J. BIOL. ENVIRON. SCI.,
2012, 6(18), 219-231

Original Research Article

The Effects of Hypoxia and Opioid Receptor Agonists Treatment in Cortical B50 Neuronal Cells in Culture

Augustine Oseloka Ibegbu[1*], Lorna Fyfe[1], Douglas McBean[1] and Ian Mullaney[2]

[1] School of Health Sciences, Queen Margaret University, Musselburgh, Edinburgh, EH21 6UU, Scotland, UNITED KINGDOM
[2] School of Pharmacy, Division of Health Sciences, Murdoch University, South Street, Murdoch, 6150, WESTERN AUSTRALIA

Received: 04.05.2012; Accepted: 06.12.2012; Available Online: 25.01.2013

ABSTRACT

Hypoxia has been implicated in nerve cell deaths in many neurological disorders and opioid receptor agonists have some positive benefits on the nervous system. The aim of the present work was to investigate the effects of hypoxia and opioid receptor agonists' treatment on the morphology of B50 cells cultured in hypoxia using neuronal pattern and pattern formation as a case study. The B50 cells were cultured in normal incubator (21%O_2; 5% CO_2) as the control group and hypoxic incubator (5%O_2; 5% CO_2) as the experimental group and three opioid receptor agonists namely DAMGO (μ), DSLET (δ) and ICI-199,441 (κ) were administered to the cells for 48 hours as treatment against hypoxia after 48 hours of culture at 10μM, 50μM and 100μM concentrations. Neuronal morphology and wellbeing was assessed using same field morphological assessment and lactate dehydrogenase leakage (LDH). The result showed groups of dead and degenerating B50 neuronal cells, altered neuronal pattern and pattern formation and some significant changes ($P<0.05$) in cellular levels of LDH leakage in normal, hypoxic cells and cells treated with different agonists. The changes in morphology, neuronal pattern and LDH release indicate that hypoxia induced morphological and cellular changes in B50 cells in hypoxia and opioid agonists have some potential benefits in the treatment of hypoxia-induced changes in B50 cells in culture.

Key Words: Hypoxia, B50 Neuronal cells, neuronal morphology, neuronal pattern, opioid agonists, neurodegeneration

INTRODUCTION

Hypoxia can lead to oxidative stress which has been implicated in nerve cell death that occurs in a variety of neurodegenerative disorders like dementias, multiple sclerosis, Alzheimer's disease and Parkinson's disease (Maher, 2001; Benzi et al., 1994). Neuronal loss, neuritic and cytoskeletal lesions represent the major dementia-associated abnormalities in Alzheimer's disease (de la Monte et al., 2000). The loss of protein kinase C activity has been coupled to the severity of the damage although the functional relationship between oxidative stress, protein kinase C and cell death is unknown (Maher, 2001). Hypoxia leads to metabolic cellular processes in which oxidative species such as superoxide radical anions, hydrogen peroxide and lipid peroxides are generated intracellularly (Scandalios, 1997; Chen & Buck, 2000). These reactive species, if not eliminated, may damage DNA, proteins or membrane lipids and cause oxidative cell death. Endogenous antioxidative enzymes as well as endogenous small molecule antioxidants are required for cells to survive (Scandalios, 1997; Chen & Buck, 2000; Semenza 2005), while exogenous small molecule antioxidants have been shown to effectively prevent oxidative cell death in cultured cells (Busciglio & Yankner, 1995; Nakao et al., 1996).

Hypoxic stress results in a rapid and sustained inhibition of protein synthesis that is partially mediated by eukaryotic initiation factor 2 alpha phosphorylation by the phospho-endoplasmic reticulum kinase (Blais et al., 2004). Severe hypoxia has been shown to induce apoptotic cell death in developing brain neurons whereas mild hypoxia has been demonstrated to stimulate neurogenesis (Bossenmeyer-Pourie et al., 2002). Hypoxia threatens brain function throughout the entire life span starting from early foetal age until death and although the physiological consequences of brain hypoxia are well documented, the molecular mechanisms involved are still not well understood (Zhu et al., 2005; Rossler et al., 2001; Semenza, 2006; Semenza, 2007). It has been shown that hypoxia may have severe detrimental effects on most cells and especially on neuronal cells. Some studies have suggested that hypoxia can induce cellular adaptive responses that overcome apoptosis or cell death leading to reduced hypoxic cell injury, damage or cell death (Yun et al., 1997; Banasiak et al., 2000). These adaptive responses of cells to hypoxia may involve activation of some ion channels, as well as induction of specific gene

[*] Corresponding author: Aoibegbu@abu.edu.ng Present Address: Department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University Zaria, Kaduna State, 81006, NIGERIA

expression which may help to suppress or limit the effects of hypoxia in these cells (Yun et al., 1997). For example, adenosine triphosphate (ATP)-sensitive potassium ion (K^+) channels are activated by hypoxia in some cortical cerebral neuronal cells, and this may play a role in cell survival during hypoxia (Yamada and Inagaki, 2002). This may explain why some cells may survive and adapt to the hypoxic environment than others. Also, hypoxia-induced basic fibroblast growth factor and nerve growth factor expression appear to be associated with prevention or delay of neuronal cell apoptosis in hypoxic conditions (Yun et al., 1997; Banasiak et al., 2000).
Hypoxia-induced oxidative stress can also cause neurite retraction leading to neurodegeneration, while hypoxia-like injury can cause neuronal loss. Both oxidative stress and hypoxic injury could contribute to neurodegeneration similar to that found in Alzheimer's disease (de la Monte et al., 2000). It has been shown that ischemia results in severe focal and global damage of brain tissue accompanied by biochemical and molecular alterations, while hypoxia results in depletion of cellular and tissue energy and consequent death of the cells involved (Rodrigo et al., 2005).

