDCP) 2011). According to the World Health Organization, 85% of the world's population lives in the cities of industrialized countries and inhabitants of cities spend 90% of their time indoors (WHO 2009).

The CO_2 level of inhaled air often is used as an indicator of indoor air quality. ASHRAE recommends that the CO_2 level be below 0.07% in indoor environments (ASHRAE 2004, Stellman 1998, CDCP 2011). According to ASHRAE, the upper limit of CO_2 is 0.1% in indoor environments. Acceptable upper limits for indoor CO_2 concentration vary. According to United Kingdom standards, indoor CO_2 levels should not exceed 0.15% (ASHRAE 2004, CDCP 2011).

Brain development begins *in utero* and continues until the end of adolescence in mammals

Correspondence: Nazan Uysal, Dokuz Eylul University, School of Medicine, Department of Physiology, Division of Behavioral Physiology, Izmir, Turkey. Tel: +90-232-4124460, Fax: +90-232-4124459, E-mail: nazan.uysal@deu.edu.tr

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(Rice and Barone 2000). The period of most rapid brain growth occurs during the first two postnatal weeks in rats and during the first three years of life in humans (Felderhoff-Mueser et al. 2004). During development, the brain is vulnerable to both external and internal factors. For example, maternal dietary deprivation during development can affect the fetal brain adversely, and exercise and ample food can affect it positively (Uysal et al. 2005a,b, 2011). It is possible that children especially could be affected adversely by air pollution. In our earlier study, central nervous system complaints were reported mainly by adolescents, while eye-related complaints were reported mainly by adults (Acikgoz et al. 2013).

Insulin-like growth factor-1 (IGF-1) is a neuroprotective growth factor that plays a role in brain development (Rubovitch et al. 2010). It has been shown that IGF-1 is correlated positively with reduced anxiety and enhanced cognitive functions (Aleman and Torres-Aleman 2009, Llorens-Martin et al. 2010, Mitschelen et al. 2011, Aksu et al. 2012). IGF-1 is produced by many organs, but liver produces approximately 70% of the total circulating IGF-1 (Torres-Aleman 2010). Circulating IGF-1 crosses the brain-blood barrier and regulates hippocampal IGF-1 levels, which affects behavior (Trejo et al. 2007, Yan et al. 2011). We have found that exposure to poor air quality decreased serum IGF-1 levels and that voluntary exercise did not modify this state in adolescent mice (Uysal et al. 2013).

We report here our investigation of the effects of air quality on the levels of IGF-1 in the blood, hippocampus, prefrontal cortex (PFC) and amygdala, and the concomitant effects on anxiety behavior, spatial learning and memory.

Materials and methods

Animals

All experiments were performed in accordance with the guidelines and with the approval of the Animal Care and Use Committee of the Dokuz Eylul University School of Medicine. Four-month-old male and female Wistar albino rats were obtained from our Experimental Animal Laboratory. The animals were maintained with a 12 h light/dark cycle (lights on at 07:00 h) and food and water were available *ad libitum*. Experiments were carried out between 9:00 and 11:00 AM each day.

The estrous cycle was monitored by vaginal smears to ensure pregnancy and female rats were placed in the air quality apparatus the day after mating. CO_2 concentration below 0.065 % is accepted as normal for indoor environments. We use one within normal levels of CO_2 (0.05% CO_2) and two high levels of CO_2 levels (0.1 and 0.3% CO_2) the latter are above the normal limit defined by ASHRAE) (ASHRAE 2004). The pregnant rats were segregated into three groups of eight animals: 1) exposed to 0.05% CO_2 atmosphere, 2) exposed to 0.1% CO_2 atmosphere, and 3) exposed to 0.3% CO_2 atmosphere (Fig. 1). All groups included both males and females. The birth date of each animal was defined as postnatal day 0. Appropriate CO_2 levels were maintained

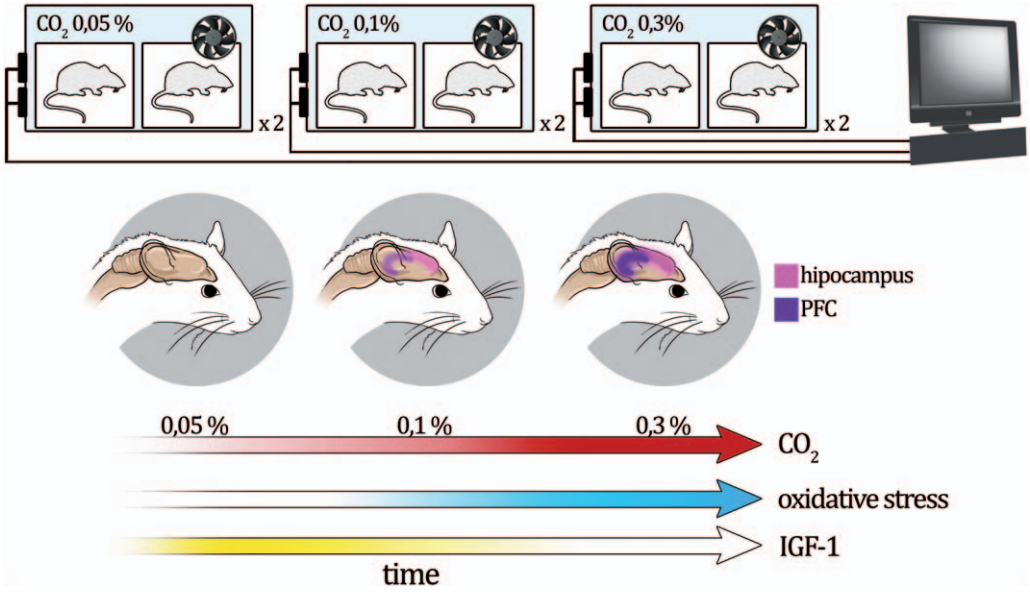

Fig. 1. Schematic diagram of experiment.

for each group until they reached 38 days old. When the animals reached 38 days old, behavior, learning and memory tests were conducted and assessed using the Noldus Ethovision XT video tracking system. After the behavioral experiments, blood samples were drawn by cardiac puncture under light ether anesthesia, then animals were sacrificed and the PFC and hippocampal regions were separated after removing the brain. The thymus and adrenal glands also were removed to assess tissue atrophy and hyperplasia, respectively.

