ADRENERGIC AND GLUTAMERGIC RECEPTOR GENE EXPRESSION AND THEIR FUNCTIONAL REGULATION IN THE CEREBRAL CORTEX OF HYPOXIA INDUCED NEONATAL RATS: ROLE OF OXYGEN, EPINEPHRINE AND GLUCOSE SUPPLEMENTATION

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BY

FINLA CHATHU

DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 022, KERALA, INDIA.

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DEPARTMENT OF BIOTECHNOLOGY

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

COCHIN- 682 022, INDIA Phone : 0484-2576267 (O), 0485-2812428 (R) Mob: 94470 12428
Email: cspaulose@cusat.ac.in, paulosecs@yahoo.co.in Fax: 91-0484-2576267, 2577595

DR. C.S.PAULOSE
HEAD
DIRECTOR, CENTRE FOR NEUROSCIENCE

CERTIFICATE

This is to certify that the thesis entitled "**ADRENERGIC AND GLUTAMERGIC RECEPTOR GENE EXPRESSION AND THEIR FUNCTIONAL REGULATION IN THE CEREBRAL CORTEX OF HYPOXIA INDUCED NEONATAL RATS: ROLE OF OXYGEN, EPINEPHRINE AND GLUCOSE SUPPLEMENTATION**" is a bonafide record of the research work carried out by **Ms. Finla Chathu**, under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

(C.S. PAULOSE)

Cochin 682 022
2-02-07

CENTRE FOR NEUROSCIENCE * UNIVERSITY OF SCIENCE AND TECHNOLOGY * COCHIN 682 022 KERALA INDIA * DEPARTMENT OF BIOTECHNOLOGY *

Dr. C.S. PAULOSE M.Sc., Ph.D. FIMSA, FGSI
DIRECTOR, CENTRE FOR NEUROSCIENCE
HEAD, DEPT. OF BIOTECHNOLOGY
Cochin University of Science & Technology
Cochin-682 022, Kerala, India

DECLARATION

I hereby declare that this thesis entitled "**ADRENERGIC AND GLUTAMERGIC RECEPTOR GENE EXPRESSION AND THEIR FUNCTIONAL REGULATION IN THE CEREBRAL CORTEX OF HYPOXIA INDUCED NEONATAL RATS: ROLE OF OXYGEN, EPINEPHRINE AND GLUCOSE SUPPLEMENTATION**" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance and supervision of **Dr. C. S. Paulose**, Director, Centre for Neuroscience, Reader & Head, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

Cochin - 682 022
Date: February 2, 2007.

FINLA CHATHU.
Reg. No. 2858

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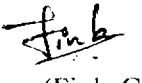

(Finla Chathu)

Dedicated To My Beloved Parents and Dearest Brother.....

ABBREVIATIONS USED IN THE TEXT

AADC	Aromatic amino acid decarboxylase
5-HIAA	5-Hydroxy indole - 3 acetic acid
5-HT	5-Hydroxy tryptamine
5-HTP	5-Hydroxy tryptophan
ACh	Acetylcholine
AMPA	Amino-3-hydroxy-5-methysoxazole-4-propionic acid
AR	Adrenergic receptor
ATP	Adenosine triphosphate
B_{max}	Maximal binding
bpm	Beats per minute
cAMP	cyclic Adenosine monophosphate
CAT	Catalase
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CP	Cerebral palsy
CPR	Cardio pulmonary resuscitation
CT	Threshold cycle
DA	Dopamine
DEPC	Diethyl pyro carbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECD	Electrochemical detector

EDTA	Ethylene diamine tetra acetic acid
EEG	Electro encephalogram
EPI	Epinephrine
GABA	Gamma aminobutyric acid
GDH	Glutamate dehydrogenase
Glu	Glutamate
GOD	Glucose oxidase
GPCRs	G protein-coupled receptors
GPx	Gluthatione peroxidase
H/H	Hypercapnic hypoxia
H_2O_2	Hydrogen peroxide
HBOT	Hyperbaric oxygen therapy
HI	Hypoxic-ischemia
HIE	Hypoxic-ischemic encephalopathy
HIF	Hypoxia-induced factor
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
i.p	Intra-peritoneally
ICU	Intensive care units
IP_3	Inositol triphosphate
KA	Kainate
K_d	Dissociation constant
K_m	Michaelis Constant
mRNA	Messenger Ribonucleic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form

NBM	Nucleus basalis magnocellularis
NE	Norepinephrine
NICU	Neonatal intensive care units
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
O.D	Optical density
p	Level of significance
PEA	Pulseless electrical activity
PFC	Prefrontal cortical
PKA	Protein kinase A
pO_2	Partial pressure of Oxygen
POD	Peroxidase
PTX	Pertussis toxin
PVL	Periventricular leukomalacia
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M.	Standard error of mean
SIDS	Sudden infant death syndrome
SOD	Superoxide dismutase
V_{max}	Maximal Velocity

CONTENTS

Introduction

Hypoxia is one of the major causes of damage to the foetal and neonatal brain. Newborn babies are frequently exposed to hypoxia and ischemia during the perinatal period as a result of stroke or problems with delivery or respiratory management after delivery (William *et al.*, 2005). Although systemic and cerebrovascular physiologic factors play an important role in the initial phases of hypoxic-ischemic injuries, the intrinsic vulnerability of specific cell types and systems in the developing brain is more important in determining the effect of damage and functional disability. In mild cases, hypoxia causes inattentiveness, poor judgment and motor in-coordination. If the lack of oxygen to the brain is limited to a very brief period of time, damage with varying levels reverse to function, depending on the extent of injury. Cerebral hypoxia refers to a condition in which there is decrease in oxygen supply to the brain in spite of adequate blood flow. Drowning, strangling, choking, suffocation, cardiac arrest, head trauma, carbon monoxide poisoning and complications of general anesthesia create conditions that lead to cerebral hypoxia. Hypoxia affects the central nervous system (CNS) both functionally and morphologically (Flynn *et al.*, 1977; Nelson & Lynch, 2004).

Foetuses, that experience injuries in the womb, premature births and birth complications, live rest of their lives in fear of growth and development (Mark, 1993). The acute interruption or reduction of cerebral blood flow, induced by several factors and clinical pathologies, reduces available oxygen to the nervous system. As the placenta stops growing during the final months of pregnancy, it becomes tough and fibrous, causing blood vessels degeneration, making the foetus more susceptible to hypoxia (Heinz, 1970, Hein & Kobilka 1995). Furthermore, the weight of the foetus

pressing down into the pelvis can compress blood vessels supplying the placenta, producing additional placental failure (Briend, 1979). Practice contractions near birth give the foetus periodic "squeezes," decreasing oxygen level even further, (Joseph, 1947) while birth itself is so hypoxic that "hypoxia of a certain degree and duration is a normal phenomenon in every delivery," not just in more severe cases. The effects on the foetus due to extreme hypoxia are dramatic: normal foetal breathing stops, foetal heart rate accelerates, then decelerates, and the foetus thrashes about frantically in a life and death struggle to liberate itself from its terrifying asphyxiation (Peter & Peth, 1980). Sometimes continuous seizures (status epilepticus) occur as a result of hypoxia (Lucas, 2002). This causes either focal or global brain damage, with characteristic biochemical and molecular alterations that can result in permanent or transitory neurological sequelae or even death (Rodrigo *et al.*, 2005).

Hypoxia has been implicated in CNS pathology in a number of disorders including stroke, head trauma, neoplasia, vascular malformations and neurodegenerative diseases. Hypoxia in newborn infants results in severe lifelong consequences. The brain, lungs, heart and kidneys are particularly sensitive to low oxygenation (Li & Jackson, 2002). Brain cells are extremely sensitive to oxygen deprivation and begin to die within five minutes after oxygen supply has been blocked. Cerebral cortex is comprised of layers of neurons exhibiting distinct morphologies and synaptic connections (McConnell, 1991). It is a standard practice to resuscitate newborn infants, both term and premature, who are asphyxiated at birth, with 100% oxygen. In addition, a small number of these newborns will require the administration of epinephrine (10μg/kg) and intravenous fluids, which include 10% glucose (500mg/ kg body wt) as part of their initial resuscitation. The safety in the use of 100% oxygen in resuscitation has been raised in several studies. Brain damage due to an episode of cerebral hypoxia remains a major problem in the human infant

(Tuor *et al.*, 1996). Every year thousands of newborn infants require some form of resuscitation immediately after birth. Over the past decades, neonatal resuscitation programme have been well developed, but some of the procedures employed in these programme are not based on scientific evidence (Nong *et al.*, 2000).

In some animals, splanchnic innervation is immature at birth; yet adrenal catecholamine secretion has been shown to occur during physiological stresses, such as hypoxia (Roger, 2004). Hedner *et al.*, (1980) reported that hypoxia causes an increased catecholamine synthesis in the immature rat adrenal gland, presumably mediated by an increased tyrosine hydroxylase activity induced via splanchnic nerves during hypoxia. Circulating catecholamines originate to a large extent from the adrenal medulla and serve vital functions in relation with cardiovascular, respiratory and metabolic responses to the stress associated with birth (Lagercrantz, 1996). The biological actions of the catecholamines are initiated through their interaction with two different types of specific cell membrane receptors, α- and β-adrenergic receptors. Epinephrine has both α- and β-adrenergic stimulating properties; of which α-adrenergic mediated vasoconstriction is the most important action (Melanie *et al.*, 2002). Vasoconstriction elevates the perfusion pressure during chest compression, enhancing delivery of oxygen to the heart and brain (Berkowitz *et al.*, 1991). α_2-adrenoceptors mediate regulatory influence over various physiological, behavioural and endocrine functions and are implicated in conditions such as hypertension, anxiety, endogenous depression and cognitive functions (Maze & Tranquilli, 1991). Stress applied to laboratory animals' results in a decreased density of α_2-adrenergic autoreceptors in the hippocampus and amygdala, reflecting down-regulation in response to elevated circulating endogenous catecholamines (Rusnak *et al.*, 1998).

Investigations on the CNS responses to oxygen deprivation are of obvious importance in revealing mechanisms that participate in coordinated behavior of

respiratory and vasomotor responses to hypoxia. Adaptation to continued moderate hypoxia in the rat brain includes structural and metabolic changes. Brain injury in newborns can cause deficits in motor and sensory function (Frances *et al.*, 2001). A large amount of investigation has focused on cytokine and hypoxia-ischemia-mediated injury to the developing cortex and periventricular white matter as the cause of the neurodevelopmental handicaps suffered by infants who have experienced perinatal brain injury. Energy failure, free radical, cytokine and excitatory amino acid release, and caspase-dependant cell death are known to contribute to injury in the neo-cortex, striatum and periventricular white matter (Back *et al.*, 1998; Cheng *et al.*, 1998). However, the degeneration of thalamus and other non-forebrain structures after hypoxia-ischemia is studied less frequently. Injury to somatosensory thalamus has been described in human newborns after hypoxic-ischemia (Barkovich, 1995; Roland *et al.*, 1998) and contribute to sensory motor deficits in infants with perinatal brain injury and cerebral palsy. Damage to the brain during development affects typical patterns of neuronal connectivity (Finlay *et al.*, 1979). The foetal brain can protect itself from hypoxia by increasing cerebral blood flow for a period between one and three hours, but as the brain becomes increasingly acidotic, the blood pressure falls, inducing ischemic injury. Apoptosis, which involves activation of genetically determined cell-suicide programme, has been observed in postmortem brain tissue from infants after hypoxic-ischemic insults (Pulera *et al.*, 1998, Yue *et al.*, 1997). Comparison of adult and immature animal models of hypoxic-ischemia suggests that apoptosis is more prevalent in the immature brain (McDonald *et al.*, 1997, Li *et al.*, 1998). Nakajima *et al.*, (2000) reported that the relative numbers of apoptotic versus necrotic cells in a rodent model of hypoxic ischemia indicate that many regions such as the cerebral cortex and basal ganglia contain high numbers of apoptotic cells for over 7 days after hypoxia-ischemia.

Excitotoxicity refers to death of neurons and certain other cells mediated by excessive stimulation of extracellular excitatory amino acid receptors mainly glutamate (Choi & Rothman, 1990). Glutamate dehydrogenase (GDH) catalyses the reductive interconversion of α-ketoglutarate and L-glutamic acid. Glutamate is a putative neurotransmitter and a precursor of the inhibitory gamma-aminobutyric acid (GABA). It is reported that GDH plays an important regulatory role of glutamate pathway in brain neural network disturbances and neuronal degeneration (Biju & Paulose, 1998). Selected neuronal circuits as well as certain populations of glia such as immature periventricular oligodendroglia die from excitotoxicity triggered by hypoxic ischemia. These patterns of neuropathologic vulnerability are associated with clinical syndromes of neurologic disability such as the extrapyramidal and spastic diplegia forms of cerebral palsy. The cascade of biochemical and histopathologic events triggered by hypoxic ischemia can extend for days to weeks after the insult is triggered, creating the potential for therapeutic interventions (Michael *et al.*, 2001). Normally these receptors mediate physiologic excitatory effects of the dicarboxylic acid glutamate, one of the most ubiquitous and versatile neurotransmitters in the brain. When excessively stimulated by combinations of elevated synaptic levels of glutamate and membrane depolarization associated with ischemia, channels associated with these receptors allow a lethal flow of Ca^{2+} and sodium to enter neurons. Excitotoxicity appears to be even more intimately involved in the pathogenesis of cell destruction from hypoxic ischemia in the developing brain than in the adult. Although we focus here on neuronal systems, recent evidence suggests that immature white matter can also be damaged by excitotoxicity triggered through glutamate receptors by hypoxic-ischemia (Follett *et al.*, 2000, Gressens, 1999). Developmental differences in the function and expression of glutamate receptors dictate the response of the newborn brain to injury (Deng *et al.*, 2004). N-methyl-D-aspartic acid (NMDA) activity in the

neonatal rat brain is required for cell survival and the factors that regulate apoptosis in the neonatal brain that play important roles in the final development of the somatosensory cortex (Anand & Scalzo, 2000).

Cerebral palsy (CP) means "brain paralysis" refers to motor or postural abnormalities that are noted during early development. These anomalies are thought to be associated with prenatal, perinatal or postnatal events of varying etiologies (often multifactorial in nature). CP generally is considered to be a static encephalopathy that is nonprogressive in nature. The immature brain has only a limited number of ways of responding to acute or chronic injury and these essentially consist of neuronal and white matter loss and glial proliferation. These changes occur over many days and weeks. By the time a child presents with cerebral palsy during the first years of life, the neuropathological effects of any hypoxic-ischemic injury or other injury will have become modified by these changes and by further postnatal brain development (Blair & Stanley, 1988). During the perinatal period and infancy (first 2 years post natal), there are several incidences that can cause brain damage. Complications with the endocrine system due to hypoxia include respiratory distress syndrome, hypoglycemia or hypothyroidism (Nelson & Ellenberg, 1986). Nevertheless CP is secondary to prenatal, perinatal or neonatal insult; or is secondary to neuronal damage at the cellular level in the neurotransmitter or receptor systems. The global effects are the result of impaired communication between the brain and the muscles which decreased the control of movements that causes poor motor coordination, balance and abnormal movements. As a result, these motor difficulties are secondary to brain damage or abnormal brain development. In the individual who has cerebral palsy and epilepsy, this disruption may be spread throughout the brain and cause varied symptoms all over the body as in tonic-clonic seizures, or is confined to just one part of the brain and cause more specific symptoms, as in partial seizures. Neonatal and infantile

seizures suggest underlying structural brain disease with the possibility of adverse motor consequences (Singhi *et al.*, 2003).

Acetylcholine (ACh) is one of the principal neurotransmitters of the parasympathetic system. Extensive evidence supports the view that cholinergic mechanisms modulate learning and memory formation. Evidence for cholinergic regulation of multiple memory systems, noting that manipulations of cholinergic functions in many neural systems can enhance or impair memory for tasks generally associated with those neural systems (Paul, 2003). The magnitude of ACh release in different neural systems regulates the relative contributions of these systems to learning. ACh is the neurotransmitter that is released by stimulation of the vagus nerve, which alters heart muscle contractions. It is important for the movement of other muscles as well. The cholinergic innervation of the cerebral cortex has been extensively investigated because of its role in arousal, learning and memory (Metherate *et al.*, 1992, Voytko *et al.*, 1994). Multiple neuropsychological tests have revealed neuropsychological dysfunction, which is largely due to brain hypoxia. Glucose acts directly on the brain to regulate neural processing, a function that seems incompatible with the traditional view that brain glucose levels are high and invariant except under extreme conditions. However, recent data suggest that the glucose levels of the brain extracellular fluid are lower and more variable than previously supposed (Oltmanns *et al.*, 2004).

In the present work, the role of oxygen, epinephrine and glucose supplementation in regulating neurotransmitter contents, adrenergic and glutamate receptor binding parameters in the cerebral cortex of experimental groups of neonatal rats were investigated. The study of neurotransmitters and their receptors in the cerebral cortex and the EEG pattern in the brain regions of neonatal rats were taken as index for brain damage due to hypoxia, oxygen and epinephrine. Real-Time PCR

work was done to confirm the binding parameters. Second messenger, cyclic Adenosine Monophosphate (cAMP) was assayed to find the functional correlation of the receptors. Behavioural studies were carried out to confirm the biochemical and molecular studies. The efficient and timely supplementation of glucose plays a crucial role in correcting the molecular changes due to hypoxia, oxygen and epinephrine. The addictive neuronal damage effect due to oxygen and epinephrine treatment is another important observation. The corrective measures from the molecular study brought to practice will lead to maintain healthy intellectual capacity during the later developmental stages, which has immense clinical significance in neonatal care.

OBJECTIVES OF THE PRESENT STUDY :

1. To induce hypoxia and supplement glucose, epinephrine and oxygen in the neonatal rats.

2. To measure the blood glucose level in the serum of experimental groups of neonatal rats.

3. To measure the neurotransmitters content in the cerebral cortex and adrenals of the experimental groups of neonatal rats using High Performance Liquid Chromatography.

4. To study the glutamate dehydrogenase activity in the cerebral cortex and liver of experimental groups of neonatal rats.

5. To study the cholinergic activity using acetylcholine esterase in the cerebral cortex and muscle of experimental groups of neonatal rats.

6. To study the adrenergic receptor, α_2-AR, β-AR and glutamate receptor binding parameters in the cerebral cortex of experimental groups of neonatal rats.

7. To study the gene expression of adrenergic receptors - α_2A-AR, β2-AR and glutamate receptor (NMDAR1) in the cerebral cortex of experimental groups of neonatal rats using Real-Time PCR.

8. To study the second messenger cAMP content in the cerebral cortex of experimental groups of neonatal rats.

9. To study the brain activity generated in the brain regions- Frontal, Temporal, Occipital and Parietal lobes of the experimental groups of neonatal rats using electroencephalogram.

10. To study the behavioural activities of the experimental groups of neonatal rats using Elevated Plus-Maze and Open-Field Test.

Literature Review

> *"A child with a slight brain defect often appears no different from a normal child. His intelligence quotient may lie in the range considered normal, but one never knows how much higher it would have been if his brain had escaped damage in the uterus or during birth."* (Windle, 1969).

IMPACT OF HYPOXIA

Brain is of special interest for hypoxia studies as it is extremely sensitive to reductions in oxygen supply. The brain damage occurs within a few minutes of hypoxia and can result in severe and complex disabilities or death (Slavin, 1994). The reason for this vulnerability is that the brain has committed high energy costs that cannot be compromised. 50–60% of the brain cells energy expenditure is devoted to transporting ions across the cell membranes in order to maintain cellular ion homeostasis (Lipton, 1999). As a result, the brain suffers energy failure after only few minutes interruption in oxygen supply.

Cerebral hypoxic ischemia appears to stimulate massive extracellular catecholamine release in the cortex, striatum and hippocampus. *In vitro* studies have also demonstrated elevated catecholamine concentrations and reduced uptake in gerbil synaptosomes during ischemia (Weinberger & Neives-Rosa, 1988). Central norepinephrine release during brain ischemia increases neuronal metabolism and exaggerates the discrepancy between impaired blood flow to ischemic tissue and an increase in the metabolic demand. Further, metabolism of excessive norepinephrine can lead to the formation of neurotoxic free radicals, whereas prevention of oxidative

deamination of catecholamines reduces hydrogen peroxide production during reperfusion (Simonson *et al.*, 1993). In addition to their direct detrimental effects, catecholamines also sensitize neurons to the excitatory amino acid glutamate, thus exacerbating the damage caused by glutamate during ischemia.

FUNCTIONAL NEUROPHARMACOLOGY OF THE SYMPATHETIC NERVOUS SYSTEM

Most organs are innervated by sympathetic and para-sympathetic divisions of the autonomic nervous system, but the adrenal medulla, sweat glands, and somatic blood vessels are regulated exclusively by the sympathetic nervous system. Preganglionic sympathetic nervous system fibers synapse within the adrenal medulla (Adams & Victor, 1993) and promote catecholamine secretion (Yoshimatsu *et al.*, 1987). Sympathetic nervous system and adrenomedullary responses are often dissociated and can be complementary (Swann *et al.*, 1991; Takahashi *et al.*, 1993). The relative independence of the sympathetic nervous system from central nervous system (CNS) regulation, its more permeable blood-nerve barrier (Kiernan,1996) and the ability of its end organs to continue functioning when autonomic nerves are interrupted, all contribute to its capacity for autonomous function. The sympathetic nervous system is regulated by the frontal cortex and hypothalamus, whereas the para-sympathetic division is controlled by the anterior and medial hypothalamus (Carpenter, 1973). Stimulation of the lateral hypothalamus increases adrenal nerve activity and the lateral hypothalamic area provides organ or site-specific regulation of sympathetic nervous system activity (Yoshimatsu *et al.*, 1993). Most dorsal hypothalamic spinal projection neurons are dopaminergic and appear to be involved in autonomic function (Cechetto *et al.*, 1988). Dopamine terminal axon density is highest in the intermediolateral cell columns of the spinal cord, where preganglionic

sympathetic nervous system neurons originate, and microelectrophoretic dopamine application there inhibits sympathetic preganglionic neurons (Lindvall *et al.*, 1983). Retrograde labeling has identified caudal lateral hypothalamic area neurons as likely sympathetic nervous system "central command neurons" (Jansen *et al.*, 1995).

The preganglionic and postganglionic synapses in the parasympathetic system releases acetylcholine (ACh) but postganglionic sympathetic nervous system neurons release norepinephrine. Norepinephrine acts only within the neuroeffector junction into which it is released (Kopin, 1985). Plasma norepinephrine levels reflect overflow from sympathetic nervous system neuroeffector junctions and there is good correlation between muscle sympathetic nerve activity and plasma norepinephrine levels (Wallin, 1984). Norepinephrine promotes its own release by means of prejunctional β-adrenoceptors; at higher concentrations. It inhibits its own release by means of prejunctional α-adrenoceptors (Sneddon *et al.*, 1996). Series of high frequency action potentials may cause norepinephrine to accumulate, potentiating its functional effects (Gonon *et al.*, 1993). Symptoms of norepinephrine toxicity include anxiety, pallor and intense diaphoresis. Epinephrine is the major catecholamine produced by the adrenal medulla; in contrast to norepinephrine, it is released into the circulation and acts at distant receptor sites. Symptoms of epinephrine toxicity include fear, anxiety, tenseness, restlessness, tremor, weakness, dizziness, pallor, respiratory difficulty and palpitations. Norepinephrine constitutes no more than 20% of the catecholamine content of the adrenal medulla (Weiner, 1980) and epinephrine has no significant activity at sympathetic nerve endings (deGroot & Chusid, 1988). Epinephrine is active primarily at β-adrenoceptors (Benschop *et al.*, 1996) and is much more potent than norepinephrine at β_2 receptors, where norepinephrine has minimal activity (Lefkowitz *et al.*, 1990). Second messengers for adrenoceptors are adenylate cyclase (activated by β, inhibited by α_2) and intracellular Ca^{2+} (increased

by α_1) (Motulsky & Insel, 1982). Adrenoceptors in smooth muscle and glands are predominantly α_1, whereas vasodilator β_2-adrenoceptors in skeletal muscle normally predominate over vasoconstrictor α-adreno-ceptors. Skeletal muscle contractility is increased by β_2-adrenoceptors and cardiac rate and contraction strength are both increased by β-adrenoceptors (Benschop *et al.*, 1996). High levels of D_2 receptor mRNA are expressed in the normal adrenal gland. In animals, D_2 agonists inhibit catecholamine release from adrenal glands and D_2 presynaptic receptors located on norepinephrine nerve terminals inhibit norepinephrine release if activated during nerve stimulation. In humans, D_2 agonists cause inhibition of sympathetic output that is abolished by D_2 antagonists, but only at higher degrees of sympathetic stimulation (Mannelli *et al.*, 1997). These data are consistent with previously discussed anatomical evidence for dopaminergic inhibition of sympathetic nervous system function and indicate that state-dependent factors mediate D_2 antagonist effects (Ronald, 1999).

NEUROTRANSMITTERS AND THEIR ROLE IN HYPOXIA

The brain neurotransmitters' receptor activity and hormonal pathways control many physiological functions in the body. The pharmacological challenge strategy involves administering a test agent under controlled conditions to elucidate some aspect of biological or behavioral function in the organism being studied. It is based on the assumption that true functional abnormalities may not be evident in the basal state because of the action of compensatory mechanisms. Under such circumstances, pharmacological perturbation of a specific target system may reveal information about the functional integrity of both that system and systems that modulate it (Lawrence *et al.*, 2000). Basing a treatment on symptoms alone (traditional medicine) will not provide the information needed to address the underlying brain imbalance. New

sophisticated equipment and tests are now available to evaluate neurotransmitter imbalances using a urine or blood sample. This provides a neurotransmitter baseline assessment and is useful in determining the root causes for many diseases and illnesses. Laboratory analysis can now provide precise information on brain neurotransmitter deficiencies or overloads, as well as detect hormonal and nutrient co-factor imbalances which influence neurotransmitter production. Testing helps to determine exactly which neurotransmitters are out of balance and helps to determine which therapies are needed for an individualized treatment plan. It also helps in monitoring the effectiveness of an individual's treatment.

ADRENERGIC RECEPTORS

Adrenergic receptors belong to the large family of G-protein coupled receptors. These receptors form the interface between the sympathetic nervous system as well as many endocrine and parenchymal tissues (Hein & Kobilka, 1995). The adrenergic receptors contain seven stretches of 20-28 hydrophobic amino acids that are likely to represent membrane-spanning regions. Adrenergic receptors are mainly classified into α and β-adrenergic receptors.

α-ADRENERGIC RECEPTORS

Based on the pharmacological and functional criteria, α-adrenergic receptors are further subdivided into two subclasses termed α_1 and α_2 adrenergic receptors (Hoffman & Lefkowitz , 1980, Lefkowitz & Caron, 1988). α_1 has three subclasses- α_{1A}, α_{1B}, α_{1C}. (Price *et al.*, 1994) and α_2 has $\alpha_{2A/D}$, α_{2B} and α_{2C} (Hamamdzic *et al.*, 1995).

α_1-ADRENERGIC RECEPTORS

α_1-adrenergic receptors are one of the three subfamilies of G protein coupled receptors activated by epinephrine (EPI) and norepinephrine (NE) to control important functions in many target organs. Three human subtypes (α_{1A}, α_{1B}, α_{1c}) are derived from separate genes and are highly homologous in their transmembrane domains but not in their amino or carboxyl termini. Although all three subtypes activate the same G[q]/11 signaling pathway, they also appear to interact with different protein binding partners. Recent evidence suggests they may also form dimers, and may initiate independent signals through pathways yet to be clearly elucidated. Thus, this subfamily represents a common phenomenon of a group of similar but non-identical receptor subtypes activated by the same neurotransmitter, whose individual functional roles remain to be clearly established (Hague *et al.*, 2003).

α_2-ADRENERGIC RECEPTORS

α_2 adrenergic receptors play an essential role in regulating neurotransmitter release from sympathetic nerves and from adrenergic neurons in the CNS (Bucheler *et al.*, 2002). α_2-adrenergic receptors mediate many of the physiological actions of the endogenous catecholamines, adrenaline and noradrenaline, and are targets of several therapeutic agents. α_2-adrenergic receptors are coupled by pertussis-toxin sensitive G proteins to various effectors, including adenylate cyclase and ion channels. The α_{2A}-adrenergic receptors subtype appears to mediate reduction in blood pressure following α_{2A} agonist administration (MacMillan *et al.*, 1998). Three human α_2-adrenoceptor subtype genes have been cloned and designated as $\alpha_{2\text{-}C10}$, $\alpha_{2\text{-}C4}$, and $\alpha_{2\text{-}C2}$, according to their location on human chromosomes 10, 4 and 2. They correspond to the previously identified pharmacological receptor subtypes α_{2A}, α_{2C} and α_{2B}. The receptor proteins share only about 50% identity in their amino acid sequence, but some structurally and functionally important domains are very well conserved. The most obvious

functionally important differences between the receptor subtypes are based on their different tissue distributions; e.g. the α_{2A} subtype appears to be an important modulator of noradrenergic neurotransmission in the brain. The three receptors bind most α_2-adrenergic drugs with similar affinities, but some compounds (e.g. oxymetazoline) are capable of discriminating between the subtypes. All α_2-adrenoceptors couple to the pertussis-toxin sensitive inhibitory G proteins Gi and G(o), but recent evidence indicates that other G proteins also interact with α_2-adrenoceptors, including Gs and Gq/11. Inhibition of adenylate cyclase activity, which results in decreased formation of cAMP, is an important consequence of α_2-adrenergic receptor activation (Aantaa *et al.*, 1995).

α_2-adrenergic receptors are known to have a critical role in regulating neurotransmitter release from sympathetic nerves and from adrenergic neurons in the central nervous system (Miller, 1998; Langer, 1997). Hein & Kobilka (1995) have now studied neurotransmitter release in mice in which the genes encoding the three α_2-adrenergic receptor subtypes were disrupted. They found that both the α_{2A} and α_{2C}-subtypes are required for normal presynaptic control of the transmitter release from sympathetic nerves in the heart and from central noradrenergic neurons. α_{2A}-adrenergic receptors inhibit transmitter release at high stimulation frequencies, whereas the α_{2C}-subtype modulates neurotransmission at lower levels of nerve activity (Hein *et al.*, 1999).

The pharmacological characterisation of α-adrenoceptors has been facilitated by the introduction of [^{3}H] Yohimbine. Its use has shown that the absolute affinities and rank order of potency of a number of antagonists for the α_2-adreneoceptor binding sites on human platelets differ from those on rat cerebral cortex membranes (Cheung *et al.*, 1982). EPI and NE are endogenous amines that are secreted in response to stress; they do not cross the blood-brain barrier. Epinephrine is a more potent agonist

of β- than α-adrenoceptors, whereas NE is primarily an α-adrenoceptor agonist with some β activity. This lack of specificity compromises their use as pharmacological challenge agents. The effects of these substances in anxiety disorders are not established, as they have rarely been studied in well-diagnosed patients.

β-ADRENERGIC RECEPTORS

Adrenergic receptor is a member of the large family of G protein-coupled receptors and is subjected to a complex regulation by hormones and other signaling molecules. The catecholamines, EPI and NE evoke specific responses in a variety of tissues. The β-adrenergic system has in many ways served as the premier model to investigate the processes by which external stimuli regulate cellular behavior. Stimulation of β-adrenergic receptor normally results in signaling by the heterotrimeric G_s protein, leading to the activation of adenylate cyclase, production of cAMP, and activation of cAMP-dependent protein kinase A (PKA). Gu *et al.* (2000) reported that cell death of thymocytes can be induced after stimulation of β-adrenergic receptor, or by addition of exogenous cAMP.

β-adrenergic receptors are subclassified into β_1, β_2 and β_3 (Dohlman *et al.*, 1991). The concept of two subtypes of β-adrenergic receptors termed β_1 and β_2 developed by Lands *et al.*, (1967) was initially interpreted in terms of absolute organ specificity such that the heart contained exclusively β_1 and the bronchial system contained β_2 adrenergic receptors (Lands *et al.*, 1967). Tan & Curtis-Prior (1983) proposed the presence of another subtype of β-adrenergic receptor in the rat adipocytes which was then termed as β_3- adrenergic receptors. Isoprenaline, CGP 12177, BRL 37344 and NE are used as potent agonists of β-adrenergic receptors. The antagonists of β-adrenergic receptors widely used are propranolol, atenolol, betaxolol

and practalol (Arch & Kaumann, 1993). The activation of β-ARs will activate AC and increase the cAMP content of the cell. The α-ARs and β-ARs have been well characterized pharmacologically and, more recently, the use of molecular biology techniques has provided new information about the structure and functional domains of these receptors (Berridge & Irvin, 1989).

ROLE OF CATECHOLAMINES IN MACRONUTRIENT METABOLISM

In comparison with other tissues, the brain is very vulnerable to hypoxia because of its high metabolic rate despite the low oxygen stores and small reserves of high-energy phosphates or carbohydrates. Thus, hypoxic insults result in a variety of functional changes in the central nervous system. The catecholamines that are relevant to macronutrient metabolism are epinephrine (adrenaline) and norepinephrine (noradrenaline).

Epinephrine is a hormone released from the medulla of adrenal glands. Stimuli for epinephrine release include stress and anxiety, exercise, a fall in blood glucose concentration and a loss of blood. Its release is initiated by nervous signals that arise from the hypothalamus, the integrating centre of the brain. EPI acts on tissues through adrenergic receptors (adrenoceptors) in cell membranes. They are linked with metabolic processes through a signal chain. For certain type of adrenergic receptors, the first step is the interaction of the receptor with a trimeric protein that can bind guanosine triphosphate called G-protein. There are inhibitory and stimulatory G-proteins, named for their effects on the next step in the sequence, the enzyme adenylate cyclase. Adenylate cyclase produces cyclic 3',5'-adenosine monophosphate (cAMP), which then acts on the cAMP-dependent PKA to bring about phosphorylation of key proteins including glycogen phosphorylase and hormone sensitive lipase. Therefore, epinephrine acting on β-receptors will cause mobilization

of stored fuels, glycogen and triglycerol, raising plasma concentrations of glucose and non-esterified fatty acids. This was termed by the American physiologist Walter Cannon in 1915 the 'fight or flight' response, implying that epinephrine, released in response to stress or anxiety, produces fuels that may be used to run away or stand up to an aggressor. The inhibitory effects of epinephrine, mediated by α_2 -adrenergic receptors, may be seen as moderating the effects of over stimulation *via* β-receptors. For instance, adipocytes have both β- and α_2 - adrenergic receptors, the latter presumably opposing excessive lipolysis that might be brought about by high concentrations of norepinephrine (Frayn 1995; 2003).

Norepinephrine is a neurotransmitter of the adrenergic nervous system originating from the locus coeruleus, which innervates most parts of the brain and plays a role in a variety of higher brain functions such as emotions and moods. It is known that norepinephrine is massively released by ischemia (Mordecai *et al.*, 1989). However, it is not known how the receptors for norepinephrine change after brain ischemia. It is released at the ends of sympathetic nerves (nerve terminals) in tissues. It acts on adrenergic receptors, which are identical to those acted upon by epinephrine. The stimuli for norepinephrine release are similar to those of epinephrine, and in many cases it is not clear which exerts the more important effects. Most of the norepinephrine released from sympathetic nerve terminals is taken up again by the nerve endings for degradation or re-secretion, but some always escape or spills over, and may reach the plasma. Plasma concentrations of norepinephrine are usually higher than those of epinephrine, and when norepinephrine is present at elevated concentrations (*eg.* during strenuous exercise), it is believed to act as hormone as well (Michael *et al.*, 2004).

Molecular cloning has led to the identification of three structurally and pharmacologically distinct α_2-adrenoceptor subtypes, termed α_{2A}, α_{2B} and α_{2C}. All three α_2-adrenoceptor subtypes are widely distributed in the nervous system, although the α_{2A} and α_{2C} subtypes appear to predominate in the central nervous system (MacMillan *et al.*, 1996). A few studies have reported a significant role for the α_{2C}-adrenoceptor subtype in both brain monoamine release and hypothermia (Bucheler *et al.*, 2002). Ischemia causes a number of changes that may lead to a change in the number of adrenoceptors. These are changes in release of transmitters, (Matsumoto *et al.*, 1984) including norepinephrine, levels of second messengers such as cyclic AMP, (Lin *et al.*, 1983) protein kinase, (Araki *et al.*, 1992) and the capacity for protein and RNA synthesis (Yanagihara, 1976).