Neuronal pattern and pattern formation makes it possible for neurons to organize themselves into groups called nerve fibers that carry neural signals into specific areas of the body to and from the brain. These patterns and the pattern formation itself are very important in ensuring that specific signals are carried to specific areas for effective and efficient coordinated neural response (Golubitsky et al., 2004). Neuronal fibers are arranged in bundles that receive and transmit related signals. These bundles form a neural tract e.g. the visual tract carries visual signals from the eyes to relay centers in the brain and back to the eyes for visual perception to occur (Cowan and Thomas, 2004). Asare et al. (1996) have shown that spatial differences exist in the distributional pattern of neurons in the superior frontal gyrus of 32 subjects who died of acquired immune deficiency syndrome. This gives support to the fact that, if there is a disruption in the tracts, the signal may not be properly coordinated and relayed for perfect response to occur. Genetic coding has been shown to play a role in the pattern formation in various part of the nervous system (Schmid et al., 2000). The aim of the present work was to investigate the effects of hypoxia and opioid receptor agonists' treatment on the morphology of B50 neuronal cell lines cultured in hypoxia using neuronal pattern and pattern formation as a case study.

MATERIALS AND METHODS

Neuronal culture

One group of B50 cells were cultured and maintained in a normal incubator (21%O_2; 5% CO_2) as control cells, with another batch of cells cultured in a hypoxic incubator (5%O_2; 5% CO_2) as hypoxic experimental cells. The cells were cultured in a 12-well culture plates for 48 hours and three highly potent opioid receptor agonists {DAMGO(μ), DSLET(δ) and ICI--199,441(κ)}, were selected and administered to the cells as treatment against hypoxia for 48 hours at a concentration of 10μM, 50μM and 100μM.

Morphological Studies

The frozen B50 cells were cells were raised in culture for 24, 48, 72, 96, 120 and 144 hours while 0 hour was regarded as the starting point of culture during splitting of cells for sub-culturing. At each stage of the experimental period, the cells were observed under the microscope, using same field morphological assessment in which the culture plates were examined from the centre to the sides in a quadri-point analysis method (Ellingson et al., 2007; Sato and Momose-Sato, 2007), and any change in the morphology of the cells was noted. This was repeated three times for each experiment. Micrographs of the cells were taken at the different time intervals at a magnification of 200 times (x 200), to show the morphological changes that may have occurred between the normal and hypoxic cells in culture. The cultured B50 cells were also examined using the Trypan Blue exclusion method and the total number of cells, viable cells and percentage viability were calculated.

Lactate dehydrogenase Assay

Lactate dehydrogenase (LDH) release which has been shown to be a reliable index of cellular injury (Zhang et al., 2006), was used to assess the level of neuronal injury in normal, hypoxic and treated cells, using a LDH kit

and procedure from Sigma. The working solution of LDH assay cofactor was prepared by adding 25ml of deionized, sterilized tissue culture water to bottle of lyophilized cofactors. The lactate dehydrogenase assay mixture was prepared by mixing equal amounts of LDH assay substrate, LDH assay cofactor and LDH assay dye solution. The LDH assay mixture was added at double the volume of the supernatant medium removed for assaying. The plates were covered with aluminum foil to protect it from light and incubated at room temperature for 30 minutes. The reaction was terminated by the addition of one tenth volume of 1N HCL solution to each well. The absorbance was measured at a wavelength of 490nm using Dynex MRX model of Micro plate reader and the result was calculated.

Data Analysis

The parameters were assayed two times in triplicate in the normal, hypoxic and treated experimental groups of cultured B50 neuronal cells and the results are presented as mean ± standard deviation (SD). A Students't-test was used to test the level of significance and a P-value less than 0.05 was considered to be significant. For multiple treatment data, One-Way Analysis of Variance (ANOVA) was used followed by Multiple Range Test post hoc subgroup testing to find the least significant difference (LSD) between the groups.

RESULTS

Morphological Changes

The result showed morphological changes between the normal, hypoxic and treated B50 cells. The normoxic cells showed normal neuronal cell morphology (Figs.1 and 2), while the hypoxic non-treated cells showed groups of dead and degenerating cells (Figs.6 and 7). The hypoxic opioid-treated B50 cells showed reduced cell death and healthier neuronal cells (Figs.8 and 9).

Total Cell Count and Viability Study

The time course effect of hypoxia on total cell count and viability showed that after 48 hours of culture, the normal cultured B50 cells had 6.50±0.84 x10^6; 6.01±0.85 x 10^6; 92.25 ±1.17; 90-93%, as total cell count, total viable cells, percentage viability and range of percentage viability respectively, while the hypoxic cells had 5.34±1.45 x 10^6, 3.89±1.22 x10^6, 72.01±3.39 and 67-76% as total cells count, total viable cells, percentage viability and range of percentage viability respectively (Table 1). The difference in the total cell count and total viable cells between the normoxic and hypoxic cells was statistically significant ($P<0.05$) and demonstrated that the longer the cells were cultured, the greater was the effect on the total cell count and viability of the B50 cells.

LDH Release from Opioid agonists treated B50 neuronal cells cultured in hypoxia

The LDH leakage from normal B50 cell (100%) was significantly increased ($p<0.05$) when compared with untreated hypoxic cells (587%) and treated hypoxic B50 cells with different concentration of opioid agonists. The LDH released from B50 cells treated with 100μM MOR DAMGO (143%); 50μM DOR DSLET (140%); 100μM DOR DSLET (143%); 10μM KOR (113%) and 50μM KOR (109%) were significantly decreased (($p<0.05$) from untreated hypoxic cells in culture (Table 2). The LDH leakage from hypoxic untreated cells (587%) was 5 folds higher than that from normal cells, and cells treated with 100μM MOR DAMGO (143%); 50μM DOR DSLET (140%); 100μM DOR DSLET (143%); 10μM KOR (113%) and 50μM KOR (109%), and about three folds higher in cells treated with 10μM MOR (338%) and 100μM KOR (322%).

LDH Release from Opioid agonists and antagonists treated B50 cells cultured in hypoxia

The LDH leakage from B50 cells in hypoxia treated with opioid agonists/antagonists showed that LDH released from the normal cultured B50 cells (100%) was significantly higher ($p<0.05$) in untreated hypoxic B50 cells (587%). The LDH release from hypoxic treated cells with different concentrations of opioid agonist in the presence of antagonist, showed that the LDH leakage from the hypoxic cells treated with opioid agonists/antagonists was increased between 2-8 folds when compared with the normal cultured B50 cells while

the LDH release was about the same as in those treated with 50µM DOR/ICI (416%), 50µM KOR/NOR (529%) and 100µM KOR/NOR (665%) when compared with untreated hypoxic cells (Table 3).