Air quality application apparatus

All animal cages were placed in an apparatus that controlled the air flow and maintained the CO_2 concentration at the desired level. The exhaled air of the animals was the source of carbon dioxide within the apparatus (Dirman, Antalya, Turkey) (Fig. 1).

Behavioral tests

Open field test

Anxiety behavior was assessed using the open field test monitored by a video camera installed 2.5 m above the apparatus. The apparatus is an open box 1 m^2 and 50 cm high. Rats were placed individually in the center of the open field and ambulation was measured for 5 min in a soundproof observation room illuminated by controlled light (100 lx). The analysis software divided the field into 16 squares comprising 12 peripheral squares and four central squares. All activity was recorded for 5 min. The time spent in the center was calculated, because rats spontaneously prefer the peripheral regions of the open field rather than the center. Time spent in central region was interpreted as an indicator of low anxiety, which is consistent with previous studies (Casarrubea et al. 2013, Aksu et al. 2012, Baykara et al. 2013). The ratio of the total exploratory time to the used cell percentage was measured for each animal and used to calculate an index of relieved anxiety (anxiolysis).

Elevated plus maze test

The elevated plus maze test was used to evaluate anxiety state. The elevated plus maze consists of a central 5×5 cm platform with two open arms, each 50 cm long, 10 cm wide with 0.5 cm high borders, and two closed arms with the same dimensions as the open arms, but with walls 40 cm high. All surfaces are elevated 50 cm above the floor of the apparatus. Each rat was placed individually on the platform facing the open arm and behavior was monitored for 5 min using the overhead camera The number of entries into the open and closed arms were counted and the total time spent in either the open or closed arms was measured. Time spent in the closed arms was interpreted as an indication of increased anxiety (Casarrubea et al. 2013).

Morris water maze (MWM) test

After completing the elevated plus maze test, all rats were subjected to the MWM test. The MWM was 140 cm in diameter and 75 cm high. The water level in the tank was 50 cm deep and 1.0 cm above the height of the escape platform that was hidden by the water. The experiment was conducted for each animal individually for four consecutive days (total 20 trials). Rats were placed in the water, permitted to locate the platform within 60 sec, then left on the platform for 20 sec before removing them from the tank. A different initiating point (north, south, east, west) was chosen for different days of the experiment. For the level of learning, we measured the time required to find the platform, swimming speed and total swimming distance over a 4 day period for each animal. To assess memory, we conducted a probe trial test on the 5th day. During the probe trial, the platform was removed, the test was conducted for 60 sec, and we measured the total time spent in the quadrant where the original platform was located and the total time spent in the quadrant opposite the original platform. Swimming behavior within 15 cm of the pool wall during the probe trial was defined as thigmotaxis, the tendency to remain close to walls, which is widely interpreted as an index of anxiety behavior in rats or mice (Simon et al. 1994, Huang et al. 2012, Dayi et al. 2012).

Biochemical analysis

Serum corticosterone was measured by a radioimmunoassay using a double antibody kit (ImmuChem, MP Biomedicals, Orangeburg, NY). PFC and hippocampus tissues were homogenized to measure the levels of IGF-1, malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activities. Aliquots of the homogenates were stored at −85° C until measured. All measurements were completed within 10 days. MDA was measured using the Bioxytech MDA-586 (Oxis International, Portland, OR) assay kit. SOD and GPx enzyme activities were measured spectrophotometrically using RANSOD (Randox Labs, Crumlin, UK) and Bioxytech GPx-340 kits (Oxis International, Portland, OR) adapted to the

autoanalyzer (Abbott Architect C16000, Park, IL). Results were expressed as U/mg protein. The protein content was determined using a total protein kit (Randox Labs).

IGF-1 levels in the serum and in the PFC and hippocampus homogenates were measured using commercially available ELISA kits specific for rat (Boster Immunoleader, Wuhan, China) according to the manufacturer's instructions; assay sensitivity was <5 pg/ml with a range of 62.5–4000 pg/ml.

Histological investigation

Blocks of PFC and hippocampus tissues were fixed in 10% formalin in phosphate buffer for 24 h. The PFC and hippocampus were sectioned coronally into serial 5 μm sections using a microtome (Thermo Finesse ME+, ThermoFisher Scientific, Inc., Waltham, MA). Neuron density was measured by taking three coronal sections through the PFC that corresponded approximately to plates 9 and 11, and hippocampus that corresponded approximately to plates 21, 23, 25 in the rat atlas of Paxinos and Watson (1982).

All sections were stained with cresyl violet. Sections were deparaffinized and hydrated after which they were immersed in cresyl violet solution for 20 min (21948, ScyTek, West Logan, UT). Sections then were dipped in 96% ethanol followed by treatment with xylene for 20 min and mounted in Entellan. The images were analyzed using a computer-assisted image analyzer consisting of a microscope (Olympus CX-41; Tokyo, Japan) equipped with a high resolution video camera (Olympus DP25). The number of neurons in each region was counted at 40 X magnification using a 16800 μm^2 counting frame. The counting frame was placed randomly three times on the image analyzer system monitor, the numbers of neurons were counted and the average PFC and hippocampus neuron densities were calculated.

To detect DNA fragmentation in the nuclei, the terminal deoxynucleotidyl-transferase-mediated dUTP-nick end-labeling (TUNEL) reaction was applied to the paraffin sections using an apoptosis kit according to the manufacturer's instructions (Roche, Mannheim, Germany).

Immunohistochemical staining was performed using the streptavidin/biotin method (85-9043, Invitrogen, Camarillo, CA). The immunohistochemistry procedure for IGF-1 (1/100, bs-0081R; Bioss, Woburn, MA) was performed according to the manufacturer's instructions. Three sections were used for immunohistochemical scoring of each sample by a blinded investigator. The intensity of staining for IGF was assessed using a subjective scale: (0) none; (+) mild; (++) moderate; and (+++) strong labeling.