Reports say that α_1-, α_2-, and ß- adrenoceptors are abundantly present in the cerebral cortex and hippocampus, with higher density in the cerebral cortex than in the hippocampus (Johnson & Minneman, 1985, Rouot *et al.*, 1982, Minneman *et al.*, 1979). The changes in α_2-adrenoceptors, which have been first identified as presynaptic autoreceptors but are also present as postsynaptic receptors in the brain, was largest among adrenoceptors by ischemia (U'Prichard *et al.*, 1979). It is suggested that the mechanisms that regulate the receptors are different among various types of receptors, and the vulnerability of the cells to ischemia is also different (Tsunetaka *et al.*, 1995).

HYPOXIA AND ADRENERGIC NEUROTRANSMITTERS

The chronic effects of catecholamines on hepatic glucose metabolism have not been well studied, especially in settings in which other counter regulatory hormones are also elevated. Chronic β-adrenergic stimulation does not increase

glucose production in humans. The acute administration of a β receptor-stimulating agent profoundly affects insulin-mediated glucose metabolism; however, little is known about the impact of chronic beta receptor stimulation on glucose metabolism and insulin sensitivity (Scheidegger *et al.*, 1984). Norepinephrine did not amplify the stress hormone-induced increase in hepatic glucose production. In the acute setting norepinephrine is considerably less potent than epinephrine in stimulating hepatic glycogenolysis (Connolly *et al.*, 1991; Stevenson *et al.*, 1991). Although, acutely, epinephrine has more potent stimulatory effects on gluconeogenesis than glucagon (Stevenson *et al.*, 1987, 1991), its chronic stimulatory effects on this process appear to be less substantive than that of glucagon when multiple stress hormones are increased. Epinephrine also plays a central role by enhancing hepatic glycogenolysis. Despite its very potent acute effects on gluconeogenic precursor supply, these actions play a relatively minor role chronically. Cortisol augments hepatic glycogen stores despite marked increases in other counterregulatory hormones. In addition, it maintains the gluconeogenic precursor supply, thus supporting the glucagon- and, to a lesser extent, epinephrine-mediated increase in gluconeogenesis. Interestingly, circulating norepinephrine does not play a major role in augmenting hepatic glucose metabolism during chronic stress; no hormone plays the central role in the chronic enhancement in glucose metabolism during stress. Rather, these hormones complement one another to allow an efficient stimulation of hepatic metabolism. The relative importance of a given stress hormone in a particular stress cannot be addressed, because the impact of an individual hormone will depend on the specific stress and the accompanying endocrine functions (Owen *et al.*, 1997).

GLUTAMATE RECEPTORS

The majority of excitatory synapses are glutamergic (Glu), in which Glu transmits the signal through postsynaptic ionotropic [N-methyl-D-aspartic acid (NMDA), amino-3-hydroxy-5-methysoxazole-4-propionic acid (AMPA), and kainate (KA)] and metabotropic receptors (Bettler & Mulle, 1995). Glu is a fast excitatory transmitter in the CNS and has been shown, with GABA, to interact primarily with receptors in the synaptic cleft (Dingledine *et al.*, 1999). The extracellular accumulation of glutamate results in neuronal death by activating ionotropic glutamate receptors sensitive to NMDA or AMPA–kainate (Choi, 1988). The presence of G protein-coupled glutamate receptors (metabotropic Glu receptors) has been described, and since 1991 (Conn & Pin, 1997), eight receptors have been discovered and classified into three groups based on their linkage to second messenger systems and their pharmacology: group I acts *via* the phosphoinositol system, and groups II and III inhibit adenylyl cyclase. In addition, the stimulation of receptors of these three groups directly influences voltage-gated Ca^{2+} and K^{+} channels through their G proteins, but their physiological correlate has not yet defined.

There are several reports of presynaptic localization of GluRs and their involvement in transmitter release. The fact that NMDA releases Glu (Pittaluga *et al.*, 1996), Dopamine (DA) (Kuo *et al.*, 1998) and NE (Pittaluga & Raiteri, 1992) from axon terminals and facilitate transmitter release *via* NMDA receptors. In addition, presynaptic AMPA receptor activation results in an increase of Glu release, provided that the receptor's fast desensitization was prevented by cyclothiazide (Barnes *et al.*, 1994; Desai *et al.*, 1994). Montague *et al.* (1994) suggested that Glu and NE release from cortical synaptosomes was in correlation with NMDA-induced production of nitric oxide (NO), an endogenous chemical that is able to inhibit basal membrane transporters, thereby increasing the concentration and life-span of transmitters (e.g.,

Glu and NE) released into the extracellular space. The inhibition of neuronal NO synthase by 7-nitroindazole protects against NMDA-mediated excitotoxic lesions but not against those evoked by AMPA or KA (Schulz *et al.*, 1995).

Glutamate functions as a fast excitatory transmitter in the mammalian brain. Recent experiments in a variety of preparations have shown that either blockade of synaptic transmission or the specific antagonism of postsynaptic glutamate receptors greatly diminishes the sensitivity of central neurons to hypoxia (Rothman & Olney, 1986). Glutamate triggers neuronal death when released in excessive concentrations by over excitation of its receptors (Vizi, 2000). Cell death due to excitotoxicity occurs in many types of cells in the newborn brain and the initial trigger may be impairment of the uptake of glutamate by glia, resulting in over activation of the receptors (McDonald & Johnston, 1990). It is reported that any sort of disturbances in the metabolic pathway of glutamate causes physiological and cognitive disorders (Preetha *et al.*, 1996).

The excitatory amino acid Glu is the most prevalent transmitter in the brain; its effect on postsynaptic receptors is limited by uptake process (Erecinska, 1987) and by diffusion of Glu from the cleft. The removal of Glu from the extracellular fluid, limitation of its action occurs by uptake and by diffusion (Tong & Jahr, 1994). This is accomplished by a transporter in the plasma membrane of both neurons and astrocytes (Brooks-Kayal *et al.*, 1998; Gelagashvili & Schousboe, 1998). Electrophysiological evidence was obtained that the block of Glu transporters potentiates postsynaptic excitation of Glu receptors (Tong & Jahr, 1994). The cellular uptake of Glu is driven by the electrochemical gradients of Na^+ and K^+ and is accompanied by voltage and pH changes. Hypoxia increase GABA levels in neurons by ATP depletion-induced activation of glutamate decarboxylase and by inhibiting GABA transaminase.

Hypoglycemia, which also depletes ATP, reduces neuronal levels of GABA and its precursor glutamate (Madl & Royer, 2000). Under hypoxia or ischemia, the release of aspartate, glutamate, glycine, alanine, taurine, and GABA increased mainly by a Ca^{2+}-independent mechanism. However, ischemia highly potentiated the reduction of the energy charge, as compared with hypoglycemia or hypoxia alone. Addition of glucose metabolites, pyruvate and malate, attenuated neuronal death after exposure to glutamate or H_2O_2 (Desagher *et al.*, 1997; Ruiz *et al.*, 1998).

GABA RECEPTORS

Gamma- aminobutyric acid, also known as GABA was discovered over 40 years ago as a key inhibitory neurotransmitter in the brain (Bazemore *et al*, 1957, Krnjevic & Phillis, 1963). Since then, evidence has accumulated that this amino acid function as a neurotransmitter not only in the central nervous system but also in the peripheral nervous system, including the mesenteric plexus (Amenta, 1986), major pelvic ganglia (Akasu *et al.*, 1999), sympathetic ganglia, encompassing the rat superior cervical ganglion (Wolff *et al.*, 1986; Kasa *et al.*, 1988,) and abdominal prevertebral ganglia (Parkman & Stapelfeldt, 1993). In the mammalian central nervous system (CNS), GABA is the most important inhibitory neurotransmitter occurring in 30-40% of all synapses. Three types of GABA receptors have been identified: $GABA_A$ and $GABA_C$ receptors are ligand-gated Cl^- channels, while $GABA_B$ receptors are G protein coupled (Chebib & Johnston, 1999). $GABA_A$ receptors are ligand-gated Cl^- channels that consist of a heteromeric mixture of protein subunits forming a pentameric structure and $GABA_B$ receptors couple to Ca^{2+} and K^+ channels *via* G proteins and second messengers (Johnston, 1996). In the central nervous system, application of GABA reduces excitability by a combination of $GABA_A$ and $GABA_B$ receptor activation, leading to membrane re-polarization,

reduced Ca^{2+} influx and suppression of neurotransmitter release. The genetic diversity of multiple $GABA_A$ receptor subunits permits the assembly of a vast number of receptor heteromeric isoforms. Apparently, the subunit composition determines the pharmacological profile of the resulting receptor subtypes (Barnard *et al.*, 1998). Mechanisms that modulate the stability and function of postsynaptic $GABA_A$ receptor subtypes and that are implicated in functional plasticity of inhibitory transmission in the brain are of special interest (Luscher & Keller, 2004).

Tissue, perfused with artificial cerebrospinal fluid (aCSF) at 37^0C with zero glucose and gassed with 95% nitrogen and 5% carbon dioxide, showed a fivefold increase in glutamate release with little effect on GABA release. Preconditioning, with three 5-min periods of hypoxia/hypoglycemia preceding continuous hypoxia/hypoglycemia, significantly decreased glutamate release whilst significantly elevating GABA release. These results suggest that GABA reduce the release of glutamate and consequently decrease the neurotoxic effects of glutamate (Johns *et al.*, 2000).

DOPAMINE RECEPTORS

Dopamine (DA) receptors are divided into two families designated D_1 and D_2. D_1 receptors activate G_s proteins and D_2 receptors activate G_i proteins (Missale *et al.*, 1998). Stimulation of D_2 receptors results in the inhibition of the release of DA from dopaminergic nerves. Activation of these presynaptic receptors inhibits the release from their respective nerve terminals of other neurotransmitters, such as NE, ACh, and GABA (Hársing & Zigmond, 1997), from the striatum. Although D_2 receptors are coupled to inhibition of adenylyl cyclase in some cell types (Onali *et al.*, 1985).

Acute stress increases dopamine release and metabolism in a number of brain areas (Zangen *et al.*, 1999). Dopaminergic innervation of the medial and dorsolateral prefrontal cortex appears to be particularly vulnerable to stress and relatively low intensity levels of stress are capable of promoting significant responses. The prefrontal dopaminergic neurons have a number of higher functions including attention and 'working' memory, and the acquisition of coping patterns in response to stress (Castellano *et al.*, 1999). Amphetamines and cocaine agonize these receptors and have a similar effect as stress, resulting in symptoms such as anxiety, panic, hypervigilence, exaggerated startle reflexes and paranoia (Horger *et al.*, 1999). NMDA and opiate receptors are plentiful in this area and stress-induced innervation of the fronto-cortical neurons is prevented if these receptors are selectively blocked. This increase of dopamine from the dendrites of dopamine neurons may be due to an alteration in GABA regulation of the dopamine neurons. As with noradrenergic systems, single or repeated exposures to stress potentiates the capacity of a subsequent stressor to increase dopamine function in the forebrain without altering basal dopamine turnover, suggesting that the receptors have been hyper-sensitized (Basso *et al.*, 1999).

An uncontrolled rise in extracellular dopamine has also been implicated as an important cause of pathogenesis in the hypoxic/ischemic brain (Globus *et al.*, 1988). The consequences of severe hypercapnic hypoxia (H/H) combined with brain ischemia, mostly as a result of secondary hypotension (Volpe *et al.*, 1985), are well documented to be associated with neuronal damage as a frequent cause of the chronic handicapping conditions of cerebral palsy, mental retardation, and epilepsy (Vannucci, 1997). Compelling evidence exists that in the newborn brain, specific structures and/or tissues are especially vulnerable to injury, creating syndromes of functional disabilities. In term newborns, a specific pattern of symmetric basal

ganglia and adjacent cortex injury has been revealed as the structural substrate for extrapyramidal cerebral palsy (Menkes & Curran, 1994, Hoon *et al.*, 1997, Roland *et al.*, 1998). It has been proposed that neurons that are connected in already established neuronal circuits seem to be especially vulnerable to excitotoxic damage based on a hyperactivity of the major excitatory glutamatergic input (Johnston *et al.*, 2001). However, the dopaminergic system is also sensitive to O_2 deprivation in the immature brain (Gordon *et al.*, 1990; Pastuszko *et al.*, 1993; Nakajima *et al.*, 1996). Obviously, there is no "oxygen reserve" that protects DA release and metabolism from decrease in O_2 pressure, because in the newborn piglet brain, even a small reduction of the brain tissue pO_2 causes a significant increase in the striatal extracellular DA concentration in a dose-dependent relationship. An increase of aromatic amino acid decarboxylase (AADC) activity, indicating an increase of mesostriatal dopaminergic activity in newborn piglets (Huang *et al.* 1994) is known to be associated with pronounced neuronal injury as a result of hypoxic-ischemic brain (Globus *et al.*, 1987, Ren *et al.*, 1997).

ADENOSINE RECEPTORS

Blood *et al.*, (2003) reported that in the near-term foetal sheep, adenosine mediates a decrease of cerebral metabolic rate during acute moderate hypoxia *via* the adenosine A_1 receptor activation. Furthermore, an inhibition of adenosine A_1 receptors during severe asphyxia resulted in an increased neuronal cell death accompanied by delayed suppression of neural activity and increased cerebral metabolism (Hunter *et al.*, 2003). Adenosine inhibits the evoked release of many neurotransmitters, both from peripheral nerves and in the CNS. The inhibitory effect of adenosine on NE (Fredholm & Dunwiddie, 1988) and ACh (Sperlágh *et al.*, 1997) release has been particularly well described and proved to be mediated by adenosine

A_1 receptors. Adenosine A_1 receptors have the general structure expected of G protein-linked receptors, and there is evidence that G_i proteins are involved in the inhibitory effects of adenosine on neurotransmitter release, inhibiting cAMP production and N-type Ca^{2+} channels and activating K^+ permeability. In addition, there is some evidence that the activation of high-affinity adenosine A_{2A} receptors increases the release of different transmitters (Gu & MacDermott, 1997; Cunha *et al.*, 1994; Sebastiao & Ribeiro, 1992) and has an effect on Gs protein and subsequently increases cAMP level. In contrast, its stimulation reduces the release of GABA from the recurrent collaterals of striatopallidal neurons (Kirk & Richardson, 1994).

Sensory and cognitive dissociations resulting from dopaminergic hyperfunction produce a state of fear and anxiety *via* direct anatomic connections from cortical brain structures to the limbic system principally through mesolimbic pathways (Iturriaga *et al.*, 1996). This disinhibition of mesolimbic dopamine neurons causes the bizarre behavioral and cognitive symptoms experienced by patients in schizophrenia and by extension, with delirium (Harrison, 1999). Delirium resulting from dopaminergic hyperfunction is characterized by global disorders of cognition and wakefulness by impairment of psychomotor behavior (Miller *et al.*, 1991). Major cognitive functions such as perception, deductive reasoning, memory, attention and orientation are all globally disordered. Excessive motor activity frequently accompanies severe cases of delirium and, when this occurs, the resulting constellation of symptoms is called 'agitated delirium' (Crippen, 1994). Integrative brain failure in the ICU environment is almost always associated with a hemodynamic or metabolic decompensation, either intra-or extracranial. The ICU environment provides a repository of typical predisposing factors of a hemodynamic or metabolic nature, including acute or chronic organic brain vascular insufficiency, endocrine insufficiency, acute or chronic cardiopulmonary decompensations, multiple organ-

system insufficiency, relative hypoxia, poor tissue perfusion, multiple medications, and finally sleep–wake cycle disruption caused by immobilization, anxiety and pain (Crippen, 1995).

If excessive responses to dopaminergic systems contribute to the aforementioned manifestations, the neuroleptic drugs that decrease neurodopamine activity such as haloperidol should alleviate some of the symptoms, particularly hypervigilence and paranoia. Haloperidol is a butryphenone structurally similar to droperidol with mechanisms of action similar to piperazine-based phenothiazines (Settle & Ayd, 1983). Haloperidol is a dopamine antagonist and benzodiazepines are GABA agonists. Theoretically, there should be a synergistic relationship between the two when used in a conjoined fashion. In addition, butryphenones such as haloperidol suppress spontaneous movements and complex behavior patterns which result from disharmonious brain function, with minimal CNS depressive effect (Tesar *et al.*, 1985). There is little or no ataxia, incoordination or dysarthria at ordinary doses. Haloperidol appears to exert a diffuse depressive effect by inhibiting dopaminergic receptors and reuptake of neurodopamine in the subcortical, midbrain and brainstem reticular formation (Todd & Grace, 1999). A unique effect of haloperidol is a relatively strong suppression of spontaneous musculoskeletal hyperactivity and behaviour that results from hyperdopaminergic brain function without pronounced sedation or hypotension (Wagner *et al.*, 1997). Haloperidol produces less sedation than other phenothiazines, with very little effect on heart rate, blood pressure and respiration. It appears to have a very wide range between therapeutic doses and the dose which precipitates extrapyramidal reactions (Gerlach & Larsen, 1999). It is thought that haloperidol's molecular structure is changed in some fashion when given orally, increasing the possibility of extrapyramidal reactions (Rosebush & Mazurek, 1999).

SEROTONIN RECEPTORS

During brain development, serotonin (5-hydroxytryptamine; 5-HT) provides essential neurotrophic signals (Justin *et al.*, 2004). 5-HT is known to play an important role in several physiological functions (Jackson & Paulose, 2000). A root cause of sudden infant death syndrome (SIDS) may be disturbances of serotonin levels in key pacemaker cells in the brain. In babies, the normal response to hypoxia is to gasp, which wakes the baby and resets the breathing mechanism. That reflex, which kicks in when a baby isn't getting enough oxygen for any reason, is governed by a set of pacemaker neurons in the respiratory neural network (Tryba *et al.*, 2006). 5-HT is one of many vasoactive substances postulated to participate in the development of hypoxia-induced pulmonary hypertension. Pulmonary vasoactive responses to hypoxia are intensified by 5-HT (Eddahibi, *et al.*, 1997). Several subtypes of signal-transducing 5-HT receptors have been characterized pharmacologically and cloned. Depending on their subtype, these receptors act on G-proteins and thereby activate phospholipase C or adenylate cyclase (Fanburg & Lee, 1997). By analogy with other signaling molecules, it is generally assumed that these receptors operate at the cell surface, without necessarily mediating the uptake of 5-HT. In addition, 5-HT is internalized into a variety of cell types, including platelets, neurons, mast cells, endothelial cells, and smooth muscle cells, through an active transport mechanism that is powered by a transmembrane Na^+/Cl^- gradient (Junod, 1972).

ACETYLCHOLINE RECEPTORS

Acetylcholine is one of the principal neurotransmitters of the parasympathetic system. Extensive evidence supports the view that cholinergic mechanisms modulate learning and memory formation. Evidence for cholinergic regulation of multiple memory systems, noting that manipulations of cholinergic functions in many neural

systems can enhance or impair memory for tasks generally associated with those neural systems. The magnitude of ACh release in different neural systems may regulate the relative contributions of these systems to learning. ACh is the neurotransmitter that is released by stimulation of the vagus nerve, which alters heart muscle contractions. It is important for the movement of other muscles as well. ACh induces movement by the locomotion of an impulse across a nerve that causes it to release neurotransmitter molecules onto the surface of the neighbouring cell. ACh is critical for an adequately functioning memory. Studies of ACh release, obtained with *in vivo* microdialysis samples during training, together with direct injections of cholinergic drugs into different neural systems, provide evidence that release of ACh is important in engaging these systems during learning, and that the extent to which the systems are engaged is associated with individual differences in learning and memory (Paul, 2003).

Hypoxia impairs brain function by incompletely defined mechanisms. Mild hypoxia, which impairs memory and judgment, decreases ACh synthesis, but not the levels of ATP or the adenylate energy charge. The decreases in glucose incorporation into ACh and into the amino acids with hypoxic hypoxia (15% or 10% O_2) or hypoxic hypoxia with 5% CO_2 were very similar to those with the two lowest levels of anemic hypoxia. Thus, any explanation of the brain's sensitivity to a decrease in oxygen availability must include the alterations in the metabolism of the amino acid neurotransmitters as well as ACh (Gibson & Peterson, 1981).

MUSCARINIC RECEPTORS

There are at least three muscarinic receptor subtypes (M_1, M_2 and M_3) involved in the modulation of transmitter release (Caulfield, 1993; Caulfield & Birdsall, 1998). This receptor diversity to some extent explain the diverse range of

signal transduction mechanisms; these include inhibition of Ca^{2+} influx (Allen & Brown, 1993; 1996) and adenylyl cyclase, stimulation of guanylyl cyclase, activation of phospholipase C and direct inhibition of Ca^{2+} channels and activation of K^+ channels (Felder, 1995). There is reasonably good evidence that the M_2 receptors expressed on cholinergic (Aubert *et al.*, 1995; Allen & Brown, 1996) and noradrenergic varicosities play a physiologically important role in the modulation of transmitter release. The muscarinic receptors that inhibit NE release appear to be of the M_2 subtype in the periphery and CNS. In contrast, there are muscarinic receptors, apparently of the M_1 subtype, that increase the release of NE (Raiteri *et al.*, 1990 a,b) expressed on noradrenergic axon terminals in the periphery. The M_1 receptor is generally coupled to PTX-insensitive G protein. Its activation results in formation of inositol trisphosphate and diacylglycerol. In contrast, the M_2 receptor is coupled *via* PTX-sensitive G protein to the N-type Ca^{2+} channel (Hille, 1992). The relative importance of these inhibitory and stimulatory muscarinic receptors may vary in noradrenergic neurons from different locations.

HYPOXIA & MEDICATIONS

Drugs are rarely indicated in resuscitation of the newly born infant (Burchfield, 1999). Bradycardia in the newly born infant is usually the result of inadequate lung inflation or profound hypoxia, and adequate ventilation is the most important step in correcting bradycardia. Administer medications if, despite adequate ventilation with 100% oxygen and chest compressions, the heart rate remains <60 bpm. Epinephrine administration is indicated when the heart rate remains <60 bpm after a minimum of 30 seconds of adequate ventilation and chest compressions. Epinephrine is particularly indicated in the presence of asystole.

Epinephrine has both α- and β-adrenergic-stimulating properties; however, in cardiac arrest, α-adrenergic-mediated vasoconstriction is the important mode of action Zaritsky & Chernow (1984). Vasoconstriction elevates the perfusion pressure during chest compression, enhancing delivery of oxygen to the heart and brain (Berkowitz *et al.*, 1991). Epinephrine also enhances the contractile state of the heart, stimulates spontaneous contractions, and increases heart rate. The recommended intravenous or endotracheal dose is 0.1 to 0.3 ml/kg of a 1:10 000 solution (0.01 to 0.03 mg/kg), repeated every 3 to 5 minutes as indicated. The data regarding effects of high-dose epinephrine for resuscitation of newly born infants is inadequate to support routine use of higher doses of epinephrine. Higher doses have been associated with exaggerated hypertension but lower cardiac output in animals (Berg *et al.*, 1996, Burchfield *et al.*, 1993). The sequence of hypotension followed by hypertension likely increases the risk of intracranial hemorrhage, especially in preterm infants (Pasternak *et al.*, 1983).

Adrenaline (epinephrine) 1 mg after every cycle of three unsuccessful shocks or after every three minutes of CPR (Cardio Pulmonary Resusciation) during a non-shockable arrest improves cerebral and coronary blood flow. In experimental animals, it increases peripheral resistance by αadrenergic stimulation, thereby preventing arterial collapse during the release phase of cardiac compression. It also increases myocardial contractility and rate by ß adrenergic stimulation after restoration of an effective heartbeat, or if in apparent PEA cardiac contraction is present but impalpable. Perhaps surprisingly, its benefit for survival in man is still debatable; no randomised controlled trial has been attempted to support its use. High dose adrenaline has no clear advantage and may be deleterious (Vandycke & Martens, 2000).

NEONATAL RESUSCITATION DURING HYPOXIA

Neonatology, perinatology and neonatal resuscitation developed to a great extent during the 1970's in response to an epidemic of litigation involving birth brain injury; foetal monitoring was detecting foetal distress in utero, and specialized perinatal intensive care promised great improvement in neonatal morbidity and mortality. One third of all neonates receive some form of resuscitation treatment. About 6% to 10% of all neonates are "morbid" and need NICU care – many of these are preemies; again, NICU mortality is extremely rare; however, in terms of neurological and mental disability, especially in NICU babies, long-term morbidity is anything but rare (Hack *et al.*, 2002). The life-saving procedures of neonatal resuscitation and NICU care are much less successful in preserving brains. If resuscitation does not result in a five minute Apgar of 7 or more, neurological impairment is likely (Thorngren-Jerneck & Herbst, 2001).

The term "resuscitation" implies restoration of deficient life support systems, especially respiration; in the depressed newborn, that deficiency is in the placenta and cord, as the lungs have not yet begun to function. The rationale on which current resuscitation is based is that early detection of foetal asphyxia combined with rapid delivery and rapid establishment of pulmonary respiration (reversal of asphyxia) will prevent brain injury. If brain damage (neuron necrosis) has occurred in utero, resuscitation will not heal it; however, overt brain damage seldom is evident at birth, and it often appears after resuscitation. Hypoxic ischemic encephalopathy usually is diagnosed hours after birth when the child convulses; germinal matrix hemorrhage in preemies develop a day or two after birth; mental and behavioral problems may not surface for years.

The general consensus is that birth "asphyxia" is the cause of the brain damage; hypoxia is a more precise term, although asphyxia implies arrest of respiration – respiration includes oxygen supply and removal of carbon dioxide. Iatrogenic resuscitation usually corrects this asphyxia promptly by initiating pulmonary ventilation; most organs survive superbly, all except the brain. This strongly implies that there are other factors active in neonatal "depression" besides hypoxia and acidosis that must be corrected during "resuscitation". The placenta is much more than a respiratory organ. Correction of the placental / cord deficiency that caused the depression, and support of placental function are thus rational priorities in revival of a depressed neonate, just as they are in the "resuscitation" of the "distressed" foetus *in utero*.

In utero, the normal blood supply of the foetal brain is relatively hypoxic. Umbilical vein blood is fairly well oxygenated, but it is mixed in the inferior vena cava and in the heart with de-oxygenated blood from the venae cavae; this is then circulated systemically. The color of a normal newborn is purple – it has been purple for nine months – circulating a mixture of hemoglobin (blue) and oxyhemoglobin (red). It turns pink only after the foetal circulation is changed to the adult circulation, and is combined with aeration of the lungs. The foetal brain thus grows and develops with a copious blood supply that is only partially oxygenated, but which readily removes products of aerobic and anaerobic respiration and excretes them through the placenta. The foetal kidneys and gut thrive on blood with the same oxygen partial pressure as the blood flowing to the placenta to be oxygenated. The newborn brain and other organs are therefore relatively immune to pure hypoxic injury (Kirks & Thorne, 1998) as long as organ and placental perfusion are copious.

The same basic principles apply to the adult brain; five minutes or more of cardiac arrest will produce some brain damage or brain death; occlusion of a cerebral artery rapidly results in infarction (death) of the supplied tissue. On the other hand, five minutes or more of pure anoxia (e.g. breathing pure nitrogen) will produce unconsciousness that is fully reversible without brain damage provided that brain perfusion is not impaired. The integrity of the newborn brain is maintained (by perfusion and oxygenation) at normal (physiological) birth; therefore the physiological mechanisms that ensure these functions (perfusion and oxygenation) should be supported and/or duplicated during resuscitation if brain damage is to be avoided.

The severely depressed/asphyxiated newborn typically shows not only sign of breathing, but also lack of muscle tone and reflexes needed to initiate breathing, as well as signs of hypoxia such as cyanosis; in the most severe cases, pallor indicates vasomotor collapse. Such a child has obviously suffered a major respiratory insult prior to or during birth; the cause of that insult and its specific effects are factors that must be corrected, if possible, in the resuscitation process. In any and every case of newborn depression, if a child is born alive – with a heart beat and a pulsating cord – the placental life support system has not failed completely; utilization of this system in resuscitation and transition to "adult" life support systems in the depressed newborn is essential in restoring the physiological state – health – without the incursion of organ damage, primary or secondary, from "birth asphyxia." With early detection of foetal distress and with rapid delivery, the neonate's central nervous system should be undamaged at birth; the objective of therapy should be that it remains so.

The switch from placental to lung "breathing" is only a portion of the whole; the switch from placental alimentation and placental excretion to the newborn's

alimentary and excretory organs is also part of "natural" resuscitation. To initiate and establish the newborn functions of the lungs, gut, kidneys, and other systems, including the brain, continuous copious perfusion of these organs is required; a large transfusion of placental blood during natural childbirth "resuscitates," or more correctly "activates" all these organ systems as the massive flow of blood through the placenta (40% of the foetal cardiac output) is diverted to these organs during physiological closure of the cord vessels.

Cord closure abruptly halts the placental supply of glucose to the brain (used in aerobic and anaerobic respiration); the neonatal liver (glycogen stores) must begin to maintain blood glucose levels. A major portion of the liver's blood supply is from the hepatic portal vein that derives its blood from the mesenteric arteries. If the gut (and hence the liver) is not "copiously perfused," hypoglycemia may result in a neonatal convulsion. Deficient perfusion of the liver may also be a factor in bilirubin excretion and "physiological" jaundice. Copious perfusion of the neonatal kidneys with adequate blood pressure is required for solute excretion, fluid, electrolyte and acid-base regulation after the placenta ceases to function. During the third stage of labor while the cord is pulsating, warm blood from the placenta courses through the newborn. After cord closure, temperature regulation is suddenly required of the neonate; switch of blood flow to and from the epidermis requires a copious amount of blood to regulate heat loss and heat retention.

In the foetus, pulmonary circulation is minimal; after the adult circulation is established, the entire cardiac output flows through the lungs. A major portion of the placental transfusion is utilized in establishing pulmonary blood flow after birth. Jaykka (1965) demonstrated that perfusion of the foetal lung "erected" the alveoli and actually initiated aeration; the high colloid osmotic pressure of the circulating blood

rapidly absorbs amniotic fluid from the erected alveoli. Thus adequate "copious perfusion" of the lungs result in pulmonary oxygenation before any muscular respiratory effort occurs. Respiratory effort is reflexively controlled through the central nervous system; hypoxia and increased concentration of carbon dioxide are strong stimulants for receptors. For the reflex to function, copious perfusion of the reflex circuit is required, as is copious perfusion of the respiratory muscles (Jaykka, 1957).

ROLE OF GLUCOSE IN REGULATING ENERGY DEMAND

Glucose is the major source of energy for organ function. In the human foetus, oxidation of glucose accounts for approximately 80% of foetal oxygen consumption, demonstrating that glucose is the major substrate for foetal oxidative metabolism (Jane & McGowan 1999). Reports say that there occurs a close association between hypoxia and the emergence of glucose intolerance, but the experimental evidence of a causative role for hypoxia in this metabolic dysfunction is lacking (Oltmanns *et al.*, 2004). Hypoxic respiratory diseases are frequently accompanied by glucose intolerance. One of the factors mediating this effect could be an elevated release of epinephrine (Kerstin *et al.*, 2004). The cerebral metabolic rate for glucose (CMRGlu) increased 70-80% after 2 min of hypoxia but then returned to nearly the normal rate by the end of the 30-min period of hypoxia. Glycolytic flux appeared to be facilitated in both groups initially but was inhibited as the hypoxic period continued. This slowing of glycolysis after 15 or 30 min of hypoxia appears to be modulated by the regulatory enzyme phosphofructokinase. A significant amount of the glucose entering the brain during the posthypoxic period appears to be used for metabolite synthesis rather than energy production (Kintner *et al.*, 1983). Hypoxic-ischemic insult in the perinatal period in humans is a significant risk factor for the development of epilepsy

later in life. Hypoxia is a leading cause of neonatal encephalopathy and is frequently associated with seizures (Jensen *et al.*, 1991).

ROLE OF ATP IN REGULATING ENERGY DEMAND

As a major consumer of energy, the brain is very susceptible to the effects of hypoxia, especially those parts of the brain – such as the hippocampus – that are crucial for cognitive function. There is no irreversible loss of neuronal/synaptic function, as long as nerve cells have an adequate supply of glucose and ATP (from anaerobic glycolysis) to maintain the minimal Na^{+} -K^{+} pump activity and protein synthesis essential for cell survival. These conditions are not met when both oxygen and glucose are deficient, as in strokes. Then the cell's protective mechanisms cannot cope with massive Ca^{2+} influx and it succumbs to the deleterious effects of Ca^{2+} overload (Krešimir, 1999). Of the approximately 130 million infants born worldwide each year, it is estimated that four million infants die during the first month of life. In animals, hypoxia is signaled at three levels: an immediate systemic response which involves central and peripheral chemoreceptors, an immediate/chronic gene response initiated by cellular oxygen signals and an immediate emergency or crisis response signaled by changes in energy metabolite concentrations (Peter & Howard, 2002).

The two most important causes of infant's death were "intrapartum asphyxia and birth trauma" (intrapartum hypoxia) which resulted in neonatal "hypoxia", and "spontaneous preterm labour" leading to "immaturity related" births. The number of deaths coded as being due to the latter would have been even higher if infants weighing 500 to 999 g at birth were included. Death rates in preterm infants were particularly high in cities, towns and rural areas where neonatal high care facilities are very limited. The large number of deaths associated with perinatal hypoxia in all three groups suggested problems and inadequacies in care of women in labour and the

resuscitation of newborn infants. Many of these deficiencies were identified as modifiable factors in all regions. Specifically, only a few infants were recorded as having died as a result of poor neonatal resuscitation and care. Given the large number of neonatal deaths due to "hypoxia", it is inconceivable that this is a true reflection of the actual circumstances. There is probably poor insight into the deficiencies in the basic management of newborn infants as well as a lack of knowledge on neonatal resuscitation and care compared to intrapartum care (Robert *et al.*, 2005).

ATP is a fast transmitter in sympathetic ganglia and at the sympatho-effector junction. In primary cultures of dissociated rat superior cervical ganglion neurons, ATP elicits noradrenaline release in an entirely Ca^{2+}-dependent manner. Nevertheless, ATP-evoked noradrenaline release was only partially reduced (by ~50%) when either Na^{+} or Ca^{2+} channels were blocked, which indicates that ATP receptors themselves mediated transmembrane Ca^{2+} entry (Stefan, 1999).

All kinds of biochemical reactions are linked to energy transfer, therefore each physiological function, as well as each pathological disorder or therapy, must have a consequence for biological energy. The adaptive changes related to hypoxia or energy deficit have been divided into defense and rescue phases. The defense phase occurs immediately after a decline in oxygen and consists of channel arrest, decreased Na^{+}/K^{+}-ATPase activity, urea synthesis, gluconeogenesis, protein synthesis and proteolysis (a highly ATP-consuming process), in such a way that ATP demand equals ATP production. Then the rescue phase involves transcriptional effects [hypoxia-induced factor (HIF)], HIF-mediated activation of genes for sustained survival at low ATP turnover (increased glycolytic enzymes, decreased enzymes involved in aerobic-linked metabolism) and, finally, production of tertiary cell signaling messengers (fos and jun). The consequences of cellular deficit and the mechanisms underlying adaptation to this situation can be understood from the results

of numerous studies, both in hypoxia and in ischemia. Such adaptations must rely on a permanent adjustment between energy demand and ATP synthesis (Stefan, 1999).

DEVELOPMENTAL CHANGES DUE TO HYPOXIA

Hypoxia occurs when oxygen availability drops below the levels necessary to maintain normal rates of metabolism. Because of its high metabolic activity, the brain is highly sensitive to hypoxia. Severe or prolonged oxygen deprivation in the brain contributes to the damage associated with stroke and a variety of other neuronal disorders. Conversely, the extreme hypoxic environment found in the core of many brain tumors supports the growth of the tumor and the survival of tumor cells. Normal cells exposed to transient or moderate hypoxia are generally able to adapt to the hypoxic conditions largely through activation of the hypoxia-inducible transcription factor HIF. HIF-regulated genes encode proteins involved in energy metabolism, cell survival, erythropoiesis, angiogenesis, and vasomotor regulation. In many instances of hypoxia or hypoxia and ischemia, the induction of HIF target genes may be beneficial. When these same insults occur in tissues that are normally poorly vascularized, such as the retina and the core of solid tumors, induction of the same HIF target genes can promote disease. Major new insights into the molecular mechanisms that regulate the oxygen-sensitivity of HIF, and in the development of compounds with which to manipulate HIF activity, are forcing serious consideration of HIF as a therapeutic target for diverse CNS disorders associated with hypoxia (Freeman & Barone, 2005).

