ISCHEMIA IN NEURONAL PC12 CELLS: ROLE OF FREE RADICAL GENERATION AND CELLULAR DAMAGE.

by

Nirmala Cunniah Govinda

A Thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

© Copyright by Nirmala C. Govinda (2001)
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-63193-1
I. ABSTRACT

ISCHEMIA IN NEURONAL PC12 CELLS: ROLE OF FREE RADICAL GENERATION AND CELLULAR DAMAGE

Nirmala Cunniah Govinda
Master of Science, 2001
Department of Physiology
University of Toronto

Oxidative stress causing impaired cell function underlies many neurodegenerative disorders. I used an in-vitro model, neuronal PC12 cells to study the effects of Oxygen-Glucose-Deprivation (OGD/GD/OD). Cytotoxicity in culture medium (DMEM) was significantly higher following OGD/GD, immediately and 24hrs later, than the typically used – balanced salt solution (BSS). Cell death significantly decreased with addition of a metabolic substrate, inhibition of glycolysis significantly increased cell death. Free radical generation (ROS) studied with 5’6’CMH2DCF-DA, using confocal-microscopy and flow-cytometry, showed a significant early and persistent increase during OGD and early reperfusion, in DMEM. Lipid-peroxidation (TBARS assay) was increased in DMEM following OGD. Changes in mitochondrial morphology during OGD, coincided with the rise in ROS. NGF, hypothermia and anti-oxidants improved cell survival. These results indicate that oxidative stress may be an important mediator of cytotoxicity in neuronal PC12 cells during OGD in DMEM. Neuroprotective treatments must target strategies to decrease oxidative stress in the brain.
II. ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Linda Mills, for her supervision and allowing me the freedom to set my own course yet with enough guidance to keep me on the right track. Her patience, optimism, encouragement and confidence in me is what motivated me to keep striving and moving ahead. Her thorough proof reading and help in making this manuscript are duly acknowledged.

I am also very grateful to Dr. James Eubanks, for his support and guidance during my work, going far beyond being a supervisory committee member. I cannot count the innumerable occasions when I have dropped by his office with my queries, which have always been answered with thorough in depth analysis. His dedication, knowledge and patience with students, during course work has impressed me and other students alike.

I would like to thank my friend and colleague, Ziping Zhu, for having been so much of a help to me and others in the lab. His eagerness and enthusiasm to help is his biggest asset. I would like to thank my other colleagues Leah Doumanovskia for teaching me the principles of tissue culture when I was new to the lab and Rebecca Amer for help in the statistics.

I am extremely grateful to my husband Chandrashekar (Chad) Masti, for his immense support throughout my days as a graduate student. The countless late nights and weekends that I have spent would not have been possible without his cooperation and help. His help in the formatting and the putting together of my whole thesis are greatly appreciated. I would also like to thank Dr. Garth (Schlichter lab) for his help in the formatting.

Last but not the least, I would like to thank my parents, who though far away from me, are there everyday in my thoughts and prayer. Their confidence in me, encouragement and principles that they imbibed in me, have shaped my whole outlook to life and will always guide me. I would also like to thank my sister Dr. Namitha, brother Sujit and their families for their moral support during my work.
III. TABLE OF CONTENTS

I. Abstract ........................................................................................................... ii
II. Acknowledgements ........................................................................................ iii
III. Table of Contents .......................................................................................... iv
IV. Table of Figures ............................................................................................. vii
V. List of Tables .................................................................................................. viii
VI. List of Abbreviations .................................................................................... ix
VII. List of Appendixes ........................................................................................ xii