Pattern formation

The results from the morphological study showed the B50 neuronal cells in the normal incubator with normal neuronal pattern and tract formation resembling those nerve fibers in normal neuronal development in the body (Figs.3, 4 and 5), when compared to the disoriented pattern found in B50 cells cultured under hypoxia (Figs.6 and 7). The B50 neuronal pattern and pattern formation was shown to be greatly improved in cells treated with opioid receptor agonists (Figs. 8 and 9), when compared to the abnormal pattern found in hypoxic cells (Figs. 3, 4 and 5).

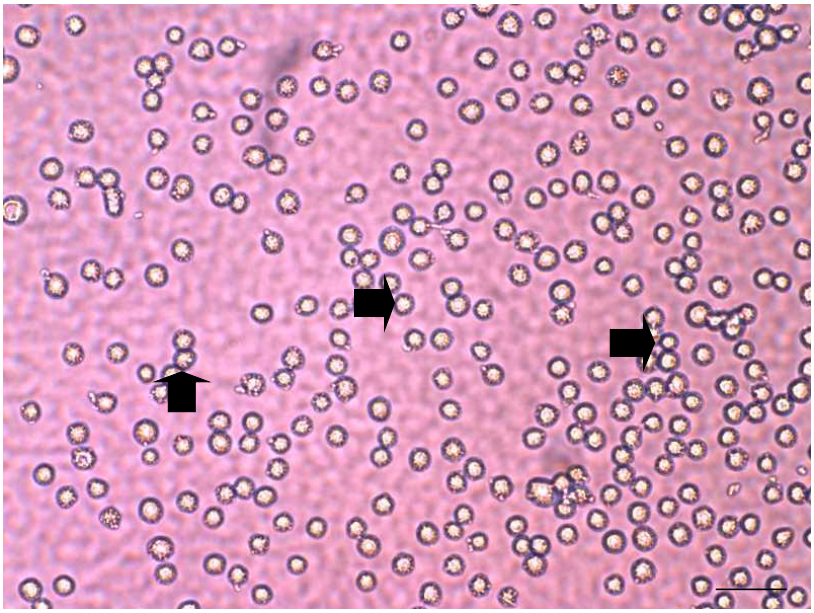

Figure 1. Representative of B50 cells at 0hrs with normal B50 cells (arrow) at the point of starting the culture at 21% O_2 and 5% CO_2. B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx40 magnification.

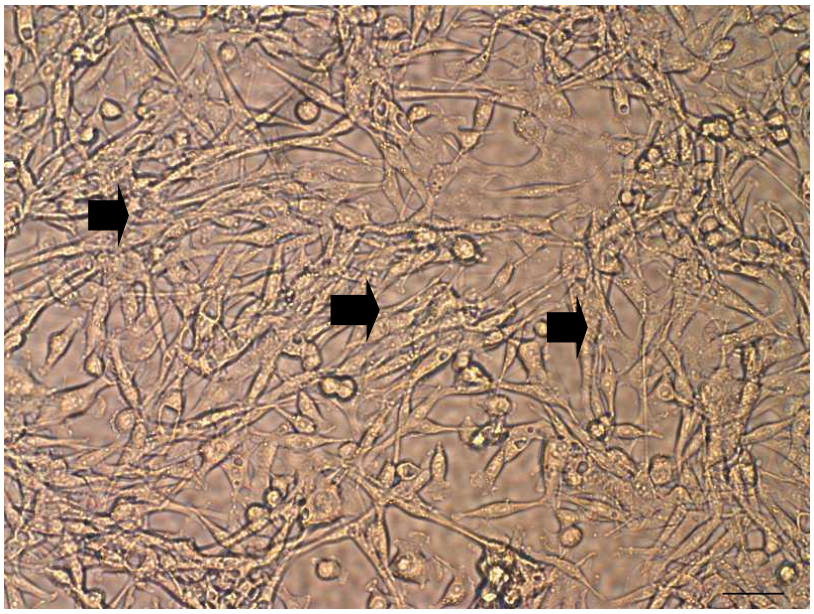

Figure 2. Representative of B50 cells at 48hrs of normal culture (21%O_2 and 5% CO_2) with B50 cells (arrow). B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with IBM Image Solutions®. Scale bar =5mmx40 magnification.

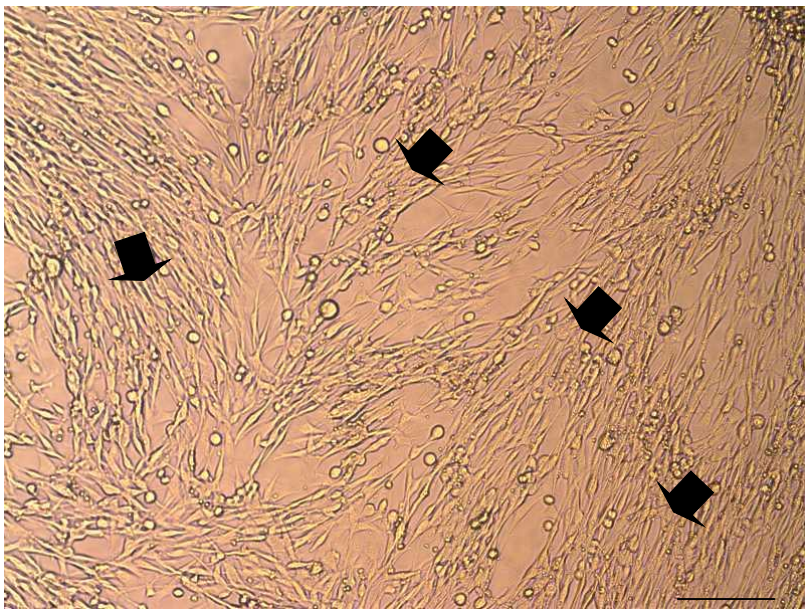

Figure 3. Representative of normal B50 cells at 48hrs of culture (21%O_2 and 5% CO_2) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx20 magnification.