EFFECT OF HYPER OXYGENATION

Oxygen availability plays a pivotal role in many cellular processes, and therefore it is not surprising that most biological systems elaborate a variety of

mechanisms for sensing oxygen and maintaining pO_2 homeostasis (Semenza, 1999; Lopez-Barneo *et al.*, 2001). In neuronal cells, responses to a decrease in oxygen availability or hypoxia include both facilitation and inhibition of neurotransmitter release (Gibson & Peterson, 1981; Gibson *et al.*, 1991). For example, hypoxia increase catecholamine releases (Hirsch & Gibson, 1984) or inhibits acetylcholine release (Gibson & Peterson, 1981; Freeman *et al.*, 1987) from brain cells. In a peripheral chemosensory organ, the mammalian carotid body, hypoxia stimulates catecholamine release from specialized O_2-chemoreceptor (glomus) cells, whether present in the intact organ (Fidone *et al.*, 1982; Donnelly, 1993), in tissue slices (Pardal *et al.*, 2000) or as isolated cells or cell clusters *in vitro* (Urena *et al.*, 1994; Montoro *et al.*, 1996; Jackson & Nurse, 1997). Hypoxia also stimulates catecholamine release from neonatal adrenal chromaffin cells (Mojet *et al.*, 1997; Thompson *et al.*, 1997) and from PC-12 cells (Taylor *et al.*, 2000; Kumar *et al.*, 1998), an O_2-sensitive cell line derived from the adrenal medulla. In particular, hypoxia causes inhibition of K^+ channels, leading to increased membrane depolarization or action potential frequency, entry of extracellular calcium and amine secretion (Lopez-Barneo *et al.*, 2001).

When blood supply and oxygen become compromised, local neurons die or become damaged in a pattern consistent with the injury. In this immediate area where blood and oxygen loss has occurred, the neurons die quickly. The surrounding neurons also react to the decreased oxygen levels by shutting down to conserve energy in an attempt to survive. This often results in an exaggeration of the symptoms experienced by brain-damaged patients. Presently, there is little information available on whether resuscitation using room air is equal to or even better than that using 100% oxygen (Nong *et al.*, 2000). Newborns and particularly pre-term infants are at high risk of oxidative stress and they are easily susceptible to free radical oxidative

damage. While no known treatments are yet able to resuscitate dead neurons, hyperbaric oxygen therapy (HBOT) serves to re-oxygenate the dormant neurons and restore a portion of their previous activity (Satoskar *et al.*, 1997). The clinical settings in which oxygen toxicity occurs are broadly divided into two groups; one is in which the patient is exposed to very high concentrations of oxygen for short duration, like in HBOT and the second is in which lower concentrations of the oxygen are used but for longer duration. These two can result in the so called 'acute' and 'chronic' oxygen toxictiy, respectively (Edmonds *et al.*, 1992). The acute toxicity has predominant CNS effects, while chronic toxicity has predominant pulmonary effects (Clark, 1982). Hyperbaric medicine is considered extremely safe under appropriate supervision and utility. Toxic effects of oxygen are observed at extremely high doses over prolonged periods. Hyperbaric oxygen treatment increases the relative dose of oxygen; thus susceptible patients need to be recognized and modifications made to prevent the manifestations of oxygen toxicity. Oxygen derived free radicals had been suggested by Gerschman *et al.*, (1954) as being the probable aetiological factor in the development of these toxic effects. Oxygen free radicals are reactive species that although crucial to normal biological processes, can lead to injury and cell death. They are implicated in the pathogenesis of many neonatal diseases such as perinatal asphyxia, bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis, intracranial hemorrhage, pulmonary hypertension and persistence of ductus arteriosus. Birth is associated with transition to a hyperoxic environment in comparison with uterine environment, which leads to increased generation free radicals. The newborn has undeveloped antioxidant systems and therefore, at increased risk of free radical oxidative injury. The understanding of neonatal factors involved in the pathogenesis of "oxygen free radical diseases" will lead to the

development of new therapies for prevention and treatment of these neonatal diseases (Rodrigues, 1998).

The key to successful neonatal resuscitation is establishment of adequate ventilation. Reversal of hypoxia, acidosis and bradycardia depends on adequate inflation of fluid-filled lungs with air or oxygen (de Burgh Daly, 1979 & 1986). Although 100% oxygen has been used traditionally for rapid reversal of hypoxia, there is biochemical evidence and preliminary clinical evidence to argue for resuscitation with lower oxygen concentrations (Rootwelt *et al.*, 1993; Ramji *et al.*, 1993). Current clinical data, however, is insufficient to justify adopting this as routine practice. If assisted ventilation is required, deliver 100% oxygen by positive-pressure ventilation. If supplemental oxygen is unavailable, initiate resuscitation of the newly born infant with positive-pressure ventilation and room air (Saugstad *et al.*, 1998).

FREE RADICAL RELEASE AND TOXICITY

Free radicals and reactive oxygen species (superoxide and hydrogen peroxide) cause tissue damage only when the radicals exceed the brain's endogenous antioxidant defenses. Newborns and particularly pre-term infants are at high risk of oxidative stress and they are very susceptible to free radical oxidative damage. Free radicals are produced as a result of mitochondrial oxi-reductive processes and also produced by the action of enzymes such as xanthine/urate oxidase at extra-mitochondrial sites. These free radicals cause lipid peroxidations, especially in the cell membranes, inactivate cellular enzymes, inhibit nucleic acids and protein synthesis.

Neuronal membranes of the brain constitute high amount of polyunsaturated fatty acids (PUFA). About 20% of the dry weights of the brain constitute essential

fatty acids. Hence any change in the relative content of fatty acids affects cognitive function and behaviour (Yehuda *et al.*, 1997). The neonatal brain is especially at risk of free radical mediated injury because neuronal membranes are rich in polyunsaturated fatty acids and the human newborn has a relative deficiency of brain superoxide dismutase and glutathione peroxidase (Buonocore *et al.*, 2001).Normally, various antioxidant enzymes protect the body from these free radicals, but in hyperoxic situations, there is explosive free radical production leading to swamping of the enzyme systems and as a result free radicals escape inactivation (Chawla & Lavaniya, 2001). Roberto *et al.*, (2005) reported that hyperoxia with 100% oxygen after hypoxia-ischemia can cause more damage in the cerebral cortex than room air in newborn rats.

Flamm *et al.*, (1978) correlated the generation of free radicals with cell damage in cerebral ischemia. Free radicals are highly reactive molecules that initiate radical chain reactions and damage cellular macromolecules, including proteins, DNA and lipids, ultimately leading to cell death. Free radicals have been implicated in neuronal cell death in acute CNS injury and in chronic neurodegenerative diseases (Coyle & Puttfarcken, 1993; Chan, 1994). There are a number of potential sources for free radicals generation in the ischemic brain. This comprises leaks from mitochondrial respiratory chain; sequences catalyzed by cyclo-oxygenase and lipooxigenase, peroxidation of lipid membrane, auto-oxidation of various small molecules, including catecholamines, by the microsomal cytochrome P450 reductase system (Freeman & Crapo, 1982) and xanthine oxidase reactions. The brain and nervous system is especially prone to oxidant damage for a number of reasons (Ozben, 1998): the membrane lipids are especially rich in polyunsaturated fatty acid side-chains, which are prime targets for free radicals attack; the brain has only moderate amounts of catalase (CAT), superoxide dismutase (SOD) and gluthatione

peroxidase (GPx) and also is relatively lacking in vitamin E; some areas of the brain are rich in iron ions which are released from injured cells or from bleeding in the reperfused area and may enhance lipid peroxidation. One particular role of oxygen free radicals in brain injury appears to involve reperfusion after cerebral ischemia (Chan, 1996). Reoxygenation during reperfusion provides oxygen to sustain neuronal viability and also provides oxygen as a substrate for numerous enzymatic oxidation reactions that produce reactive oxidants. In addition, reflow after occlusion often causes an increase in oxygen to levels that cannot be utilized by mitochondria under normal physiological flow conditions.

BEHAVIOURAL CHANGES ASSOCIATED WITH HYPOXIA

Chronic hypoxia in advanced chronic obstructive pulmonary disease (COPD) result in altered and reduced neuropsychological functioning, which, in turn, leads to memory impairment even when other mental faculties remain unaffected (Sandhu, 1986). Multiple neuropsychological tests with these patients have revealed neuropsychological dysfunction, which is largely due to brain hypoxia. Reversal of cognitive dysfunction has been reported after oxygen therapy (Heaton *et al.*, 1983; Krop *et al.*, 1973) and even abnormalities in electroencephalograms have been shown to improve (Brezinova *et al.*, 1979).

The temporal lobes and the Hesh gyrus receive auditory information, modulate memory and language skills and relay information to the cortex where cognitive judgments are made and motor responses are integrated (Davidson & Irwin, 1999). The thalamus and basal ganglia act as relay stations between lower centers and the cortex (Kropotov & Etlinger, 1999). The brainstem enables endurance and

survival capabilities, modulating heart rate, respiratory function and autonomic actions (Reid & Milsom, 1998). The pineal gland is thought to modulate sleep-wake cycles (Barrera-Mera & Barrera-Calva, 1998). The hippocampal area including the mammillary bodies modulates spatial memory formation, declarative memory, working memory, memory indexing/storage, relating expectancy to reality and internal inhibition. Memory is recorded in several parts of the brain at same time as 'memory molecules' for storage. These molecules are modulated by limbic system, especially the mammillary bodies. Bilateral hippocampal resection results in short-term anterograde amnesia (Wise & Murray, 1999). The hippocampus has receptors for neurosteroids, both mineralocorticoid and glucocorticoid. The mineralocorticoid receptors (high affinity) are agonized by aldosterone, and antagonized by spironolactone. The glucocorticoid receptors (low affinity) are agonized by dexamethasone. There are no known antagonists to glucocorticoid receptors. The locus coeruleus is a small structure on the upper brainstem under the fourth ventricle and is involved in the regulation of wakefulness, attention and orientation (Smythies, 1997).

Some parts of the brain that are especially involved in higher cognitive functions (including consciousness) must be very dependent on a rich supply of energy – presumably because they are extremely active. In the first place, as their neurons continually generate many synaptic and action potentials, resulting in large inward and outward fluxes of ions, cellular and ionic homeostasis can be preserved only by the ATP-consuming Na^+-K^+ pump, which maintains the trans-membrane Na^+ and K^+ gradients and thus indirectly supports such vital transport processes as uptake of sugars and amino acids. Even more than for the pump, 60% of ATP consumption is utilized for protein synthesis (Hochachka, 1996), presumably required to maintain the cell's structure, as well as the rapid turnover of enzymes, receptors, and other proteins

involved in neurotransmitter release, action, and transport. These processes are crucial for synaptic transmission and plasticity and the closely related cognitive processes of memory, learning, and selective attention. Hypoxia or hypoglycemia has almost immediate effects on behavior and brain function. Though dramatic, they are fully reversible if the hypoxia or hypoglycemia is not sustained. But longer or more severe energy deprivation leads to irreversible functional and indeed cellular damage (cell death) – which develop only after a delay of some days (Pulsinelli *et al.*, 1982). Extensive evidence indicates that peripheral or direct central glucose administration enhances cognitive processes in rodents and humans. These behavioral findings suggest that glucose acts directly on the brain to regulate neural processing, a function that seems incompatible with the traditional view that brain glucose levels are high and invariant except under extreme conditions. However, recent data suggest that the glucose levels of the brain extracellular fluid are lower and more variable than previously supposed. In particular, the level of glucose in the extracellular fluid of a given brain area decreases substantially when a rat is performing a memory task for which the brain area is necessary. Together with results identifying downstream effects of such variance in glucose availability, the evidence leads to new thinking about glucose regulation of brain functions including memory (Ewan *et al.*, 2002).

BRAIN WAVE ACTIVITY AND SEIZURES AS A RESULT OF HYPOXIA

In severe encephalopathy there is an initial period of irritability or high arousal, often accompanied by seizures and apneic spells, for the first 24 hours. The earlier the seizures, the more severe the insult. This stage is followed by increasing coma with extreme hypotonia and progressive decline in brainstem function. Brainstem involvement is the best indicator of severe encephalopathy and the signs include abnormal eye movements and interference with sucking, swallowing which

often persists as the bulbar and pseudo-bulbar palsy of the severe quadriplegic. Ongoing apnea and cardio-respiratory arrest may bring death at 2 to 3 days of age. When such cases "recover" the incidence of severe neuro-developmental abnormality is 100%. In a variety of clinical settings, an EEG-based monitoring system is considered to be optimal for the detection of an impending failure of cerebral oxygen supply (Prior & Brierley, 1980). In addition, there are other neurological structures implicated in CP. The frontal lobe is in charge of voluntary motion. The left lobe controls the motor movements involved in language (speech and writing). The right lobe is usually involved in non-verbal activities. Damage to one frontal lobe usually results in a person's inability to move the opposite side of his body. Moreover, damage to the frontal lobes can also cause the inability to initiate or respond to speech even though language can still be understood.

The last structure is the parietal lobe. The parietal lobe is a structure where sensory information, such as touch, pressure, muscles, temperature and pain, is processed. Damage to one parietal lobe usually results in a loss of sensation in the opposite side of the body as well as being unable to feel touch, temperature, and pain. The most frequent clinical syndrome, caused by lesions in the cerebral cortex and underlying white matter, is spastic paralysis (spastic cerebral palsy), which accounts for approximately 50% of all cerebral pasly cases (Miyahara & Mobs, 1995).

Seizures occur commonly in neonatal intensive care units (NICUs). They are an important clinical consequence of central nervous system diseases in the newborn including brain haemorrhage, stroke, meningitis and hypoxic-ischemic encephalopathy (Stephen *et al.*, 2005). Seizures in the newborn are often clinically unsuspected. Consequently, the extent of the electrographic seizures burden in the sick baby can be greatly underestimated (McBride *et al.*, 2000). A seizure affect the entire

brain (generalized seizure), or it may be confined to one neural region (partial seizure). Autonomic changes are the most common symptoms of simple partial seizures but they go unrecognized. As effective seizure control in the neonate requires abolition of both clinical and electrographic seizures, EEG monitoring is necessary.

Neonatal seizures are paroxysmal alterations in neurological function. This can be behavioural, motor or autonomic (Volpe 2000). Early pioneering work of 1970s by Meldrum, (1978) and Wasterlain & Plum, (1973) suggested that prolonged seizures and status epilepticus in mature and immature animals produced an energy failure leading to severe brain cell injury. Later experiments in the last 2 decades, however, seem to have disproved that theory by showing that 10-day-old rats (said to be equivalent to human newborns) maintain energy production in the brain by virtue of increased glycolysis and high adenosine triphosphate (ATP) release if there are no systemic complications such as hypoxia or hypotension (Ingvar & Siesjo, 1990). Most of the literature about neonatal seizures concludes that the prognosis of a particular baby depends upon the etiology of the seizures. It is reported that certain etiologies, such as hypoxic-ischemic encephalopathy (HIE), meningitis, congenital brain abnormalities, and inborn errors of metabolism, almost uniformly have severe neurological sequelae (Richard, 1999).

From infancy to adulthood, tonic–clonic seizures and complex partial seizures of temporal or extratemporal origin often lead to sympathetic activation. Because the memory circuits originate in the temporal lobe, repeated seizure activity which involves these structures can cause difficulties with memory and intellectual function. Seizures typically activate sympathetic nerve activity, increasing the heart rate and blood pressure, although parasympathetic activation or sympathetic inhibition predominates during partial seizures (Orrin, 2004). Brain requires continuous supply

of oxygen for energy utilization and efficient functioning. Hypoxia leads to disruption of this energy utilization, resulting in neuronal functional failure, cerebral palsy and neuro-developmental delay with characteristic biochemical and molecular alterations that can result in permanent or transitory neurological sequelae or even death. Structural and functional integrity of brain depends on regular oxygen and glucose supply.

In the present study, we investigated the role of glucose, epinephrine and oxygen supplementation in adrenergic and glutamate receptor regulation in the cerebral cortex of hypoxic neonatal rats. Gene expression studies using Real-Time PCR were done to confirm the receptor data. EEG was measured to analyse the cerebral activity during hypoxic insult. Behavioural studies were done after one month in all experimental groups of neonatal rats to confirm the behavioural changes in later stages of life in these rats. This is the first molecular study on hypoxic neonates showing that glucose supplementation has significant impact in controlling hypoxia induced functional damage at the adrenergic and glutamate receptors, which has immense therapeutic application in the neonatal care.

Materials and Methods

BIOCHEMICALS AND THEIR SOURCES

Biochemicals used in the present study were purchased from Sigma Chemical Co., USA. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India.

Chemicals used in the study

Biochemicals: (Sigma Chemical Co., USA.)

(±)Norepinephrine, (±)epinephrine, dopamine, 5-hydroxy tryptamine, 5-hydroxy indole acetic acid, homovanillic acid, sodium octyl sulfonic acid, citric acid, D-glucose, Catechol, Acetylthiocholine iodide, calcium chloride, atenolol, phentolamine, propranolol, glutamate, yohimbine, ascorbic acid, Tris HCl, ethylene diamine tetra acetic acid-EDTA.

Radiochemicals

Levo-[N-methyl-^{3}H]Epinephrine (Sp. activity 68.6 Ci/mmol) was purchased from NEN life sciences products Inc., Boston, U.S.A. DL-[4-^{3}H] Propranolol (29.0 Ci/mmol) [*o-methyl*-^{3}H]Yohimbine (Sp. activity 83.0 Ci/mmol) & L-[G-^{3}H]Glutamic acid (Sp. activity 49.0 Ci/mmol), Cyclic AMP (^{3}H) assay system were obtained from Amersham Life science, U.K.

ANIMALS

Adult Wistar rats were purchased from Amrita Institute of Medical Sciences, Kochi. Neonatal rats of four days old were weighed and used for experiments. All

groups of neonatal rat were maintained with their mothers under optimal conditions 12 hour light and 12 hour dark periods and were fed standard food and water *ad libitum*.

Induction of Acute Hypoxia in Neonatal Rats

Wistar neonatal rats of 4-days old (body weight, 6.06 ± 0.45g) were used for the experiments and were grouped into seven as follows: (i) Control neonatal rats were given atmospheric air (20.9% oxygen) for 30 minutes (C); (ii) Hypoxia was induced by placing the neonatal rats in a hypoxic chamber provided with 2.6% oxygen for 30 minutes (Hx); (iii) Hypoxic neonatal rats were injected 10% dextrose (500mg/ Kg body wt) intra-peritoneally (i.p.) (Hx+G). (iv) Hypoxic neonatal rats were supplied with 100% oxygen for 30 minutes (Hx+O); (v) Hypoxic neonatal rats were injected 10% dextrose (500mg/ Kg body wt) i.p. and treated with 100% oxygen for 30 minutes (Hx+G+O); (vi) Hypoxic neonatal rats were injected epinephrine (0.1µg/ Kg body wt) i.p. and treated with 100% oxygen for 30 minutes (Hx+E+O); (vii) Hypoxic neonatal rats, 10% dextrose (500mg/ Kg body wt) and epinephrine (0.1µg/ Kg body wt) were injected i.p. and then treated with 100% oxygen for 30 minutes (Hx+G+E+O). The experimental animals were maintained in the room temperature for one week.

Neonatal experimental rats were kept for one month for behavioural studies. Body weights were measured before the experiment.

Tissue preparation

Control and experimental neonatal rats were sacrificed by decapitation. The brain regions and body parts were dissected out quickly over ice according to the procedure of Glowinski & Iversen (1966) and the tissues were stored at -70^{0}C for various experiments.

All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

Principle: Glucose oxidase (GOD) catalyses the oxidation of glucose in accordance with the following equation:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{(GOD)} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} \quad \text{4-aminoantipyrene} \xrightarrow{(\text{Peroxidase})} \text{Coloured complex} + H_2O$$

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510nm in a spectrophotometer (Milton Roy Genesys 5 Spectronic).

Quantification of Neurotransmitters and its Metabolites in the Cerebral Cortex and Adrenals of Experimental Groups of Neonatal Rats.

The neurotransmitters and their metabolites were assayed according to Paulose *et al.*, (1988). The cerebral cortex were homogenised in 0.4 N perchloric acid and adrenals in 1N perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4°C (Heraeus refrigerated centrifuge) and the clear supernatant was filtered through 0.22 μm high performance liquid chromatography (HPLC) grade filters and used for HPLC analysis.

Norepinephrine (NE) and epinephrine (EPI), Dopamine (DA), 5-hydroxy tryptamine (5-HT), 5-hydroxy indole acetic acid (HIAA), homovanillic acid (HVA) were determined in HPLC with electrochemical detector (HPLC-ECD) (Waters, USA) fitted with CLC-ODS reverse phase columns of 5 μm particle size. The mobile phase consisted of 0.05M sodium phosphate dibasic, 0.03M citric acid, 0.1mM EDTA, 0.6mM sodium octyl sulfonate & 15% methanol. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22 μm filter (Millipore) and degassed. A Waters (model 515, Milford, USA) pump was used to deliver the solvent at a rate of 1 ml/minute. The neurotransmitters and their metabolites were identified by amperometric detection using an electrochemical detector (Waters, model 2465) with a reduction potential of +0.8 V. Twenty microlitre aliquots of the acidified supernatant were injected into the system for detection. The peaks were identified by relative retention times compared with external standards and quantitatively estimated using an integrator (Empower software) interfaced with the detector. Data from cerebral

cortex and adrenals of the control and experimental groups of neonatal rats were tabulated and statistically analysed.

Glutamate contents in the cerebral cortex of experimental groups were quantified by displacement method using modified procedure of Kurioka *et al.*, (1981).The incubation mixture contained 40nM [^{3}H]Glutamate with and without glutamate at a concentration range of 10^{-9}M to 10^{-4}M. The unknown concentrations were determined from the standard displacement curve using appropriate dilutions and calculated for µmoles/g wt. of the tissue.

GLUTAMATE DEHYDROGENASE ASSAY

GDH activity was measured in the crude extract of brain and liver (Kaur & Kanungo, 1970)). Sample extracts were prepared by making 5% homogenate of the tissue in cold distilled water and the supernatant fluid was collected after centrifugation at 10,000xg for 20 minutes. The enzyme activity was measured in supernatant fluid as follows. The reaction mixture in the experimental and reference cuvettes contain 0.04M triethanolamine buffer pH 8.0, 2.6mM EDTA, 105mM Ammonium acetate and 100µl of the enzyme sample extract of appropriate concentrations. The reaction mixture of 1ml volume was assayed at 366nm using Milton Roy Genesis spectrophotometer by adding saturating concentrations of α-ketoglutarate and 10mM NADH. Decrease in optical density (O.D) due to the oxidation of NADH was measured at 15 second intervals for two minutes at room temperature. The decrease in absorbance was linear during the course of the assays. One unit of enzyme activity is equal to the change in O.D of 0.1 in 100 seconds at 366nm. Activity of enzyme was expressed as specific activity represented by Units/mg protein. Kinetic parameters V_{max} and K_m, were calculated from the data of

GDH activity measured at substrate concentrations of 0.5mM, 1mM, 2mM and 4mM of α-ketoglutarate.

ACETYLCHOLINE ESTERASE ASSAY

Acetylcholine esterase assay was done using the spectrophotometric method of Ellman *et al.*, (1961). The homogenate (10%) was prepared in sodium phosphate buffer (30mM, pH 7.0). One ml of 1% Triton x 100 was added to the homogenate to release the membrane bound enzyme and centrifuged at 10,000 rpm for 30 minutes at 4^0C. Different concentrations (0.05mM to 0.8mM) of acetylthiocholine iodide were used as substrate. The mercaptan formed as a result of the hydrolysis of the ester reacts with an oxidising agent 5, 5' -dithiobis (2-Nitrobenzoate) absorbs at 412 nm. Activity of enzyme was expressed as specific activity represented by Enzyme Units/mg protein. Kinetic parameters V_{max} and K_m, were calculated from the data of ACh activity.

Adrenergic Receptor Binding Studies using [^{3}H] radioligands

The adrenergic receptor (AR) binding status in the cerebral cortex were studied using [^{3}H]Epinephrine binding with epinephrine, [^{3}H]Yohimbine binding with phentolamine, [^{3}H]Propranolol binding with propranolol for general-AR, α_2-AR and β-AR . The assay was done according to the modified procedure of U'Prichard & Snyder (1977). The brain tissues were homogenized in 20 volumes of cold 50mM Tris-HCl buffer pH 7.7. The homogenate was centrifuged twice at 50,000xg for 10 minutes. The pellet resuspended in appropriate volume of incubation buffer containing 0.1% Ascorbic acid, 1mM Catechol, 0.1mM EDTA-Na_2, 10μM DTT, 50mM Tris-HCl

and 10mM $MgCl_2$.Binding assay was done using different concentrations of radioligands.

[^{3}H]Epinephrine Binding Studies

The Epinephrine (general-AR) binding status was studied using [^{3}H]Epinephrine and epinephrine. Membrane binding assays were done using different concentrations *i.e.*, 0.1 – 5.0 nM of [^{3}H]Epinephrine in the incubation buffer, pH 7.7 in a total volume of 250µl containing (0.2-0.3mg) protein concentrations. Non-specific binding was determined using 100µM unlabelled epinephrine. The tubes were incubated at 37^{0}C for 15 min and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 50mM Tris- HCl (pH 7.7). Bound radioactivity was counted with cocktail-T in a liquid scintillation counter (Wallac 1409). The non-specific binding determined showed 30-40% in all our experiments.

[^{3}H]Yohimbine Binding Studies

α_2-AR membrane binding assays were done using α_2 antagonist-[^{3}H]Yohimbine and α_1 antagonist- unlabelled phentolamine. Different concentrations i.e., 1.0–12.0nM of [^{3}H]Yohimbine in the incubation buffer, pH 7.7 in a total volume of 250µl containing (0.2-0.3mg) protein concentrations were used for the assay. Non-specific binding was determined using 100µM unlabelled phentolamine. The tubes were incubated at 37^{0}C for 15 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 50mM Tris- HCl (pH 7.7). Bound radioactivity was counted with cocktail-T in a liquid scintillation counter (Wallac 1409). The non-specific binding determined showed 30-40% in all our experiments.

[^{3}H]Propranolol Binding Studies

β-AR membrane binding assays were done using different concentrations i.e., 0.25 – 8nM of [^{3}H]Propranolol in the incubation buffer, pH 7.7 in a total volume of 250µl containing (0.2-0.3 mg) protein concentrations. Non-specific binding was determined using 100µM unlabelled Propranolol. The tubes were incubated at 37^{0}C for 15 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 50mM Tris- HCl (pH 7.7). Bound radioactivity was counted with cocktail-T in a liquid scintillation counter (Wallac 1409). The non-specific binding determined showed 30-40% in all our experiments.

Glutamate binding Studies using [^{3}H] radioligand

Glutamate receptor binding assay was done according to the modified procedure of Rao & Murthy (1993) using different concentrations i.e., 20–350nM of [^{3}H]Glutamate in the incubation buffer, containing 25mM Tris HCl and 5mM $MgCl_2$, pH 7.4 in a total volume of 250µl containing (0.2 -0.3mg) protein concentrations. Non-specific binding was determined using 500µM unlabelled glutamate. The incubation was carried out at 30^{0}C for 30 minutes and the reaction was stopped by centrifugation at 27,000xg for 15 minutes. The pellet and the wall of the tubes were washed with ice-cold distilled water to remove unbound radioactive. 50µl of 1M KOH was added and kept for overnight digestion. Bound radioactivity was counted with cocktail-T in a liquid scintillation counter (Wallac 1409). The non-specific binding determined showed 30-40% in all our experiments.

PROTEIN DETERMINATION

Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was

proportional to the amount of protein, which was read in a spectrophotometer at 660nm.

Analysis of the receptor binding data

Linear regression analysis for Scatchard plots

The receptor binding parameters determined using Scatchard analysis (Scatchard, 1949). The maximal binding (B_{max}) and equilibrium dissociation constant (K_d) were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis using Sigma plot computer software. This is called a Scatchard plot. The B_{max} is a measure of the total number of receptors present in the tissue and the K_d represents affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity or the "strength" of binding.

Nonlinear regression analysis for displacement curve

Competitive binding data were analysed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). The concentration of competitor that competes for half the specific binding was defined as EC_{50} which is same as IC_{50} (Unnerstall, 1990). The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors (Cheng & Prusoff, 1973).

The data of the competitive binding assays are represented graphically with the negative log of concentration of the competing drug on X-axis and percentage of the radioligand bound on the Y-axis. The steepness of the binding curve can be quantified with a slope factor, often called a Hill slope. A one-site competitive binding curve that follows the law of mass action has a slope of -1.0. The slope factor is negative because curve goes downhill. If slope factor differs significantly from -1.0, then the binding does not follow the law of mass action with a single site, suggesting a two-site model of curve fitting.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Isolation of RNA

RNA was isolated from the brain regions of sham and partially pancreatectomised rats using the Tri reagent (MRC., USA). Tissue (25-50mg) homogenates were made in 0.5ml Tri Reagent. The homogenate was kept in the room temperature for 5 minutes and centrifuged at 12,000x*g* for 15 minutes at 4^0C. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000x*g* for 8 minutes at 4^0C. RNA precipitate forms a pellet on the bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000x*g* for 5 minutes at 4^0C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2µl of RNA was made up to 1 ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 42µg.

cDNA Synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit in 0.2ml microfuge tubes. The reaction mixture of 20µl contained 0.2µg total RNA, 10X RT buffer, 25X dNTP mixture, 10X Random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25^0C for 10 minutes and 37^0C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Fosterity, CA, USA designed using Primer Express Software Version (3.0).

Quantitative Real-Time Assay

Real–Time PCR assays were performed in 96-well plates in a ABI 7300 Real–Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase "polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled Tag probe (designed by Applied Biosystems). Endogenous control (β-actin) was labeled with a report dye (VIC). All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher dye at the 3' end. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20µl contained 25ng of total RNA-derived cDNAs, 200nM each of the forward primer, reverse primer and TaqMan probe for adrenergic- (α_{2A} AR and β_2 AR) gene, glutamergic- NMDAR1 gene, endogenous control (β-actin) and 12.5µl of TaqMan 2X Universal PCR MasterMIX (Applied

Biosystems). The volume was made up with RNAse free water. Each run contained both negative (no template) and positive controls.

The thermocycling profile conditions were as follows:

50°C -- 2 minutes ---- Activation

95°C -- 10 minutes ---- Initial Denaturation

95°C -- 15 seconds ---- Denaturation 40 cycles

50°C -- 30 seconds --- Annealing

60°C -- 1 minutes --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$).

Assay of cyclic Adenosine Monophosphate (cAMP)

cAMP assay kit (Amersham, England) was used. The assay is based on the competition between unlabelled cAMP and a fixed quantity of tritium labeled compound for binding to a protein which has a high specificity and affinity for cAMP. The amount of labeled protein - cAMP - complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated.

Free [^{3}H] cAMP + Binding protein = Bound [^{3}H] cAMP-binding protein

cAMP + cAMP-binding protein

Separation of the protein bound cAMP from unbound nucleotide is achieved by adsorption of the free nucleotide on to a coated charcoal followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

Tissue preparation for cAMP assay.

The brain tissues were homogenized in acidic ethanol (1ml 1NHCl/100 ml ethanol) and allowed to stand for 5 minutes at room temperature. The homogenate was centrifuged at 13,000xg for 5minutes at 4^{0}C. The supernatant was collected. The pellet was washed with ethanol:water (2:1) and again centrifuged. The supernatant were combined and evapourated to dryness at 55^{0}C under vacuum. The residue was dissolved in 0.5 ml of Tris/ EDTA buffer pH 7.5 containing 0.05M Tris and 4mM EDTA. The suspension was centrifuged to remove insoluble residues and the supernatant was used directly for the assay.

cAMP Assay Protocol

The tubes were placed on a water bath at 0^{0}C. The assay mixture consisted of different concentrations of standard, [^{3}H] cAMP and binding protein in case of standards; buffer, [^{3}H] cAMP and binding protein for zero blank and unknown samples, [^{3}H] cAMP and binding protein for determination of unknown samples. The mixture was incubated at +2^{0}C for 2 hours. Cold charcoal reagent was added to the

tubes and the tubes were immediately centrifuged at 12,000g for 2 minute at 2^0C. Aliquots of the supernatant was immediately transferred to scintillation vials and mixed with cocktail-T and counted in a liquid scintillation counter. (Wallac, 1409). C_0/C_x is plotted on the Y-axis against picomoles of inactive cAMP on the X- axis of a linear graph paper, where C_0 is the counts per minute bound in the absence of unlabelled cAMP and C_x is the counts per minute bound in the presence of standard or unknown unlabelled cAMP. From the C_0/C_x value for the sample, the number of picomoles of unknown cAMP is calculated.

EEG analysis

The electroencephalograph were analysed according to the procedure of Hughes *et al.*, (1983) and recorded using Neurocare™ Wingraph Digital EEG system. The brain waves recorded on the EEG is used to understand the slow waves and neurophysiological mechanisms in the experimental groups of rats. The frequency of the brain waves reflects the responsiveness of the neurons to the stimulus. Spontaneous electrical activities was measured by placing electrodes in the left and right lobes of frontal, temporal, parietal, occipital areas of the scalp of experimental rats, reference electrodes were placed on the ear and ground reference on the trunk. Each electrodes were placed 10-20 percent away from the neighbouring electrodes. Brain wave activity were analysed from the EEG recorded data of control and experimental rats.

BEHAVIOURAL STUDY

1) Elevated Plus-Maze

The elevated plus-maze is a widely used animal model of anxiety that is based on two conflicting tendencies; the rodent's drive to explore a novel environment and it's aversion to heights and open spaces. Four arms are arranged in the shape of a cross. Two arms have side walls and an end wall ("closed arms") - the two other arms have no walls ("open arms"). Closed arm walls can be individually inserted into guide rails.The open arms are surrounded by small ledges to prevent the animal from falling from the maze. The maze is fastened to a light-weight support frame. Thus "anxious" animals will spend most time in the closed arms while less anxious animals will explore open areas longer.

Procedure – Animals are placed individually into the center of elevated plus-maze consisting of two open arms (38L x 5W cm) and two closed arms (38L x 5W x 15H cm), with a central intersection (5 cm x 5 cm) elevated 50 cm above the floor. Movement through the maze is detected. The test lasts for a total of 5 minutes. The data are subsequently reduced to the following parameters for each arm and the center: basic movements (beam breaks), distance traveled (cm), time spent (s), number of full entries, number of pokes into, number of head dips over the side of the open arm (Pellow *et al.*, 1985).

2) Open field test

Measurement of general activity (open-field), simultaneous measurement of locomotion, rearing and stereotyped head movements (general activity, behaviour, learning etc). Rodents naturally avoid bright light and open spaces. When placed into

a brightly lit open field, rats and mice tend to remain in the periphery of the apparatus or against the walls (thigmotaxis).

Procedure – Animals are removed from the home cage and placed individually into an open field arena (Plexiglas cage, 41 cm (L) x 41 cm (W) x 38 cm (H). Animals remain the open field for 10-45 min. Each trial began with placing the animal in the center of the open field (to maximize the initial fear response). At the beginning of each trial, the animal was briefly covered by a cardboard box of similar size to the animal's body length and width. When the box was lifted, the animal was allowed to freely ambulate. After each trial, the animal was returned to its home cage, which was placed within the testing room. In order to minimize interference with the animal's behavior, the experimenter remained at the same location in the room during all trials (Cannizzaro *et al.*, 2001). Data were collected as an indication of activity in the center and periphery of the arena. The data were subsequently reduced to the following parameters for the center and periphery: basic movements (beam breaks), distance traveled (cm), time spent (s), number of repetitive beam breaks (i.e. stereotypic movement). Data were collected and analyzed in time bins (e.g. every minute) or as a total over the course of the experiment (Kitay, 1961).

STATISTICAL ANALYSIS

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). Empower software were used for HPLC analysis. Relative Quantification Software were used for analyzing Real-Time PCR results.

Results

Body weight and Blood glucose level.

There was no significant change in the body weight and blood glucose levels in the control and experimental groups of neonatal rats (Table-1).

Catecholamines and its metabolite content in the cerebral cortex of control and experimental groups of neonatal rats.

NE and EPI contents in the cerebral cortex showed a significant increase Hx ($p<0.01$); Hx+O ($p<0.001$); Hx+E+O and Hx+G+E+O ($p<0.01$) compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the NE, EPI contents near to C. DA and HVA contents significantly decreased in Hx ($p<0.01$), Hx+O ($p<0.001$), Hx+E+O ($p<0.01$) and Hx+G+E+O ($p<0.001$) compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the DA and HVA contents near to C (Table-2).