Statistical analysis

Differences in the behavioral and biochemical parameters among the groups were analyzed using the one-way ANOVA test. Post hoc comparisons were done using the Bonferroni test. Differences between each learning day in the MWM were analyzed using the GLM-repeated measure. Correlations among groups were calculated using Pearson correlation analysis. Results are presented as mean $\pm$ SEM; $p \leq 0.05$ was considered significant.

Results

Open field test

In the open field test, the total movment time was reduced in the 0.3% CO_2 atmosphere group compared to the other groups; these rats also were significantly less active in the open field test ($F_{2-22} = 11.99$, $p < 0.001$) (Fig. 2A).

Elevated plus maze test

The total number of entries into the center cells were reduced in the group that had been exposed to a 0.3% CO_2 atmosphere compared to the group exposed to 0.05% CO_2 atmosphere ($F_{2-22} = 4.61$, $p < 0.05$) (Fig. 2B). The groups exposed to 0.3% CO_2 and 0.1% CO_2 atmospheres spent more time in the closed branches of the elevated plus maze device ($F_{2-22} = 12.46$, $p < 0.0001$ for both) (Fig. 2C). The 0.3% CO_2 and 0.1% CO_2 atmosphere groups also spent less time in the open arms; their anxiety measurements were greater than for the 0.05 % CO_2 atmosphere group ($F_{2-22} = 12.90$, $p < 0.0001$ for both) (Fig. 2D).

Morris water maze test

We found also that spatial learning and memory were impaired in the 0.3% CO_2 atmosphere group. For the 0.1% and 0.05% CO_2 atmosphere groups, the mean latency for locating the MWM platform declined progressively over the 4 days of the experiment. Furthermore, rats in the 0.3% CO_2 atmosphere group were unable to locate the platform at all. The 0.3% CO_2 atmosphere group showed longer escape latencies than the other groups on the first, third

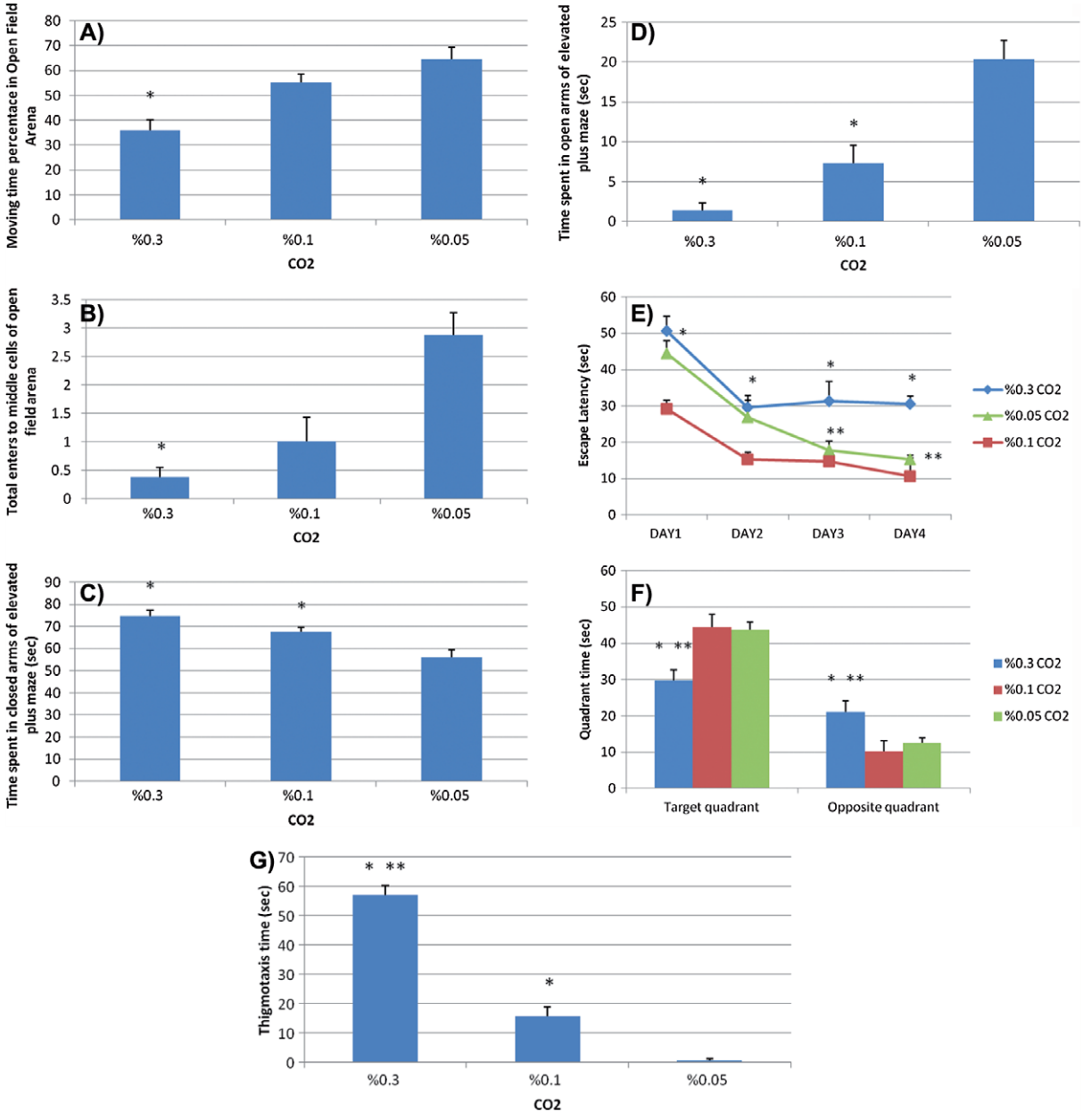

Fig. 2. Behavioral results. A) Percentage of total movement time during open field test. B) Number of open field arena entries (middle cell). C) Total number of closed arm entries in elevated plus maze. D) Total number of open arm entries in elevated plus maze. E) Daily change of escape latency in MWM test, F) Time spent in target and opposite quadrant during MWM probe trial G) Duration of thigmotaxis in MWM probe trial. *$p < 0.05$ compared to 0.05% CO_2 atmosphere group. **$p < 0.05$ compared to 0.1% CO_2 atmosphere group.