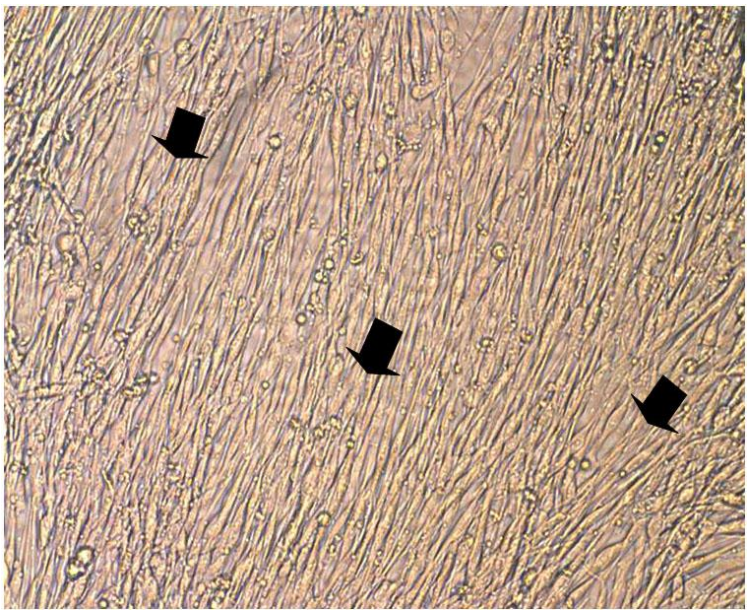

Figure 4. Representative of normal B50 cells at 96hrs of culture (21%O_2 and 5% CO_2) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

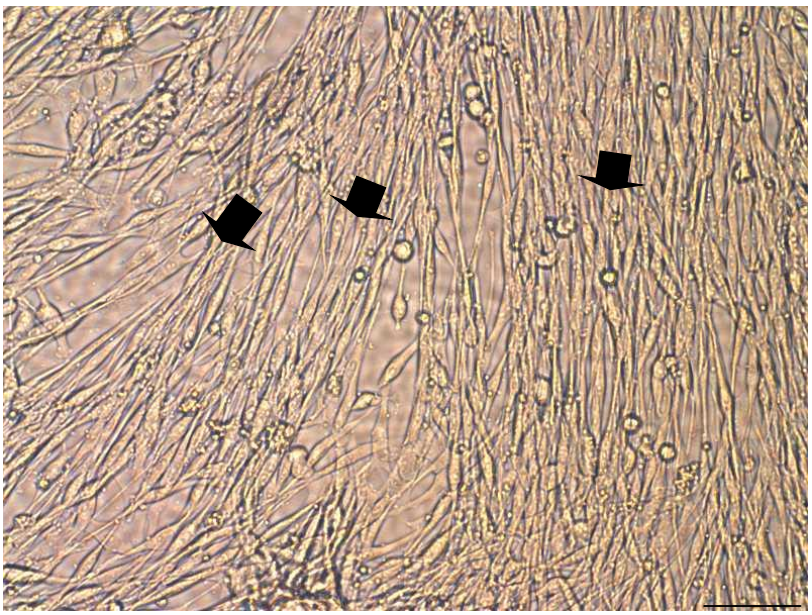

Figure 5. Representative of normal B50 cells at 96hrs of culture (21%O_2 and 5% CO_2) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification

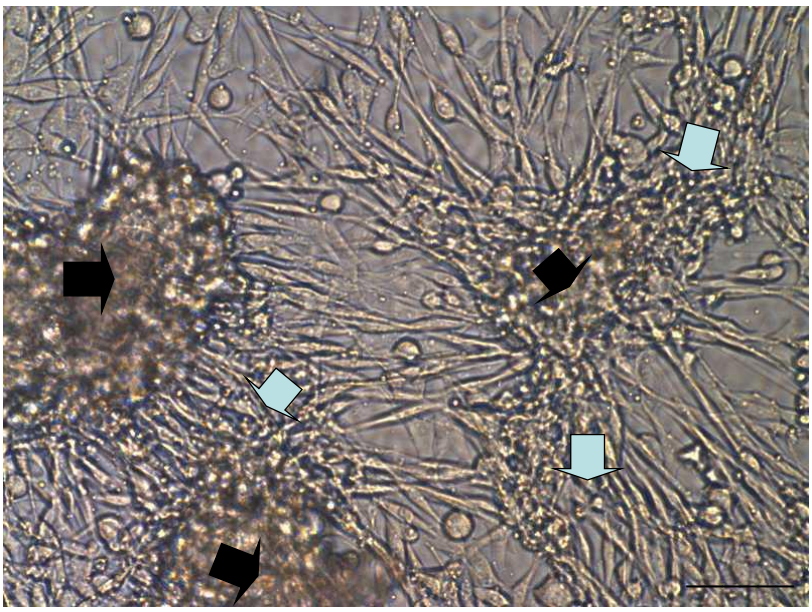

Figure 6. Representative of hypoxic B50 cells at 96hrs of culture (5%O_2 and 5% CO_2), with groups of degenerating cells (black arrow) and altered pattern of cell arrangement (blue arrow). Groups of degenerating and altered nerve arrangement were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

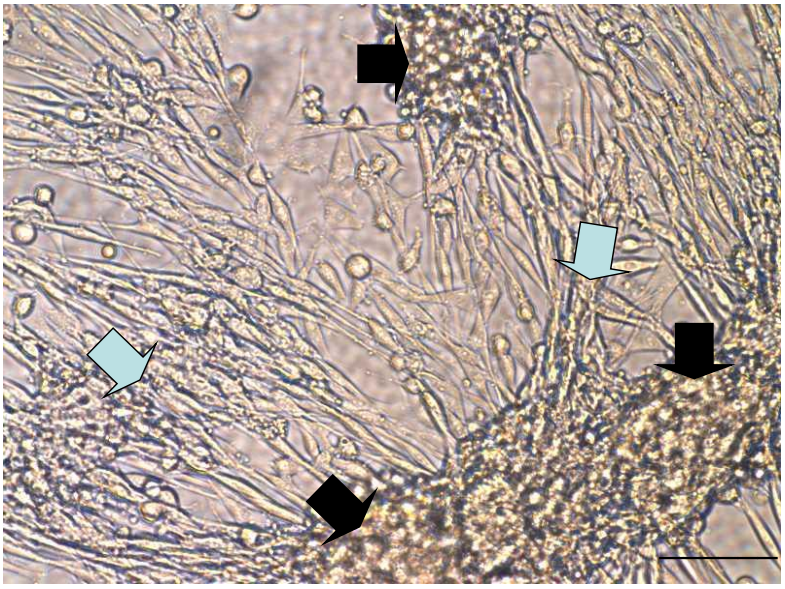

Figure 7. Representative of hypoxic B50 cells at 96hrs of culture (5%O_2 and 5% CO_2), with groups of degenerating cells (black arrow) and altered pattern of cell arrangement (blue arrow). Groups of degenerating and altered nerve arrangement were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