INTRODUCTION ............................................................................................... 1
1 Overview Of Stroke, Ischemia And Oxygen-Glucose Deprivation (OGD) .......... 1
2 Cellular And Molecular Mechanisms Of Damage In Ischemia And Reperfusion 2
  2.1 Mitochondria ............................................................................................... 2
  2.2 Protein Synthesis ....................................................................................... 3
  2.3 Cytoskeleton ............................................................................................... 3
  2.4 Ion Channels In Ischemia .......................................................................... 4
3 In Vitro Ischemia – Oxygen-Glucose Deprivation (OGD) ................................ 5
4 Cytotoxicity In Anoxia And Hypoglycemia .................................................... 6
  4.1 Anoxia ........................................................................................................ 6
  4.2 Hypoglycemia ............................................................................................. 6
5 Rat Pheochromocytoma PC12 Cell Line .......................................................... 7
  5.1 Undifferentiated PC12 Cells And Differentiated (Neuronal) PC12 Cells .... 8
  5.2 PC12 Cells And Glutamate ........................................................................ 9
  5.3 PC12 Cells In Ischemia ............................................................................. 10
  5.4 PC12 Cells In Hypoxia ............................................................................. 10
  5.5 PC12 Cells In Hypoglycemia ................................................................... 11
  5.6 Amino Acid Utilization In PC12 Cells ....................................................... 12
  5.7 PC12 Cells And Catecholamines/Eicosanoids ......................................... 12
6 Role Of Glutamate, Calcium And Free Radicals In Ischemia ......................... 13
  6.1 Extracellular Glutamate ........................................................................... 13
  6.2 Intracellular Calcium ................................................................................ 14
  6.3 Free Radical Generation During Ischemia And Reperfusion .................... 15
    6.3.1 Reactive Oxygen Species In Ischemia And Reperfusion .................... 15
    6.3.2 Reactive Nitrogen Species In Ischemia And Reperfusion .................... 18
7 Free Radical Induced Damage – Lipids, Proteins And DNA .......................... 19
  7.1 Lipids ........................................................................................................ 19
  7.2 Proteins ...................................................................................................... 20
  7.3 DNA .......................................................................................................... 20
8 Role Of Iron In Oxidative Stress .................................................................... 21
9 Neuroprotective Strategies ............................................................................. 22
  9.1 NGF .......................................................................................................... 22
  9.2 Effect Of A Decrease In Temperature On Ischemia (Hypothermia) ......... 22
  9.3 Role Of Anti-Oxidants In Ischemia ......................................................... 23
10 Summary Of Introduction ............................................................................. 24
11 Hypothesis ..................................................................................................... 24
12 Specific Aims .................................................................................................. 24
METHODS
1 PC12 Cell Culture ........................................................................... 26
1.1 Growth, Differentiation And Maintenance Of PC12 Cells ............. 26
1.2 Doxycycline Treatment .................................................................. 26
1.3 Plating Of Cells ........................................................................... 28
1.4 Experimental Solutions .................................................................. 28
1.5 Method Of Inducing OGD/OD/GD .................................................. 30
1.6 Reperfusion .................................................................................. 30
2 Analysis Of Cell Death: LDH Assay/PI Staining .............................. 30
2.1 Immediate LDH Measurements .................................................... 31
2.2 Delayed LDH Measurements ....................................................... 31
2.3 PI Staining .................................................................................. 31
3 Confocal Microscopy, Fluorescence Analysis And Image Processing 34
3.1 Confocal Microscopy .................................................................... 34
3.2 Mitochondrial Morphology Study .................................................. 34
3.3 Study Of ROS Generation Using 5'6'CMH2DCF-DA ..................... 34
3.4 Image Analysis ............................................................................. 35
3.5 Image Processing .......................................................................... 36
4 Flow Cytometric Evaluation Of ROS Generation .............................. 36
5 Thiobarbituric Acid Reactive Substances Assay (TBARS) .................. 36
6 Statistical Assays ............................................................................ 37
RESULTS
1 General Morphological Features Of Differentiated PC12 Cells ......... 38
2 Culture Medium Increases OGD Induced Cytotoxicity ....................... 41
3 Oxygen Deprivation Does Not Increase Cell Death ........................... 46
4 Glucose Deprivation Increases Cytotoxicity ..................................... 49
5 Undifferentiated PC12 Cells Respond Similarly To OGD Induced Cell Death 52
6 Effect Of Oxygen-Glucose Deprivation On Neuronal Cell Death (NGF 25ng/ml) 55
7 Morphological Changes During OGD In Neuronal Cells ................. 59
8 Morphological Changes During Early Reperfusion ........................... 59
9 Mitochondrial Morphology During OGD ........................................... 64
10 Mitochondrial Morphology During Early Reperfusion ..................... 64
11 Comparison Of The Effects Of OGD/OD/GD for 5 Hours ............... 71
12 Effect Of L-Glutamine On OGD Induced Cell Death ....................... 74
13 Effect Of 2 DDG On OGD Induced Cell Death ............................... 77
14 Ferric Nitrate as A Source Of Oxidative Stress ............................... 81
15 Confocal Microscopic Measurement Of ROS During OGD .......... 84
16 Flow Cytometry Confirms The Increase In Free Radical Generation 89
17 Generation of ROS During Early Reperfusion .................................. 92
18 Lipid Peroxidation after OGD ......................................................... 97
19 Effect of Anti-Oxidants On OGD Induced Cell Death ....................... 97
20 Inhibition Of Nitric Oxide During OGD And Reperfusion Increases Cytotoxicity 103
21 Ischemic Preconditioning (IPC) Using BSS On OGD Induced Cell Death 106
22 Hypoxic (HPC)/Ischemic Preconditioning (IPC) Using DMEM .......... 106
23 Response Of Cells In 2X NGF To OGD Induced Neuronal Cell Death 112
24 Response Of Cells In 4X NGF To OGD Induced Neuronal Cell Death 112
25 NGF Is Neuroprotective In OGD In A Dose Dependent Manner ........ 118
26 Role Of NGF Induced Protection Against Serum Deprivation ........... 118
27 Hypothermia (24°C) During OGD Is Neuroprotective ....................... 123
28 Hypothermia (24°C) During OD Is Neuroprotective ......................... 123
Summary of Results