Serotonin and its metabolite content in the cerebral cortex of control and experimental groups of neonatal rats.

5-HT and 5- HIAA in cerebral cortex showed a significant decrease ($p<0.001$) in Hx, Hx+O, Hx+E+O, Hx+G+E+O compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed 5-HT and 5-HIAA content to near C. Glucose treatment to epinephrine and oxygen treated rats -Hx+G+E+O, did not reverse the 5-HT and 5- HIAA content change observed in Hx+E+O (Table-3).

Catecholamines and metabolite content in the adrenals of control and experimental groups of neonatal rats.

NE, EPI, DA contents in adrenals showed a significant increase in Hx, Hx+O ($p<0.001$), Hx+E+O ($p<0.001$) and Hx+G+E+O ($p<0.01$) compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the NE, EPI, DA contents near to C (Table-4).

Serotonin and its metabolite content in the adrenals of control and experimental groups of neonatal rats.

5-HT and 5-HIAA contents in adrenals did not show any significant change in the adrenals among the groups when compared to C (Table-5).

Glutamate content in the cerebral cortex of control and experimental groups of neonatal rats.

Glutamate content in the cerebral cortex showed a significant increase in Hx ($p<0.01$); Hx+O ($p<0.001$); Hx+E+O ($p<0.05$) and Hx+G+E+O ($p<0.01$) compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the glutamate content near to C (Table-6).

Glutamate dehydrogenase activity in the cerebral cortex and liver of control and experimental groups of neonatal rats.

Cerebral Cortex

Glutamate dehydrogenase assay in the cerebral cortex showed that the V_{max} increased significantly ($p<0.001$) in Hx, Hx+O, Hx+E+O and Hx+G+E+O and the K_m showed no significant change among the groups when compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the Glutamate dehydrogenase activity near to C (Table-7).

Liver

Glutamate dehydrogenase assay in the liver showed that the V_{max} increased significantly ($p<0.001$) in Hx, Hx+O, Hx+E+O and Hx+G+E+O compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the glutamate dehydrogenase activity near to C. K_m showed a significant decrease ($p<0.01$) in Hx and Hx+O; Hx+E+O ($p<0.05$) compared to C. Glucose treatment to Hx- Hx+G, Hx+G+O and Hx+G+E+O reversed the K_m near to C (Table-8).

Acetylcholine esterase activity in the cerebral cortex and muscle of control and experimental groups of neonatal rats.

Cerebral Cortex

Activity of Acetylcholine esterase decreased in the cerebral cortex with a significant decrease in the V_{max} in Hx ($p<0.001$); Hx+O, Hx+E+O and Hx+G+E+O ($p<0.01$) compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the

Acetylcholine esterase activity near to C. K_m showed no significant change in the experimental groups of rats compared to C (Table-9).

Muscle

Activity of Acetylcholine esterase in the muscle with a significant decrease in the V_{max} in Hx, Hx+O, Hx+E+O and Hx+G+E+O ($p<0.001$) compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the Acetylcholine esterase activity near to C. K_m did not show any significant change in the experimental groups of rats compared to C (Table-10).

Receptor alterations in the cerebral cortex of control and experimental groups of neonatal rats.

a) Epinephrine receptors Analysis

[^{3}H]Epinephrine binding parameters

Epinephrine binding in the cerebral cortex showed that the B_{max} increased significantly in Hx ($p<0.05$), Hx+O and Hx+E+O ($p<0.001$) and Hx+G+E+O ($p<0.05$) when compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the epinephrine binding parameter near to C. K_d showed significant increase ($p<0.001$) in Hx+O while all other groups showed no significant change compared to C (Fig 1-7; Table 11-17).

Displacement Analysis of [^{3}H] Epinephrine by Epinephrine

The competition curve between [^{3}H] Epinephrine against epinephrine fitted for two-sited model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ decreased in Hx+E+O indicating a shift towards higher affinity. Hx+G+E+O showed an increase in $Ki_{(H)}$ showing a shift towards lower affinity. $Ki_{(L)}$ showed a decrease in Hx+O, Hx+G+O and Hx+G+E+O Showed a shift towards higher affinity (Fig-8; Table-18).

b) α_2-adrenergic receptors Analysis

[^{3}H]Yohimbine binding parameters

[^{3}H]Yohimbine binding to α_2-adrenergic receptors showed that the B_{max} decreased significantly in Hx ($p<0.01$); Hx+O ($p<0.001$); Hx+E+O ($p<0.01$) and Hx+G+E+O ($p<0.001$) compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the α_2-adrenergic receptors binding parameter near to C. K_d showed no significant change among the experimental groups (Fig 9-15; Table 19-25).

Displacement Analysis of [^{3}H]Yohimbine by phentolamine

The competition curve between [^{3}H]Yohimbine against phentolamine fitted for two-sited model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ increased in Hx indicating a shift in high affinity towards low affinity. $Ki_{(L)}$ remained same in all experimental groups (Fig-16; Table-26).

Real-Time PCR analysis of α_{2A}-AR

α_{2A} subunit of adrenergic receptor mRNA showed a significant decrease in the gene expression in Hx, Hx+O, Hx+E+O and Hx+G+E+O compared to C. Glucose treatment to Hx+G, Hx+G+O showed a reversal of gene expression towards control level compared to Hx, Hx+O, Hx+E+O and Hx+G+E+O. Hx+O showed a decreased expression compared to the other groups of experimental neonatal rats. Real-Time PCR results confirmed the α_{2A}-AR receptor binding parameter data for the various experimental groups of neonatal rats (Fig-17; Table-27).

c) β-adrenergic receptors Analysis

[^{3}H] Propranolol binding parameters

[^{3}H]Propranolol binding to β-adrenergic receptors showed that B_{max} increased significantly in Hx ($p<0.05$); Hx+O and Hx+E+O ($p<0.001$); Hx+G+E+O ($p<0.01$) when compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the β-adrenergic receptors binding parameter near to C. The K_d showed no significant change in all the experimental groups. (Fig 18-24; Table 28-34)

Displacement Analysis of [^{3}H] Propranolol by propranolol

The competition curve between [^{3}H] Propranolol against propranolol fitted for two-sited model in all the groups with Hill slope value away from Unity. Hx+E+O Hx+G+E+O showed an increase in $Ki_{(H)}$ value indicating a shift towards lower affinity. $Ki_{(L)}$ showed an increase in Hx and Hx+E+O, indicating a shift towards lower affinity (Fig-25; Table-35).

Real-Time PCR analysis of β_2-AR receptor

β_2 subunit of adrenergic receptor mRNA showed a significant increase in the gene expression in Hx, Hx+O, Hx+E+O and Hx+G+E+O compared to C. Glucose treatment to Hx+G, Hx+G+O showed a reversal of gene expression to C. Hx+E+O showed the maximum gene expression compared to all other groups of experimental neonatal rats. Real-Time PCR results confirmed the AR receptor binding parameter data for the various experimental groups of neonatal rats (Fig-26; Table-36).

d) Glutamate receptors Analysis

[^{3}H] Glutamate binding parameters

[^{3}H] Glutamate binding showed that the B_{max} increased significantly in Hx, Hx+O, Hx+E+O and Hx+G+E+O ($p<0.001$) compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the glutamate receptors binding parameter near to C. The K_d showed no significant change in all the experimental groups. (Fig 27-33; Table 37-43)

Displacement Analysis of [^{3}H] Glutamate by glutamate

The competition curve between [^{3}H] Glutamate against glutamate fitted for two-sited model in all the groups with Hill slope value away from Unity. $Ki_{(H)}$ showed a decrease in Hx+O and Hx+G+O denoting a shift in the towards higher affinity (Fig-34; Table-44).

Real-Time PCR analysis of Glutamate receptor

NMDAR1 subunit of Glutamate receptor mRNA showed a significant increase in Hx, Hx+O, Hx+E+O and Hx+G+E+O compared to C. Glucose treatment to Hx+G, Hx+G+O showed a reversal of gene expression towards control level compared to Hx, Hx+O, Hx+E+O and Hx+G+E+O. Hx+E+O. Hx+O showed an increased expression compared to the other groups of experimental neonatal rats. A real-Time PCR result confirms the glutamate receptor binding parameter data for the various experimental groups of neonatal rats. (Fig-35; Table-45)

cAMP content in the cerebral cortex of experimental groups of neonatal rats

cAMP content showed a significant increase ($p<0.001$) in Hx, Hx+O and Hx+E+O compared to C. Glucose treatment to Hx- Hx+G and Hx+G+O reversed the cAMP content near to C. (Fig-36, Table-46).

EEG Analysis

The frequency of the brain waves recorded in the EEG showed the neuronal response or excitability to the hypoxic insult to which the animal is exposed.

Frontal Lobe

The brain waves in the frontal left lobe of Hx showed an abnormal wave patterns and Hx+E+O showed abnormal spiking in the left and right lobes. Hx+O and Hx+G+E+O showed changes in its wave pattern while Hx+G and Hx+G+O showed a wave pattern similar to C (Fig 37 & 38).

Temporal Lobe

Hx, Hx+O, Hx+E+O and Hx+G+E+O showed abnormal spiking in the temporal lobes compared to C. Hx+G and Hx+G+O showed a wave pattern significant to C (Fig 39 & 40)

Parietal Lobe

An abnormal brain wave pattern was observed in Hx, Hx+O, Hx+E+O and Hx+G+E+O, whereas Hx+G and Hx+G+O showed a wave pattern similar to C (Fig 41 & 42).

Occipital Lobe

Hx+O, Hx+E+O and Hx+G+E+O showed an irregularity in the brain waves of left and right lobes compared to C. Hx, Hx+G and Hx+G+O showed a wave pattern similar to C (Fig 43 & 44).

Behavioural Study

Body weight of experimental animals used for Behavioural studies

One month old rats after all experimental treatments were used for behavioural study. Body weight of experimental animal used for behavioural studies showed a significant decrease in Hx ($p<0.01$) and Hx+G+E+O ($p<0.001$) when compared to C, Whereas there was no significant change in Hx+O, Hx+E+O, Hx+G and Hx+G+O when compared to C (Table-47).

I. Elevated Plus-Maze

a) Open arm entry attempts by control and experimental groups of neonatal rats

The experimental groups showed a significant decrease in the attempt taken for open arm entry - Hx and Hx+G ($p<0.05$); Hx+O ($p<0.01$); Hx+G+O ($p<0.05$); Hx+E+O ($p<0.001$) and Hx+G+E+O ($p<0.01$) compared to C (Fig-45, Table-48).

b) Closed arm entry attempts by control and experimental groups of neonatal rats

There was a significant increase in the number of entries made into closed arm by Hx, Hx+O, Hx+G+E+O ($p<0.01$); while Hx+G, Hx+E+O, Hx+G+O showed no significant change compared to C (Fig-46, Table-49).

c) Percentage arm entry attempts by control and experimental groups of neonatal rats

A significant decrease in the percentage arm entry by Hx, Hx+O, Hx+E+O Hx+G+E+O ($p<0.001$); Hx+G, Hx+G+O ($p<0.05$) were observed in the experimental groups compared to C (Fig-47, Table-50).

d) Total arm entry attempts by control and experimental groups of neonatal rats

The total arm entry showed a significant decrease in Hx+O ($p<0.01$); Hx+E+O ($p<0.001$); Hx+G+E+O ($p<0.05$) were observed in the experimental groups

compared to C. Hx, Hx+G, Hx+G+O showed no significant change compared to C (Fig-48, Table-51).

e) Time spent in open arm by control and experimental groups of neonatal rats

There was a significant decrease in time spent in open arm by Hx and Hx+O ($p<0.01$); Hx+E+O ($p<0.001$); Hx+G+E+O ($p<0.05$) compared to C. Hx+G and Hx+G+O showed no significant change compared to C (Fig-49, Table- 52).

f) Time spent in closed arm by control and experimental groups of neonatal rats

Time spent in closed arm showed a significant increase in Hx, Hx+O ($p<0.01$), Hx+E+O ($p<0.001$); Hx+G+E+O ($p<0.05$), when compared to C. Hx+G and Hx+G+O showed no significant change compared to C (Fig-50 Table-53).

g) Percentage Time spent in open arm by control and experimental groups of neonatal rats

Percentage of time spent in open arm showed a significant decrease in Hx ($p<0.0$), Hx+O ($p<0.01$), Hx+E+O ($p<0.001$), Hx+G+E+O ($p<0.05$), when compared to C. Hx+G and Hx+G+O showed no significant change compared to C (Fig-51, Table-54,).

h) Head Dipping attempts by control and experimental groups of neonatal rats

There was a significant decrease in head dipping attempt in Hx ($p<0.01$), Hx+G ($p<0.05$); Hx+O ($p<0.01$); Hx+E+O ($p<0.001$); Hx+G+O ($p<0.05$) and Hx+G+E+O ($p<0.001$) compared to C (Fig-52, Table-55).

i) Stretched Attend Posture by control and experimental groups of neonatal rats

Hx+E+O and Hx+G+E+O showed a significant decrease ($p<0.01$) in stretched attend posture compared to C, while Hx, Hx+O, Hx+G and Hx+G+O showed no significant change (Fig-53, Table-56).

j) Grooming attempts by control and experimental groups of neonatal rats

There was a significant decrease in Hx, Hx+E+O and Hx+O($p<0.001$); Hx+G+E+O, ($p<0.01$) in grooming attempts compared to C, while Hx+G showed no significant change compared to C (Fig-54, Table-57).

II. Open Field Test

a) Crossing attempts by control and experimental groups of neonatal rats

There was a significant decrease in Crossing attempts by Hx, Hx+O ($p<0.05$), Hx+E+O ($p<0.01$); Hx+G+E+O ($p<0.05$), compared to C. Hx+G and Hx+G+O showed no significant change compared to C (Fig-55, Table-58).

b) Walking Time by control and experimental groups of neonatal rats

A significant decrease in the time of walk was shown by Hx ($p<0.01$), Hx+O ($p<0.001$), Hx+E+O ($p<0.05$), Hx+G+E+O ($p<0.05$) showed a compared to C. Hx+G and Hx+G+O showed no significant change compared to C (Fig-56, Table-59).

c) Resting Time by control and experimental groups of neonatal rats

A significant increase in the resting time were observed in Hx ($p<0.01$), Hx+O ($p<0.001$), Hx+E+O ($p<0.05$) and Hx+G+E+O ($p<0.01$) compared to C. Hx+G and Hx+G+O showed no significant change in the behaviour compared to C (Fig-57, Table-60).

d) Episodes of Rearings by control and experimental groups of neonatal rats

Episodes of rearings showed a significant increase in Hx+E+O ($p<0.001$), Hx+G+E+O ($p<0.01$) compared to C. Hx , Hx+O, Hx+G and Hx+G+O showed no significant change compared to C (Fig-58, Table-61).

e) Episodes of Head Sniffing and Washing by control and experimental groups of neonatal rats

Episodes of head sniffing and washing showed a significant increase in Hx, Hx+O and Hx+E+O ($p<0.01$), compared to C. Hx+G, Hx+G+O and Hx+G+E+O showed no significant change in the behaviour compared to C (Fig-59, Table-62).

Discussion

In the brain, the autonomic nervous system regulates and maintains body function and responds to external stimuli. It consists of two mutually inhibitory subsystems: those nerves which activate tissues- the sympathetic or arousal system and those which slow structures down for rest and repair- the parasympathetic or quiescent system. The sympathetic is ergotropic that releases energy and the parasympathetic is trophotropic, which is conserving energy. The two sides of our autonomic system reflect the two main processes in life "growth" or "protection." Perinatal hypoxia/ischemia is a major cause of cerebral palsy, mental retardation, epilepsy, various motor and behavioral disorders (Brown *et al.*, 1974). Vulnerability of the neonatal rat brain to hypoxia/ischemia appears to peak at the end of the first postnatal week and then progressively diminish (Ikonomidou *et al.*, 1989).

Body weight and blood glucose level in the serum

Maintaining blood glucose level continues to be important throughout the pregnancy, but it is particularly important during early developmental stage, when an embryo's organs are forming. Oxygen is needed by cells to break down glucose and produce energy. The oxygen requirement is more during embryonic development for all the tissues. Insufficient oxygen supply cause developmental abnormalities and birth defects. Thus hypoxia has a potential to cause damage to cells (Rulin *et al.*, 2005). The results suggest that hypoxic stress for thirty minutes during four days old rats does not cause significant change in body weight and blood glucose level after one week. Also, it suggests that supplementation of 100% oxygen, epinephrine and glucose does not adversely affect the blood glucose level and body weight (Kypson &

Hait, 1978). Epinephrine stimulated mechanical performance and heart rate of hypoxic hearts, but decreased myocardial glycogen and ATP. Though glucose utilization remained unchanged, the release of lactate increased from hypoxic hearts treated with epinephrine. However, epinephrine failed to stimulate myocardial lipolysis in hypoxic hearts. These metabolic changes due to epinephrine would lead to accelerated depletion of energetic reserves in hypoxic heart and its earlier deterioration.

Neurotransmitters in the cerebral cortex and adrenals.

Hypoxia and/or ischemia in the neonatal rat have been used as a model for studying the mechanisms underlying the pathogenesis of human brain pathology during perinatal anoxia (Rice *et al.*, 1981). Monoamine metabolites have been found to decrease in the extracellular fluid during hypoxia (Masuda & Ito, 1993; Richards *et al.*, 1993; Sarna *et al.*, 1990) and to increase during the recovery period (Damsma *et al.*, 1990). Exposure of neonatal rats to transient hypoxia induces a prolong decrease of brain DA and 5-HT content as well as DA uptake activity (Hadjiconstantinou *et al.*, 1990). Brain hypoxia/anoxia is associated with excessive unregulated release of neurotransmitters, especially Glu (Choi, 1988, 1990) and activation of their receptors. Glu has excitotoxic properties after its induced release. Hypoxic stress in the neonate induces significant changes in neurotransmitter activity and functioning of the hypothalamic- pituitary- adrenal- axis. These alterations cause an impairment of cognition by interfering with working memory capacity, independently of nutritional status. The change in cognitive performance after administration of glucose, depend on the level of sympathetic activation, glucocorticoid secretion and pancreatic β-cell function, rather than simple fuelling of neural activity. The outcomes can be predicted by vulnerability in coping with stressful challenges, interacting with nutritional and

neuroendocrine status (Gibson & Green, 2002). Stress exacerbates many neuropsychiatric disorders associated with prefrontal cortical (PFC) dysfunction. Stress also impairs the working memory functions of the PFC. Birnbaum *et al.*, (1999) reported that stress increases NE release in PFC.

Increased NE and EPI content in the cerebral cortex and adrenals of experimental animals exposed to hypoxia and supplemented with epinephrine and oxygen (Hx; Hx+O; Hx+E+O; Hx+G+E+O). The results suggest that the supplementation of glucose to hypoxic rats and hypoxic rats treated with oxygen showed better regulation of altered NE and EPI content in the cerebral cortex and adrenals of other experimental groups. Our results support the decreased DA content in the Cerebral cortex of neonatal rats exposed to hypoxic stress. The increased content of DA, NE and EPI in the adrenals suggests the enhanced production of catecholamines in the under-hypoxic stress in neonatal rats. Cheung *et al.*, (2001) reported that there were no differences in systemic or splanchnic oxygen extraction or consumption at any dose of dopamine or epinephrine.

Glutamate dehydrogenase activity in the cerebral cortex and liver

Enzymatic adaptations to hypoxia have shown that there is a change in affinity of enzymes involved in aerobic and anaerobic metabolism (Lushchac *et al.*, 1997). Glutamate dehydrogenase is an enzyme which has a key role in the synthesis of D- glucose. In hypoxic state a shift in the dynamic equilibrium from tri phosphate to di-phosphate resulting from increased energy consumption partially contribute to the increase in enzyme activity (Mons *et al.*, 1998, Hawkins *et al.*, 1986). GDH induces an increase in extracellular glutamate levels in the CNS with subsequent development of excitotoxicity (Kostic *et al.*, 1989). Glutamate, a major excitatory transmitter in the

brain, is most widely distributed in the central nervous system. Its malfunction has been implicated in major psychiatric disorders such as schizophrenia, drug addiction and depression (Pomara *et al.*, 1992; Perry & Hansen 1990; Plaitakis *et al.*, 1988; Rothstein *et al.*, 1996).

Organ specific studies revealed that GDH activity is higher in the liver. Developmental changes of GDH in rat liver were reported by Iguchi *et al.*, (1992). It is important to maintain low levels (1-3μM) of extracellular glutamate as excessive receptor stimulation or excessive ammonium generated by the glutamate dehydrogenase can lead to neural injury and/or death ("excitotoxicity"). Increased extracellular glutamate has also been implicated in the onset of neurodegeneration associated with hypoxic damage (stroke). The release in glutamate following hypoxia has been suggested to be due, at least in part, to a calcium-independent mechanism following the reversal of the neuronal glutamate uptake carrier (Szatkowski & Attwell, 1994). This increase in extracellular glutamate acts afterwards post-synaptically to increase cellular calcium levels with subsequent cell death.

GDH in the liver and cerebral cortex showed an enhanced activity in animals exposed to Hx, Hx+O, Hx+E+O, Hx+G+E+O. This suggests that the hypoxic stress causes an elevated glutamate release, which in turn can lead to glutamate toxicity. The supplementation of oxygen and epinephrine enhances the glutamate toxicity even administered along with glucose. When glucose is supplemented to Hx and Hx+O, the glutamate release is regulated towards C. This suggests that the glucose supplementation reduce the possibility for glutamate toxicity. Alterations in components of glutamergic system and glutamate metabolizing enzymes are considered with reference to mental disorders such as senile dementia of Alzheimer's type and schizophrenia (Boksha, 2004).

Acetylcholine esterase in the cerebral cortex and muscle

Acetylcholine esterase activity has been used as a marker for cholinergic activity (Goodman & Soliman, 1991). Acetylcholine esterase is the enzyme catalysing the degradation of acetylcholine into choline and acetyl CoA. Other evidence indicates that glucose and acetylcholine can interact during memory formation, raising the possibility that the memory-enhancing effects of postextinction trial glucose may ultimately involve a cholinergic mechanism. Extracellular and tissue ACh contents as well as choline activity are reported to be depressed during and after a hypoxic or ischemic insult (Beley *et al.*, 1992). One mechanism by which glucose may enhance acetylcholine function is by serving as a precursor to this neurotransmitter in conditions of high acetylcholine demand. Acetylcholine is synthesized by the reaction of choline and acetyl-Co-A, and glucose serves as the main source of acetyl-Co-A (Quastel, 1978). Although high-affinity choline uptake is generally the rate-limiting step for acetylcholine synthesis (Simon *et al.*, 1976), the availability of acetyl-CoA appears to be the rate-limiting step in certain conditions, including when cholinergic neurons are activated (Bielarczyk & Szutowicz, 1989).

The cholinergic innervation of the cerebral cortex has been extensively investigated because of its role in arousal, learning and memory (Olton *et al.*, 1991; Metherate *et al.*, 1992; Voytko *et al.*, 1994). Cholinergic neurons in the nucleus basalis magnocellularis (NBM) and associated forebrain nuclei are the major sources of the extrinsic cholinergic innervation of the cortex (Mesulam *et al.*, 1983). In the present study a significant increase in AChE activity in the muscle and significant decrease in the AChE activity in the cerebral cortex of Hx, Hx+O, Hx+O+E, Hx+G+E+O were observed.

In rodents, a small portion of the cortical cholinergic innervation is also derived from intrinsic neurons (Eckenstein & Baughman, 1983). The density of cholinergic terminals is particularly high in cortical layer (Houser *et al.*, 1985). There is evidence that synaptic density is related to cognitive function (Eastwood *et al.*, 1994). For instance, acquisition of cognitive tasks corresponds to an increase in the number of synapses in the motor cortex (Kleim *et al.*, 1996) and a reduction in synaptic density in the frontal cortex in Alzheimer's disease is correlated with cognitive decline (DeKosky & Scheff, 1990; Terry *et al.*, 1991). Therefore, it is reasonable to assume that the decline in cognitive function in aging is related to the diminution in cortical synaptic number in general and in particular to the decline in cholinergic synapses (Winkler *et al.*, 1995). Enhanced AChE activity in the muscle explains that the acetylcholine released is utilized by the muscle under hypoxic stress, where there is increased demand for energy. AChE activity in the cerebral cortex is deprived, indicating an alteration in ACh synthesis that leads to impairment in behaviour and cognition. The muscles need more energy and an increased demand for glucose is generated under hypoxia. AChE activity increase in the muscle, which in turn leads to enhanced utilization of ACh leading to locomotory defects during hypoxia.

Adrenergic receptor (AR) of epinephrine, α_2- AR, β- AR

Adrenergic receptors can be subdivided into several distinct categories, based on pharmacological specificity and physiological actions. These include the α_1, α_2, $ß_1$ and $ß_2$- adrenergic receptor subtypes. Theoretically, the ß-adrenergic effects of epinephrine would produce bronchodilation that could affect the distribution of ventilation. Because epinephrine activates both α-and ß-receptors, both pulmonary vasoconstriction (α) and vasodilation (ß) are possible. Bucheler *et al.*, (2002) reported

that the two functionally distinct α_2-adrenergic receptor subtypes α_{2A} and α_{2C}, operate as presynaptic inhibitory receptors regulating neurotransmitter release in the mouse CNS. These distinct categories of receptors differ not only in their specificity for various ligands but also in their specificity for coupling to G proteins and thereby respond to different second messenger systems.

It was reported that hypoxic pulmonary vasoconstriction was attenuated by a ß-adrenergic receptor–mediated vasodilation caused by reflex release of catecholamine from the adrenal gland and sympathetic nerves (David *et al.*, 1997). Prior to the development of sympathetic nerve function, adrenal catecholamine plays a predominant role in enabling the neonate to survive hypoxia. Interference with the release of adrenal amines invariably increased mortality during hypoxia. In contrast, interference with sympathetic neural release of catecholamines did not affect the ability of 1-day-old rats to withstand hypoxia, indicating that survival during low PO_2 conditions is not dependent on the sympathetic innervation at that stage of development. After functional development of the sympathetic nerves and disappearance of non-neurogenic adrenomedullary responses, the neonatal rats became partially dependent upon catecholamines derived from sympathetic terminals (Seidler & Slotkin 1985).

The present study suggests that hypoxia causes an up-regulation of epinephrine and β-adrenergic receptor in the cerebral cortex of hypoxic experimental groups and those treated with oxygen and epinephrine (Hx, Hx+O, Hx+E+O, Hx+G+E+O) whereas α_2 showed a down regulation in the above experimental groups. The glucose supplementation to hypoxic neonatal rats and those treated with oxygen (Hx+G; Hx+G+O) showed a reversal of receptor activity of epinephrine, β_2 and α_2AR. Real-Time PCR results showed an up-regulation of β2-AR gene expression and a

down –regulation of α_{2A} -AR which are in concordant with the receptor binding studies.

In the periphery, the adrenergic system plays an important role in regulating sympathetic function (Ani *et al.*, 2006 a,b) and cardiovascular system. Increased sympathetic discharge to the heart increases the rate and force of contraction mediated through β_1 receptors. Circulating adrenaline also acts on cardiac tissue. In addition, it acts on α_1 adrenoceptors in arterial smooth muscles, stimulating vasoconstriction, and on β_2 adrenoceptors in vascular beds of skeletal muscles, stimulating vasodilation. β_1 and β_2 receptors often coexist, but one subtype normally predominates. β_2 receptors mediate relaxation of smooth muscle (including vascular beds, bronchus, intestine and uterus); they mediate glycogenolysis and glucogenesis in the liver and regulate cell metabolism in skeletal muscles; they inhibit the activity of leukocytes and other blood cells; and they are found in the heart. The receptors are located presynaptically in nerves, where they facilitate neurotransmitter release and in the brain, where they regulate a variety of physiological processes. The β-adrenergic receptor stimulation evokes an increase in cAMP levels, which then activates cAMP dependent protein kinase through G proteins. Conversely, agonist activation of α_2-adrenergic receptor leads primarily to inhibition of cAMP *via* a distinct G protein, Gi (Bylund, 1988).

Glutamate receptor binding parameters in the cerebral cortex of experimental neonatal rats

Glucose in brain supplies energy essential for maintenance of the nervous system. Deficiency in glucose that results from hypoglycemia or ischemic insults can trigger neuronal injuries. Disturbance of ionic homeostasis results in membrane depolarization and massive release of neurotransmitters, including glutamate (Siesjo, 1988; Erecinska & Silver, 1989). Neurons can display many different kinds of

glutamate receptors on their surface, some of which are useful targets for treating neurologic diseases. Glutamate receptors can be divided into ionotropic and metabotropic receptors. Activation of ionotropic glutamergic receptors leads to greater permeability of the cell membrane to the sodium (Na^+) and calcium (Ca^{2+}) cations.

The extracellular accumulation of glutamate results in neuronal death by activating ionotropic glutamate receptors sensitive to NMDA or AMPA-kainate (Choi, 1988). In addition, neurons impaired of energy metabolism appear to be highly sensitive to excitotoxicity (Simon *et al.*, 1984; Cebers *et al.*, 1998). NMDA receptor antagonism appreciably affects both ventilatory phases of hypoxia. The inability to uphold ventilation in the depressant phase suggests that the NMDA glutamate-mediated pathway is operative in shaping the late hypoxic ventilatory response. The role of the glutamergic pathway is thus be extended beyond the hitherto recognized early ventilatory stimulation of hypoxia (Tarakanov *et al.*, 2004). The contribution of glutamate to synaptic transmission, plasticity and development is well established; current evidence is based on diverse approaches to decipher function and malfunction of this principal transmitter (Riedel *et al.*, 2003).

NMDA receptors are assembled from 5 subunits belonging to two families--NMDAR1 and NMDAR2. NMDAR2 family has four members-A, B, C, and D. Studies employing *in situ* hybridization and immunocytochemical techniques have shown that NR1 subunits are ubiquitously distributed throughout the central nervous system whereas the four NR2 subunits display differential expression patterns in several mammalian species including humans (Moriyoshi *et al.*, 1991, Petralia *et al.*, 1994, Rigby *et al.*, 1996) In general, within the cerebral cortex, hippocampus, and cerebellum, NMDA receptors are mainly distributed in neuronal cell bodies and dendrites. NR1 and NR2 are also found intracellular membranes such as mitochondria

and rough endoplasmic reticulum whereas in synapses they appear to be limited to the postsynaptic membrane and density (Moriyoshi *et al.*, 1991; Sheng *et al.*, 1994).

It is studied that the mechanisms of amino acid release that occur *in vivo* upon oxidative stress, hypoxia or ischemia is frequently associated with the impairment of energy metabolism (Rego *et al.*, 1996). Glutamate appears to be remarkably potent and rapidly acting neurotoxin. Exposure to 100μM glutamate for 5 min is enough to destroy large numbers of cultured cortical neurons. By the way, glutamate neurotoxicity is blocked by antagonist compounds and attenuated by antagonists added after glutamate exposure (Choi, 1990).

The results suggest that the glutamate receptors get overactivated during hypoxia in (Hx, Hx+O, Hx+E+O, Hx+G+E+O) and the toxicity can be reduced by the supplementation of glucose along with oxygen or without oxygen to the sufferers of hypoxic insult during early neonatal period. The Real-Time PCR study also supports this receptor data.

Intracellular free Ca^{2+} and reactive oxygen species (ROS) have been well documented as causative mediators of excitotoxicity (Choi, 1988; Lundgren *et al.*, 1992; Coyle & Puttfarcken 1993; Li *et al.*, 1998). However, administration of high glucose before hypoxic– ischemia has been reported to reduce brain damage (Kraft *et al.*, 1990; Vannucci *et al.*, 1996). Increasing glucose entry into neurons was shown to protect neurons from glutamate neurotoxicity (Ho *et al.*, 1995), stroke or seizure (Lawrence *et al.*, 1995, 1996), and mitochondrial toxins (Dash *et al.*, 1996).

Hypoxia causes irreversible neuronal damage within a shorter period than ischemia, with both free radicals and glutamate suggested to be involved in tandem in the neurotoxicity induced by hypoxia, whereas glutamate alone is involved in ischemic neurotoxicity (Omata *et al.*, 2000). NMDA neurotoxicity and oxidative stress have been well documented as mechanisms underlying hypoxic–ischemic brain

injuries. The neonatal rat administrated with high glucose is resistant to hypoxic–ischemic injuries (Palmer & Vannucci, 1993). Glucose entered into cells likely enhances mitochondrial potentials that play a central role in regulation of $[Ca^{2+}]i$ and ROS. These in contrast to the beneficial effects of high glucose, systemic administration of glucose before ischemia emphasize brain damage in the adult animals after hypoxic–ischemic injuries (Sieber & Traystman, 1992; So *et al.*, 1999).

Second messenger cAMP in the cerebral cortex of experimental neonatal rats

Hypoxia appears to activate adenylate cyclase directly and independent of any hormone-receptor interactions (Delpiano & Acker 1991). Biochemically, adrenergic receptors couple to several well-characterized signal transduction systems. Both β_1 and β_2-adrenergic receptors stimulate adenylyl cyclase (Emorine *et al.*, 1989), whereas α_2-adrenergic receptors have been classically shown to inhibit adenylyl cyclase in tissues (Ruffolo *et al.*, 1988). Agonist activation of either the β_1 or β_2-adrenergic receptor subtype classically leads to the generation of cyclic adenosine monophosphate (cAMP) by stimulating the enzyme adenylyl cyclase; this pathway is mediated by the G protein Gs. Conversely, agonist activation of α_2-adrenergic receptor leads primarily to inhibition of adenylyl cyclase *via* a distinct G protein, Gi (Bylund, 1988). Neurotransmitters, neuropeptides, chemokines and many other molecules signal through G protein-coupled receptors (GPCRs). Neonatal HI-induced brain damage is associated with specific changes in the GPCR desensitization machinery (Lombardi *et al.*, 2004). The adrenergic receptor subtypes involved in cyclic AMP responses to norepinephrine showed a large response to both α_1-receptor activation (increases in inositol phosphate accumulation) and α_2-receptor activation (decreases in forskolin-stimulated cyclic AMP accumulation) were observed in slices

of rat cerebral cortex and primary neuronal and glial cultures from rat brain (Brian & Kenneth, 1991).

The functions of G protein-coupled receptors are subject to dynamic regulation by a number of mechanisms. Covalent modification of the receptor (i.e., phosphorylation by various kinases) has been implicated in the regulation of β-adrenergic receptor function (Sibley *et al.*, 1987). Phosphorylation of β-adrenergic receptor is closely associated with impaired receptor function, (Benovic *et al.*, 1985, 1986) correlating with a decreased ability to couple to its G protein Gs. The paradigm in which receptor phosphorylation reactions have been examined is the phenomenon known as desensitization. Desensitization refers to the attenuation of responsiveness to a drug or hormone in its continued presence. This phenomenon can markedly diminish the therapeutic efficacy and duration of action of a drug.

Second messenger assay showed that the cAMP gets significantly enhanced in the experimental groups of neonatal rats except Hx+G and Hx+G+O. this support the receptor data showing up-regulation of β-AR and glutamate and down regulation of α_2-AR is mediated through cAMP pathway.

Electroencephalogram (EEG)

Neonatal electroencephalography presents some of the most difficult challenges in EEG interpretation. It differs significantly in many ways from EEG of neonate and older children. Technologically, acquisition of a neonatal EEG is significantly more difficult and different than an adult EEG. There are numerous features that are age-specific and change almost week-to-week in the preterm infant. Some features may be normal at one age and abnormal if they persist for several weeks. Many of these features also have different implications in neonates as compared to older individuals.

Seizures occur commonly in neonatal intensive care units and they are the important clinical evidence for CNS diseases in the newborn including brain hemorrhage, stroke, meningitis and hypoxic-ischemic encephalopathy (Stephen *et al.*, 2005). A seizure is the most frequent sign of neurological dysfunction in the neonate. Since seizures are the only sign of a central nervous system disorder, their recognition is very important. O'Meara *et al.*, (1995) reported that several infants had electrographic seizures with reduced or no clinical manifestations.