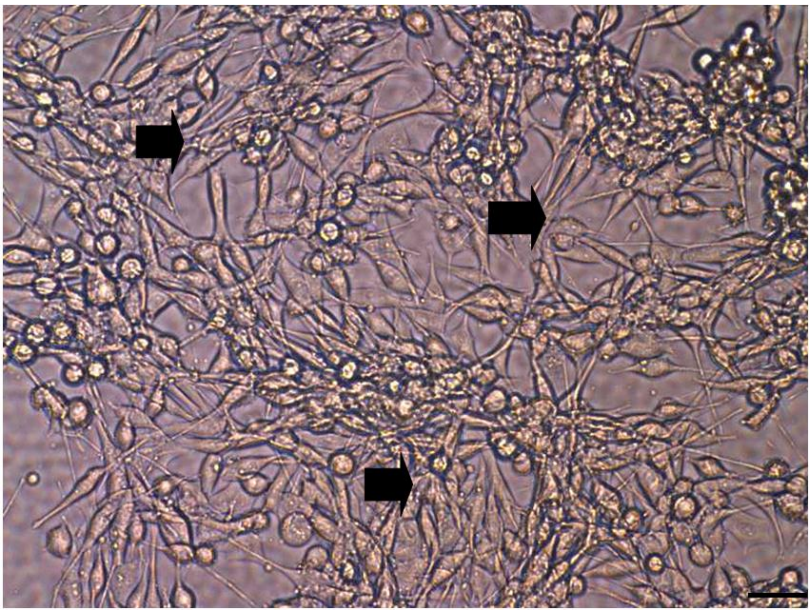

Figure 8. Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 100uM DAMGO against hypoxia for 48hrs making-up 96hrs of culture (5%O_2 and 5% CO_2), showing many normal cells (black arrow). The cells were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

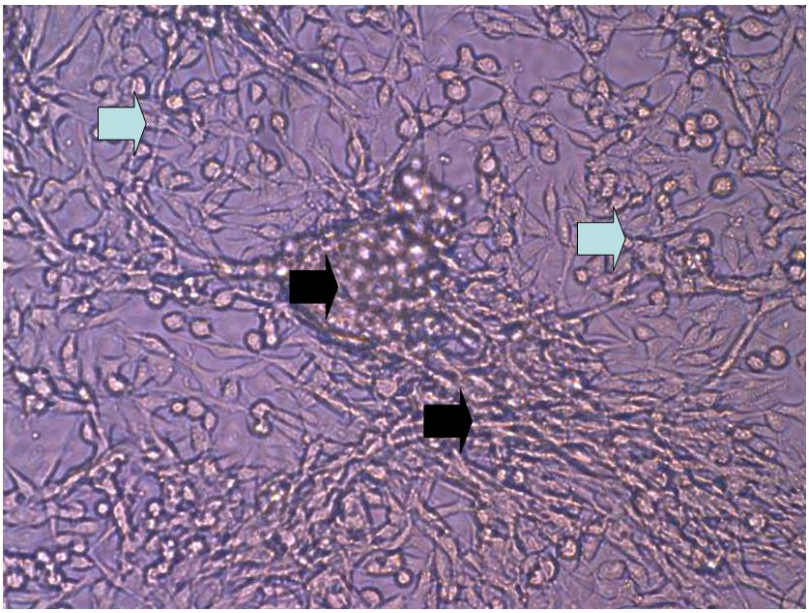

Figure 9. Representative of B50 cells pre-treated in 100uM DSLET against hypoxia for 96hrs of culture (5%O_2 and 5% CO_2), showing few degenerated cells (black arrow), and some normal cells (blue arrow). The cells were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

Table 1. The time effect of hypoxia on total cell count and viability in B50 cells.

Time(hours)/Type of treatment (n=6)	Total cell count ($x10^6$)cells/ml	Total Viable Cells ($x10^6$)cells/ml	Viability (%)
0 HRS	**2.67 ± 1.64**	**2.5 ± 1.4**	**93.6 ± 2.8**
Range	(1.2-5.0)	(1.1-4.7**)**	(89.5-97.4**)**
24 HRS			
NORMAL	**3.5 ±0.9**	**3.05 ±0.8**	**87.1 ± 0.7**
Range	(2.4-4.6**)**	(2.1-4.0)	(86.1-88.1)
HYPOXIA	**2.8 ± 0.4**	**1.96 ± 0.2**	**70.9 ± 5.4**
Range	(2.1-3.1)	(1.6-2.3)	(63.3-76.2)
48HRS			
NORMAL	**6.5±0.8**	**6.0±0.9**	**92.3±1.2**
Range	(5.2-7.4)	(4.7-6.9)	(90.4-93.6)
HYPOXIA	**5.34±1.45**	**3.9±1.2**	**72.0±3.4**
Range	(3.4-7.5)	(2.3-4.7)	(67.7-76.0)
72HRS			
NORMAL	**18.8±5.1**	**17.1±4.4**	**91.2±3.8**
Range	(10.7-26.8)	(9.4-22.4)	(82.8-95.4)
HYPOXIA	**12.6±4.9***	**9.10±2.62***	**75.3±11.6**
Range	(5.3-21.2)	(4.3-12.8)	(60.4-94.4)
96HRS			
NORMAL	**35.4±12.9**	**32.9±12**	**93.3±2.3**
Range	(16.9-51.0)	(16.1-48.5)	(89.5-95.3)
HYPOXIA	**12.5±4.0***	**10.9±4.2***	**85.7±7.0**
Range	(8.19)	(6-17)	(76.1-93.3)
120HRS			
NORMAL	**6.8±2.7**	**6.3±2.6**	**92.2±2.8**
Range	(3.4-9.9)	(3.0-9.3)	(88.2-94.3)
HYPOXIA	**7.5±1.1**	**6.1±0.7**	**81.5±4.4**
Range	(6.1-8.8)	(5.3-6.7)	(76.1-86.9)
144HRS			
NORMAL	**46.5±9.3**	**44.0±9.1**	**94.5±0.8**
Range	(36.6-57.2)	(34.4-54.2)	(93.7-95.7)
HYPOXIA	**14.9±6.0***	**11.6±6.4***	**74.2±13.6**
Range	(9.1-26.4)	(5.1-24.0)	(55.6-90.9)

Data show a comparative time effect of hypoxia on total cell count, total viable cells and percentage viability in B50 cells in culture using trypan blue exclusion method from three different experiments repeated twice (n=6). The normal cells cultured in (21% O_2; 5% CO_2) and hypoxic cells in (5% O_2; 5% CO_2), showed a significant increase (*$P<0.05$ versus normoxic cells; Student's t-test) in total cell count and total viable cells at 72, 96 and 144 hours. The data expressed as mean ±SD (n=6) and Range.