DISCUSSION

1. PC12 Cells In Ischemia (OGD)
2. PC12 Cells In Hypoxia (OD)
3. PC12 Cells In Hypoglycemia (GD)
4. Undifferentiated PC12 Cells And OGD
5. Cellular And Mitochondrial Morphology During OGD And Reperfusion
6. Glycolytic Inhibition With 2-Deoxy-D-Glucose (2DDG)
7. L-Glutamine Decreases OGD Induced Cell Death
8. Ferric Nitrate Is Not A Significant Cause Of Cell Death In DMEM
9. Free Radical Generation During OGD And Reperfusion
10. Free Radical Induced Damage To Lipid
11. Role Of Anti-Oxidants During OGD And Reperfusion
12. Role Of Nitric Oxide (NO) Generation In OGD And Reperfusion
13. Ischemic (IPC) And Hypoxic (HPC) Preconditioning
14. Role Of NGF In Protection Against OGD And Serum Withdrawal
15. Hypothermia Is Protective Against OGD/OD In This Model
16. Catecholamine Oxidation Causing Free Radical Generation And Damage
17. Proposed Mechanisms Of Cytotoxicity In My Model of Ischemia and Reperfusion
18. Future Directions And Possible Implications

References
IV. TABLE OF FIGURES

Figure 1: Undifferentiated And Differentiated PC12 Cells ......................................................... 27
Figure 2: Composite Figure Representing Methods And Techniques ............................................. 29
Figure 3: Neuronal PC12 Cells with PI+ nuclei and GFP+ mitochondria ........................................ 33
Figure 4: PC12 GFP(tet) Cell Line .............................................................................................. 40
Figure 5: Effect Of OGD On Neuronal Cell Death ....................................................................... 43
Figure 6: Comparison Of The Effects Of OGD In DMEM And BSS .............................................. 45
Figure 7: Oxygen Deprivation Does Not Increase Cell Death ...................................................... 48
Figure 8: Glucose Deprivation Significantly Increases Cell Death ............................................... 51
Figure 9: Undifferentiated Cells Respond Similarly To OGD ...................................................... 54
Figure 10: Response Of OGD On Neuronal Cell Death In 25 ng/ml NGF ..................................... 57
Figure 11: Morphological Changes During OGD ......................................................................... 61
Figure 12: Morphological Changes In Early Reperfusion ............................................................ 63
Figure 13: Mitochondrial Morphology During Early OGD ........................................................... 66
Figure 14: Mitochondrial Morphology At The End Of OGD ......................................................... 68
Figure 15: Mitochondrial Morphology In Early Reperfusion ....................................................... 70
Figure 16: Comparison Of The Effects Of OGD/OD/GD For 5 Hrs ............................................... 73
Figure 17: Effect Of L-Glutamine Supplementation During OGD ................................................ 76
Figure 18: Effect Of Glycolytic Inhibition During OGD Using 2 Deoxy-D-glucose ....................... 79
Figure 19: Effect Of Addition Of Iron To BSS During OGD ......................................................... 83
Figure 20: Changes In ROS Generation During OGD ................................................................. 86
Figure 21: Fluorescent Measurements Of ROS During OGD ....................................................... 88
Figure 22: Flow Cytometric Measurements Of ROS At The End Of OGD ..................................... 91
Figure 23: Changes In ROS During Early Reperfusion ............................................................... 94
Figure 24: Fluorescent Measurements Of ROS During Early Reperfusion ................................. 96
Figure 25: Lipid Peroxidation At 5 hrs Of OGD (TBARS Assay) .................................................. 99
Figure 26: Effect Of Anti-oxidants During OGD And Reperfusion ............................................. 101
Figure 27: Effect Of Nitric Oxide Inhibition During OGD And Reperfusion .............................. 105
Figure 28: Ischemic Preconditioning Using BSS ........................................................................ 109
Figure 29: Ischemic/Hypoxic Preconditioning In DMEM .......................................................... 111
Figure 30: Effect Of 2X NGF On OGD Induced Cell Death ....................................................... 114
Figure 31: Effect Of 4X NGF On OGD Induced Cell Death ....................................................... 116
Figure 32: Dose Dependent Protection Of NGF Against OGD .................................................... 120
Figure 33: Protective Effect Of NGF On Serum Withdrawal Induced Cell Death ....................... 122
Figure 34: Effect Of Hypothermia On OGD Induced Cell Death ............................................... 125
Figure 35: Protective Effect Of Hypothermia In OD .................................................................. 127
Figure 36: Summary Of Results ............................................................................................... 129
Figure 37: Auto-oxidation Of Dopamine ..................................................................................... 156
Figure 38: Enzymatic Oxidation Of Dopamine ......................................................................... 157
Figure 39: Probable Models To Explain Cytotoxicity During OGD/OD/GD ............................... 161
Figure 40: Proposed Mechanism Of Cytotoxicity in OGD/OD/GD: A New Model .................... 164
Figure 41: Implications Of This Model ...................................................................................... 168
V. LIST OF TABLES