Although infants have been noted to have greater relative right or left frontal EEG as early as the neonatal period, other ways in which these newborns differ have not been reported (Field *et al.*, 2002). EEG measured during the neonatal period helps to analyse behavioral, physiological and biochemical changes of the individual as well as predict the possibility of neurodegenerative defects. A clinical seizure was considered to arise from a specific epileptic basis if it occurred simultaneously and was consistently synchronized to an electrographic seizure displayed on the coincident EEG. Many parts of the brain are immature at birth. This immaturity implies selective vulnerability, as well as selective resistance to specific disease processes. Rapid brain growth imposes rigid constraints. Therefore, an event that interferes with the developmental cascade has the potential for long term effects. In newborn rats, seizures inhibit brain protein synthesis (Jorgensen *et al.*, 1986), reduce brain size and delay developmental mile-stones (Wasterlain *et al.*, 1990, Holmes *et al.*, 1998). Recurrent seizures during the neonatal period also result in deficits in learning and memory when animals are studied as adults, despite lack of cell loss (Neill *et al.*, 1996). Neonatal seizures arise focally and often become generalized. Seizures contain rhythmic activity that can vary in frequency from approximately 0.5 to 8Hz and this activity is often very sharp (Stephen *et al.*, 2005). Generalized spike and wave activity

that is often seen in older children and adults is extremely rare in neonates (Clancy, 1995).

Behavioural activities of the experimental neonatal rats using Elevated Plus-Maze and Open-Field Test.

The differences in the anxiety and locomotors related behavioural activities observed in the experimental groups of rats predict the chance for occurrence of significant behavioural abnormalities as a result of hypoxic impact. Neonatal hypoxia-ischemia in the preterm human leads to selective injury to the subcortical developing white matter, which results in periventricular leukomalacia (PVL), a condition associated with abnormal neurodevelopment. Maturation-dependent vulnerability of late oligodendrocyte progenitors is thought to account for the cellular basis of this condition. A high frequency of cognitive and sensory deficits with decreasing gestational age suggests pervasive abnormalities of cortical development. In a neonatal rat model of hypoxic-ischemic injury that produces the characteristic pattern of subcortical injury associated with human PVL, selective sub plate neuron death is seen. The premature sub plate neuron death occurs after thalamic axons have reached their targets in cortex. Sub plate neuron cell death in PVL provides another mechanism for abnormal neurodevelopment and deficits in motor function after neonatal hypoxia-ischemia (Patrick *et al.*, 2003).

Elevated Plus-Maze test is used to test a drug's anxiogeneic or anxiolytic properties, memory impairment and general motor activities (Shah & Treit, 2004). The following parameters were measured to analyse the behavioural changes of the experimental rats using elevated plus-maze: open arm entry, closed arm entry, percentage arm entry, total arm entry, time spent in open arm, time spent in closed

arm, percentage of time spent in open arm, head dipping, stretched attend posture and grooming. Open field test is used to measure the locomotor activity of experimental animals to determine the drug effects (Halina & Roza, 2006). The response of experimental animal groups to crossing, time of walk, resting time, episodes of rearings, head sniffing and washing were measured.

The results showed that the experimental rats exhibit significant abnormalities in its behavioural pattern which may be due to cortical neuronal damage as a result of hypoxic stress. Supplementation of oxygen and epinephrine separately and in combination or along with glucose (as the traditional way of resuscitation practiced) seem to produce anxiogenic behaviour in the neonates exposed to hypoxia. The glucose supplemented to hypoxic and hypoxic rats oxygen treated showed a behavioural pattern very similar to control. Thus our study suggests the supplementation of glucose first alone or together with oxygen can regulate the possible anxiogenic and locomotory defect shown in neonates after hypoxic insult.

Cerebral palsy (CP) means "brain paralysis" refers to motor or postural abnormalities that are noted during early development. These anomalies are thought to be associated with prenatal, perinatal or postnatal events of varying etiologies (often multifactorial in nature). CP generally is considered to be a static encephalopathy that is non-progressive in nature. However, the clinical expression of CP is subjected to change as children and their developing nervous systems mature. Despite advances in neonatal care, CP remains a significant clinical problem (Papile *et al.*, 1978). Brain injury due to vascular insufficiency depends on various factors at the time of injury, including vascular distribution to the brain, efficiency of cerebral blood flow and regulation of blood flow, and biochemical response of brain tissue to decreased oxygenation (Singhi *et al.*, 2003).

Thus our results suggest that the traditional way of administration of epinephrine and oxygen currently practiced as part of initial resuscitation cause an adverse effect in the hypoxic stress. The sequence of treatment followed is without any scientific basis. Glucose administration prior to oxygen and epinephrine treatment has shown recovery from hypoxic damage to the brain. However, epinephrine treatment to glucose treated hypoxic rat model did not show that effective recovery. The neurotransmitters, neurotransmitter synthesizing enzymes, receptor binding parameters and Real-Time PCR studies support these findings. Also, brain activity measurements by EEG and behavioural studies confirm these results. These corrective measures from the molecular study brought to practice will lead to maintain healthy and intellectual life during later developmental stages. This will have immense clinical significance in neonatal care.

Summary

1) Hypoxia was induced in neonatal rats and was supplemented with glucose, epinephrine and oxygen as a traditional mode of resuscitation during neonatal hypoxia. This experimental model was used to study the adrenergic and glutamergic receptor alterations in the hypoxic neonatal rats.

2) Blood glucose level in the serum was measured to analyse the circulating glucose level changes due to supplementation of glucose, epinephrine and oxygen to hypoxic neonatal rats compared to control.

3) The neurotransmitter contents and its metabolites were measured to identify its alteration in the cerebral cortex and adrenals due to hypoxia using High Performance Liquid Chromatography.

    a) Significant increase of NE and EPI were observed in the cerebral cortex, whereas DA and HVA showed a significant decrease. Catecholamines (NE & EPI) showed a significant increase in the adrenals of the neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen. The glucose supplementation to hypoxic rats and along with oxygen reversed the altered catecholamine contents to near control.
    b) 5-HT and 5- HIAA in cerebral cortex showed a significant decrease in the neonatal rats- hypoxic; hypoxic rats treated with oxygen;

epinephrine and oxygen; a combination of glucose, epinephrine and oxygen. The glucose supplementation to hypoxic rats and along with oxygen reversed the altered 5-HT and 5-HIAA content to near control. Adrenals showed no significant change in its contents in the experimental groups of neonatal rats.

c) Glutamate content showed a significant increase in the cerebral cortex of neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen. Glucose supplementation to hypoxic rats and along with oxygen reversed the enhanced glutamate content to near control.

4) Glutamate dehydrogenase activity in the cerebral cortex and liver showed a significant increase in the neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen. The glucose supplementation to hypoxic rats and along with oxygen reversed the enhanced glutamate content to near control. Thus glucose is suggested to regulate the glutamate toxicity in hypoxic neonatal rats.

5) Acetylcholine esterase has been used as a marker for cholinergic activity. AChE showed a significant decrease in the cerebral cortex while it showed an increase in the muscles of experimental groups of hypoxic neonatal rats. The altered AChE activity is responsible for the abnormal behavioural pattern in the neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen. The glucose supplementation to hypoxic rats and along with oxygen reversed the cholinergic activity near to control.

6) Adrenergic receptor (AR) functional status was analysed by Scatchard and displacement analysis using specific [^{3}H] ligands in the cerebral cortex. [^{3}H] Epinephrine binding against epinephrine, [^{3}H] Yohimbine binding against phentolamine, [^{3}H] Propranolol binding against propranolol for general-AR, α_2-AR and β-AR respectively. Glutamate receptor activity was studied using [^{3}H] Glutamate against glutamate.

Epinephrine, β- AR and glutamate receptor showed a significant increase in the receptor activity in the neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen, whereas, the glucose supplementation to hypoxic rats and along with oxygen showed reversal of receptor activity near to control. Glucose supplementation to hypoxic rats showed reversal of the down regulation of α_2-AR receptor activity shown in the cerebral cortex of neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen.

Receptor analyses were confirmed by studying the mRNA status of the corresponding receptor using specific primers in Real-Time PCR.

7) Second messenger cAMP study showed an up-regulation in the cerebral cortex of neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen, whereas, the glucose supplementation to hypoxic rats and along with oxygen showed reversal of cAMP content towards control.

8) Brain wave activity generated in the brain regions- Frontal, Temporal, Parietal and Occipital lobes of the experimental neonatal rats measured using

electroencephalogram showed a significant change in neurophysiological activity of brain to hypoxic stress. The glucose supplementation to hypoxic rats and along with oxygen was able to reverse the brain wave activity caused by hypoxic stress in the neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen.

9) Behavioural activities of the experimental neonatal rats using Elevated Plus-Maze and Open-Field Test, showed a significant change in the anxiogeneic and locomotory action in neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen; a combination of glucose, epinephrine and oxygen. Also, the glucose supplementation to hypoxic rats and along with oxygen was able to reverse this changes observed to near control.

Our studies suggest that hypoxic stress damages the brain function which leads to severe consequences in later stages of life. The sequence of treatment followed, if corrected by supplementation of glucose and oxygen; and if required, epinephrine, will have tremendous importance in the control of damage to the brain. These corrective measures from the molecular study brought to practice will lead to maintain healthy intellectual life during the later developmental stages. This will have immense therapeutic significance in neonatal care.

Conclusion

Our findings demonstrated that hypoxia during the neonatal period caused significant impact in the central nervous system (CNS) both functionally and behaviourally. The evaluation of these damages at molecular level is very important, especially in a critical cerebral function to reduce the damage during later developmental stages. Even though the body weight or blood glucose level is not altered after seven days of hypoxic insult, it caused immense alterations in the brain and adrenal neurotransmitter contents. The GDH activity in the cerebral cortex and liver was enhanced in the hypoxic neonatal rats explaining the glutamate excitotoxicity. AChE assay in cerebral cortex and muscle highlight that hypoxia causes memory imbalance and muscular damage. Brain requires continuous supply of oxygen for energy utilization and efficient functioning. Hypoxia leads to disruption of this energy utilization, resulting in neuronal functional failure, cerebral palsy and neuro-developmental delay. Structural and functional integrity of brain depends on regular glucose and oxygen supply. The receptor binding studies showed an up-regulation of epinephrine, β-AR and glutamate receptors. The α_2-AR is down regulated by hypoxia. The second messenger study confirms that this up-regulation is through the activation of cAMP pathway. Receptor data were confirmed by Real-Time PCR. These receptor studies suggest that hypoxic stress causes damage to brain in the neonatal rats- hypoxic; hypoxic rats treated with oxygen; epinephrine and oxygen and a combination of glucose, epinephrine and oxygen. The glucose supplementation to hypoxic rats and along with oxygen is able to reverse this damage. The brain activity test done using EEG confirmed the neurophysiological changes in

brain due to hypoxic insult. The behavioural studies using Elevated Plus-Maze and Open-Field Test suggest the anxiogenic and locomotor deformity due to hypoxia.

Thus from our results we conclude that, hypoxic neonatal rats supplemented with glucose alone and glucose plus oxygen showed reversal of adrenergic and glutamate receptor functional changes to control. This is for the first time that an investigation based on therapeutic application of glucose supplementation to hypoxia, oxygen and epinephrine were studied. The impact of this molecular study on brain adrenergic and glutamate receptor function in hypoxic neonatal rats confirmed the altered neuronal cellular functional failure due to hypoxia, oxygen and epinephrine treatment. EEG wave pattern in the brain regions showed brain activity changes in hypoxia, oxygen and epinephrine treatment. The efficient and timely supplementation of glucose plays a crucial role in correcting the molecular changes due to hypoxia, oxygen and epinephrine. The additive neuronal damaging effect due to oxygen and epinephrine treatment is another important observation. These corrective measures from the molecular study brought to practice will lead to maintain healthy intellectual capacity during the later developmental stages, which has immense clinical significance in neonatal care.

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Papers Published:

1. **Finla Chathu** and C. S. Paulose. Glutamate Toxicity in the Cerebral Cortex of Hypoxia Induced Neonatal Rats: Epinephrine, Glucose and Oxygen Effects. SFRR India Bulletein, **75**: **(2006)**.

2. V. Ani Das, **Finla Chathu** and C.S. Paulose: Decreased α_2-adrenergic receptor in the brain stem and pancreatic islets during pancreatic regeneration in weanling rats. **79**,16: 1507-1513 **(2006)**.

3. V.V. Mohanan, **Finla Chathu**, Paulose C. S: Decreased 5-HT2C receptor binding in the cerebral cortex and brain stem during pancreatic regeneration in rats. Molecular *and Cellular Biochemistry*,**272**, 165-170 **(2005)**.

Awards:

1. **Finla Chathu** and C. S. Paulose. Glutamate Toxicity in the Cerebral Cortex of Hypoxia InducedNeonatal Rats: Epinephrine, Glucose and Oxygen Effects.
   Best oral Presentation Award in the **International Conference On Free Radicals And Clinical Laboratory Medicine And SFRR-India Satellite Meeting** organised by Dept. of Biochemistry, AIMS, January 3-4th, **2007**.

2. Amee Krishnakumar, **Finla Chathu** and C. S. Paulose. Acetylcholine esterase activity in the cerebral cortex and muscle of hypoxic rats: regulatory role of glucose, oxygen and epinephrine. National conference on molecular medicine, SBTI & AIMS Kochi January 13-14th **2007. IBS award for Best Oral Presentation** in Medical Biotechnology.

Abstracts/ Presented:

1. Anu Joseph, **Finla Chathu** and C S Paulose. Enhanced Glutamate dehydrogenase activity in the Cerebral Cortex and Liver of Hypoxic Rats: Effect of Oxygen, Epinephrine and Glucose treatment. Society for Reproductive Biology and Comparative Endocrinology [SRBCE] XXV **National Symposium on Reproductive Biology and Comparative Endocrinology.** January 15 - 17th, **2007.**

2. Amee Krishnakumar, **Finla Chathu** and C. S. PauloseAcetylcholine esterase activity in the cerebral cortex and muscle of hypoxic rats: regulatory role of glucose, oxygen and epinephrine. **National conference on molecular medicine, SBTI & AIMS Kochi** January 13 - 14th, **2007.**

3. **Finla Chathu** and C. S. Paulose. Glutamate Toxicity in the Cerebral Cortex of Hypoxia InducedNeonatal Rats: Epinephrine, Glucose and Oxygen Effects. **International Conference On Free Radicals And Clinical Laboratory Medicine And SFRR-India Satellite Meeting** organised by Dept. of Biochemistry, AIMS, January 3-4th, **2007.**

4. **Finla Chathu** and C. S. Paulose: Down-regulation of $Alpha_2$ Adrenergic Receptor Activity in the Cerebral Cortex of Hypoxia Induced Neonatal Rats. Biotechnology and Economic Development- A Kerala Scenario and Annual Award Session, Society for Biotechnologists (INDIA) at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram (March 25-26th, **2006**).

5. **Finla Chathu**: Enhanced Adrenergic Receptor Gene Expression in the Cerebral Cortex of Hypoxic Neonatal Rats: Epinephrine, Glucose and Oxygen Effects- Kerala Science Congress, CESS Thiruvananthapuram (January 29 – 31st, **2006).**

6. **Finla Chathu**, Ani Das V. and Paulose C. S. Alpha$_2$-adrenergic receptor down-regulation in the cerebral cortex and brain stem during pancreatic regeneration in rats. International Conference on Biotechnology and Neuroscience, Cochin University of Science and Technology, Cochin (Dec. **2004**).

7. G. Gireesh, S. Reas Khan, **Chathu Finla** and C.S. Paulose. Decreased glutamate decarboxylase and increased glutamate dehydrogenase activity in the cerebellum of alcoholic rats. IAN, SNCI, International Conference, University of Hyderabad, (May. **2004**).

8. **Finla Chathu**, Asha Abraham and C. S. Paulose, Changes in monoamine contents in the corpus striatum of streptozotocin – induced diabetic rats as a function of age – Geriatric Society of India, Annual Conference at Kochi, India, (Nov **2003**).

9. Renuka T.R. and C. S. Paulose. Differential expression of M1 and M3 muscarinic receptors in the cerebral cortex during pancreatic regeneration IMSACON (**Finla Chathu** presented for Renuka T. R) at Kochi (Sept. **2003**).

Table-1

Body Weight and Blood Glucose level of Control and Experimental Groups of Neonatal Rats

Animal status	Body weight (g)		Blood Glucose level (mg/dl)
	Initial day of Experiment	Final day of Experiment	
C	6.99 ± 0.42	12.53 ± 0.05	123.40 ± 0.59
Hx	7.11 ± 0.35	13.18 ± 0.55	125.40 ± 1.74
Hx+G	7.37 ± 0.25	13.45 ± 0.67	120.47 ± 1.01
Hx+O	7.09 ± 0.06	13.45 ± 0.40	127.73 ± 3.62
Hx+G+O	7.19 ± 0.11	13.39 ± 0.32	123.73 ± 2.83
Hx+E+O	7.14 ± 0.02	12.93 ± 0.37	123.37 ± 1.09
Hx+G+E+O	7.07 ± 0.12	12.93 ± 0.19	123.37 ± 1.71

Values are Mean ± S.E.M of 4-6 separate experiments

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-2

Catecholamines and Metabolite Content (nmoles/g wet wt.) in the Cerebral Cortex of Control and Experimental Groups of Neonatal Rats.

Animal status	NE	EPI	DA	HVA
C	336.56± 2.76	543.50 ± 7.95	1127.7 ± 9.91	306.68 ± 11.17
Hx	536.86 ± 4.67$^{**\varphi\varphi\dagger\dagger b}$	638.33 ± 8.19$^{***\varphi\varphi\varphi\dagger\dagger\dagger}$	727.33 ± 4.2$^{**\varphi\varphi\dagger\dagger}$	242.94 ± 5.06$^{**\varphi\varphi\dagger\dagger}$
Hx+G	372.45 ± 2.55	540.72 ± 7.81	1055.7± 1.58	316.50 ± 4.02
Hx+O	673.47 ± 3.99$^{***\varphi\varphi\varphi\dagger\dagger\dagger a\ b\ c}$	639.55 ± 12.01$^{***\varphi\varphi\varphi\dagger\dagger\dagger}$	546.08 ± 6.23$^{***\varphi\varphi\varphi\dagger\dagger\dagger}$	255.31 ± 6.03$^{**\varphi\varphi\dagger\dagger}$
Hx+G+ O	353.90 ± 6.30	545.52 ± 5.33	1041.21 ± 7.52	296.32 ± 8.63
Hx+E+O	551.75 ± 7.14$^{**\varphi\varphi\dagger\dagger b}$	615.83 ± 12.45$^{**\varphi\varphi\dagger\dagger}$	754.55 ± 5.65$^{**\varphi\varphi\dagger\dagger}$	227.87± 6.91$^{**\varphi\varphi\dagger\dagger}$
Hx+G+E+O	567.67 ± 4.17$^{**\varphi\varphi\dagger\dagger}$	603.44 ± 4.45$^{**\varphi\varphi\dagger\dagger bb}$	594.38 ± 5.39$^{***\varphi\varphi\varphi\dagger\dagger\dagger}$	240.55 ± 7.80$^{**\varphi\varphi\dagger\dagger}$

Values are Mean ± S.E.M of 4-6 separate experiments

$^{**}p<0.01$, $^{***}p<0.001$ when compared to C
$^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ when compared to Hx+G
$^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to Hx+G+O
$^{a}p<0.05$ compared to Hx
$^{b}p<0.05$, $^{bb}p<0.01$ when compared to Hx+E+O
$^{c}p<0.05$ compared to Hx+G+E+ O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated Hx+G+E+O

Table-3

Serotonin and its Metabolite content (nmoles/g wet wt.) in the Cerebral Cortex of Control and Experimental Groups of Neonatal Rats

Animal status	5-HT	5-HIAA
C	277.23 ± 9.83	674.98 ± 12.32
Hx	151.20 ± 5.62 $^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger}$	513.14 ± 12.56 $^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger}$
Hx+G	250.91 ± 6.10	631.15 ± 11.47
Hx+O	217.55 ± 4.07 $^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger}$	528.20 ± 5.80 $^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger\ b}$
Hx+G+ O	269.13 ± 4.07	666.21 ± 7.32
Hx+E+O	151.46 ± 6.67 $^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger}$	475.52 ± 11.12 $^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger}$
Hx+G+E+O	158.17 ± 8.56 $^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger}$	545.42 ± 8.05 $^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger\ b}$

Values are Mean ± S.E.M of 4-6 separate experiments

*** $p<0.001$ when compared to C
$^{\varphi\varphi\varphi}$ $p<0.001$ when compared to Hx+G
††† $p<0.001$ when compared to Hx+G+O
$^{b}p<0.05$ when compared to Hx+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table 4

Catecholamines and Metabolite content (nmoles/g wet wt.) in the Adrenals of Control and Experimental Groups of Neonatal Rats.

Animal status	NE	EPI	DA	HVA
C	536.25 ± 11.75	4737.72 ± 7.76	140.69 ± 6.57	10.67 ± 0.24
Hx	656.30 ± 2.51$^{**\varphi\varphi\dagger\dagger bb}$	5654.61± 6.89$^{***\varphi\varphi\varphi\dagger\dagger\dagger bb\,b}$	301.32 ± 3.04$^{***\varphi\varphi\varphi\dagger\dagger\dagger ccc}$	7.35 ± 2.97
Hx+G	537.05 ± 1.49	4931.63 ± 5.65	143.05 ± 0.93^{aaa}	8.86 ± 0.03
Hx+O	655.35 ± 2.14$^{**\varphi\varphi\dagger\dagger bb}$	5569.42 ± 4.36$^{***\varphi\varphi\varphi\dagger\dagger\dagger bb\,b}$	512.5 ± 4.73$^{***\varphi\varphi\varphi\dagger\dagger\dagger aaa ccc}$	9.49 ± 1.91
Hx+G+ O	555.35 ± 2.35	4736.32 ± 6.85	145.01 ± 6.37^{aaa}	9.35 ± 0.15
Hx+E+O	743.44 ± 2.94$^{***\varphi\varphi\varphi\dagger\dagger\dagger}$	6849.90 ± 12.45$^{***\varphi\varphi\varphi\dagger\dagger\dagger}$	507.22 ± 6.04$^{***\varphi\varphi\varphi\dagger\dagger\dagger aaa ccc}$	10.79 ± 1.80
Hx+G+E+O	644.33 ± 1.19$^{**\varphi\varphi\dagger\dagger bbb}$	5427.32 ± 5.55$^{***\varphi\varphi\dagger\dagger bbb}$	188.22 ± 12.32$^{*\varphi\dagger\dagger aaa}$	21.11 ± 2.45$^{*a\dagger\varphi}$

Values are Mean ± S.E.M of 4-6 separate experiments

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to C
$^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ compared to Hx+G
$^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared to Hx+G+O
$^{a}p<0.05$, $^{a\,a}p<0.01$, $^{a\,a\,a}p<0.001$ compared to Hx
$^{b}p<0.05$, $^{bb}p<0.01$, $^{bb\,b}p<0.001$ compared to Hx+E+O
$^{c}p<0.05$), $^{cc}p<0.01$, $^{c\,c\,c}p<0.001$ compared to Hx+G+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated Hx+G+E+O

Table-5

Serotonin and its Metabolite content (nmoles/g wet wt.) in the Adrenals of Control and Experimental Groups of Neonatal Rats

Animal status	5-HIAA	5-HT
C	55.90 ± 6.31	17.64 ± 3.13
Hx	57.90 ± 3.42	11.68 ± 1.17
Hx+G	57.35 ± 2.05	22.05 ±1.35
Hx+O	65.62 ± 4.10	20.20 ± 1.80
Hx+G+ O	60.62 ± 4.52	22.55 ± 0.15
Hx+E+O	54.15 ± 1.45	22.32 ± 5.52
Hx+G+E+O	60.65 ± 2.25	22.25 ± 2.95

Values are Mean ± S.E.M of 4-6 separate experiments

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-6

Glutamate content (μmoles/g wt of tissue) in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	Glutamate content
C	12.53 ± 1.33
Hx	21.21 ± 1.26 $^{**\ \varphi\varphi\ \dagger\dagger}$
Hx+G	13.49 ± 1.68
Hx+O	28.37 ± 2.48 $^{***\ \varphi\varphi\varphi\dagger\dagger\dagger\ aa\ bbb\ cc}$
Hx+G+O	14.08 ± 0.45
Hx+E+O	18.52 ± 0.73 *
Hx+G+E+O	22.29 ± 1.24 $^{**\ \varphi\varphi\ \dagger\dagger}$

Values are Mean ± S.E.M of 4-6 separate experiments

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ when compared to C
$^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ when compared to Hx+G
$^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to Hx+G+O
$^{aa}p<0.01$ when compared to Hx
$^{bbb}p<0.001$ when compared to Hx+E+O
$^{cc}p<0.01$ when compared to Hx+G+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-7

Glutamate Dehydrogenase Activity in the Cerebral Cortex of Control and Experimental Groups of Neonatal Rats

Animal status	Cerebral Cortex	
	V_{max} (Enyme Units/mg protein)	K_m (mM)
C	6.66 ± 0.83	0.80 ± 0.10
Hx	11.33 ± 0.16$^{***\ \varphi\varphi\varphi\dagger\dagger\dagger}$	0.63 ± 0.06
Hx+G	7.26 ± 0.39	0.80 ± 0.10
Hx+O	16.83 ± 0.16$^{***\ \varphi\varphi\varphi\dagger\dagger\dagger\ aaa\ bbb\ ccc}$	0.58 ± 0.04
Hx+G+ O	7.66 ± 0.33	0.72 ± 0.02
Hx+E+O	10.16 ± 0.16$^{***\ \varphi\varphi\varphi\dagger\dagger\dagger}$	0.78 ± 0.08
Hx+G+E+O	11.16 ± 0.17$^{***\ \varphi\varphi\varphi\dagger\dagger\dagger}$	0.83 ± 0.08

Values are Mean ± S.E.M of 4-6 separate experiments

*** $p<0.001$ when compared to C
$^{\varphi\varphi\varphi}$ $p<0.001$ when compared to Hx+G
††† $p<0.001$ when compared to Hx+G+O
$^{aaa}p<0.001$ when compared to Hx
$^{bbb}p<0.001$ when compared to Hx+E+O
$^{ccc}p<0.001$ when compared to Hx+G+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-8

Glutamate Dehydrogenase Activity in the Liver of Control and Experimental Groups of Neonatal Rats

Animal status	Liver	
	V_{max} (Enyme Units/mg protein)	K_m (mM)
C	14.16 ± 0.16	0.33± 0.03
Hx	$21.20 \pm 0.65^{***\ \varphi\varphi\varphi\dagger\dagger\dagger\ bb}$	$0.18 \pm 0.01^{**}$
Hx+G	15.25 ± 0.14	0.28 ± 0.02
Hx+O	$24.33 \pm 0.16^{***\ \varphi\varphi\varphi\dagger\dagger\dagger\ bbb}$	$0.19 \pm 0.01^{**}$
Hx+G+ O	15.46 ± 29	0.25 ± 0.02
Hx+E+O	$18.50 \pm 0.28^{***\ \varphi\varphi\varphi\dagger\dagger\dagger}$	$0.22 \pm 0.02^{*}$
Hx+G+E+O	$19.51 \pm 0.50^{***\ \varphi\varphi\varphi\dagger\dagger\dagger}$	0.26 ± 0.03

Values are Mean ± S.E.M of 4-6 separate experiments

$^{*}p<0.05, ^{**}p<0.01, ^{***}p<0.001$ when compared to C
$^{\varphi\varphi\varphi}p<0.001$ when compared to Hx+G
$^{\dagger\dagger\dagger}p<0.001$ when compared to Hx+G+O
$^{bb}p<0.01$ and $^{bbb}p<0.001$ when compared to Hx+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-9

Acetylcholine Esterase Activity in the Cerebral Cortex of Control and Experimental Groups of Neonatal Rats

Animal status	Cerebral Cortex	
	V_{max} (Enyme Units/mg ptn)	K_m (mM)
C	6.90± 0.72	0.20 ± 0.04
Hx	4.03± 0.17 ***	0.22 ± 0.06
Hx+G	6.02 ± 0.38	0.20 ± 0.10
Hx+O	4.70 ± 0.16 **	0.20 ± 0.04
Hx+G+ O	5.45± 0.28	0.22 ± 0.02
Hx+E+O	4.75± 0.16 **	0.22 ± 0.08
Hx+G+E+O	4.89 ± 0.16 **	0.20 ± 0.08

Values are Mean ± S.E.M of 4-6 separate experiments

**p<0.01, *** p<0.001 when compared to C

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-10

Acetylcholine Esterase Activity in the Muscle of Control and Experimental Groups of Neonatal Rats

Animal status	Muscle	
	V_{max} (Enyme Units/mg protein)	K_m (mM)
C	3.52 ± 0.12	0.125 ± 0.10
Hx	5.01 ± 0.13 ***	0.10± 0.06
Hx+G	4.02 ± 0.08	0.125 ± 0.10
Hx+O	5.20 ± 0.11 ***	0.10 ± 0.04
Hx+G+ O	4.75± 0.18	0.125 ± 0.12
Hx+E+O	5.30 ± 0.86 ***	0.125 ± 0.08
Hx+G+E+O	5.10 ± 0.76 ***	0.10 ± 0.08

Values are Mean ± S.E.M of 4-6 separate experiments

*** $p<0.001$ when compared to C

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-1

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine in the Cerebral Cortex of Control and Hypoxic Neonatal Rats

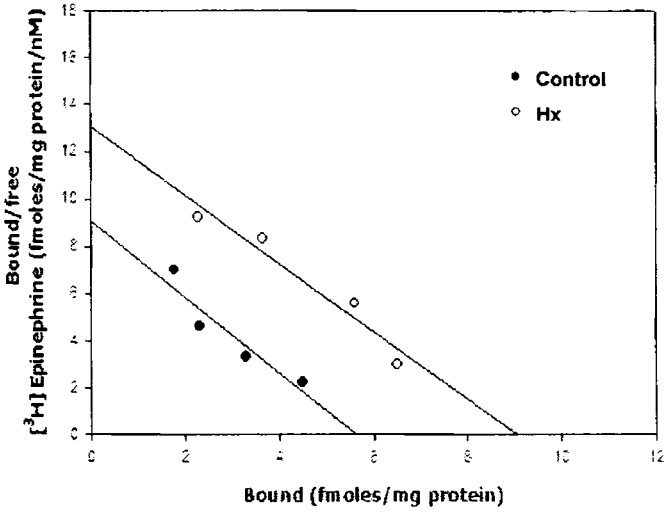


Table-11

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine in the Cerebral Cortex of Control and Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	5.87 ± 0.28	0.55±0.05
Hx	9.18 ± 0.52*	0.65±0.01

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats – C; Hypoxic rats- Hx
* $p<0.05$ when compared to C

Figure-2

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine in the Cerebral Cortex of Control and Glucose Treated Hypoxic Neonatal Rats

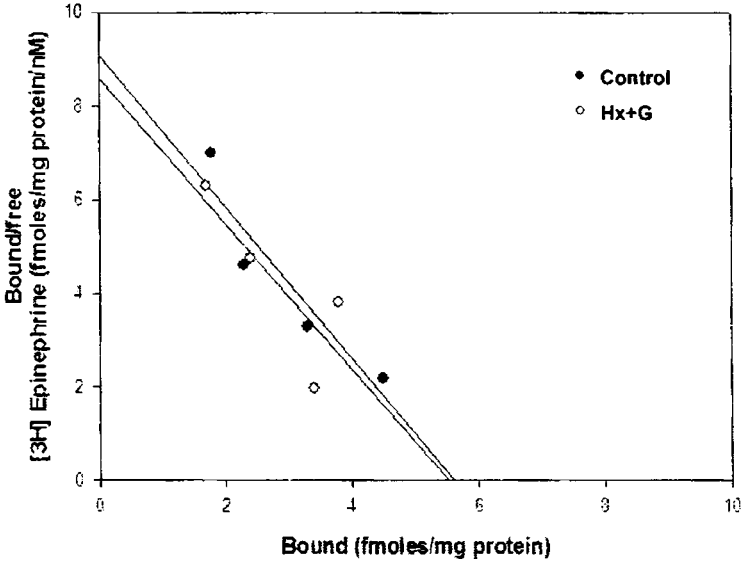


Table-12

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine in the Cerebral Cortex of Control and Glucose Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	5.87 ± 0.28	0.55±0.05
Hx+G	6.70 ±0.37	0.78±0.04

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats glucose treated - Hx+G

Figure -3

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine in the Cerebral Cortex of Control and Oxygen Treated Hypoxic Neonatal Rats

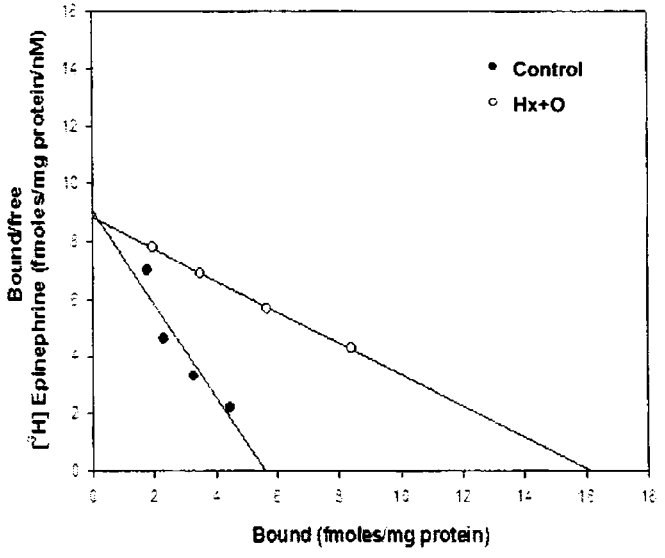


Table-13

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine in the Cerebral Cortex of Control and Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	5.87 ± 0.28	0.55±0.05
Hx+O	12.78 ±1.54***	1.49 ±0.10***

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats oxygen treated - Hx+O
*** $p<0.001$ when compared to C

Figure-4

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine In the Cerebral Cortex of Control and Glucose + Oxygen Treated Hypoxic Neonatal Rats

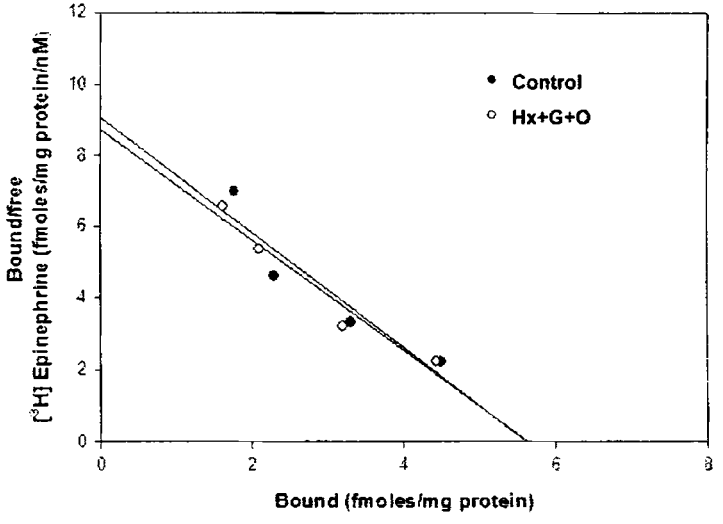


Table-14

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine In the Cerebral Cortex of Control and Glucose+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	5.87 ± 0.28	0.55±0.05
Hx+G+O	5.90 ± 0.21	0.65 ±0.06

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats – C; Hypoxic rats glucose and oxygen treated - Hx+G+O

Figure-5

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine In the Cerebral Cortex of Control and Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

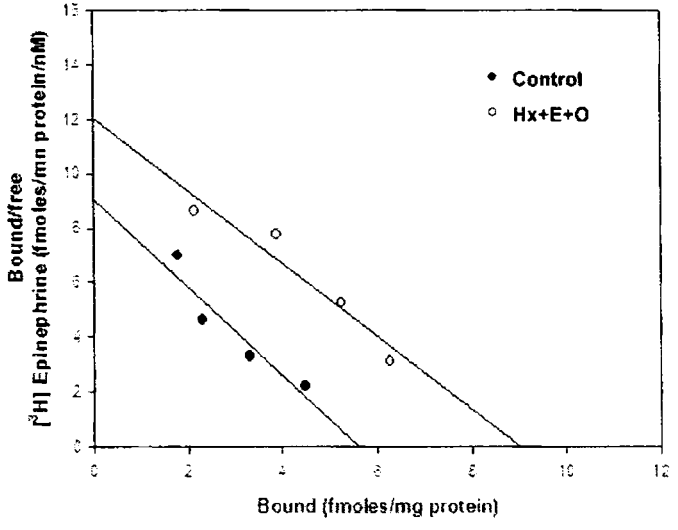


Table-15

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine In the Cerebral Cortex of Control and Epinephrine+ Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	5.87 ± 0.28	0.55±0.05
Hx+E+O	10.56 ±0.53***	0.89 ± 0.08