and fourth days of training (first day, $F_{2-25} = 10.55$, $p < 0.001$; third day, $F_{2-25} = 5.91$, $p < 0.009$; fourth day, $F_{2-25} = 10.89$, $p < 0.001$). Rats in the 0.1% CO_2 atmosphere group showed shorter escape latencies on the third and fourth training days than rats in the 0.3% CO_2 atmosphere group ($p < 0.05$) (Fig. 2E). In probe trials (quadrant times), time spent in the target and opposite quadrants was used to evaluate long-term memory. Rats in the 0.3% CO_2 atmosphere group spent significantly less time in the target quadrant ($F_{2-24} = 3.51$, $p < 0.05$) and more time in the opposite quadrant than other groups ($F_{2-24} = 3.83$, $p < 0.05$ for all) (Fig. 2F). We found also that for both the 0.3% CO_2 and 0.1% CO_2 atmosphere groups, the duration of thigmotactic behavior was significantly longer than for the 0.05% CO_2 group ($F_{2-22} = 84.77$, $p < 0.0001$ for both). In addition, the duration of thigmotaxis of rats in the 0.3 % CO_2

atmosphere group was longer than for the 0.1% CO_2 atmosphere group ($p < 0.001$) (Fig. 2G).

Biochemical analysis

Tissue analysis of the 0.3% CO_2 and 0.1 % CO_2 atmosphere groups revealed decreased hippocampus and PFC volumes, and blood IGF-1 levels compared to the 0.05% CO_2 atmosphere group (hippocampus IGF-1, $F_{2-18} = 23.59$, $p < 0.0001$; PFC IGF-1, $F_{2-18} = 8.85$, $p < 0.05$; blood IGF-1, $F_{2-19} = 21.77$, $p < 0.0001$) (Fig.4A). Immunohistochemical evaluation based on the intensity of IGF-1 immunoreactivity in the PFC and hippocampus is given in Tables 2 and 3. IGF-1 reactivity was reduced in the PFC, hippocampus and amygdala of the 0.3% CO_2 atmosphere group compared to the 0.05% CO_2 atmosphere group (Fig. 3A, B, C).

While SOD enzyme activity in the PFC was increased in the 0.3% CO_2 atmosphere group, it was decreased in the 0.1% CO_2 atmosphere group ($F_{2-20} = 21.54$, $p < 0.0001$). Enzyme activity in the hippocampus SOD was greater than in the 0.3% CO_2 atmosphere group ($F_{2-20} = 4.88$, $p < 0.05$). GPx enzyme activity in the PFC was decreased in the 0.3% CO_2 atmosphere group compared to the 0.05% CO_2 group ($F_{2-20} = 5.44$, $p < 0.05$). GPx enzyme activity in the hippocampus was greater in both the 0.3% CO_2 and 0.1% CO_2 atmosphere groups than in the 0.05% CO_2 atmosphere group ($F_{2-20} = 12.55$, $p < 0.003$ for both). PFC and hippocampus MDA levels were elevated in the 0.3% CO_2 atmosphere group (PFC, $F_{2-20} = 10.13$, $p < 0.001$; hippocampus, $F_{2-20} = 9.34$, $p < 0.002$) (Table 1).

The resting blood corticosterone level was higher in the 0.3% CO_2 and 0.1% CO_2 atmosphere group than in the 0.05% CO_2 group ($F_{2-20} = 7.00$, $p < 0.007$) (Fig. 5A). The ratio of adrenal weight:body weight was increased and body weight was decreased in the 0.3% CO_2 and 0.1% CO_2 atmosphere groups compared to the 0.05% CO_2 group (adrenal weight, $F_{2-20} = 16.87$, $p < 0.0001$ for both; body weight, $F_{2-20} = 37.06$, $p < 0.0001$ for both) (Fig. 5B, C). The ratio of thymus weight:body weight was decreased in the 0.3% CO_2 atmosphere group compared to the 0.05% CO_2 atmosphere group ($F_{2-20} = 4.30$, $p < 0.05$) (Fig. 5C).

Histological investigation

Cresyl violet staining revealed that most of the neurons were shrunken with darkly stained pycnotic nuclei in the amygdala, PFC and hippocampus of the 0.3% CO_2 atmosphere group. The density of neurons was significantly decreased in the PFC and hippocampus of 0.3% CO_2 atmosphere group compared to the 0.05% CO_2 atmosphere group ($F_{2-20} = 6.19$, $p < 0.05$) (Fig. 3A, B, C; Tables 2, 3,4).

TUNEL staining showed fewer TUNEL positive cells in the PFC, amygdale and hippocampus of 0.05% CO_2 and 0.1% CO_2 atmosphere groups than in the 0.3% CO_2 atmosphere group. Quantification and statistical analysis of TUNEL staining showed that the number of TUNEL positive cells was increased significantly in the 0.3% CO_2 atmosphere group compared to the 0.05% CO_2 and 0.1% CO_2 atmosphere groups (hippocampus, $F_{2-20} = 60.09$, $p < 0.0001$; PFC, $F_{2-20} = 47.75$, $p < 0.0001$; amygdala, $F_{2-20} = 58.24$, $p < 0.002$) (Fig. 3A, B, C; Tables 2, 3, 4).

Correlation analysis

We found a strongly negative correlation between blood IGF-1 levels and percentages of TUNEL positive neurons in the PFC, hippocampus, blood IGF-1 levels and TUNEL positive neurons in the CA1 region of the hippocampus ($n = 24$, $r = -0.765$, $p < 0.001$). Also we found a strongly negative correlation between blood IGF-1 levels and TUNEL positive neurons in the PFC ($n = 24$, $r = -0.744$, $p < 0.002$) (Fig. 4B). In addition, we found a strongly negative correlation between the TUNEL positive neuron percentage in the PFC and time spent in the open arms of the elevated plus maze ($n = 24$, $r = -0,796$, $p < 0.001$). There was a negative correlation between time spent in the open arms of the elevated plus maze and blood corticosterone levels ($r = -0.495$, $p < 0.05$). There was a positive correlation between duration of thigmotaxis and blood corticosterone levels ($r = 0.515$, $p < 0.05$), and a positive correlation between TUNEL positive cells of the PFC and blood corticosterone levels ($r = 0.540$, $p < 0.05$).