1. Effect of 5 hrs of OGD on neuronal cell death (model paradigm) .........................58
2. Effect of 2DDG on OGD induced cell death ..........................................................80
3. Effect of SOD and Catalase on OGD induced cell death ........................................102
4. Neuroprotective effect of NGF (4X) on OGD induced cell death .............................117
VII. LIST OF ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
Anova: Analysis of variance
ATP: Adenosine triphosphate
BDNF: Brain derived neurotrophic factor
BFGF: Basic fibroblast growth factor
BMAA: β-N-methyl-α, β-diaminopropionic acid
BOAA: L-Oxalyl-α, β-diaminopropionic acid
BSS: Balanced salt solution
Ca²⁺: Calcium
CaM: Calmodulin
CICR: Calcium induced calcium release
2DDG: 2DeoxyDGlucose
DA: Dopamine
DCF: Dichlorofluorescein
DMEM: Dulbecco’s modified eagles medium
EC: Extracellular
eIF: Elongation initiation factor
eNOS: Endothelid nitric oxide synthase
FAD: Flavin adenine dinucleotide
Fe₃O₉: Ferric Nitrate
FeSO₄: Ferrous sulphate
FMN: Flavin mono nucleotide
FSC: Forward angle light scatter
GD: Glucose Deprivation
GF: Growth factor
GFP: Green fluorescent protein
GSH: Glutathione
H₂O₂: Hydrogen peroxide
HNE: Hydroxynonenal
6OHDA: 6-hydroxy dopamine
Hr: Hour
IC: Intracellular
iNOS: Inducible nitric oxide synthase
IP: Inositol triphosphate
KA: Kainic Acid
KRH: Krebbs Ringer Hepes
L-NAME: L-nitroarginine methyl ester
MAO: Monoamine oxidase
MCA: Middle cerebral artery
MDA: Malondialdehyde
mGluR: Metabotropic glutamate receptor
Min: Minute
MPTP: 1-methyl-1,2,3,6-tetrahydropyridine
mRNA: messenger RNA
MTP: Mitochondrial transition pore
NE: Norepinephrine
NGF: Nerve Growth Factor
NMGA: N-methyl D-aspartate
nNOS: Neuronal nitric oxide synthase
NO: Nitric oxide radical
NOS: Nitric oxide synthase
O2-: Superoxide radical
OD: Oxygen Deprivation
OGD: Oxygen Glucose Deprivation
OH: Hydroxyl radical
ONOO-: Peroxynitrite radical
PARP: Poly ADP ribose polymerase
PC12 cells: Pheochromocytoma cells
PG: Prostaglandins
PI: Propidium Iodide
PLC: Phospholipase C
ROS: Reactive oxygen species
SOD: Superoxide dismutase
SSC: Side angle light scatter
TBARS: Thiobarbituric acid reactive substances assay
TH: Tyrosine hydroxylase
VO: Vessel occlusion
XD: Xanthine dehydrogenase
XO: Xanthine oxidase
VII. LIST OF APPENDIXES

1. Components of DMEM (Dulbecco's Modified Eagle Medium) ........................................ 170
2. Components of BSS (Balanced salt solution) .......................................................... 171
3. Components of KRH (Krebbs Ringer Hepes) ......................................................... 172
4. Preparation of Collagen ......................................................................................... 173
INTRODUCTION

1 Overview Of Stroke, Ischemia And Oxygen-Glucose Deprivation (OGD)

Stroke is defined as a circulatory disturbance resulting in neurological impairments that are sustained for at least a 24 hour time period. Stroke continues to be the third most common cause of death in industrialized nations, behind only cardiovascular disease and cancer (Thompson D. W. and Furlan A. J., 1997) accounting for approximately 10% of all mortalities in developed nations (Camaratta P. J., 1994). It is presently the leading cause of disability in adults making a huge economic impact on health expenditure.

Ischemia refers to a reduction in cerebral blood flow to levels incompatible with tissue viability, usually used in the context of animal models of stroke. The magnitude of the problem has stimulated much research on animal models and techniques occluding vessels to or within the cranium for inducing ischemic brain injury (Molinari G. F., 1986). The validity of any animal model of cerebral ischemia is ultimately determined by its ability to reproducibly yield a lesion similar to that observed in the human stroke condition. Extensive research with neuronal primary cultures has also been performed in vitro using different methods of oxygen and glucose deprivation by placing neuronal cultures into anaerobic chambers and exchanging culture medium with oxygen-free defined ionic composition media (Marcoux F. W. et al., 1989, Goldberg M. P. and Choi D. W., 1990).

Ischemia disrupts neuronal function by a partial interruption of oxidative metabolism. Depressed oxidative metabolism may disrupt neuronal function by altering signal transduction systems. The causal link between these cellular transduction signals and subsequent impairment of cell function or cell death have not been defined (reviewed Lipton P., 1999, Caroll J. M., 1992). The cellular response to ischemia may provide important clues towards understanding the critical processes that lead to impaired cell function and elucidate the role of altered oxidation in neuronal cell death or abnormal function under a variety of conditions.