Table 2. The effect of opioid agonist treatment on LDH release in cultured B50 cells in hypoxia.

Type of Treatment	Measured optical density(OD)	Percent of control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	1.214±0.511	587±51.1*
DAMGO(μ)10μM	0.699±0.421	338±42.1*#
DAMGO(μ)50μM	0.344±0.210	167±21.0#
DAMGO(μ)100μM	0.296±0.112	143±11.2#
DSLET(δ) 10μM	0.346±0.201	167±20.1#
DSLET(δ) 50μM	0.290±0.112	140±11.2#
DSLET(δ) 100μM	0.296±0.221	143±22.1#
ICI-199441(κ) 10μM	0.234±0.221	113±22.1#
ICI-199441(κ) 50μM	0.224±0.223	109±22.3#
ICI-99441(κ)100μM	0.666±0.251	322±25.1*#

Table 2; Cells were cultured for 48hrs and then treated with different concentrations of opioid agonists for 48hrs for a total of 96hrs of culture. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells ($21\%O_2;5\%CO_2$) was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; *P<0.05 versus untreated hypoxic cells; #P<0.05 versus agonist treated hypoxic cells; Student's t-test).

Table 3. The effect of opioid agonist/antagonist treatment on LDH release from cultured B50 cell in hypoxia.

Type of Treatment	Measured optical density(OD)	Percent of control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	1.214±0.511	587±51.1*
DAMGO(μ)10μM	0.699±0.421	338±42.1*
DAMGO/CTAP10μM	0.726±0.335	351±33.5*
DAMGO(μ)50μM	0.344±0.210	167±21.0
DAMGO/CTAP50μM	0.535±0.252	259±25.2*#
DAMGO(μ)100μM	0.296±0.112	143±11.2
DAMGO/CTAP100μM	0.752±0.305	364±30.5*#
DSLET(δ) 10μM	0.346±0.201	167±20.1
DSLET/ICI-174864 10 μM	0.530±0.256	256±25.6*#
DSLET(δ) 50μM	0.290±0.112	140±11.2
DSLET/ICI-174864 50 μM	0.859±0.345	416±34.5*#
DSLET(δ) 100μM	0.296±0.221	143±22.1
DSLET/ICI-174864 100 μM	0.644±0.225	312±22.5*#
ICI-199441(κ) 10μM	0.234±0.221	113±22.1
ICI-199441/NOR 10μM	1.698±0.501	821±50.1*#
ICI-199441(κ) 50μM	0.224±0.223	109±22.3
ICI-199441/NOR 50 μM	1.093±0.552	529±55.2*#
ICI-99441(κ)100μM	0.666±0.251	322±25.1
ICI-199441/NOR 100 μM	1.375±0.446	665±44.6*#

Table 3; The effect of opioid agonist/antagonist treatment on LDH release from B50 cells cultured in hypoxia ($5\%O_2$; $5\%CO_2$), using LDH assay. Cells were cultured for 48hrs and then treated with different concentrations of opioid agonists for 48hrs for a total of 96hrs of culture. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells ($21\%O_2;5\%CO_2$) was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; *P<0.05 versus untreated hypoxic cells; #P<0.05 versus agonist/antagonist treated hypoxic cells; Student's t-test).

DISCUSSION

The results presented in this study showed hypoxia-induced changes in the neuronal B50 cell morphology, pattern and LDH of neuronal viability. The results showed that hypoxia has an effect on neuronal morphology, pattern and pattern forming abilities of B50 neuronal cells when compared to normal B50 cells in culture. The neuronal pattern is very important for an organized and integrated neuronal activity which could be disrupted if the pattern is disorganized or disoriented as shown in hypoxic cells in this study. This is because the normal neuronal organization involves the grouping of nerve fibers to form nerve bundles of which functionally related nerve bundles ultimately become grouped together and form specific nerve tracts. These linkages of neurons are possible through synapses where signals are passed between cells. The disruption of the neuronal patterns as shown during hypoxia in this study, indicate that hypoxia could result in the alteration of signal transmission between neuronal cells since some of the neuronal cells are dead and degenerating. These changes in the pattern could also explain the changes in the morphology since the neuronal pattern of arrangement is a part of neuronal morphology.

The changes in morphology, neuronal pattern and LDH that occur during hypoxia in B50 cells in culture were in agreement with the study by Titus et al. (2007), which showed that simulated hypobaric hypoxia, resembling that found in high altitude hypoxia, severely affects the morphology of the central nervous system (CNS) and results in several physiological changes. They showed that these effects specifically within the hippocampus may closely be associated with learning and memory and proposed that insult to this region also affects cognition.

The effects of opioid administration on LDH release from B50 cells in hypoxia showed significant reductions in LDH leakage in hypoxia-treated B50 cells in culture. The release of intracellular LDH into the extracellular fluids has been documented as a reliable indicator of neuronal injury and damage (Zhang et al., 2006). Results showed that there was a concentration-dependent decrease in LDH release from opioid treated B50 cells in hypoxia. The cells treated with DAMGO and DSLET had LDH release decreased with increases in the concentration of the agonists while ICI-199441, had LDH release increased as the concentration of the agonist increased. The effect of the agonists in lowering the LDH release from B50 cells was blocked by the selective mu (μ), delta (δ) and kappa (κ), (CTAP, ICI-174864 and NOR) opioid antagonists suggesting that the effect of inhibition of the LDH release from B50 cells was mediated through the (μ, δ and κ) opioid receptors, indicating the involvement of $G_{i/o}$ in the protective activities of the opioid receptor agonists.