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats epinephrine and oxygen treated - Hx+E+O
*** $p<0.001$ when compared to C

Figure-6

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine In the Cerebral Cortex of Control and Glucose+Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

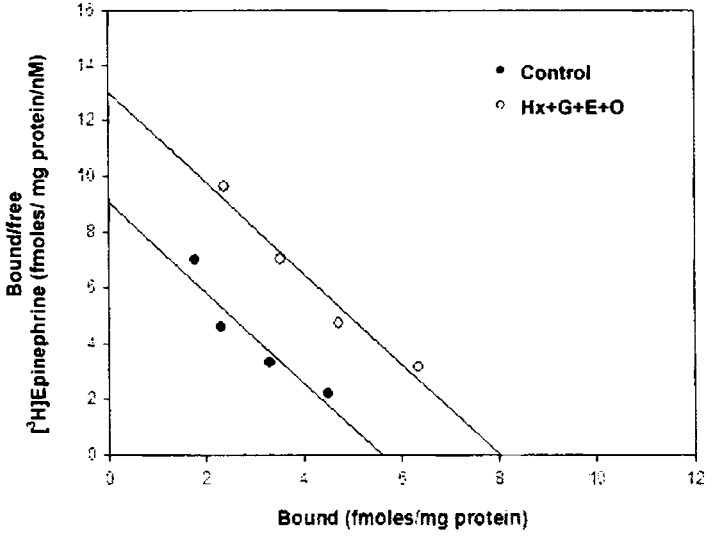


Table-16

Scatchard analysis of [^{3}H]Epinephrine Binding Against Epinephrine In the Cerebral Cortex of Control and Glucose+Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	5.87 ± 0.28	0.55±0.05
Hx+G+E+O	8.91±0.41*	0.71±0.09

Values are Mean ± S.E.M of 4-6 separate experiments

B_{max} - Maximal binding; K_d - Dissociation constant

Control rats – C; Hypoxic rats Glucose, Epinephrine and Oxygen treated - Hx+G+E+O

* $p<0.05$ when compared to C

Figure-7

Binding parameters of [^{3}H]Epinephrine Against Epinephrine in the Cerebral Cortex of Experimental Groups of Neonatal Rats

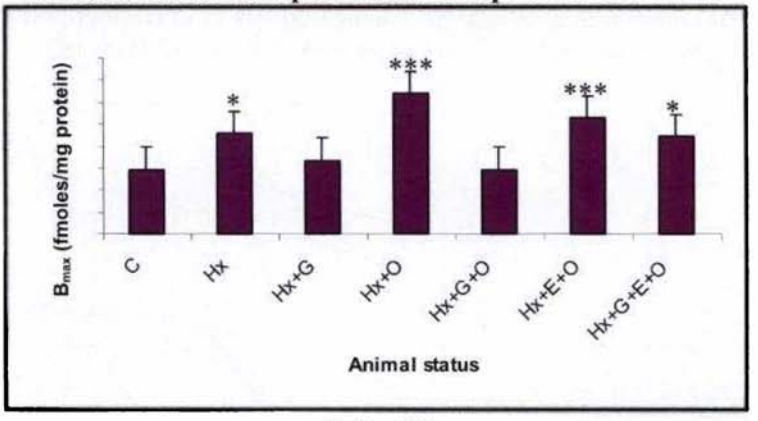


Table -17

Binding parameters of [^{3}H]Epinephrine Against Epinephrine in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	5.87 ± 0.28	0.55±0.05
Hx	9.18 ± 0.52$^{*\,\varphi\dagger}$	0.65±0.01
Hx+G	6.70 ±0.37	0.78±0.04
Hx+O	12.78 ±1.54$^{***\ \varphi\varphi\varphi\dagger\dagger\dagger\ abc}$	1.49 ±0.10$^{***\ \varphi\varphi\varphi\ \dagger\dagger\dagger\ aa\ bb\ cc}$
Hx+G+O	5.90 ± 0.21	0.65 ±0.06
Hx+E+O	10.56 ±0.53$^{***\ \varphi\varphi\varphi\dagger\dagger\dagger}$	0.89 ± 0.08
Hx+G+E+O	8.91±0.41$^{*\varphi\,\dagger}$	0.71±0.09

Values are Mean ± S.E.M of 4-6 separate experiments

B_{max} - Maximal binding; K_d - Dissociation constant

* p<0.05, *** p<0.001 when compared to C

^{a} p<0.01, ^{aa}p<0.001 when compared to Hx

$^{\varphi}$ p<0.05, $^{\varphi\varphi\varphi}$ p<0.001 when compared to Hx+G

† p<0.05, ††† p<0.001 when compared to Hx+G+O

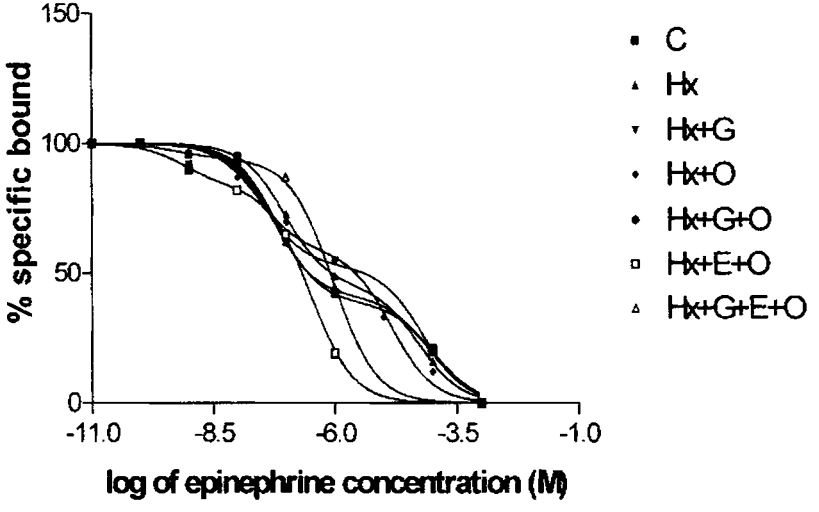
^{b} p<0.05, ^{bb} p<0.001 when compared to Hx+E+O

^{c} p<0.01, ^{cc} p<0.001 when compared to Hx+G+E+O

Control rats - C; Hypoxic rats- Hx; Hypoxic rats glucose treated - Hx+G, Hypoxic rats oxygen treated - Hx+O; Hypoxic rats glucose and oxygen treated - Hx+G+O; Hypoxic rats epinephrine and oxygen treated - Hx+E+O; hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure -8

Displacement of [^{3}H]Epinephrine Against Epinephrine in the Cerebral Cortex of Experimental groups of Neonatal rats



Values are mean of 4-6 separate experiments

Control rats – C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-18

Displacement of [^{3}H]Epinephrine Against Epinephrine in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
C	Two-site	-7.02	-3.77	3.12×10^{-8}	5.64×10^{-5}	-0.61
Hx	Two-site	-6.93	-4.19	3.88×10^{-8}	2.14×10^{-5}	-0.51
Hx+G	Two-site	-7.31	-4.09	1.61×10^{-8}	2.67×10^{-5}	-0.50
Hx+O	Two-site	-7.42	-4.74	1.25×10^{-8}	5.99×10^{-6}	-0.37
Hx+G+O	Two-site	-7.09	-6.23	2.67×10^{-8}	4.5×10^{-7}	-0.64
Hx+E+O	Two-site	-7.86	-3.86	4.50×10^{-9}	1.92×10^{-5}	-0.28
Hx+G+E+O	Two-site	-6.20	-5.84	2.09×10^{-7}	4.81×10^{-7}	-0.14

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA).
Ki - The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity).
EC_{50} is the concentration of the competitor that competes for half the specific binding.

Control rats – C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-9

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Hypoxic Neonatal Rats

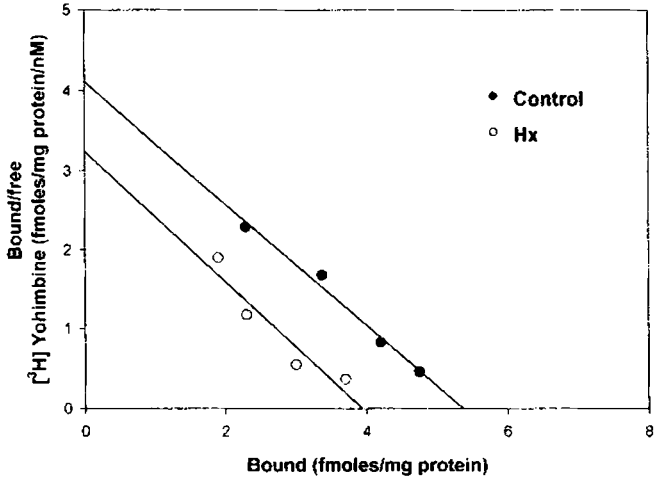


Table -19

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	7.66 ± 0.16	3.02 ± 0.07
Hx	5.90 ± 0.21 **	3.3 ± 0.12

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats – C; Hypoxic rats- Hx
** $p<0.01$ when compared to C

Figure-10

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Glucose Treated Hypoxic Neonatal Rats

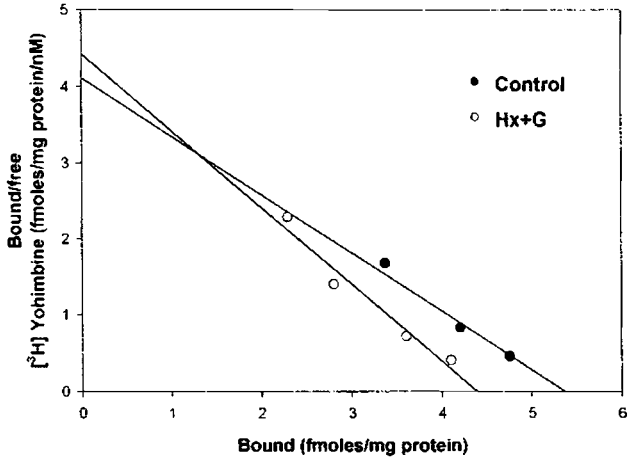


Table-20

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Glucose Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	7.66 ± 0.16	3.02 ± 0.07
Hx+G	6.66 ± 0.16	3.20 ± 0.42

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats glucose treated - Hx+G

Figure-11

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Oxygen Treated Hypoxic Neonatal Rats

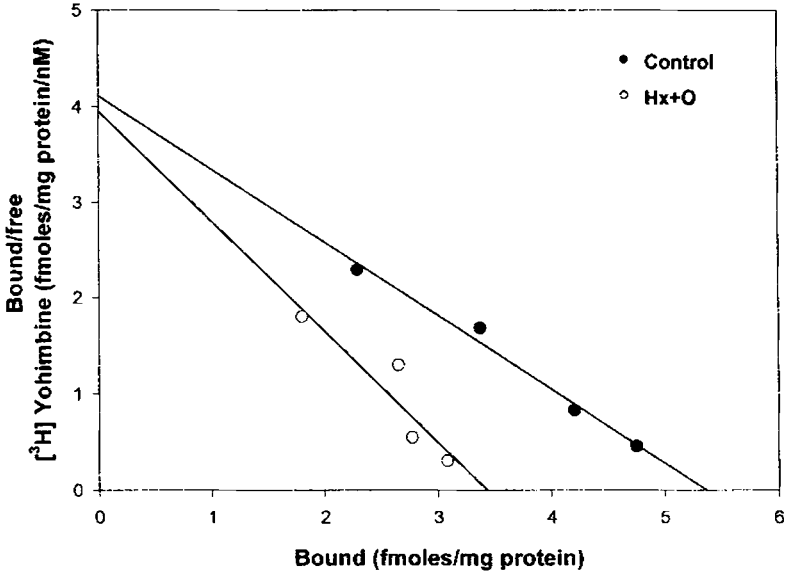


Table-21

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	7.66 ± 0.16	3.02 ± 0.07
Hx+O	4.96 ± 0.24 ***	2.67 ± 0.19

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C ; Hypoxic rats oxygen treated - Hx+ O
*** $p<0.001$ when compared to C

Figure-12

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Glucose+Oxygen Treated Hypoxic Neonatal Rats

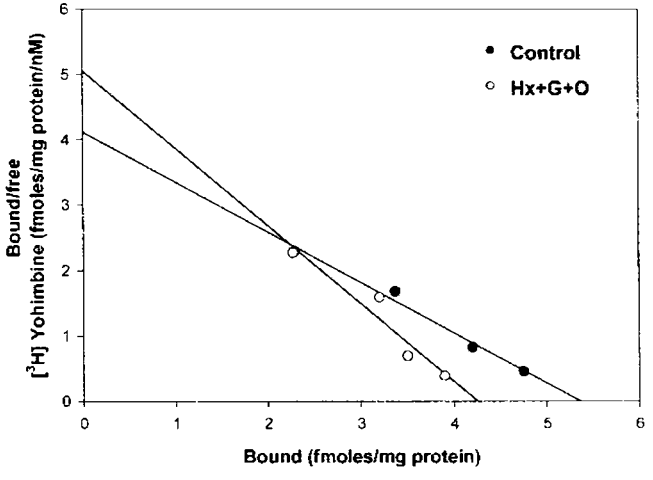


Table-22

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Glucose+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	7.66 ± 0.16	3.02 ± 0.07
Hx+G+ O	6.60 ± 0.41	3.47 ± 0.22

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats -- C; Hypoxic rats glucose and oxygen treated - Hx+G+O

Figure-13

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine In the Cerebral Cortex of Control and Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

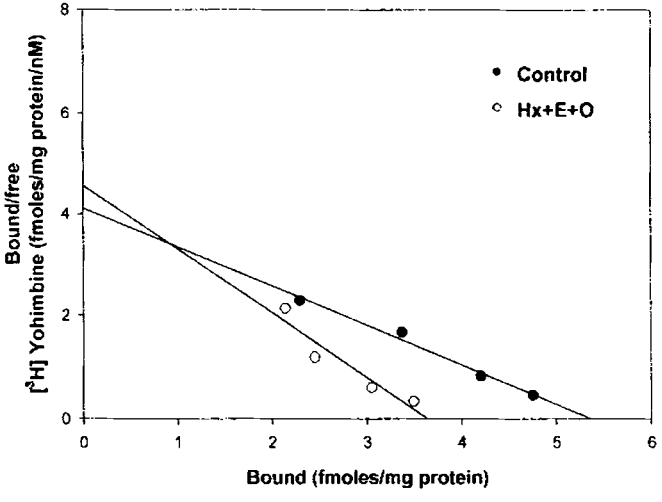


Table-23
Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine In the Cerebral Cortex of Control and Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	7.66 ± 0.16	3.02 ± 0.07
Hx+E+O	5.83 ± 0.26 **	2.97 ± 0.33

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats epinephrine and oxygen treated - Hx+E+O
** $p<0.01$ when compared to C

Figure-14

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Glucose+Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

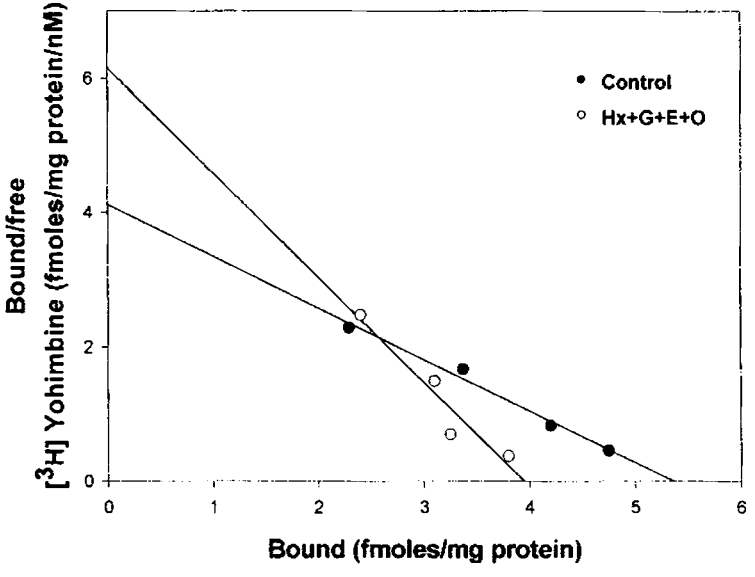


Table-24

Scatchard analysis of [^{3}H]Yohimbine Binding Against Phentolamine in the Cerebral Cortex of Control and Glucose+Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	7.66 ± 0.16	3.02 ± 0.07
Hx+G+E+O	5.53 ± 0.26 ***	2.90 ± 0.33

Values are Mean ± S.E.M of 4-6 separate experiments

B_{max} - Maximal binding; K_d - Dissociation constant

Control rats–C; Hypoxic rats Glucose, Epinephrine and Oxygen treated - Hx+G+E+O

***$p<0.001$ when compared to C

Figure-15

Binding parameters of [^{3}H]Yohimbine Against Phentolamine in the Cerebral Cortex of Experimental Groups of Neonatal Rats

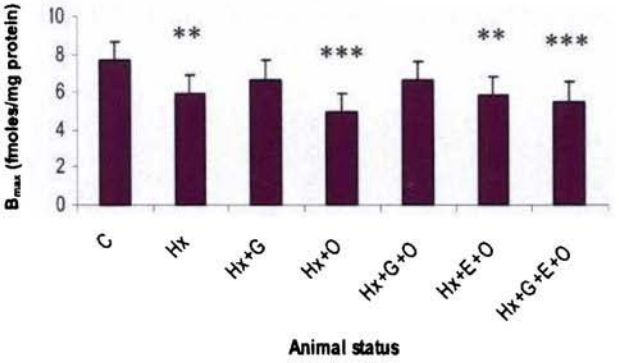


Table -25

Binding parameters of [^{3}H]Yohimbine Against Phentolamine in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	7.66 ± 0.16	3.02 ± 0.07
Hx	5.90 ± 0.21 **	3.3 ± 0.12
Hx+G	6.66 ± 0.16	3.20 ± 0.42
Hx+O	4.96 ± 0.24 *** φφ††	2.67 ± 0.19
Hx+G+ O	6.60 ± 0.41	3.47 ± 0.22
Hx+E+O	5.83 ± 0.26 **	2.97 ± 0.33
Hx+G+E+O	5.53 ± 0.26 *** φ†	2.90 ± 0.33

Values are Mean ± S.E.M of 4-6 separate experiments

B_{max} - Maximal binding; K_d - Dissociation constant

** $p<0.01$, *** $p<0.001$ when compared to C

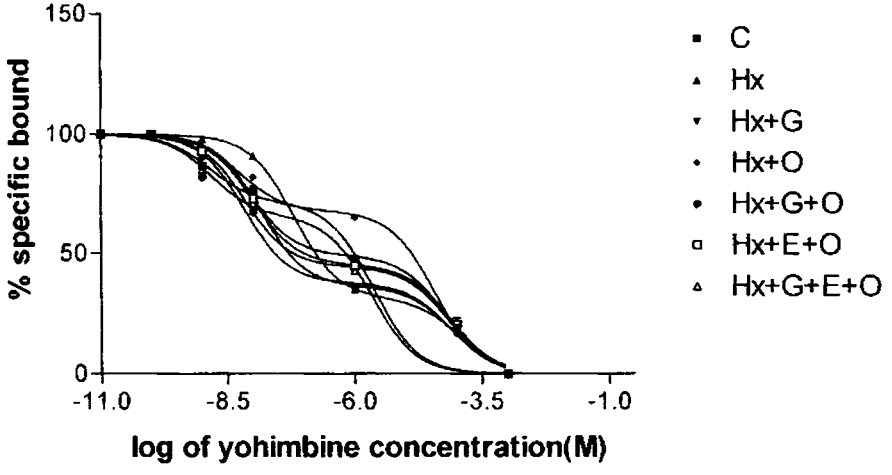
φ $p<0.05$, φφ $p<0.01$ when compared to Hx+G

† $p<0.05$, †† $p<0.01$ when compared to Hx+G+O

Control rats - C; Hypoxic rats- Hx; Hypoxic rats glucose treated - Hx+G, Hypoxic rats oxygen treated - Hx+O; Hypoxic rats glucose and oxygen treated - Hx+G+O; Hypoxic rats epinephrine and oxygen treated - Hx+E+O; hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O.

Figure-16

Displacement of [^{3}H] Yohimbine against Phentolamine in the Cerebral Cortex of Experimental Groups of Neonatal Rats



Values are mean of 4-6 separate experiments

Control rats – C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-26

Displacement of [^{3}H] Yohimbine against Phentolamine in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
C	Two-site	-7.88	-4.04	4.30×10^{-9}	3.04×10^{-5}	-0.29
Hx	Two-site	-7.09	-3.83	2.69×10^{-8}	4.91×10^{-5}	-0.38
Hx+G	Two-site	-7.63	-3.71	7.74×10^{-9}	6.42×10^{-5}	-0.26
Hx+O	Two-site	-8.06	-4.31	2.81×10^{-9}	1.62×10^{-5}	-0.29
Hx+G+ O	Two-site	-8.00	-3.38	3.33×10^{-9}	4.84×10^{-5}	-0.38
Hx+E+O	Two-site	-7.91	-4.00	4.06×10^{-9}	3.33×10^{-5}	-0.26
Hx+G+E+O	Two-site	-7.99	-3.95	3.40×10^{-9}	1.7×10^{-5}	-0.26

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA).
Ki - The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity).
EC_{50} is the concentration of the competitor that competes for half the specific binding.

Control rats – C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-17

Real-Time PCR amplification of the α_{2A} subunit of Adrenergic receptor mRNA from the Cerebral Cortex of Control and Experimental Groups of Neonatal Rats

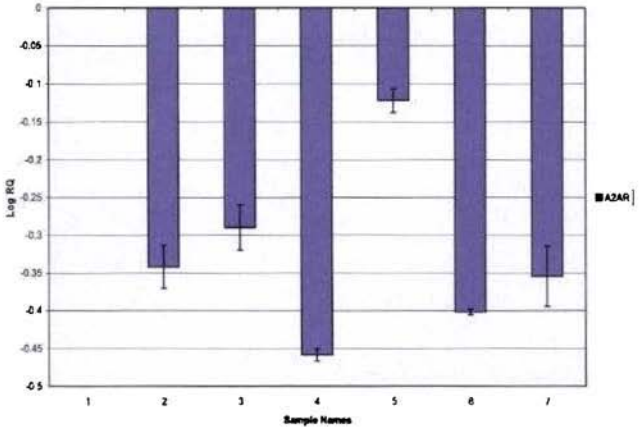


Table-27

Real-Time PCR Amplification of the α_{2A} subunit of Adrenergic receptor mRNA from the Cerebral Cortex of Experimental Groups of Neonatal Rats

Sample No.	Animal status	RQ value
1	C	0
2	Hx	-0.34 ± 0.02
3	Hx+G	-0.29 ± 0.03
4	Hx+O	-0.46 ± 0.01
5	Hx+G+O	-0.12 ± 0.02
6	Hx+E+O	-0.40 ± 0.01
7	Hx+G+E+O	-0.35 ± 0.04

Values are Mean ± S.D of 4-6 separate experiments

Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the ΔΔCT method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

1 - Control rats Hypoxic rats
2 - Hypoxic rats glucose treated
3 - Hypoxic rats oxygen treated
4 - Hypoxic rats glucose and oxygen treated
5 - Hypoxic rats glucose and oxygen treated
6 - Hypoxic rats epinephrine and oxygen treated
7 - Hypoxic rats glucose, epinephrine and oxygen treated

Figure-18

Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Hypoxic Neonatal Rats

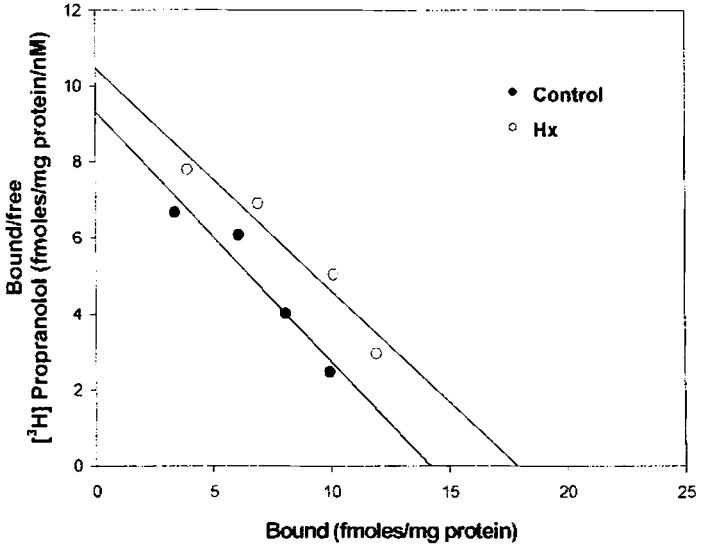


Table-28
Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	14.51 ± 0.28	1.71± 0.26
Hx	17.66 ± 0.66 *	1.73 ± 0.02

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats – C; Hypoxic rats- Hx
* $p<0.05$ when compared to C

Figure-19

Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Glucose Treated Hypoxic Neonatal Rats

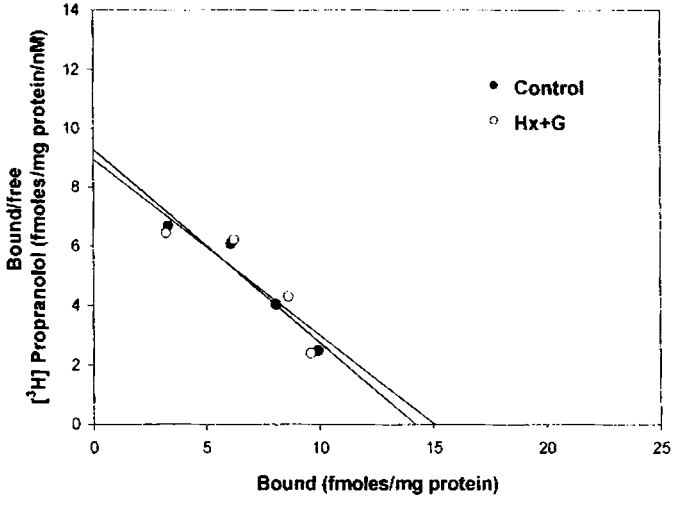


Table-29

Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Glucose Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	14.51 ± 0.288	1.71± 0.26
Hx+G	15.73 ± 0.64	1.72± 0.01

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats – C; Hypoxic rats glucose and oxygen treated - Hx+G

Figure-20

Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Oxygen Treated Hypoxic Neonatal Rats

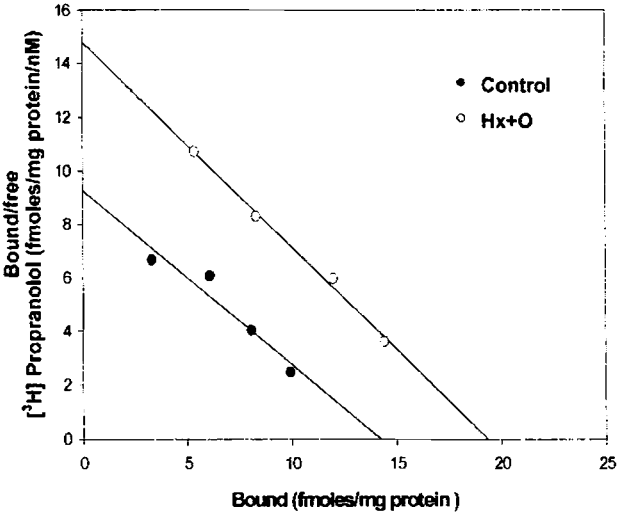


Table-30

Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	14.51 ± 0.28	1.71± 0.26
Hx+O	22.07 ± 0.64 ***	1.74 ± 0.05

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C ; Hypoxic rats oxygen treated - Hx+O
*** $p<0.001$ when compared to C

Figure-21

Scatchard analysis of [^{3}H] Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Glucose+Oxygen Treated Hypoxic Neonatal Rats

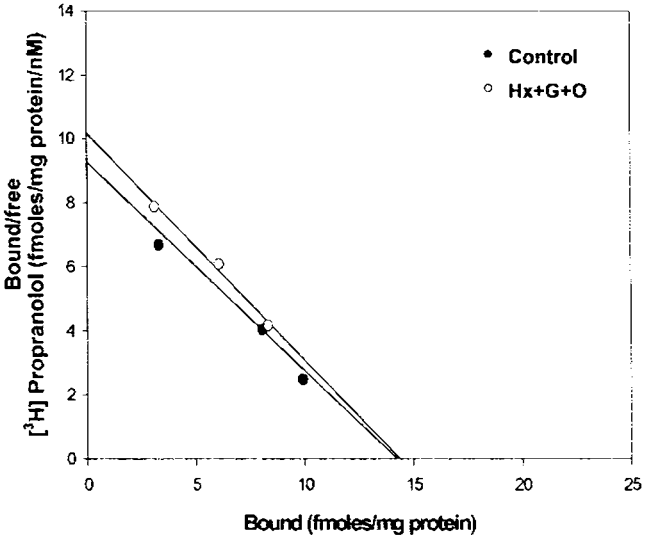


Table-31

Scatchard analysis of [^{3}H] Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Glucose+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	14.51 ± 0.28	1.71± 0.26
Hx+G+ O	15.13± 1.04	1.73 ± 0.01

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats -- C; Hypoxic rats glucose and oxygen treated - Hx+G+O

Figure-22

Scatchard analysis of [^{3}H] Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

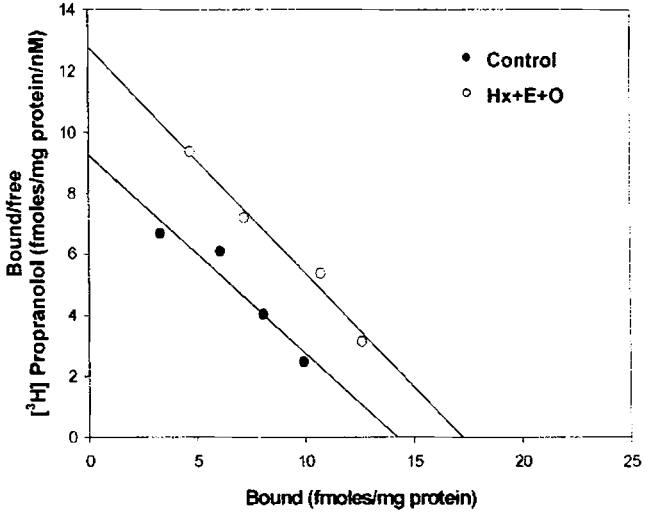


Table-32

Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	14.51 ± 0.28	1.71± 0.26
Hx+E+O	20.02 ± 0.50 ***	1.75 ± 0.01

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats epinephrine and oxygen treated - Hx+E+O
*** $p<0.001$ when compared to C

Figure-23

Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Glucose+Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

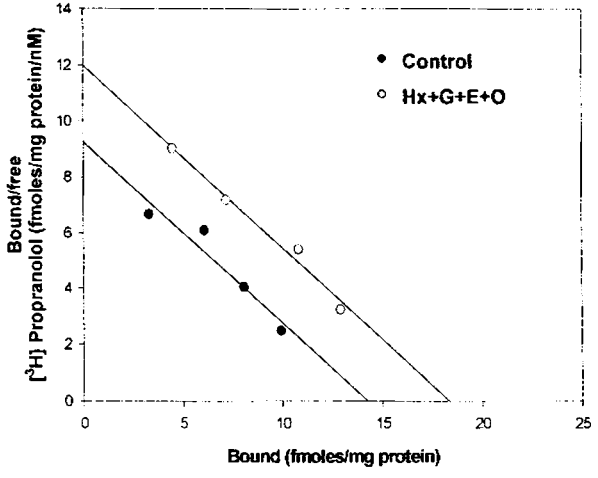


Table-33

Scatchard analysis of [^{3}H]Propranolol Binding Against Propranolol in the Cerebral Cortex of Control and Glucose+Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	14.51 ± 0.28	1.71± 0.26
Hx+G+E+O	15.73 ± 0.63 **	1.73 ± 0.01

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats-C; Hypoxic Rats Glucose, Epinephrine and Oxygen Treated - Hx+G+E+O
** $p<0.01$ when compared to C

Figure-24
Binding parameters of [^{3}H]Propranolol Against Propranolol in the Cerebral Cortex of Experimental Groups of Neonatal Rats

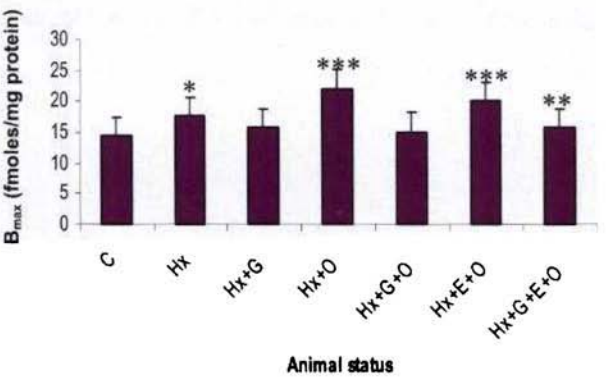


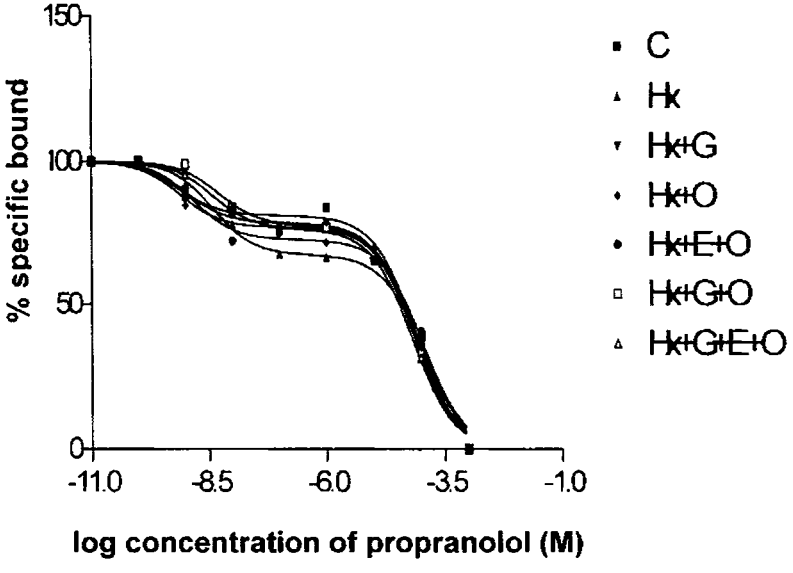
Table-34
Binding parameters of [^{3}H]Propranolol Against Propranolol in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	14.51 ± 0.288	1.71± 0.26
Hx	17.66 ± 0.66 *†	1.73 ± 0.02
Hx+G	15.73 ± 0.64	1.72± 0.01
Hx+O	22.07 ± 0.64 $^{***\varphi\varphi\varphi\dagger\dagger\dagger abc}$	1.74 ± 0.05
Hx+G+ O	15.13± 1.04	1.73 ± 0.01
Hx+E+O	20.02 ± 0.50 $^{***\varphi\varphi\dagger\dagger\dagger}$	1.75 ± 0.01
Hx+G+E+O	15.73 ± 0.63 $^{**\varphi\varphi\dagger\dagger}$	1.73 ± 0.01

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
** p<0.01, *** p<0.001 when compared to C
$^{\varphi}$ p<0.05, $^{\varphi\varphi}$ p<0.01 when compared to Hx+G
† p<0.05, †† p<0.01when compared to Hx+G+O
^{a} p<0.01 when compared to Hx
^{b} p<0.05 when compared to Hx+E+O
^{c} p<0.05 when compared to Hx+G+E+O
Control rats - C; Hypoxic rats- Hx; Hypoxic rats glucose treated - Hx+G, Hypoxic rats oxygen treated - Hx+O; Hypoxic rats glucose and oxygen treated - Hx+G+O; Hypoxic rats epinephrine and oxygen treated - Hx+E+O; hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-25

Displacement of [^{3}H]Propranolol against Propranolol in the Cerebral Cortex of Experimental Groups of Neonatal Rats



Values are mean of 4-6 separate experiments

Control rats – C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-35

Displacement of [^{3}H]Propranolol against Propranolol in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
C	Two-site	-8.67	-4.01	3.1×10^{-9}	9.61×10^{-5}	-0.18
Hx	Two-site	-8.20	-3.86	9.5×10^{-9}	1.30×10^{-4}	-0.32
Hx+G	Two-site	-8.71	-4.04	7.0×10^{-9}	9.00×10^{-5}	-0.21
Hx+O	Two-site	-8.78	-4.01	3.1×10^{-9}	9.73×10^{-5}	-0.23
Hx+G+ O	Two-site	-7.99	-4.06	9.5×10^{-9}	8.61×10^{-5}	-0.22
Hx+E+O	Two-site	-8.48	-3.84	7.0×10^{-8}	1.4×10^{-4}	-0.27
Hx+G+E+O	Two-site	-7.92	-4.07	7.0×10^{-8}	8.71×10^{-5}	-0.23

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA).
Ki - The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity).
EC_{50} is the concentration of the competitor that competes for half the specific binding.