The approach in the present experiments was to compare between 'complete ischemia' like conditions and 'partial ischemia', a representation of the partial reduction in oxidative metabolism that occurs with ischemia in vivo, in Alzheimer's disease or in Parkinson's disease. There is extensive
literature on the use of the oxygen sensitive PC12 cell line in ischemia/hypoxia/hypoglycemia (Czyzyk-Krzeska M. F. et al., 1994, Raya S. A. et al., 1993 and Pereira C. et al., 1998). PC12 cells are unique in that after treatment with NGF, they express neuronal properties in the absence of support cells such as glia. Using cell cultures also facilitated examination of the cellular mechanisms underlying cytotoxicity. Finally, in my experiments, the availability of a PC12 cell line stably transfected with GFP targeted to mitochondria permitted examination of mitochondrial morphology in live neurons during OGD and reperfusion in culture medium (DMEM) or in the more generally used ‘physiological-balanced salt solution’ (BSS).

2 Cellular And Molecular Mechanisms Of Damage In Ischemia And Reperfusion.

Ischemic cell death is initiated by changes that result directly from inhibition of oxidative phosphorylation. These changes include decreased pH, decreased ATP, initiation of free radical production by the mitochondrial chain, increased cell Na\(^+\) and membrane depolarization as a result of the loss of ATP substrate for the Na\(^+\)-K\(^+\) pump (reviewed Lipton P., 1999). Ischemia can cause plasma membrane damage, mitochondrial damage, inhibition of protein synthesis and cytoskeletal damage ultimately leading to cell damage and death (Brown A. W. et al., 1972, Yamamoto K. T. et al., 1990, Neumar R. W. et al., 1998 and Hu R. J. et al., 1991). Multiple cellular and molecular mechanisms are involved in ischemia, the ones relevant to my work are briefly discussed below.

2.1 Mitochondria


1. Inhibition of mitochondrial oxidative phosphorylation will lower ATP levels, increase free radical production and remove the Ca\(^{2+}\) buffering ability of the organelle. These could be because of direct effects on the tricarboxylic acid cycle (TCA) cycle enzymes, on the mitochondrial electron transport, lipid composition of mitochondrial membrane or long term opening of the


3. Release of cytochrome c and/or at least one more protein from the inter-membrane space is now recognized as a critical step in apoptosis, probably by activating caspases and inhibiting the respiratory chain. Such a release is effected by opening of the MTP or by direct effects on the integrity of the outer mitochondrial membrane (Eskes R. B. et al., 1998, Reed J. C., 1997).

2.2 Protein Synthesis

Protein synthesis is extremely sensitive to cell energy charge and ion contents (Lipton P. and Heimbach C. J., 1978, Raley Susman K. M. and Lipton P., 1990), both of which can be expected to alter as a consequence of ischemia. For example, changes in the two initiation factors eIF-2 and eIF-4 have been positively implicated in the decreased synthesis of proteins. Factor eIF-4G is a substrate for calpain and there is a substantial loss in its immunoreactivity after global ischemia. This is very likely due to calpain mediated proteolysis (Neumar R. W. et al., 1995, Neumar R. W. et al. 1995). The eIF-4 family of proteins are also inactivated for a long duration by free radical attack (Jornot L. and Junod A. F., 1989). Increased cytosolic Ca²⁺ activates calpain-mediated breakdown of eIF-4 (Marin P. et al., 1997), it might activate PKR which phosphorylates eIF-2 (Jagus R. and Gray M. M., 1994).

2.3 Cytoskeleton

The Cytoskeleton is extremely sensitive to ischemia, resulting in gross disruption of cytoskeletal molecules thereby affecting transport within the cell. Microtubule dissociation is caused by MAP2 phosphorylation, proteolysis, dissociation of the Microtubule stabilizing protein (STOP) and proteolysis of tubulin. All of these are activated by Ca²⁺, as well as PKC and other kinases (Brugg B. and Matus A., 1991, Hoshi M. et al., 1992, Mandelkow E., 1995, Mitchison T. and Kirschner M, 1984). Spectrin provides major linkages between the cell membrane and membrane-associated proteins which in turn are involved in maintaining integrity and localization of integral membrane proteins (Bennett V., 1990, Bennett V., 1992, Hu R. J. and Bennett V., 1991). It is very susceptible to calpain (Hu R. J. and Bennett V., 1991) and to caspase-3 like proteases which cleave it at different sites (Nath R. et al.,
Ankyrin plays a major role in linking membrane proteins such as Na⁺-K⁺-ATPase and Na⁺ channels to the cytoskeleton. Thirty minutes of global ischemia followed by 60 minutes of reperfusion caused a loss of immunoreactivity of Ankyrin B and breakdown of Ankyrin R, possibly mediated by calpain. This could lead to malfunction of the Na⁺ pump or other membrane proteins and contribute to cell death (Harada K. et al., 1997, Palmer G. C. et al., 1985). Phosphatidylcholine precursor CDP-choline is protective in ischemia indicating that membrane damage may be critical (Kakihana M. et al., 1988, Patel P. M. et al., 1994). This molecule acts as a precursor to phosphatidylcholine incorporation into cell membranes (Dorman R. V. et al., 1983) and it dramatically increases the net liberation of free fatty acids (FFA) during ischemia (Kakihana M. et al., 1988, Trovarelli G. et al., 1981), presumably by activating the synthesis of phospholipid. Short exposures to several different free radical species cause severe irreversible damage to the Na⁺ pump (Chen J. W. et al., 1992, Huang W. H. et al., 1992, Shattock M. J. and Matsura H., 1993) and also make it more susceptible to proteolytic attack (Huang W. H., 1992).