Discussion

We found that the 0.1 and 0.3% CO_2 animals spent more time in the closed branches in the elevated plus maze test and entered the center cells of open-field-arena less often than the 0.05% CO_2 animals (Fig. 2B and C). We suggest that exposure to a high CO_2 atmosphere contributed to increased anxiety behavior in rats. We also found a positive correlation between spending more time in closed branches in the elevated-plus-maze test and entering less often into the center cells of the open-field-arena, and elevated plasma corticosterone levels. Our findings are consistent with previous studies that showed a positive correlation between anxiety and elevated

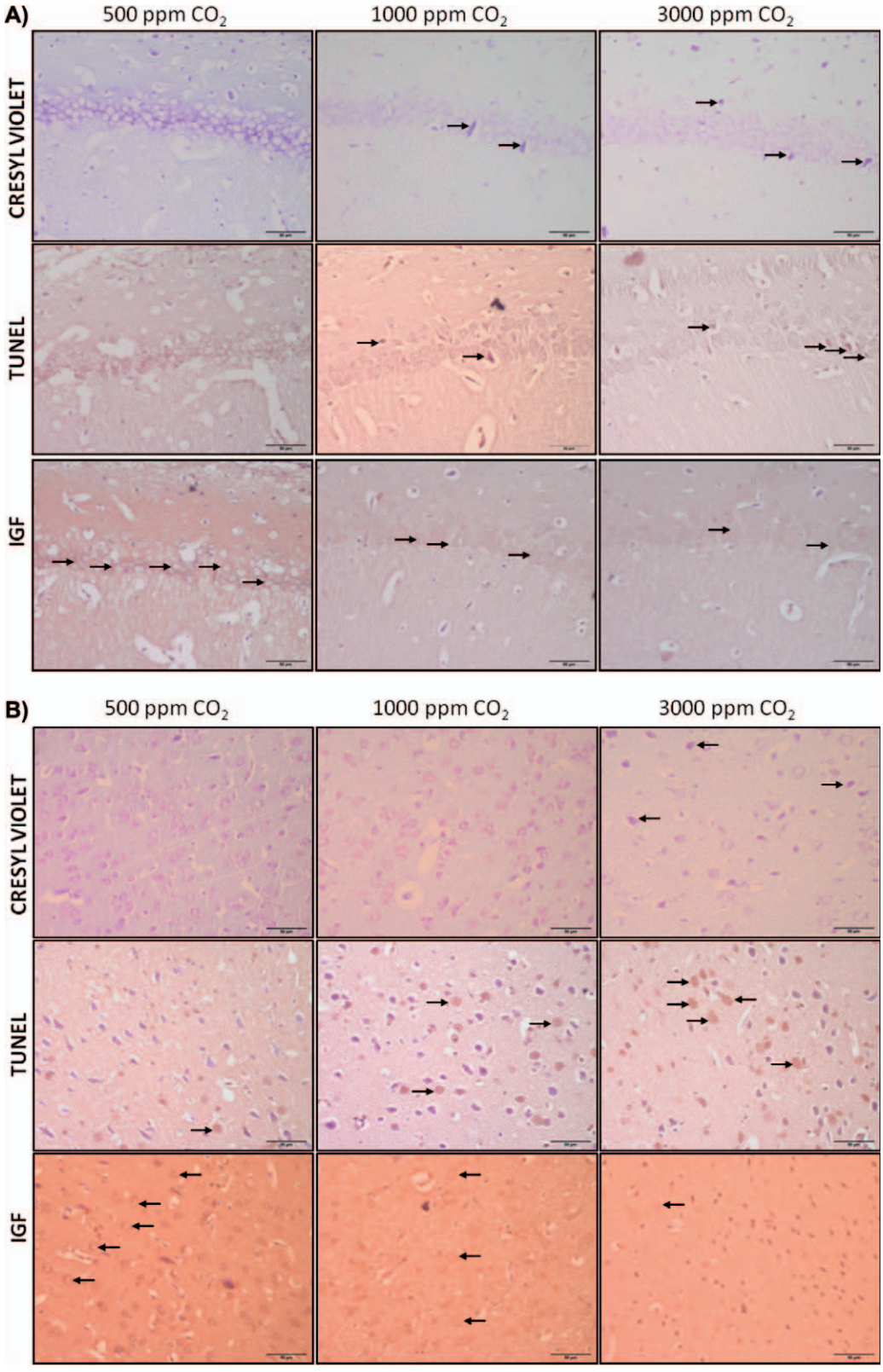

Fig. 3. Light microscopy images of rat brain hippocampal and PFC sections. A) Representative photomicrographs of TUNEL and IGF-1 positive neurons of hippocampal CA1 region. B) Representative photomicrographs of TUNEL and IGF-1 positive neurons of the PFC. C) Representative photomicrographs of TUNEL and IGF-1 positive neurons of the amygdala.