Control rats – C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-26

Real-Time PCR Amplification of the β_2 subunit of Adrenergic receptor mRNA from the Cerebral Cortex of Experimental Groups of Neonatal Rats

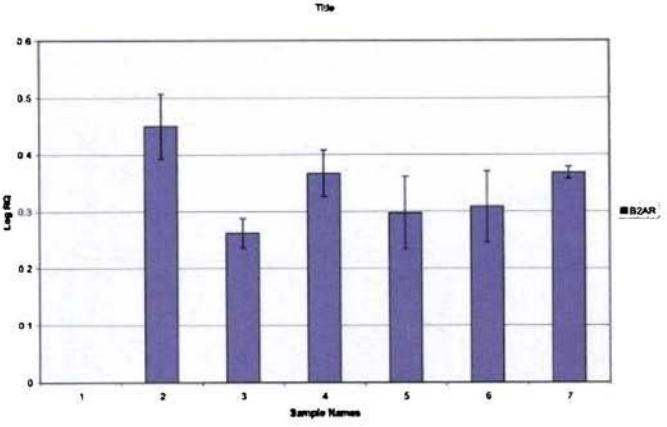


Table-36

Real-Time PCR Amplification of the β_2 subunit of Adrenergic receptor mRNA from the Cerebral Cortex of Experimental Groups of Neonatal Rats

Sample No.	Animal status	RQ value
1	C	0
2	Hx	0.31± 0.06
3	Hx+G	0.26 ± 0.03
4	Hx+O	0.36 ± 0.04
5	Hx+G+O	0.29 ± 0.06
6	Hx+E+O	0.49 ± 0.04
7	Hx+G+E+O	0.36 ± 0.01

Values are Mean ± S.D of 4-6 separate experiments

Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the ΔΔCT method normalized with β-actin CT value as the internal control and Control CT value as the calibrator.

1 - Control rats Hypoxic rats
2 - Hypoxic rats glucose treated
3 - Hypoxic rats oxygen treated
4 - Hypoxic rats glucose and oxygen treated
5 - Hypoxic rats glucose and oxygen treated
6 - Hypoxic rats epinephrine and oxygen treated
7 - Hypoxic rats glucose,epinephrine and oxygen treated

Figure-27

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Hypoxic Neonatal Rats

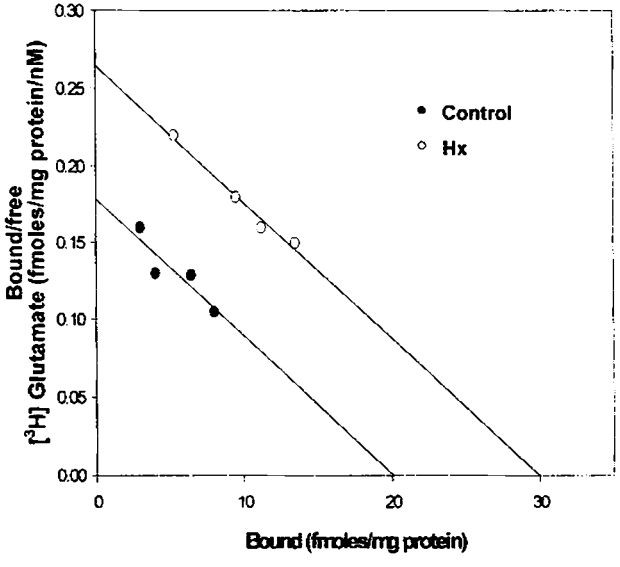


Table-37

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	23.51± 0. 28	112.50 ± 0.09
Hx	34.66 ± 0.92***	112.97 ± 0.71

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats – C; Hypoxic rats- Hx
*** $p<0.001$ when compared to C

Figure-28

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Glucose Treated Hypoxic Neonatal Rats

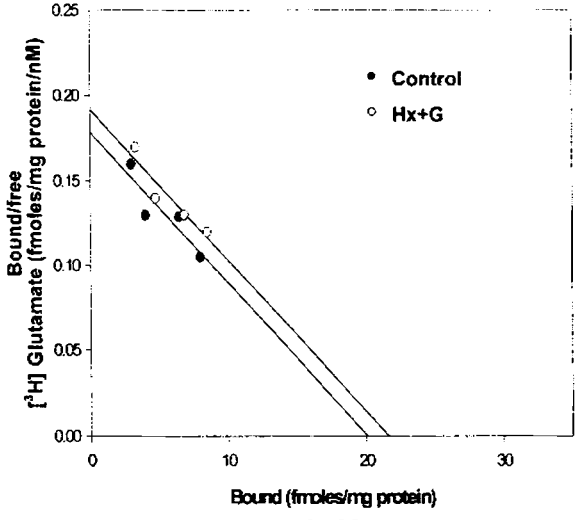


Table-38

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Glucose Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	23.51± 0. 28	112.50 ± 0.09
Hx+G	24.83 ± 0.57	114.38 ± 1.86

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats glucose treated - Hx+G

Figure-29

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Oxygen Treated Hypoxic Neonatal Rats

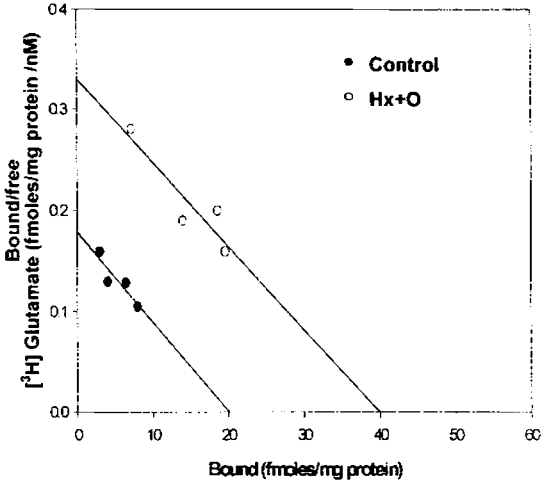


Table-39

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	23.51± 0. 28	112.50 ± 0.09
Hx+O	42.66 ±1.20***	113.83 ± 1.58

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats oxygen treated - Hx+O
*** $p<0.001$ when compared to C

Figure-30

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Glucose+Oxygen Treated Hypoxic Neonatal Rats

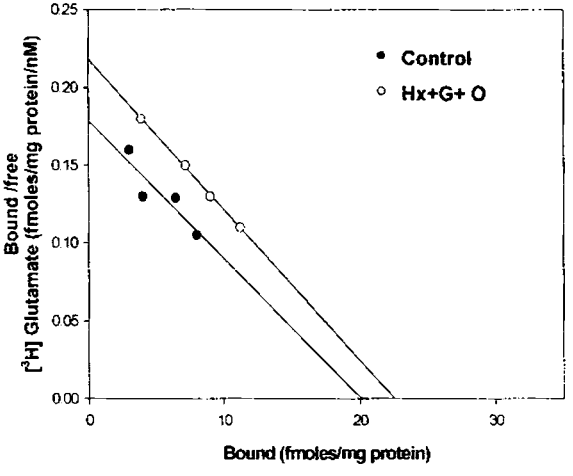


Table-40

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Glucose+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	23.51± 0. 28	112.50 ± 0.09
Hx+G+O	25.50 ± 0.57	116.73 ± 1.36

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats – C; Hypoxic rats glucose and oxygen treated - Hx+G+O

Figure-31

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

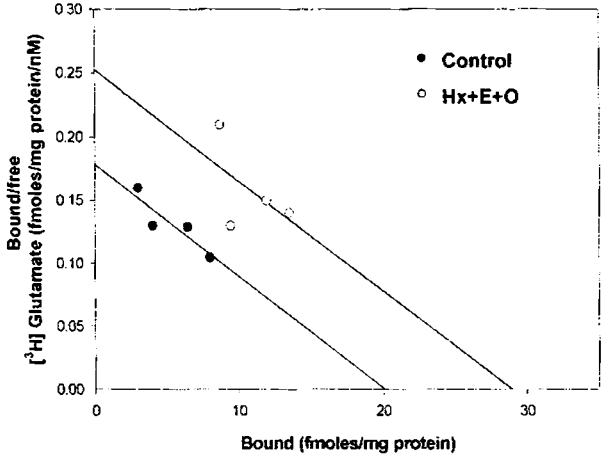


Table-41

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	23.51± 0. 28	112.50 ± 0.09
Hx+E+O	33.33 ± 1.66***	112.93 ± 1.83

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats - C; Hypoxic rats epinephrine and oxygen treated - Hx+E+O
*** $p<0.001$ when compared to C

Figure-32

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Glucose +Epinephrine + Oxygen Treated Hypoxic Neonatal Rats

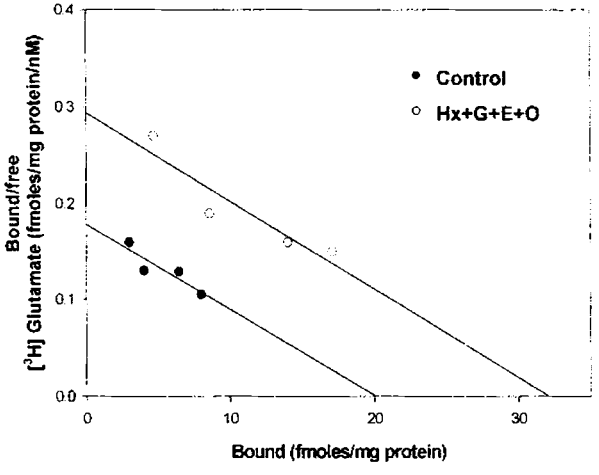


Table-42

Scatchard analysis of [^{3}H]Glutamate Binding Against Glutamate in the Cerebral Cortex of Control and Glucose+Epinephrine+Oxygen Treated Hypoxic Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	23.51± 0. 28	112.5 ± 0.09
Hx+G+E+O	37.83 ± 0.16***	116.8 ± 1.90

Values are Mean ± S.E.M of 4-6 separate experiments
B_{max} - Maximal binding; K_d - Dissociation constant
Control rats – C; Hypoxic rats Glucose, Epinephrine and Oxygen treated - Hx+G+E+ O
*** $p<0.001$ when compared to C

Figure-33

Binding parameters of [^{3}H]Glutamate Against Glutamate in the Cerebral Cortex of Experimental Groups of Neonatal Rats

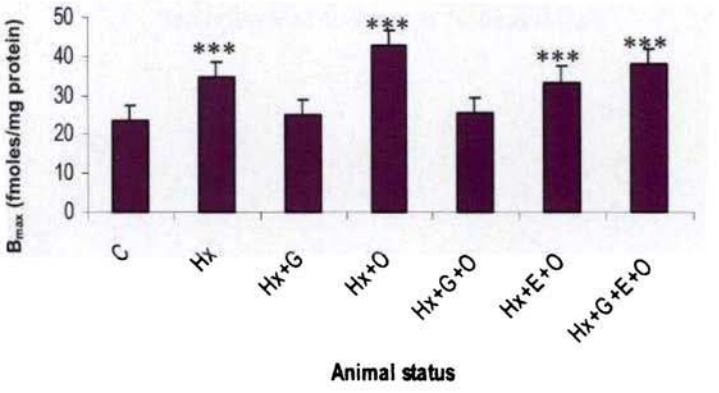


Table-43

Binding parameters of [^{3}H]Glutamate Against Glutamate in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
C	23.51± 0. 28	112.50 ± 0.09
Hx	34.66 ± 0.92$^{***\,\varphi\dagger\,c}$	112.97 ± 0.71
Hx+G	24.83 ± 0.57	114.38 ± 1.86
Hx+O	42.66 ±1.20$^{***\varphi\,\dagger\,a\,b\,cc}$	113.83 ± 1.58
Hx+G+O	25.50 ± 0.57	116.73 ± 1.36
Hx+E+O	33.33 ± 1.66$^{***\,\varphi\dagger\,c}$	112.93 ± 1.83
Hx+G+E+O	37.83 ± 0.16$^{***\,\varphi\dagger}$	116.80 ± 1.90

Values are Mean ± S.E.M of 4-6 separate experiments

B_{max} - Maximal binding; K_d - Dissociation constant

*** $p<0.001$ when compared to C

$^{\varphi}$ $p<0.001$ when compared to Hx+G

† $p<0.001$ when compared to Hx+G+O

^{a} $p<0.001$ when compared to Hx

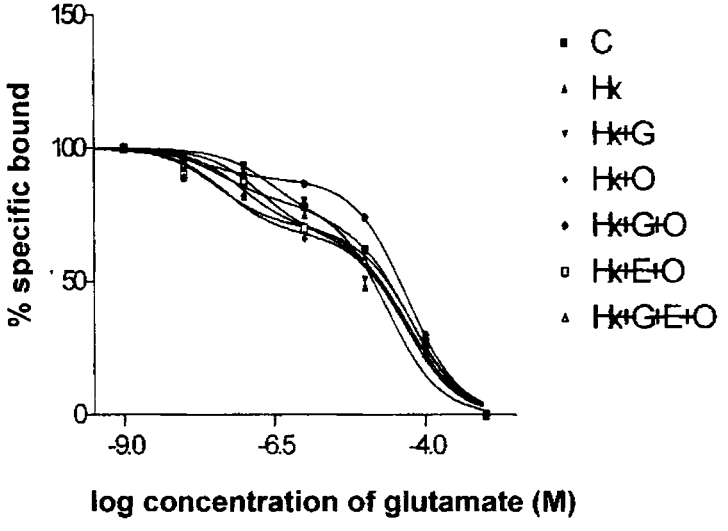
^{b} $p<0.001$ when compared to Hx+E+O

^{c} $p<0.05$, ^{cc} $p<0.01$ when compared to Hx+G+E+O

Control rats - C; Hypoxic rats- Hx; Hypoxic rats glucose treated - Hx+G, Hypoxic rats oxygen treated - Hx+O; Hypoxic rats glucose and oxygen treated - Hx+G+O; Hypoxic rats epinephrine and oxygen treated - Hx+E+O; hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O.

Fig 34

Displacement of [^{3}H]Glutamate against Glutamate in the Cerebral Cortex of Experimental Groups of Neonatal Rats



Values are mean of 4-6 separate experiments

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Table-44

Displacement of [^{3}H]Glutamate against Glutamate in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
C	Two-site	-6.29	-4.19	3.1×10^{-7}	6.05×10^{-5}	-0.31
Hx	Two-site	-6.95	-4.17	9.5×10^{-7}	6.63×10^{-5}	-0.35
Hx+G	Two-site	-6.09	-4.51	7.0×10^{-7}	3.07×10^{-5}	-0.25
Hx+O	Two-site	-7.05	-4.22	3.1×10^{-8}	6.02×10^{-5}	-0.36
Hx+G+ O	Two-site	-7.39	-4.25	9.5×10^{-8}	5.52×10^{-5}	-0.13
Hx+E+O	Two-site	-6.69	-4.09	7.0×10^{-7}	8.11×10^{-5}	-0.36
Hx+G+E+O	Two-site	-6.54	-4.19	7.0×10^{-7}	6.41×10^{-5}	-0.36

Values are mean of 4-6 separate experiments

Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA).
Ki - The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity).
EC_{50} is the concentration of the competitor that competes for half the specific binding.

Control rats – C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-35

Real-Time PCR amplification of the glutamate receptor (NMDAR1) mRNA from the Cerebral Cortex of Control and Experimental Groups of Neonatal Rats

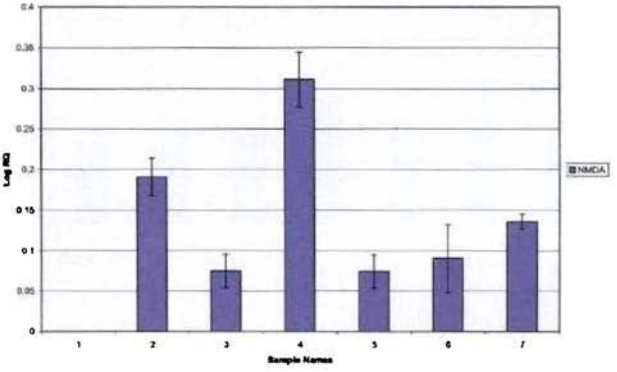


Table-45

Real-Time PCR amplification of the glutamate receptor (NMDAR1) mRNA from the Cerebral Cortex of Control and Experimental Groups of Neonatal Rats

Sample No.	Animal status	RQ value
1	C	0
2	Hx	0.19 ± 0.02
3	Hx+G	0.07 ± 0.02
4	Hx+O	0.31 ± 0.02
5	Hx+G+O	0.07 ± 0.02
6	Hx+E+O	0.90 ± 0.04
7	Hx+G+E+O	0.14 ± 0.01

Values are Mean ± S.D of 4-6 separate experiments

Relative Quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the ΔΔCT method normalized with β-actin CT value as the internal control and Control CT value as the caliberator.

1 - Control rats Hypoxic rats
2 - Hypoxic rats glucose treated
3 - Hypoxic rats oxygen treated
4 - Hypoxic rats glucose and oxygen treated
5 - Hypoxic rats glucose and oxygen treated
6 - Hypoxic rats epinephrine and oxygen treated
7 - Hypoxic rats glucose, epinephrine and oxygen treated

Figure-36

cAMP Content (pmole/mg protein) in the Cerebral Cortex of Experimental Groups of Neonatal Rats

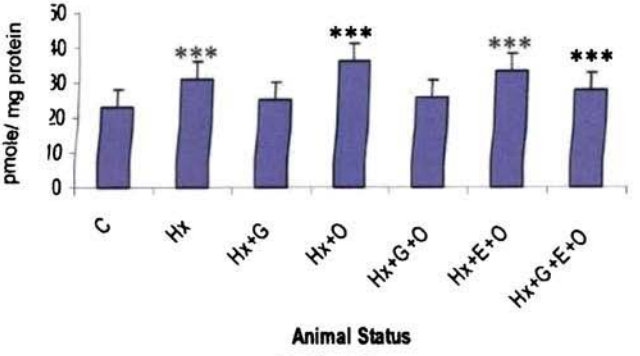


Table-46

cAMP Content (pmole/mg protein) in the Cerebral Cortex of Experimental Groups of Neonatal Rats

Animal status	cAMP (pmole/mg protein)
C	23.51 ± 0. 32
Hx	31.01 ± 0.74$^{***\varphi\dagger c}$
Hx+G	24.76 ± 0.36
Hx+O	35.76 ± 1.44$^{***\varphi\dagger abcc}$
Hx+G+O	25.58 ± 0.85
Hx+E+O	33.02 ± 0.32$^{***\varphi\dagger cc}$
Hx+G+E+O	27.34 ± 0.05***

Values are Mean ± S.E.M of 4-6 separate experiments

*** $p<0.001$ when compared to C

^{a} $p<0.001$ when compared to Hx

$^{\varphi}$ $p<0.001$ when compared to Hx+G

† $p<0.001$ when compared to Hx+G+O ,

^{b} $p<0.001$ when compared to Hx+E+O ,

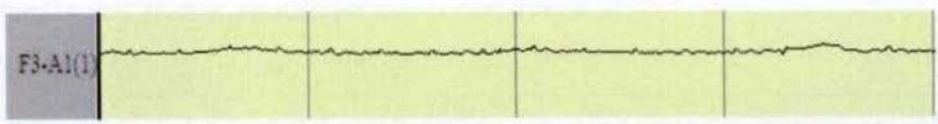
^{c} $p<0.01$, ^{cc} $p<0.001$ when compared to Hx+G+E+O

Control rats - C; Hypoxic rats- Hx; Hypoxic rats glucose treated - Hx+G, Hypoxic rats oxygen treated - Hx+O; Hypoxic rats glucose and oxygen treated - Hx+G+O; Hypoxic rats epinephrine and oxygen treated - Hx+E+O; hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O.

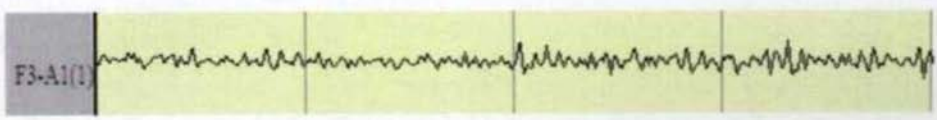
Figure-37

EEG of the left Frontal lobe (F3) of Control and Experimental Groups of Neonatal Rats

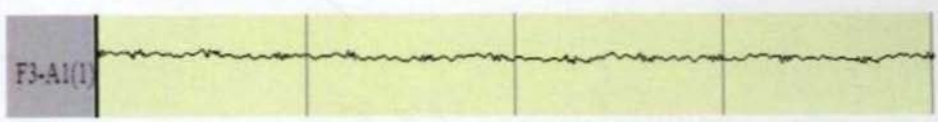
Control Rats



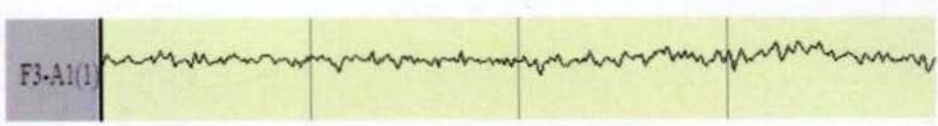
Hypoxic rats



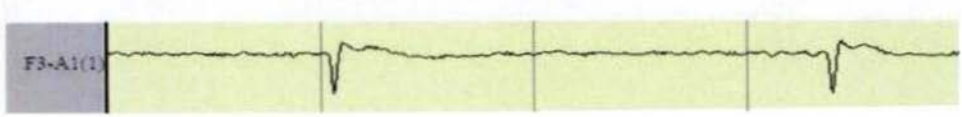
Hypoxic rats glucose treated



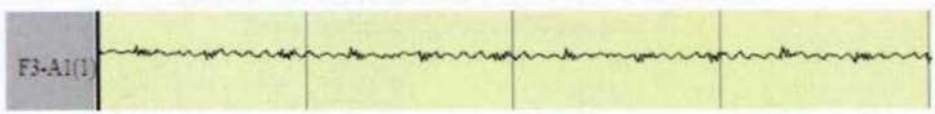
Hypoxic rats oxygen treated



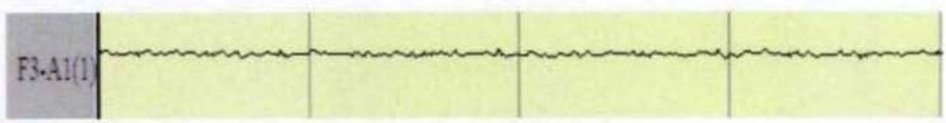
Hypoxic rats epinephrine and oxygen treated



Hypoxic rats glucose and oxygen treated



Hypoxic rats glucose, epinephrine and oxygen treated

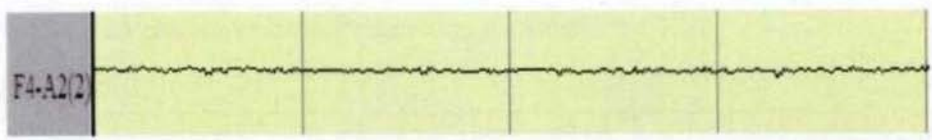


A1 Reference left

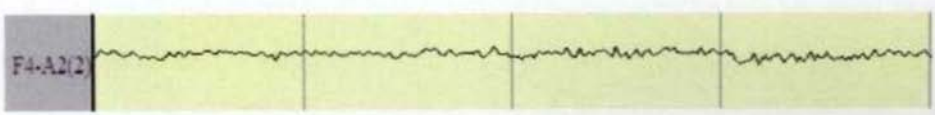
Figure-38

EEG of the right Frontal lobe (F4) of Control and Experimental Groups of Neonatal Rats

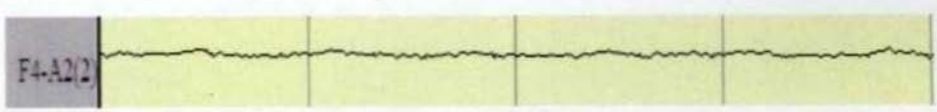
Control Rats



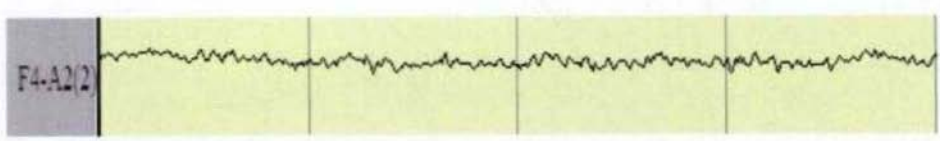
Hypoxic rats



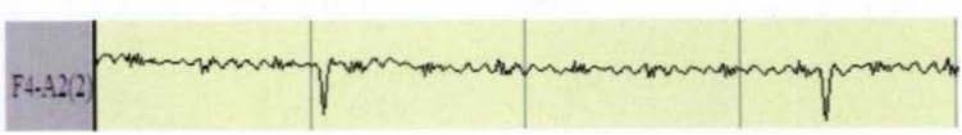
Hypoxic rats glucose treated



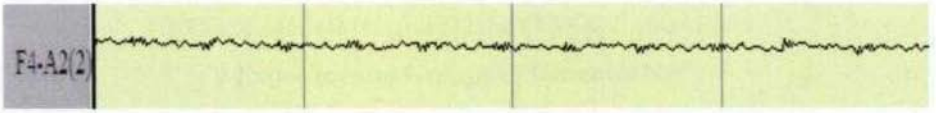
Hypoxic rats oxygen treated



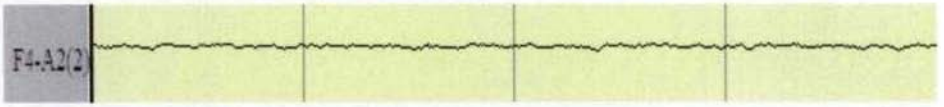
Hypoxic rats epinephrine and oxygen treated



Hypoxic rats glucose and oxygen treated



Hypoxic rats glucose, epinephrine and oxygen treated

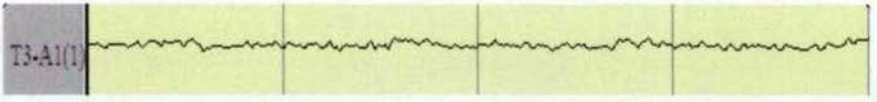


A2 Reference right

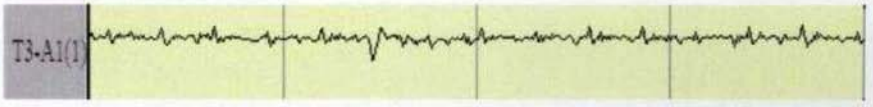
Figure-39

EEG of the left temporal lobe (T3) of Control and Experimental Groups of Neonatal Rat

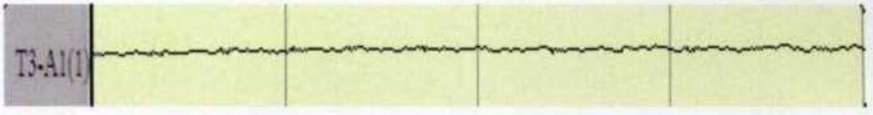
Control Rats



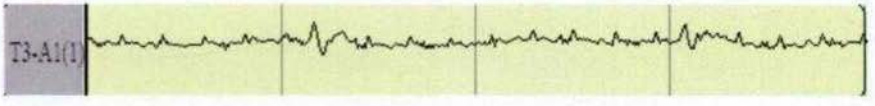
Hypoxic rats



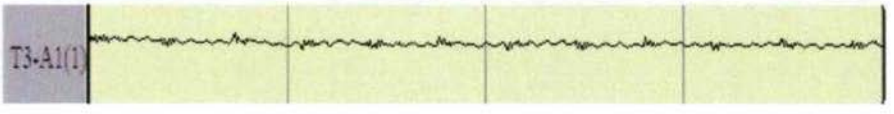
Hypoxic rats glucose treated



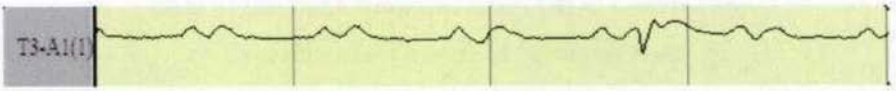
Hypoxic rats oxygen treated



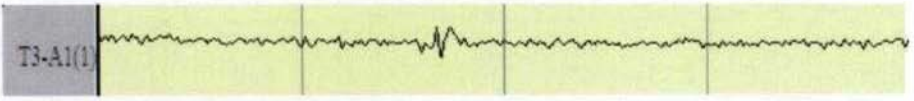
Hypoxic rats glucose and oxygen treated



Hypoxic rats epinephrine and oxygen treated



Hypoxic rats glucose, epinephrine and oxygen treated

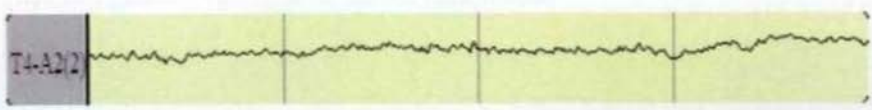


A1 Reference left

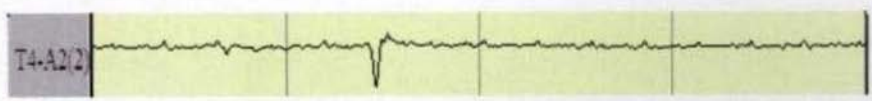
Figure-40

EEG of the right temporal lobe (T4) of Control and Experimental Groups of Neonatal Rats

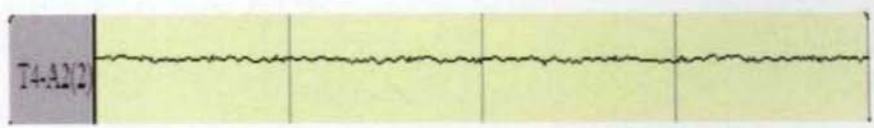
Control Rats



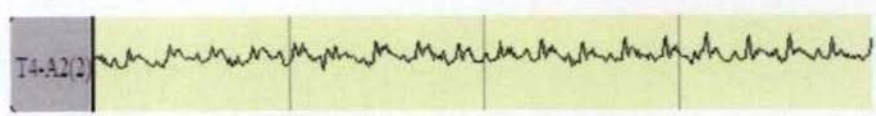
Hypoxic rats



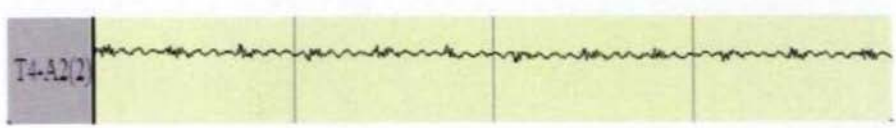
Hypoxic rats glucose treated



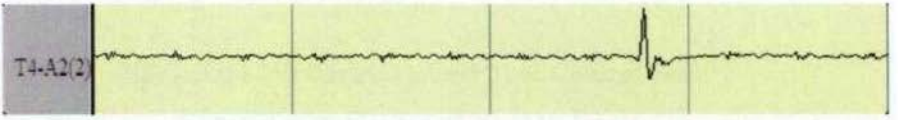
Hypoxic rats oxygen treated



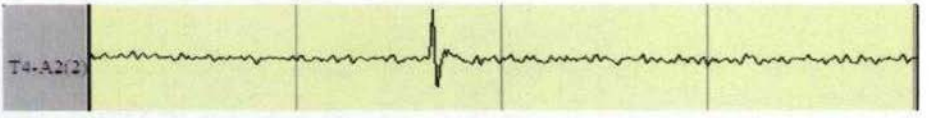
Hypoxic rats glucose and oxygen treated



Hypoxic rats epinephrine and oxygen treated



Hypoxic rats glucose, epinephrine and oxygen treated

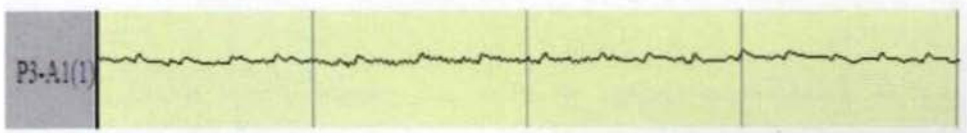


A2 Reference right

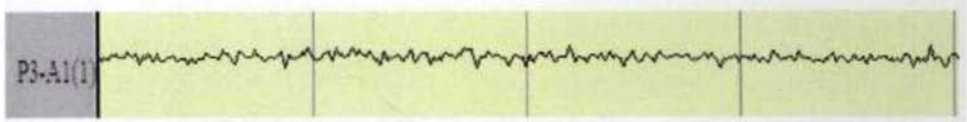
Figure-41

EEG of the left Parietal lobe (P3) of Control and Experimental Groups of Neonatal Rats

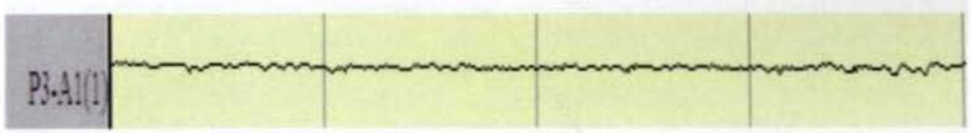
Control Rats



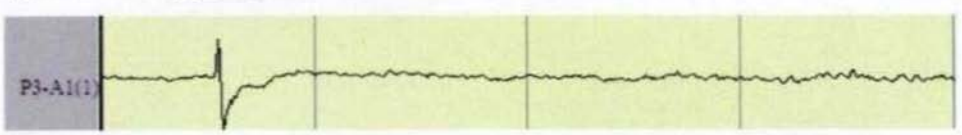
Hypoxic rats



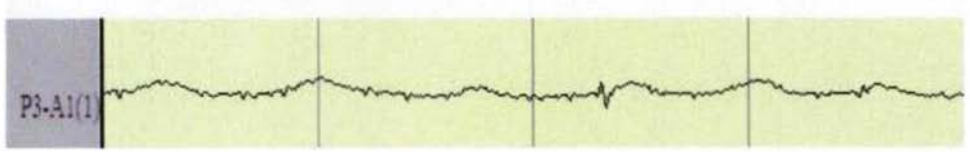
Hypoxic rats glucose treated



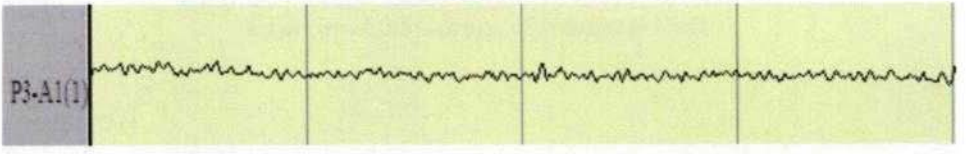
Hypoxic rats oxygen treated



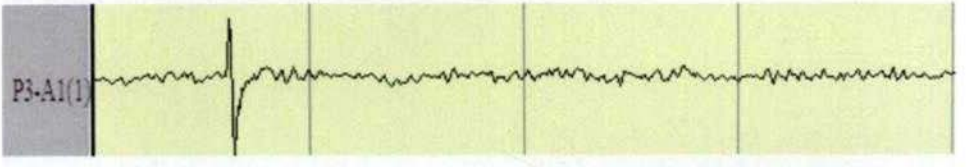
Hypoxic rats epinephrine and oxygen treated



Hypoxic rats glucose and oxygen treated



Hypoxic rats glucose, epinephrine and oxygen treated

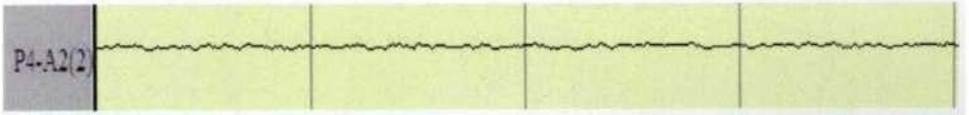


A1 Reference left

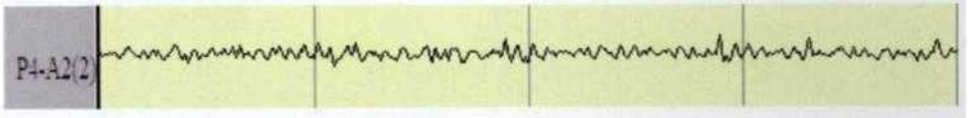
Figure-42

EEG of the right Parietal lobe (P4) of Control and Experimental Groups of Neonatal Rats