There were some relative differences in the actions of the opioid agonists and antagonists as shown in the treatment with 100μM DSLET and 50μM DSLET which had LDH release lower than those of the agonist involved, thus indicating that there may be other systems and processes involved in the regulation of opioid receptor activities in B50 cells in culture. These multiple processes could be as a result of interaction between the various opioid receptor subtypes involved, since it has been shown that the opioid receptor subtypes share a close homology in their sequences (Mayer et al., 2003). There may be the involvement of endogenous opioids in the system, which has been shown to be released during hypoxia (Zhang et al., 2000). Also there may be the involvement of interaction between the opioid agonists, the endogenous opioid and the endogenous cannabinoid system present in the cells (Schoffelmeer et al., 2007).

The B50 neuronal cell degeneration in hypoxic cultures may be linked to the loss of neuronal cells and tissues which invariably would result in defects in neuronal activities. These B50 neuronal cell losses and loss of normal neuronal pattern results in loss of neuronal mass, could lead to loss of nervous system coordination of activities and actions from the cellular level to the whole organism. The neuronal loss may affect cognition, behavioral activities, learning, and memory, and may even lead to physical defect depending on the area of the brain affected by the degeneration. Studies by Shukitt-Hale et al. (1994), had suggested that rapid or prolonged exposure to hypobaric hypoxia (HBH), is associated with psychomotor and cognitive impairments.

The results presented in this study which showed changes in the characteristics of neuronal B50 cells in hypoxia compared to the normal B50 cells supported the work of Titus et al. (2007), and Shukitt-Hale et al. (1996). They showed that there were structural changes in the neuronal cells of both human and animal models

exposed to hypoxia. These structural and morphological changes can lead to defects in memory, cognitive behaviour and loss of coordinated responses to stimuli.

The result with the B50 neuronal cells showed that cell damage increases with the increase in the exposure time to hypoxia as quantified by the trypan blue method. Thus, the more time the cells are exposed to the hypoxic environment the greater the damage to the B50 cells. This is in agreement with the findings of Titus et al. (2007), which showed that the damage to cells increases with the level of exposure to an oxygen-deprived environment and that the number of damaged cells increases following increase in altitude. Clausen et al. (2005) correlated the hippocampal morphological changes and memory dysfunction, cortical lesion volume and regional hippocampal morphological changes after controlled cortical contusion (CCC) injury in rats, and showed morphological changes in CA1, CA3, and hilus of the dentate gyrus in severely injured rats.

Sousa et al. (2000) have demonstrated that stress-induced cognitive deficits in rats do not correlate with hippocampal neuronal loss when they evaluated the effects of chronic stress on hippocampal dendrite morphology, volume of mossy fiber system, and number and morphology of synapses between the mossy fibers. They found profound changes in the morphology of the mossy fiber terminals and significant loss of synapses were detected in stress-induced cognitive conditions (Sousa et al., 2000). The study by Sousa et al. (2000) showed that stress-induced morphological changes in the hippocampus could not be correlated with cognitive changes in rat.

Yoshimura et al. (2006) have shown that neuronal polarity is essential for both structurally and functionally unidirectional signal flows from dendrites to axons. The initial event in establishing a polarized neuron is the specification of a single axon and early in neuronal development, one small neurite becomes differentiated from other neurites to form an axon. The ability of neuronal cells to proliferate, differentiate and polarize is essential for organization of the nervous system and cultured hippocampal neurons which develop a single long axon and several shorter dendrites that maintain their structural characteristics at the molecular level have been used as a model for neuronal polarization (Yoshimura et al., 2006). During maturation, hippocampal neurons dramatically change their morphology and differentiate into a single axon and many dendrites which form synaptic contacts and establish a neuronal network (Yoshimura et al 2006**;** Dotti et al., 1988). It is these neuronal networks that finally joined together into a specific pattern to form neuronal bundles that ultimately form neural tracts.

The result as presented in this study showed that hypoxia affected neuronal pattern and pattern formation in B50 cells in hypoxia when compared with the normal B50 cells in culture. This disorganization of the neuronal pattern could be as a result of the reduction and disorientation of the proteins necessary for cellular signaling through neuronal cell proliferation and differentiation. Of relevance are the findings of Zhong et al. (2007), who showed that Raf serine/threonine kinases play important role in nervous system development and when these molecules were conditionally eliminated, the result showed markedly reduced phosphorylation of ERK in neural tissues which led to growth retardation. This supports the finding in this present study.

In conclusion, the data presented here showed that hypoxia had an effect on B50 neuronal cell morphology, pattern and pattern formation which could have a deleterious effects on neuronal structure and morphology which would result in overall functional impairment of the nervous system in terms physical activities, cognitive abilities, learning and memory functions even though this may depend on the parts of the brain that are affected. This could be correlated with changes in the morphology of the brain as seen in the study with other neurodegenerative conditions like Stroke, Alzheimer's disease (AD) and Parkinson's disease (PD). The changes in morphology, neuronal pattern and LDH release indicate that hypoxia induced morphological and cellular changes in B50 cells in hypoxia and B50 neuronal cells are susceptible to damage and injurious effects of hypoxia as most brain cells while opioid agonists have some potential benefits in the treatment of hypoxia-induced changes in neuronal B50 cells in culture.

REFERENCES

Asare E, Dunn G, Glass J, McArthur J, Luthert P, Lantos P, Everall I. (1996) Neuronal pattern correlates with the severity of human immunodeficiency virus-associated dementia complex. Usefulness of spatial pattern analysis in clinicopathological studies. *Am J Pathol*. 148(1):31-38.

Banasiak KJ, Xia Y, Haddad GG. (2000). Mechanisms underlying hypoxia-induced neuronal apoptosis. Prog. Neurobiol. 62(3):215-249.

Benzi G, Gorini A, Ghigini B, Arnaboldi R, Villa RF. (1994). Modifications by hypoxia and drug treatment of cerebral ATPase plasticity. *Neurochem Res.* 19(4): 517-524.

Blais JD, Filipenko V, Bi M, Harding HP, Ron D, Koumenis C,Wouters BG & Bell JC. (2004). Activating transcription factor 4 is translationally regulated by hypoxic stress. *Moll Cell Biology*. 23(17):7469-7482.

Bossenmeyer-Pourie C, Lievre V, Grojean S, Koziel V, Pillot T, Daval JL. (2002). Sequential expression patterns of apoptosis- and cell cycle-related proteins in neuronal response to severe or mild transient hypoxia. *Neuroscience.* 114(4): 869-882.