2.4 Ion Channels In Ischemia

Ion gradients are important for any type of cell. Excitable cells like neurons use these gradients to accomplish their function of signal transfer. The maintenance of ion gradients consumes ATP which must be continuously replenished. Na⁺ and K⁺ gradients are essential for the occurrence of Na⁺ action potentials and of voltage, transmitter or second messenger gated re/hyperpolarization of the membrane (Spuler A. et al., 1996). During ischemia the marked increase in extra-cellular K⁺ accompanied by a decrease in intracellular K⁺ is due to both impaired ATPase function and opening of K⁺ channels. Both mechanisms eventually lead to membrane depolarization. In conjunction with Na⁺ influx chloride ions flow into neurons thereby contributing to cell swelling during the initial stages of ischemia (Spuler A. et al., 1996).

Ischemia evokes an influx of Ca²⁺ across the cell membrane due to extra-cellular concentrations of excitatory amino acids and through opening of voltage gated Ca²⁺ channels (see Introduction section 6.2). Increased intracellular Na⁺ concentration during ischemia may even reverse the direction of the Na⁺/Ca²⁺ exchanger thus transporting Ca²⁺ into the cell. Additional buffering capacity is provided by cytosolic Ca²⁺ binding proteins such as calmodulin, calbindin and parvalbumin. Further,
endoplasmic reticulum and the mitochondria exhibit important Ca$^{2+}$ storing capacity and are known to elevate cytosolic Ca$^{2+}$ under energy deprived conditions (Spuler A. et al., 1996).

Regulation of neuronal pH is achieved by a Na$^+$/H$^+$ antiporter and the Na$^+$ dependent Cl$^-$/HCO$_3^-$ exchanger located in the cell membrane. They utilize the Na$^+$ gradient, these regulators are hence indirectly ATP-dependent and thus reduced in their efficacy during ischemia. Accumulating lactate is another mechanism for the development of acidosis in ischemia. Acidosis leads to a decrease in synaptic transmission. The changes in K$^+$ conductance are mainly responsible for the delay in membrane depolarization and the limitation of energy expenditure in the early phase of energy depletion (Spuler A., 1996).

3 In Vitro Ischemia – Oxygen-Glucose Deprivation (OGD)

The choice of a model to examine the actions of ischemia is difficult because each approach has its own limitations. Several means of inducing in vitro ischemia are available. Recent years have seen a dramatic increase in our understanding of hypoxic/ischemic mechanisms. Much of this knowledge has arisen from in vitro studies in cultured neurons in which ischemia-like conditions are simulated using either anoxic/hypoglycemic conditions or the application of excitatory amino acids (Choi D. W., 1988, Choi D. W., 1990, Lipton S. A. and Rosenberg P. A., 1994, Tymianski M and Tator C. H., 1996). Commonly used agents for chemical hypoxia include cyanide, rotenone or antimycin A (Caroll J. M. et al., 1992).

In my model of OGD, physical methods to induce ischemia/hypoxia were used, to avoid changes in ROS generation caused by chemical hypoxia (mitochondrial inhibitors). A number of studies of ischemia in culture show a correlation between the number of disintegrated necrotic cells and membrane breakdown, measured as LDH release or uptake of vital stains like propidium iodide (Goldberg M. P. and Choi D. W., 1993, Myers K. M. et al., 1995, Strasser U. and Fischer G., 1995, Uto A. et al., 1995). I have therefore considered the percentage of maximal LDH release to represent cell death in my model of OGD/OD and GD.
4 Cytotoxicity In Anoxia And Hypoglycemia

4.1 Anoxia

Anoxia is an important aspect of ischemia and represents oxygen deprivation in the presence of glucose under in vitro experimental conditions. Organotypic hippocampal slices show a strong neuroprotective effect of NMDA receptor blockade on CA1 neuronal damage following combined oxygen-glucose deprivation: however no protective effect of NMDA and/or AMPA/Kainate receptor blockade was seen following severe oxygen deprivation alone (Newell D. W. et al., 1995). This indicates that there are different mechanisms that produce neuronal damage during severe anoxia in the presence (OD) or absence of glucose (OGD). NMDA receptor blockade is not found to prevent intraneuronal calcium accumulation, which is found to be associated with delayed neuronal death following severe anoxia (Newell D. W. et al., 1995).