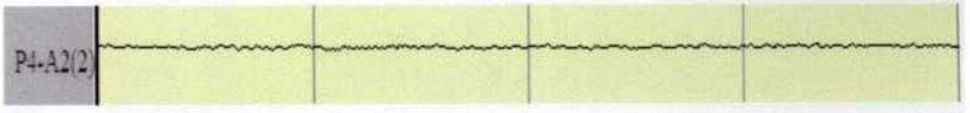
Control Rats



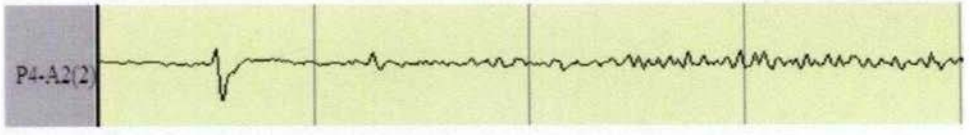
Hypoxic rats



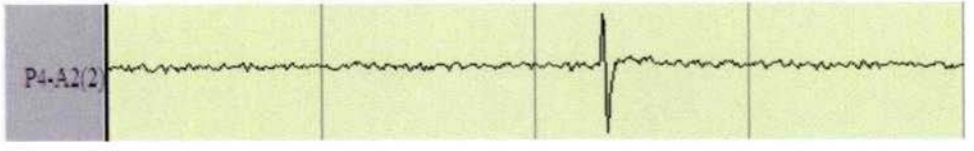
Hypoxic rats glucose treated



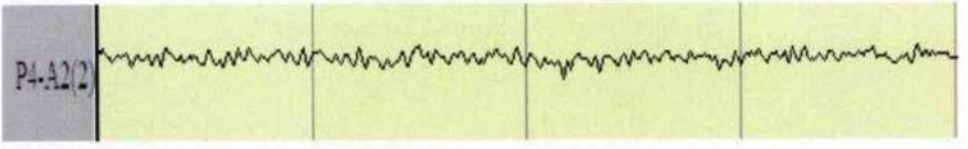
Hypoxic rats oxygen treated



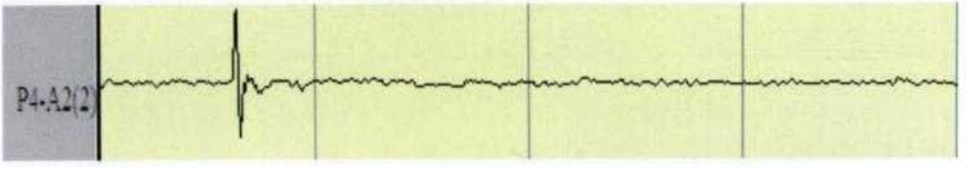
Hypoxic rats epinephrine and oxygen treated



Hypoxic rats glucose and oxygen treated



Hypoxic rats glucose, epinephrine and oxygen treated



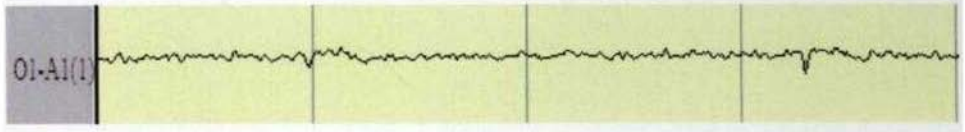
A2 Reference right

Figure-43

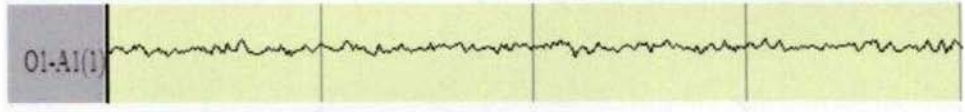
EEG of the left Occipital lobe (O1) of Control and Experimental Groups of Neonatal Rats

Control rats

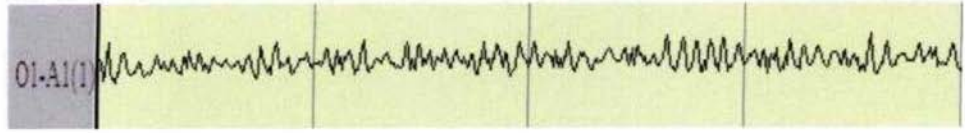
Hypoxic rats



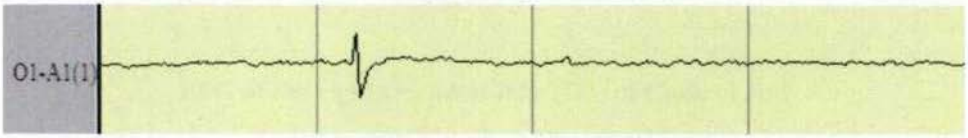
Hypoxic rats glucose treated



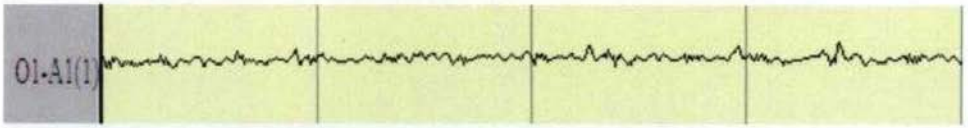
Hypoxic rats oxygen treated



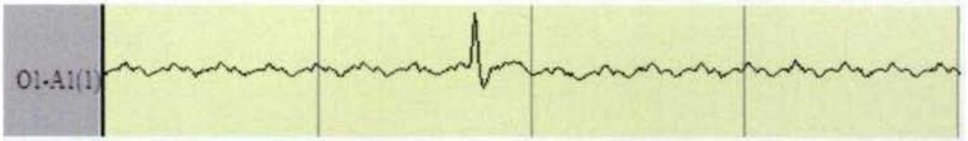
Hypoxic rats epinephrine and oxygen treated



Hypoxic rats glucose and oxygen treated



Hypoxic rats glucose, epinephrine and oxygen treated

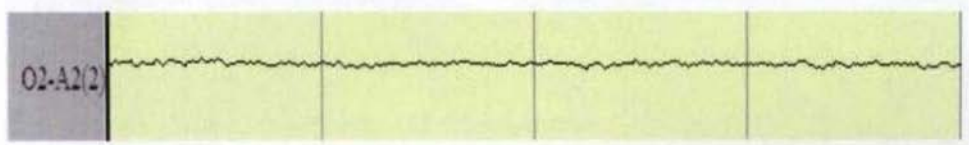


A1 Reference left

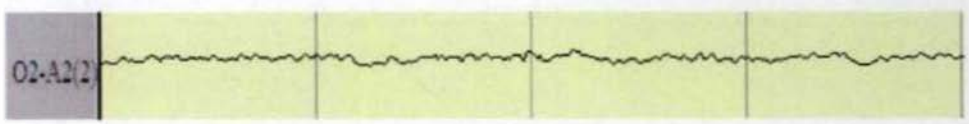
Figure-44

EEG of the right Occipital lobe (O2) of Control and Experimental Groups of Neonatal Rats

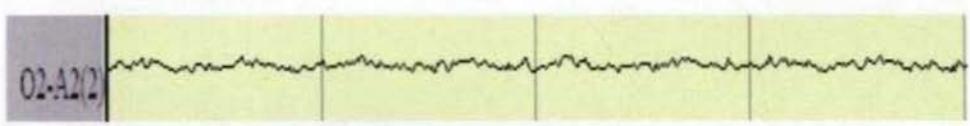
Control rats



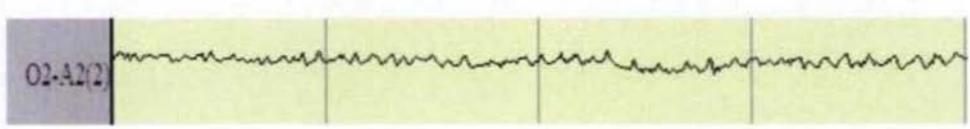
Hypoxic rats



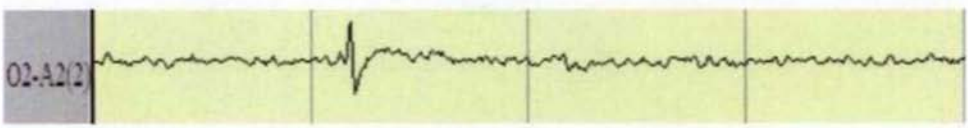
Hypoxic rats glucose treated



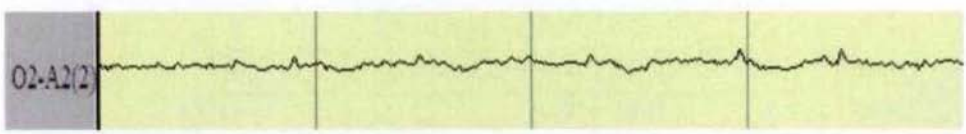
Hypoxic rats oxygen treated



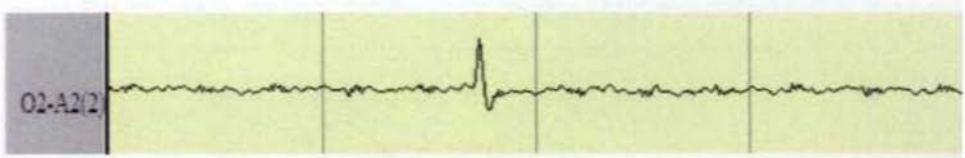
Hypoxic rats epinephrine and oxygen treated



Hypoxic rats glucose and oxygen treated



Hypoxic rats glucose, epinephrine and oxygen treated



A2 Reference right

Table-47

Body weights of experimental animals (1 month) used for behavioural study

Animal status	Body weight (g)
C	72.80 ± 0.36
Hx	68.63 ± 0.87 $^{**\varphi\varphi\dagger\dagger\dagger\ b}$
Hx+G	75.31 ± 0.67
Hx+O	*70.40 ± 0.40* $^{\varphi}$
Hx+G+O	75.86 ± 0.52
Hx+E+O	73.23 ± 1.18 ††
Hx+G+E+O	57.61 ± 0.85 $^{***\varphi\varphi\dagger\dagger\dagger\ aa\ b}$

Values are Mean ± S.E.M of 4-6 separate experiments

$^{**}p<0.01$, $^{***}p<0.001$ when compared to C
$^{\varphi}p<0.05$, $^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ compared to Hx+G
$^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ compared to Hx+G+O
$^{aa}p<0.001$ compared to Hx
$^{b}p<0.001$ compared to Hx+E+O

Control rats – C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

I) ELEVATED PLUS -MAZE

Figure-45

a) Open Arm Entry Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

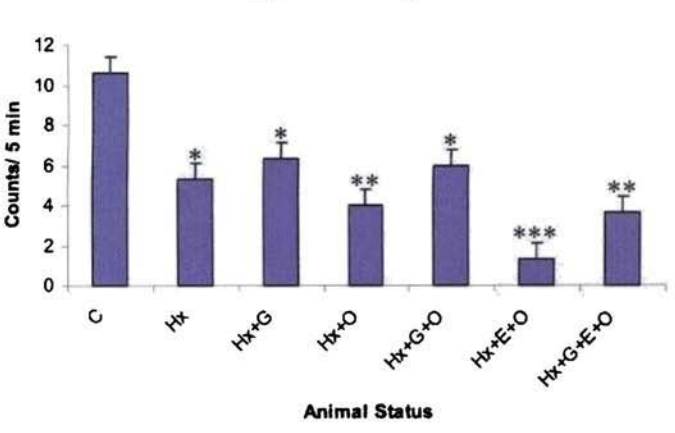


Table-48

a) Open Arm Entry Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

Animal status	Open Arm Entry (Counts/5 minutes)
C	10.66 ± 1.76
Hx	5.33 ± 0.66 *
Hx+G	6.33 ± 0.88 *
Hx+O	4.01 ± 0.57 **
Hx+G+O	6.06 ± 0.57 *
Hx+E+O	1.33 ± 0.33 ***
Hx+G+E+O	3.66 ± 1.76 **

Values are Mean ± S.E.M of 4-6 separate experiments.
Each experimental group contain 8 Groups of Rats
*p <0.05), **p<0.01, *** p <0.001 when compared to C

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-46

b) Closed Arm Entry Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

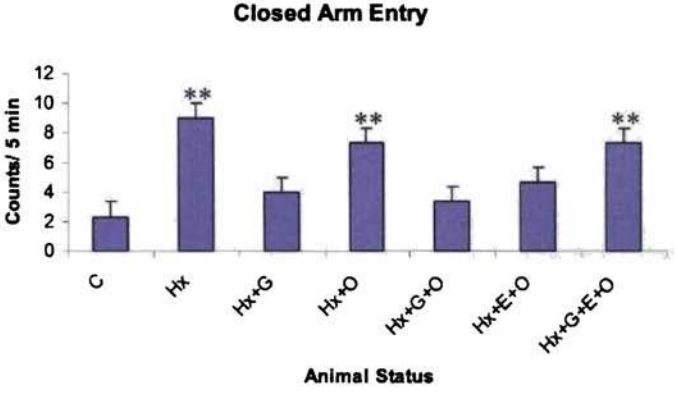


Table-49

b) Closed Arm Entry Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

Animal status	Closed Arm Entry (Counts/5 minutes)
C	2.33 ± 0.66
Hx	9.00 ± 1.53 $^{**\dagger\dagger\varphi\varphi b}$
Hx+G	4.01 ± 0.57
Hx+O	7.33 ± 0.88 $^{**\dagger\varphi}$
Hx+G+O	3.33 ± 0.33
Hx+E+O	4.66 ± 0.88
Hx+G+E+O	7.33 ± 0.88 $^{*\dagger\varphi}$

Values are Mean ± S.E.M of 4-6 separate experiments.
Each experimental group contain 8 Groups of Rats
$^{*}p<0.05$, $^{**}p<0.001$ when compared to C
$^{\varphi}p<0.05$, $^{\varphi\varphi}p<0.01$ when compared to Hx+G
$^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ when compared to Hx+G+O
$^{b}p<0.05$ when compared to Hx+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-47

c) Percentage Arm Entry Attempts by Control and Experimental Groups of Neonatal Rats.

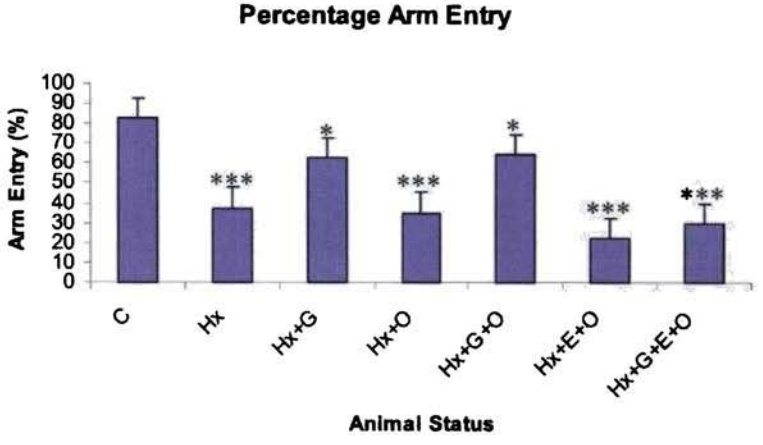


Table-50

c) Percentage Arm Entry Attempts by Control and Experimental Groups of Neonatal Rats.

Animal status	Percentage Arm Entry
C	82.66 ± 3.44
Hx	37.56 ± 1.38 $^{***\dagger\dagger\varphi\varphi}$
Hx+G	62.56 ± 4.03 *
Hx+O	35.10 ± 0.92 $^{***\dagger\dagger\varphi\varphi}$
Hx+G+O	64.23 ± 1.22 *
Hx+E+O	22.56 ± 4.30 $^{***\dagger\dagger\dagger\varphi\varphi\varphi}$
Hx+G+E+O	29.85 ± 9.18 $^{***\dagger\dagger\dagger\varphi\varphi\varphi}$

Values are Mean ± S.E.M of 4-6 separate experiments.
Each experimental group contain 8 Groups of Rats
$^{*}p<0.05$, $^{***}p<0.001$ when compared to C
$^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ when compared to Hx+G
$^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to Hx+G+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-49

e) Time Spent in Open Arm Entry Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

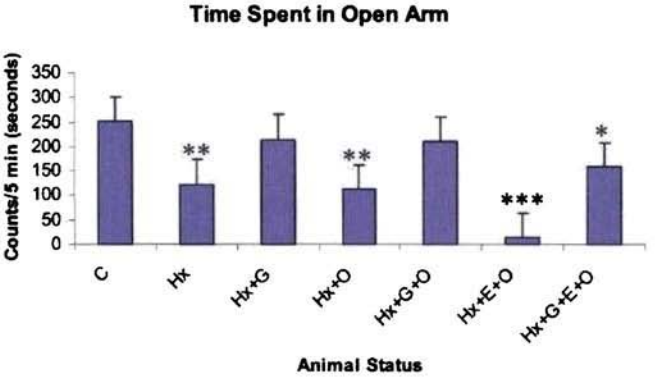


Table-52

e) Time Spent in Open Arm Entry Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

Animal status	Time spent in Open Arm (Counts/5 minutes)
C	250.67 ± 24.27
Hx	122.23 ± 2.85 $^{**\dagger\dagger\varphi\,bb}$
Hx+G	215.43 ± 3.45
Hx+O	113.33 ± 38.87 $^{**\dagger\varphi bb}$
Hx+G+O	211.67 ± 7.27
Hx+E+O	15.02 ± 5.00 $^{***\dagger\dagger\dagger\varphi\varphi\varphi aaa}$
Hx+G+E+O	158.33 ± 11.22 $^{*\,\varphi\varphi bb}$

Values are Mean ± S.E.M of 4-6 separate experiments.
Each experimental group contain 8 Groups of Rats
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ when compared to C
$^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ when compared to Hx+G
$^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to Hx+G+O
$^{aaa}p<0.001$ when compared to Hx
$^{bb}p<0.01$ when compared to Hx+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-50

f) Time Spent in Closed Arm Entry Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

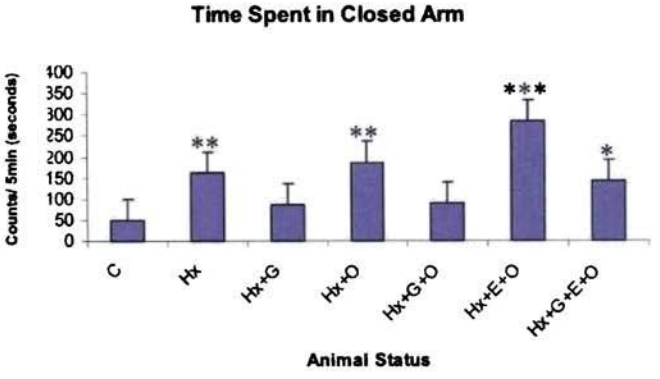


Table-53

f) Time Spent in Closed Arm Entry Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

Animal status	Time spent in Closed Arm (Counts/5 minutes)
C	49.33 ± 24.26
Hx	$162.67 \pm 13.53^{**\dagger\varphi b}$
Hx+G	84.68 ± 3.52
Hx+O	$186.67 \pm 38.87^{**\dagger\varphi b}$
Hx+G+O	88.33 ± 7.27
Hx+E+O	$285.01 \pm 5.00^{***\dagger\dagger\dagger\varphi\varphi\varphi}$
Hx+G+E+O	$141.53 \pm 11.24^{*\varphi\varphi bb}$

Values are Mean ± S.E.M of 4-6 separate experiments.
Each experimental group contain 8 Groups of Rats
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ when compared to C
$^{\varphi}p<0.05$, $^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ when compared to Hx+G
$^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ when compared to Hx+G+O
$^{b}p<0.05$, $^{bb}p<0.01$ when compared to Hx+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-51

g) Percentage of Time Spent in Open Arm Entry Attempts by Control and Experimental Groups of Neonatal Rats.

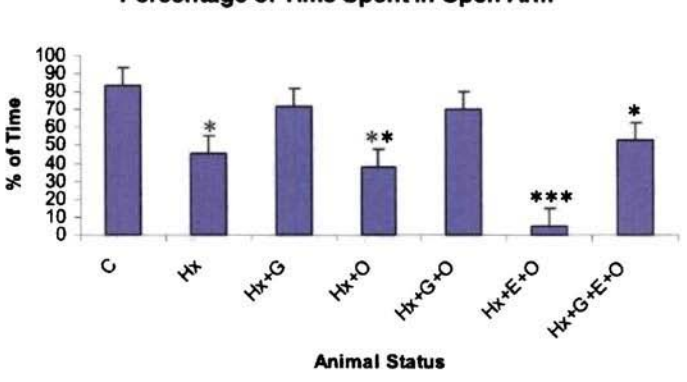


Table-54

g) Percentage of Time Spent in Open Arm Entry Attempts by Control and Experimental Groups of Neonatal Rats.

Animal status	Percentage of Time Spent in Open Arm
C	83.53 ± 8.11
Hx	$45.73 \pm 4.49^{*\dagger\varphi b}$
Hx+G	71.80 ± 1.15
Hx+O	$37.77 \pm 12.95^{**\dagger\varphi b}$
Hx+G+O	70.55 ± 2.43
Hx+E+O	$4.96 \pm 1.66^{***\dagger\dagger\dagger\varphi\varphi\varphi}$
Hx+G+E+O	$52.78 \pm 3.76^{*\varphi\varphi bb}$

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats
$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ when compared to C
$^{\varphi}p<0.01$, $^{\varphi\varphi}p<0.001$ when compared to Hx+G
$^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to Hx+G+O
$^{b}p<0.01$, $^{bb}p<0.001$ when compared to Hx+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-52

h) Head Dipping Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

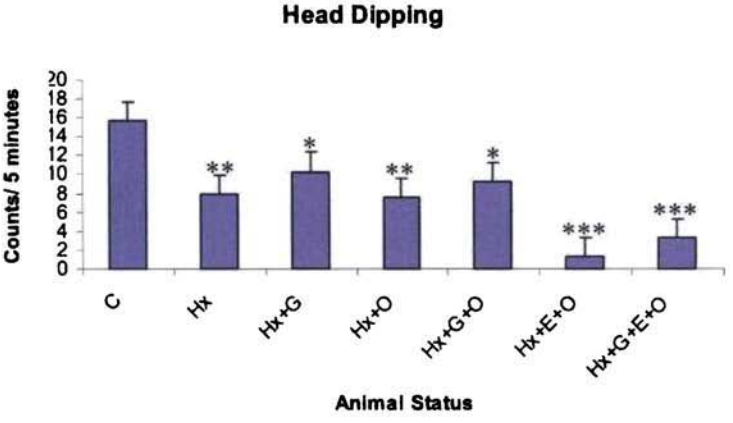


Table-55

h) Head Dipping Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

Animal status	Head Dipping (Counts/5 minutes)
C	15.67 ± 2.19
Hx	8.00 ± 1.16 **
Hx+G	10.33 ± 2.19 *
Hx+O	7.67 ± 0.88 **b
Hx+G+O	9.33 ± 0.88 *
Hx+E+O	1.33 ± 0.33 ***φ†
Hx+G+E+O	3.33 ± 0.33 ***φ†

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats

*p<0.05,** p <0.01,*** p<0.001 when compared to C
φp<0.05 when compared to Hx+G
†p<0.05 when compared to Hx+G+O
b p<0. 05 when compared to Hx+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-53

i) Stretched Attend Posture Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

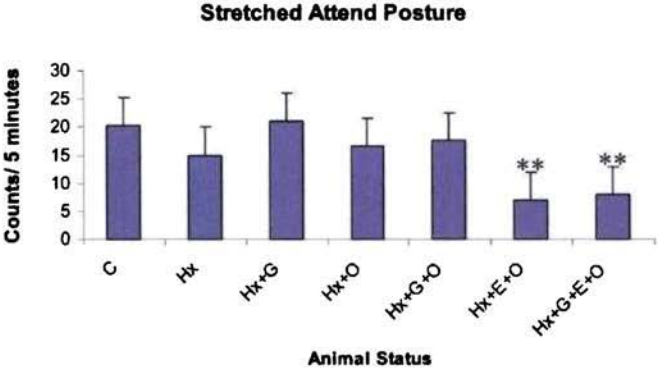


Table-56

i) Stretched Attend Posture Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

Animal status	Stretched Attend Posture (Counts/5 minutes)
C	20.33 ± 3.18
Hx	15.00 ± 1.00 ^{bc}
Hx+G	21.00 ± 2.64
Hx+O	6.66 ± 1.33 ^{bc}
Hx+G+O	17.66 ± 1.20
Hx+E+O	7.00 ± 1.52 $^{**\varphi\varphi\dagger\dagger}$
Hx+G+E+O	8.00 ± 1.15 $^{**\varphi\varphi\dagger}$

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats
** p<0.01 when compared to C
$^{\varphi\varphi}$p <0.01 when compared to Hx+G
††p<0.01 when compared to Hx+G+O
^{b} p<0. 05 when compared to Hx+E+O
^{c} p<0.05 when compared to Hx+G+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-54

j) Grooming Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

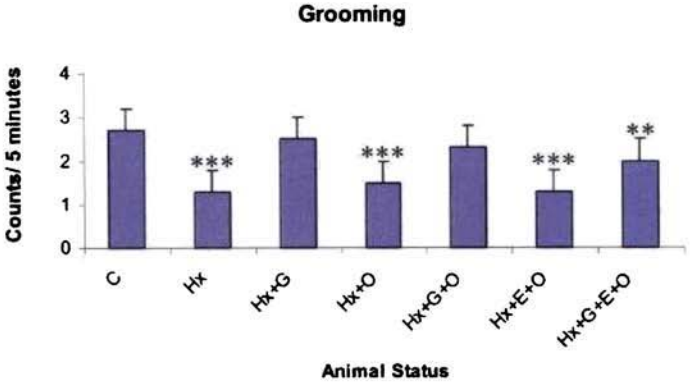


Table-57

j) Grooming Attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

Animal status	Grooming (Counts/5 minutes)
C	2.66 ± 0.11
Hx	$1.30 \pm 0.07^{***\dagger\dagger\dagger\varphi\varphi\varphi}$
Hx+G	2.53 ± 0.02
Hx+O	$1.56 \pm 0.15^{***\dagger\dagger\dagger\varphi\varphi\varphi}$
Hx+G+O	2.50 ± 0.10
Hx+E+O	$1.33 \pm 0.03^{***\dagger\dagger\dagger\varphi\varphi\varphi}$
Hx+G+E+O	$2.00 \pm 0.07^{**\dagger\varphi}$

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats

$^{**}p<0.01$, $^{***}p<0.001$ when compared to C
$^{\varphi\varphi}p<0.01$, $^{\varphi\varphi\varphi}p<0.001$ when compared to Hx+G
$^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to Hx+G+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

II) OPEN FIELD TEST

Figure-55

a) Crossing attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

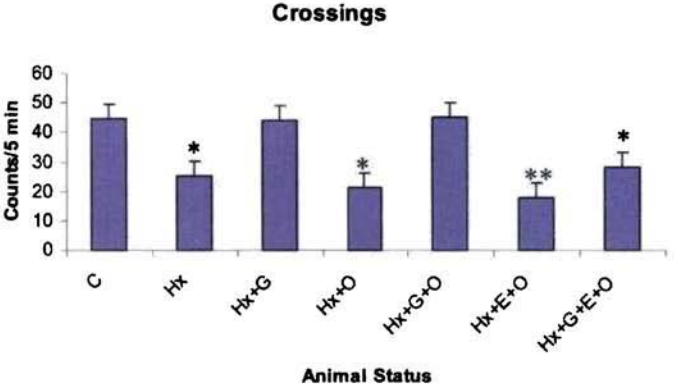


Table-58

a) Crossing attempts (Counts/5 minutes) by Control and Experimental Groups of Neonatal Rats.

Animal status	Crossings (Counts/5 minutes)
C	44.66 ± 1.45
Hx	25.33 ± 3.53$^{*\varphi\dagger}$
Hx+G	44.00 ± 1.15
Hx+O	21.16 ± 4.29$^{*\varphi\dagger}$
Hx+G+O	45.33 ± 8.81
Hx+E+O	18.00 ± 2.88$^{**\varphi\varphi\dagger\dagger}$
Hx+G+E+O	28.33 ± 1.15*

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats
$^{*}p<0.05$, $^{**}p<0.01$, when compared to C
$^{\varphi}p<0.05$, $^{\varphi\varphi}p<0.01$ compared to Hx+G
$^{\dagger}p<0.01$ when compared to Hx+G+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-56

b) Walking time (Seconds) by Control and Experimental Groups of Neonatal Rats.

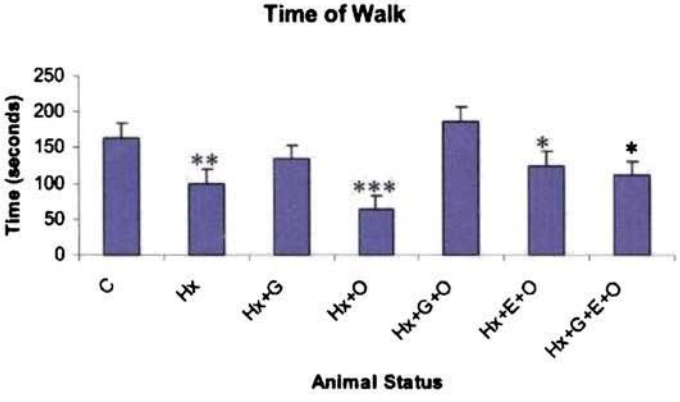


Table-59

b) Walking time (Seconds) by Control and Experimental Groups of Neonatal Rats.

Animal status	Time of Walk (Seconds)
C	163.33 ± 7.25
Hx	$100.00 \pm 10.41^{**\,\dagger\dagger\dagger}$
Hx+G	133.33 ± 8.81
Hx+O	$63.33 \pm 14.53^{***\,\dagger\dagger\dagger\,\varphi\varphi}$
Hx+G+O	186.67 ± 13.02
Hx+E+O	$125.00 \pm 2.88^{*\,\dagger\dagger}$
Hx+G+E+O	$28.33 \pm 1.15^{*\,\dagger\dagger}$

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to C
$^{\varphi\varphi}$ <0.01when compared to Hx+G
$^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ when compared to Hx+G+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-57

c) Resting time (Seconds) by Control and Experimental Groups of Neonatal Rats

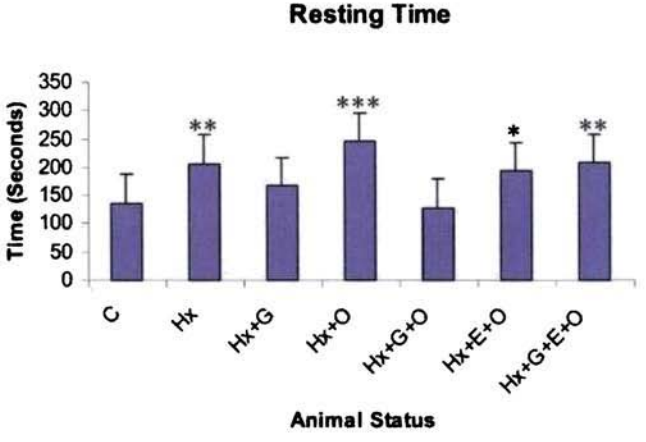


Table-60

c) Resting time (Seconds) by Control and Experimental Groups of Neonatal Rats

Animal status	Resting Time (Seconds)
C	137.33 ± 6.74
Hx	$206.67 \pm 4.41^{**\,\dagger\dagger}$
Hx+G	166.67 ± 8.81
Hx+O	$245.33 \pm 7.42^{***\,\dagger\dagger\dagger\,\varphi\varphi\,b}$
Hx+G+O	128.33 ± 26.82
Hx+E+O	$192.67 \pm 28.96^{*\,\dagger}$
Hx+G+E+O	$207.33 \pm 8.19^{**\dagger\dagger}$

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats
$^{*}p < 0.05$, $^{**}(P < 0.01$, $^{***}p < 0.001$ when compared to C
$^{\varphi\varphi}p<0.01$ when compared to Hx+G
$^{+}p < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ when compared to Hx+G+O
$^{b}p<0.05$ when compared to Hx+E+O

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated Hx+G+E+O

Figure-58

d) Episodes of Rearing (Counts/5minutes) by Control and Experimental Groups of Neonatal Rats.

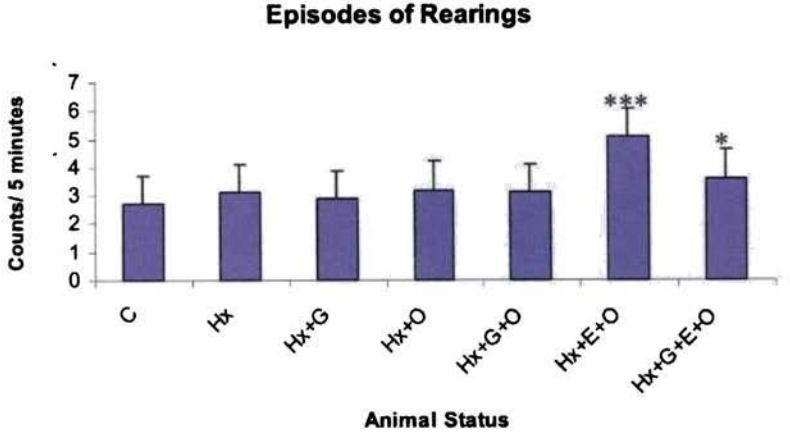


Table-61

d) Episodes of Rearing (Counts/5minutes) by Control and Experimental Groups of Neonatal Rats

Animal status	Episodes of Rearings (Counts/5 minutes)
C	2.76± 0.10
Hx	3.10 ± 0.10
Hx+G	2.92 ± 0.2
Hx+O	3.30 ± 0.31
Hx+G+O	3.1 ± 0.10
Hx+E+O	5.12 ± 0.12***
Hx+G+E+O	3.6 ± 0.10*

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats
*p<0.05, *** p<0.001 when compared to C

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx+E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O

Figure-59

e) Head Sniffing and Washing Attempts (Counts/5minutes) by Control and Experimental Groups of Neonatal Rats

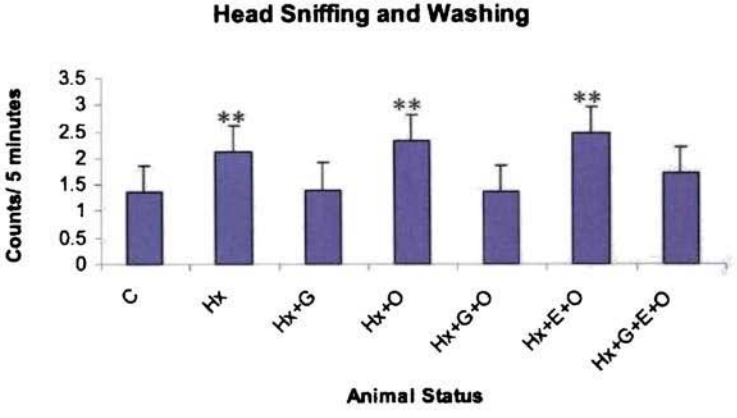


Table-62

e) Head Sniffing and Washing Attempts (Counts/5minutes) by Control and Experimental Groups of Neonatal Rats

Animal status	Head Sniffing and Washing (Counts/5 minutes)
C	1.33 ± 0.33
Hx	2.00 ± 0.01**
Hx+G	1.33 ± 0.33
Hx+O	2.33 ± 0.33**
Hx+G+O	1.33 ± 0.33
Hx+E+O	2.00 ± 0.01**
Hx+G+E+O	1.66 ± 0.66

Values are Mean ± S.E.M of 4-6 separate experiments
Each experimental group contain 8 Groups of Rats
** $p<0.001$ when compared to C

Control rats - C
Hypoxic rats- Hx
Hypoxic rats glucose treated - Hx+G
Hypoxic rats oxygen treated - Hx+O
Hypoxic rats glucose and oxygen treated - Hx+G+O
Hypoxic rats epinephrine and oxygen treated - Hx−E+O
Hypoxic rats glucose, epinephrine and oxygen treated - Hx+G+E+O