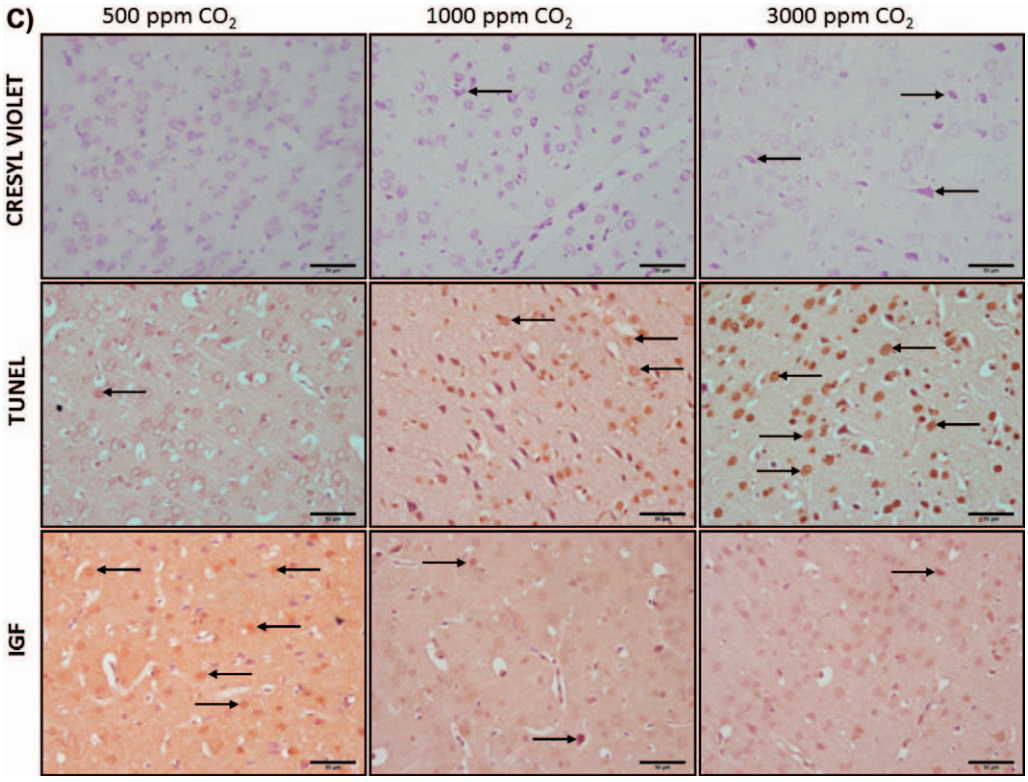

Fig. 3. (Continued)

serum corticosterone. Rats that were exposed to the 0.3% CO_2 atmosphere demonstrated impaired performance in learning and memory. The 0.1% CO_2 atmosphere group showed less learning impairment and no associated memory deficit, which suggests a dose-dependent effect of CO_2. Exposure to 0.1 and 0.3 % CO_2 levels of CO_2 contributed to decreased hippocampal and PFC neuron numbers, and blood IGF-1 levels. Our findings suggest further that exposure to high levels of CO_2 could play a role in the etiology of the anxiety state, and impaired learning and memory, all of which may be caused by structural changes in the brain.

IGF-1 plays an important role in brain development and survival (Aleman and Tores-Aleman 2009). Our earlier studies showed that decreased IGF-1 levels in the PFC and hippocampus were associated with impaired learning, memory and increased anxiety states (Aksu et al. 2012, Ozdemir et al. 2012). Many organs produce IGF-1 including brain and muscle tissue; liver produces 70% of the total circulating IGF-1 (Torres-Aleman 2010). IGF-1 crosses the blood brain-barrier so brain is affected by the concentration of circulating IGF-1 (Carro et al. 2000). Circulating IGF-1 regulates hippocampal IGF-1 levels and brain IGF-1 gene expression in adolescent rats (Yan et al. 2011). Reduced circulating IGF-1 may be an important factor in the development of cognitive dysfunction in adults and adolescents (Aleman and Tores-Aleman 2009, Aksu et al. 2012, Ozdemir et al. 2012, Baykara et al. 2013). Recently, we have shown that exposure to a 0.3% CO_2 atmosphere in adolescent mice contributed to decreased blood levels of IGF-1 (Uysal et al. 2013). Our current study is consistent with our earlier finding, because exposure to 0.3% CO_2 atmosphere caused reduced IGF-1 levels in blood and brain tissues.

IGF-1 exerts a neuroprotective effect and prevents apoptosis (Aksu et al. 2012, Wine et al. 2009). We found a negative correlation between blood IGF-1 levels and the percentage of TUNEL positive neurons in the PFC and hippocampus. Apoptotic activity was increased in mouse pup brains when exposed to an 8% CO_2 atmosphere between postnatal days two and seven (Das et al. 2009). We found that chronic exposure to both 0.3% CO_2 and 0.1% CO_2 atmospheres promoted apoptotic activity in all regions of the hippocampus and PFC.

Environmental factors can affect both brain cell proliferation and death. Exposure to 0.3% CO_2 atmosphere caused a significant reduction in neuron density in the CA1 region of the hippocampus and PFC. Spatial learning depends on neurobiological circuitry that involves the hippocampus, PFC, entorhinal cortex, parietal cortex,