One suggested mechanism is through increased intracellular Ca\(^{2+}\) due to massive neuronal depolarization and ATP depletion. Calcium may enter cells through a variety of ways, and failure of energy dependent extrusion mechanisms could cause a massive increase in intracellular Ca\(^{2+}\) (Newell D. W. et al., 1995). There seems to be an apparent distinction in the pre- and postsynaptic terminals in Ca\(^{2+}\) regulation during hypoxia. The hypoxia induced increase in intracellular Ca\(^{2+}\) in presynaptic terminals is related to diminished buffering by the mitochondria, whereas the elevated postsynaptic Ca\(^{2+}\) is related to enhanced efflux across the membrane (Gibson G. et al., 1997).

It is possible oxygen deprivation in the presence of glucose may induce anaerobic metabolism, which could lower pH. At low pH, NMDA receptors are inactivated and other factors like elevated lactate levels may come into play (Newell D. W. et al., 1995).

4.2 Hypoglycemia

It is well known that a continuous supply of glucose is necessary for the normal functioning and morphological integrity of mammalian central neurons. Brain hypoglycemia can occur when blood glucose levels fall or when blood supply to the brain is reduced, as occurs in stroke (Siesjo B. K. et al., 1989). Hypoglycemia leads to increased utilization of endogenous substrates, ATP depletion, membrane depolarization, excessive release and reduced uptake of glutamate or aspartate, loss of neuronal ion homeostasis (notably increased intraneuronal calcium), and finally irreversible brain
It is generally believed that calcium, which serves physiologically important functions as a second messenger regulating neural information coding and plasticity (Kater S. B. et al., 1988), also mediates neuronal degeneration resulting from an array of metabolic and environmental insults (Kater S. B. et al., 1988, Choi D. W., 1988, Mattson M. P., 1992). In the central nervous system, the excitatory neurotransmitter glutamate contributes to neuronal vulnerability to insults such as hypoglycemia and anoxia by enhancing calcium influx (Choi D. W., 1988). Glucose deprivation caused a highly significant, 3-fold elevation of intracellular calcium levels in both hippocampal and human cortical neurons exposed to glucose-free medium for 16 or 30 hr, respectively (Cheng B. and Mattson M. P., 1991). Incubation of cultures in medium lacking extracellular calcium resulted in highly significant protection of both rat hippocampal and human cerebral cortical neurons against hypoglycemic damage (Cheng B. and Mattson M. P., 1991). These data suggested that calcium influx was responsible for the hypoglycemic neuronal damage and that damaging levels of influx occurred through calcium channels other than the dihydropyridine sensitive L-type calcium channels (Cheng B. and Mattson M. P., 1991).

5 Rat Pheochromocytoma PC12 Cell Line

Clonal cell lines which express neuronal properties are useful model systems for studying the nervous system at the single cell and molecular levels. Such lines have been established from human and murine neuroblastomas (Kolber A. R. et al., 1974, McMorris F. A. et al., 1973), rat central nervous system tumors (Schubert D., 1974) and rat Pheochromocytoma (Greene L. A. and Tischler A. S., 1976). Clonal rat Pheochromocytoma (PC12) cells, which are derived from normal medullar chromaffin cells, are widely used as a model system for sympathetic ganglion like neurons. Clonal systems provide a useful model for the rigorous testing of hypotheses which may then be more extensively tested in vivo (Jackson G. R. et al., 1990).

PC12 cells provide an attractive culture system in which the underlying mechanisms associated with cellular response to oxygen deprivation may be explored (Caroll J. M. et al., 1992). Since its initial description and characterization in 1976 (Greene L. A. and Tischler A. S., 1976), the rat Pheochromocytoma PC12 cell line has become a commonly employed model system for studies of the development and function of peripheral and central neurons. These cells are currently a favored tool in neuroscience research for the study of nerve growth factor (NGF), neuronal differentiation, electrical
excitability and release of catecholamines (Fujita K. et al., 1989). Undifferentiated and differentiated PC12 cells have been used in neuroscience as a model for neuronal cell death caused by serum or NGF deprivation, toxins, and ischemia (Abu-Raya S. et al., 1999). Several attributes of the PC12 cells have led to their widespread popularity in neurobiological research. These include their high degree of differentiation after NGF treatment, their relative homogeneity, the availability of large numbers for biochemical and genetic analysis (Teng K. K. et al., 1993), as well as the ease of manipulation and degree of control that may be exerted over the extra-cellular milieu. All of these characteristics provide definitive advantages over animal experiments. Differentiated PC12 cells also have particular advantages for examining the response of cells to hypoxia/ischemia as they do not require a layer of glial cells for survival. Finally as a population, PC12 cells are more uniform than can generally be obtained in primary neuronal cultures making it easier to select cells and to draw generalized conclusions (Gibson G. et al., 1997).