Busciglio J and Yankner BA. (1995). Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons *In Vitro*. *Nature*. 378:776-779.

Chen Y and Buck J. (2000). Cannabinoids protect cells from oxidative cell death: a receptor-independent mechanism. *J Pharmacol Exp Ther*. 293(3):807-812

Clausen F, Lewen A, Marklund N, Olsson Y, McArthur DL, Hillered L. (2005). Correlation of hippocampal morphological changes and Morris water maze performance after cortical contusion injury in rats**.** *Neurosurgery*. 57(1):154-163.

Cowan JD and Thomas PJ. (2004). Pattern Formation in Visual Systems. *Phys. Rev. Lett.* 92:188101.

de la Monte SM, Neely TR, Cannon J, Wands JR. (2000). Oxidative stress and hypoxia-like injury cause Alzheimer-type molecular abnormalities in central nervous system neurons. *Cell and Molecular Life Sciences.* 57(10):1471-1481.

Dotti CG, Sullivan CA, Banker GA. (1988). The establishment of polarity by hippocampal neurons in culture. *J Neurosci.* 8(4):1454-68.

Ellingson BM, Ulmer JL, Schmit BD. (2007). Morphology and Morphometry of Human Chronic Spinal Cord Injury Using Diffusion Tensor Imaging and Fuzzy Logic. *Ann Biomed Eng.* 36(2):224-236.

Golubitsky M, Shiau L-J, Torok A. (2004). Symmetry and Pattern Formation on the Visual Cortex. *Torok, SIAM J. Appl. Dynam*. Sys. 2: 97–143.

Maher, P. (2001). How Protein kinase C Activation Protects Nerve Cells from Oxidative stress-induced cell death. *The Journal of neuroscience*. 21(9) 2929-2938.

Mayer P, Kroslak T, Tischmeyer H and Höllt V. (2003)**.** A truncated δ opioid receptor, spontaneously produced in human but not rat neuroblastoma cells, interferes with signalling of the full-length receptor. *Neuroscience Letters.* 344(1): 62-64.

Nakao N, Grasbon-Frodl EM, Widner H, Brundin P. (1996). Antioxidant treatment protects striatal neurons against excitotoxic insults. *Neuroscience.* 73(1):185-200.

Rodrigo J, Fernandez AP, Serrano J, Peinado MA, Martinez A. (2005). The role of free radicals in cerebral hypoxia and ischemia. *Free Radic Biol Med*. 39(1):26-50.

Rossler J, Schwab M, Havers W, Schweigerer L. (2001). Hypoxia promotes apoptosis of human neuroblastoma cell lines with enhanced N-myc expression. *Biochem Biophys Res Commun*. 281(2):272-276.

Sato K and Momose-Sato Y. (2007). Optical imaging analysis of neural circuit formation in the embryonic brain. *Clin Exp Pharmacol Physiol*. 18067593 Epub.

Scandalios JG. (1997). Oxidative stress and defense mechanisms in plants: introduction. *Free Radic Biol Med.* 23(3): 471-472.

Schmid RS, Pruitt WM, Maness PF. (2000). A MAP kinase-signaling pathway mediates neurite outgrowth on L1 and requires Src-dependent endocytosis. *J. Neurosci.* 20(11): 4177-4188.

Schoffelmeer AN, Hogenboom F, Wardeh G, De Vries TJ. (2006). Interactions between CB_1 cannabinoid and mu opioid receptors mediating inhibition of neurotransmitter release in rat nucleus accumbens core. *Neuropharmacology.* 51(4): 773-781.

Semenza GL. (2005). Involvement of hypoxia-inducible factor 1 in pulmonary pathophysiology. *Chest.* 128(6 Suppl):592S-594S.

Semenza GL. (2006). Regulation of physiological responses to continuous and intermittent hypoxia by hypoxia-inducible factor 1. *Exp. Physiol.* 91(5):803-806.

Semenza GL. (2007). Hypoxia and cancer. *Cancer Metastasis Rev.* 26(2):223-224

Shukitt-Hale B, Kadar T, Marlowe BE, Stillman MJ, Galli RL, Levy A, Devine JA, Lieberman HR. (1996). Morphological alterations in the hippocampus following hypobaric hypoxia. *Hum Exp Toxicol.* 15(4):312-319.

Shukitt-Hale B, Stillman MJ, Welch DI, Levy A, Devine JA, Lieberman HR. (1994). Hypobaric hypoxia impairs spatial memory in an elevation-dependent fashion. *Behav Neural Biol.* 62(3):244-252.

Sousa N, Lukoyanov NV, Madeira MD, Almeida OF, Paula-Barbosa MM. (2000). Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioural improvement. *Neuroscience.* 97(2):253-266.

Titus AD, Shankaranarayana Rao BS, Harsha HN, Ramkumar K, Srikumar BN, Singh SB, Chattarji S, Raju TR. (2007). Hypobaric hypoxia-induced dendritic atrophy of hippocampal neurons is associated with cognitive impairment in adult rats. *Neuroscience.* 145(1):265-278.

Yamada K and Inagaki N. (2002). ATP-sensitive K(+) channels in the brain: sensors of hypoxic conditions. *News Physiol Sci.* 17:127-130.

Yoshimura T. Arimura N, and Kaibuchi K. (2006). Signalling networks in neuronal polarization. *The Journal of Neuroscience.* 26(42):10626-10630.

Yun JK, McCormick TS, Judware R, Lapetina EG. (1997). Cellular adaptative responses to low oxygen tension: apoptosis and resistance. *Neurochem. Res.* 22(4): 517-521.

Zhang JH, Lo T, Mychaskiw G, Colohan A. (2000). Mechanisms of hyperbaric oxygen and neuroprotection in stroke. *Pathophysiology*. 12(1):63-77.

Zhang J, Qian H, Zhao P, Hong SS, Xia Y. (2006). Rapid hypoxia preconditioning protects cortical neurons from glutamate toxicity through delta-opioid receptor. *Stroke.* 37(4):1094-1099.

Zhong J, Li X, McNamee C, Chen AP, Baccarini M, Snider WD. (2007). Raf kinase signaling functions in sensory neuron differentiation and axon growth in vivo. *Nat Neurosci.* 10(5):598-607.