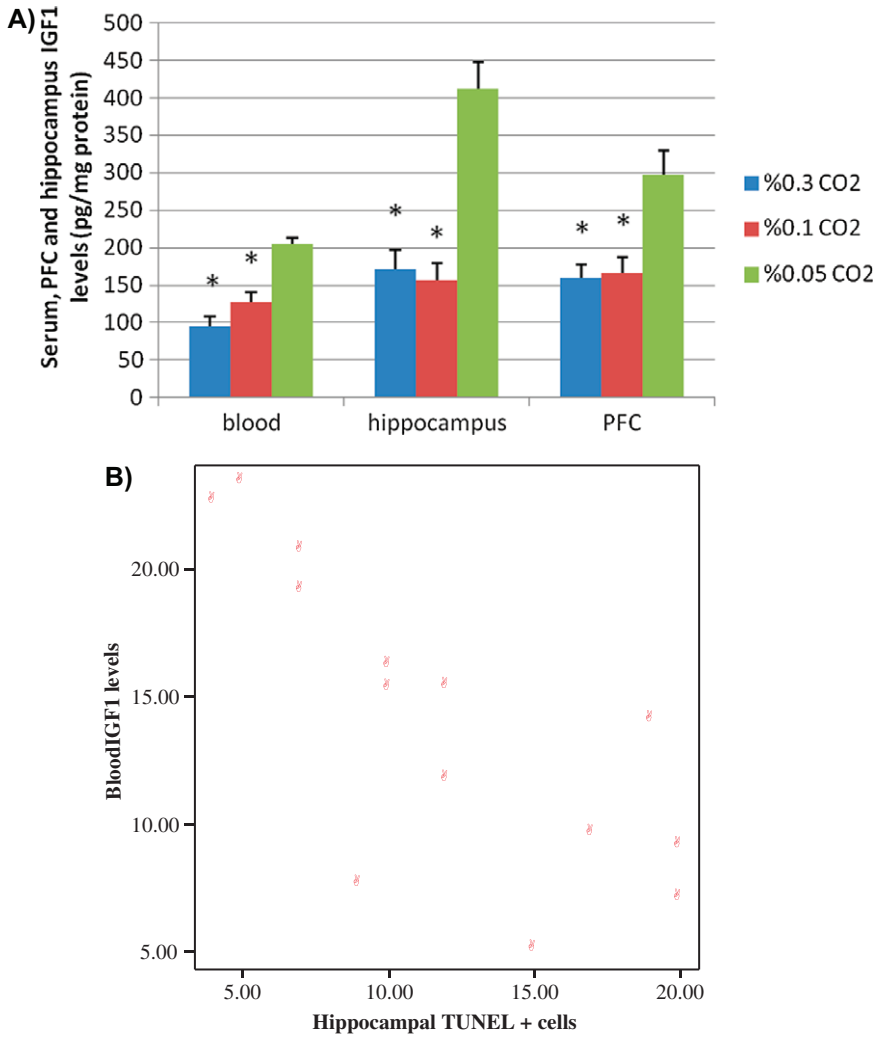

Fig. 4. IGF-1 levels. A) Blood, hippocampus and PFC IGF-1 levels. B) Correlation between blood IGF-1 levels and hippocampal TUNEL positive cells; *$p < 0.05$ compared to the 0.05% CO_2 atmosphere group.

anterior cingulate cortex, striatum and amygdala (Frankland et al. 2004). We found that exposure to a 0.3% CO_2 atmosphere impaired learning and memory in rats.

The amygdala and PFC are involved in the neurocircuitry of fear and anxiety states and the PFC is known to regulate and control the output of the amygdala (Quirk et al. 2006). We showed earlier that decreased neuron numbers and IGF-1 in the PFC were correlated with an increased anxiety state (Aksu et al. 2012). In addition, increased PFC apoptosis was correlated negatively with increased

Table 1. The effects of CO_2 levels on SOD and GPx enzyme activities and MDA levels in rat PFC and hippocampus

	PFC			Hippocampus		
	SOD (U/mg protein)	GPx (U/mg protein)	MDA (nmol/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	MDA (nmol/mg protein)
0.3% CO_2	$5.1 \pm 0.2^{a,b}$	0.012 ± 0.003^{a}	$34.3 \pm 5.3^{a,b}$	3.1 ± 0.3^{a}	0.07 ± 0.004^{a}	53.5 ± 9.8^{a}
0.1% CO_2	3.8 ± 0.4^{a}	0.022 ± 0.002	18.5 ± 1.7^{a}	2.3 ± 0.5^{a}	0.07 ± 0.003^{a}	29.9 ± 5.6^{a}
0.05% CO_2	4.3 ± 0.2	0.025 ± 0.004	12.1 ± 2.8	1.5 ± 0.2	0.05 ± 0.002	15.1 ± 2.7

N = 8 for all groups. Data are means ± SEM. $^{a}p < 0.05$ compared to the 0.05% CO_2 atmosphere group, and $^{b}p < 0.05$ compared to 0.1% CO_2 atmosphere group. SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde.

Table 2. TUNEL and IGF-1 positive cells in hippocampus

	Neuron number CA1	GD	TUNEL positive cell	IGF-1 positive cell
0.3% CO_2	27.2 ± 1.8^a	86.6 ± 2.6	$18.2 \pm 0.9^{a,b}$	1.6 ± 0.2^a
0.1% CO_2	29.2 ± 0.6	95.4 ± 2.6	10.6 ± 0.6^a	2.2 ± 0.2
0.05% CO_2	34.3 ± 1.7	91.8 ± 2.3	6.2 ± 0.7	2.6 ± 0.2

N = 8 for all group. Data are means ± SEM. $^a p < 0.05$ compared to the 0.05% and $^b p < 0.05$ compared to 0.1% CO_2 atmosphere group.

activity in the open arms of the elevated plus maze. The anxiety state is correlated positively with direct inhalation of high levels of CO_2 , which can trigger a panic attack by activating the hypothalamus-pituitary-adrenal (HPA) axis (Pine et al. 1998, Gorman et al. 2001). Inhaling air that contained 5–7% CO_2 for 15–20 min, or 13% CO_2 once using full vital capacity, is sufficient to cause anxiety (Gorman et al. 1988). We found that time spent in the open arms of the elevated plus maze were decreased and the duration of thigmotactic behavior was increased in the MWM, both of which are associated with increased anxiety behavior. CO_2 induced anxiety is thought to be modulated by GABAergic system (Bailey and Nutt 2008). Furthermore, GABA administration has been shown to decrease serum IGF-1 levels (Thanapreedawat et al. 2013).

We have shown that exposure to high CO_2 levels causes hyperactivity of the HPA axis. The plasma corticosterone level was increased by increased CO_2 exposure. It has been shown earlier that high CO_2 levels can activate the HPA axis by up to 35%, which in turn causes release of ACTH and cortisol (Argyropoulos et al. 2002, Hackbarth et al. 2000, Vahl et al. 2005). Chronic exposure to high CO_2 levels may cause excess glucocorticoid receptor activation in rodents that could be secondary to increased activity in the glucocorticoid feedback areas of the brain (Revsin et al. 2009). Hyperactivity of the HPA axis could be due to increased activity of the hypothalamic neurons that secrete corticotrophin releasing hormone; this is the current explanation for increased ACTH release from pituitary corticotrophic neurons and alterations in adrenal sensitivity (Revsin et al. 2009, Chan et al. 2002). Activation of the HPA axis contributes to thymic involution, hypertrophy of the adrenal gland and decreased overall body weight in mice (Tramullas et al. 2012). Our findings are consistent with earlier reports that exposure to high levels of CO_2 may cause adrenal hypertrophy as a result of activation of the HPA axis.

CO_2 plays important roles in various physiological reactions such as acid-base reactions. Inhalation of high levels of CO_2 may be a stressor for humans as a result of increased oxidative reactivity. CO_2 interacts with both reactive nitrogen species and ROS. Consequently, CO_2 exposure, depending on its duration and intensity, could either prevent or cause oxidative stress (Vesela and Wilhelm 2002). It has been shown that during endotoxin induced lung injury, membrane lipid injury was prevented by 20 min exposure to 0.5% CO_2 atmosphere, which reduced oxidative reactions (Nichol et al. 2010). We found that PFC and hippocampus MDA levels were increased in both the 0.3% CO_2 and 0.1% CO_2 atmosphere groups.

In a healthy body, ROS and antioxidants are in equilibrium. We found that antioxidant enzyme activity was not correlated with MDA levels, which is an indicator of lipid oxidation. The lack of correlation between the activities of antioxidant enzymes and MDA levels is relevant given the protective

Table 3. TUNEL and IGF-1 positive cells in PFC

	Neuron number PFC	TUNEL positive cell	IGF-1 positive cell
0.3% CO_2	11.9 ± 1.7^a	$21.6 \pm 2.7^{a,b}$	1.8 ± 0.4^a
0.1% CO_2	15.8 ± 2.8	12.4 ± 2.1^a	2.4 ± 0.5
0.05% CO_2	16.6 ± 2.8	7.6 ± 2.1	2.8 ± 0.4

N = 8 for all group. Data are means ± SEM. $^a p < 0.05$ compared to the 0.05% group and $^b p < 0.05$ compared to 0.1% CO_2 atmosphere group.

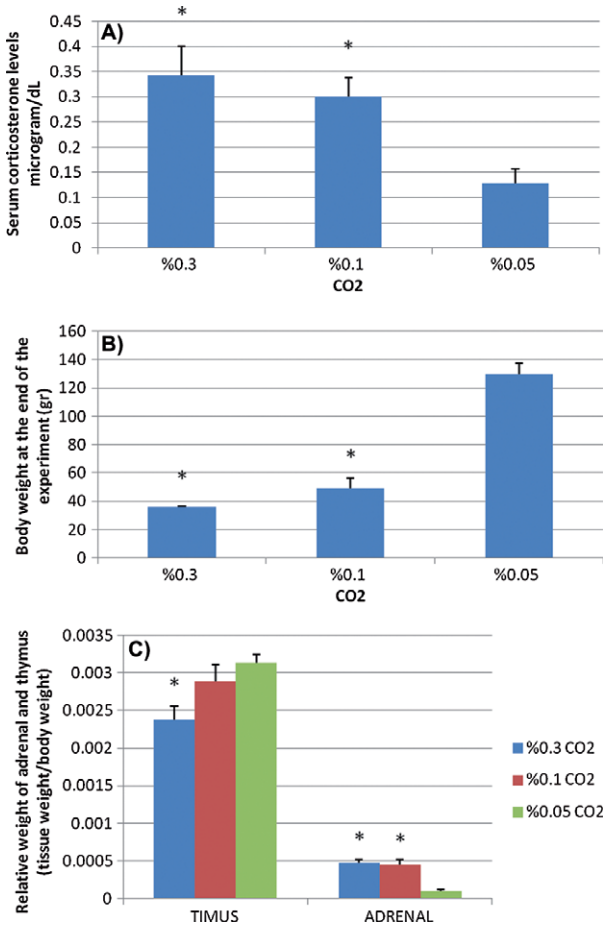

Fig. 5. A) Serum corticosterone levels. B) Body weights of groups. C) Relative adrenal/thymus weights of groups expressed as mg/body weight (g). *$p < 0.05$ compared to the 0.05% CO_2 atmosphere group.

roles of antioxidant enzymes against cellular and histological damage caused by ROS. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide and GPx converts hydrogen peroxide to H_2O (Halliwell 1992). We found that decreased GPx may explain the neuronal damage in the PFC and hippocampus under conditions of increased SOD and MDA enzyme activity in both the 0.1 and 0.3% CO_2 atmosphere groups.

It has been demonstrated that antioxidant enzymes are down-regulated in IGF-1 deficient mice (Csiszar et al. 2008). Therefore, we suggest that under conditions of decreased blood and tissue IGF-1 levels, oxidative stress and oxidative damage occur in the PFC and hippocampus. We found also that blood and brain IGF-1 levels were decreased in both the 0.1 and 0.3% CO_2 atmosphere groups.

Oxidative stress is associated with apoptosis. Oxidative stress mediates apoptosis by forming lipid hydroperoxides that are highly toxic and cause DNA fragmentation (Forrest et al. 1994). This condition causes mitochondrial damage, which can lead to release of cytochrome c, caspase activation and cell death (Leon et al. 2004). We found that TUNEL positive cells, indicators of apoptosis, were increased in the PFC and hippocampus of rats exposed to a 0.1 or 0.3% CO_2 atmosphere.

Maternal hypoxia causes intrauterine growth reduction and low birth weight (Joseph et al. 2002, Wang et al. 2009). We found that the body weights of the 0.3% and 0.1% CO_2 atmosphere groups were decreased compared to the 0.05% CO_2 atmosphere group. A possible explanation of the weight loss could be that the appetite center in the hypothalamus was affected by increased CO_2 concentration in the circulation.

Ours appears to be the first study to investigate the negative effects of chronic CO_2 exposure on memory, learning and anxiety behavior of rats during development. Our findings suggest that exposure to high CO_2 levels can cause decreased IGF-1 levels in blood, PFC and hippocampus, and increased oxidative stress. We found also that that oxidative stress increased apoptosis and neuron volume loss, which could explain the negative effects on cognitive functions and increased anxiety behavior in rats. Further studies are needed to clarify the role of IGF-1 in GABA metabolism, which also is known to be involved in neurobiological circuitries that modulate cognitive functions and anxiety.

Table 4. Neuron count and TUNEL- and IGF1-positive cells in amygdala

	Neuron number amygdala	TUNEL positive cell	IGF-1positive cell
0.3% CO_2	12.8 ± 1.2^a	$24.0 \pm 1.2^{a,b}$	1.6 ± 0.2^a
0.1% CO_2	16.0 ± 1.5	15.2 ± 1.1^a	2.2 ± 0.4
0.05% CO_2	18.8 ± 1.7	8.4 ± 0.7	2.8 ± 0.4

N = 8 for all group. Data are means ± SEM. $^a p < 0.05$ compared to the 0.05% CO_2 group and $^b p < 0.05$ compared to the 0.1% CO_2 group.

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