5.1 Undifferentiated PC12 Cells And Differentiated (Neuronal) PC12 Cells

When cultured in serum containing medium, undifferentiated PC12 cells adopt a round and phase bright morphology and proliferate to a high density. Under these conditions PC12 cells display many of the properties associated with immature adrenal chromaffin cells and sympathicoblasts. When exposed to physiological levels of NGF, these cells cease division, become electrically excitable, extend long branching neurites, and gradually acquire many characteristics of mature sympathetic neurons (Teng K. K. et al., 1993). NGF increases the expression of N type Ca\(^{2+}\) channels (Usowicz M. M. et al., 1990) and voltage dependent Na\(^+\) channels (Usowicz M. M. et al., 1990). Under serum free conditions, NGF promotes not only neuronal differentiation of PC12 cells, but also promotes their survival (Greene L. A., 1978, Rukenstein A. et al., 1991). PC12 cells synthesize and secrete dopamine (DA) and norepinephrine (NE) (Greene L. A., 1977, Greene L. A. and Rein G., 1978, Greene L. A and Tischler A. S., 1982) and release these neurotransmitters in response to a variety of stimuli, including hypoxia and ischemia, and pharmacological agents (Courtney N. D. et al., 1991, Kanthasamy A. G. et al., Koizumi S. et al., 1995, Shafer T. J. and Atchison W. D. et al., 1991). The mechanism of transmitter release has been found to involve membrane depolarization (Greene L. A. and Rein G., 1977, Greene L. A. and Tischler A. S., 1982, Kawai Y. et al., 1997) and elevation of cytosolic Ca\(^{2+}\) concentration (Shafer T. J. and Atchison W. D., 1991, Ahnert-Hilger G. et al., 1987, Bright G. R. et al., 1996, Pozzan
T. et al., 1984) that occurs in response to activation of various membrane bound receptors. In addition protein kinases also play an important role in transmitter release from PC12 cells (Ahnert-Hilger G. et al., 1987, Harris K. M. et al., 1986, Pozzan T. et al., 1984133).

5.2 PC12 Cells And Glutamate

Undifferentiated PC12 cells treated with glutamate respond with the intracellular accumulation of IP, increase in cytosolic Ca\(^{2+}\) and secretion of NE indicating the presence of functional PLC (phospholipase C) coupled mGluRs (metabotropic glutamate receptors) in these cells (Kurozumi K. et al., 1990). PC12 cells differentially express group I mGluRs (Kane M. D. et al., 1998). Activation of the metabotropic glutamate receptors may also contribute to a rise in intracellular Ca\(^{2+}\) in ischemia, as a result of mobilization of Ca\(^{2+}\) from the intracellular stores (Love S., 1999).

Although PC12 cells express high levels of NMDA receptor mRNA and this mRNA directs the synthesis of active NMDA receptors in frog oocytes, the cytotoxicity elicited by glutamate appears not to function primarily through the NMDA receptor per se. Several observations support this conclusion

(i) High concentrations of glutamate are required (1 mM).

(ii) NMDA (5 mM) is only a weak agonist of the glutamate receptor in PC12 cells requiring high glycine.

(iii) Neither competitive nor non-competitive NMDA receptor antagonists inhibit glutamate induced killing by >25%.

(iv) The effects of Mg\(^{2+}\) and high concentrations of glycine are small.

(v) BOAA (see Abbreviations) is more toxic than BMAA, whereas the latter is more specific for NMDA receptors (Schubert D. et al., 1992).

In a neuroblastoma-retina hybrid cell line an alternative mechanism for glutamate induced cell death is that glutamate competes for cystine uptake, reducing intracellular glutathione and increasing free radical generation. This is not true in PC12 cells as increasing cystine 5 fold over the normal
medium has little effect on glutamate induced cell death, cystine deletion inhibits toxicity and K⁺ depolarization enhances toxicity.

5.3 PC12 Cells In Ischemia

PC12 cells represent an excellent model system to analyze ischemic insults (Raya S. A. et al., 1993). Undifferentiated PC12 cells do not require NGF for survival and proliferation but in the presence of NGF, will differentiate to a sympathetic phenotype (Thoenen H. and Barde Y. A., 1980). Moreover in NGF-differentiated PC12 cells there is a marked increase of glucose utilization via the oxidative pathway of the Krebs cycle (Morelli A. et al., 1986) as for brain neurons. PC12 cells have multiple pathways that are capable of maintaining ATP production. They can maintain ATP concentrations in the absence of oxygen if glucose is present, and in the absence of glucose if oxygen is present (Pereira C. et al., 1998, Caroll J. M. et al., 1992). This may be due to the presence of other substrates such as glutamine, that can be used to generate ATP. An alternative explanation is that these cells have a low metabolic rate hence their energy consumption may be low (Caroll J. M. et al., 1992).

5.4 PC12 Cells In Hypoxia

Reduction of available oxygen (hypoxia) is a key feature of ischemia. Hypoxia is known to exert a diverse range of responses in cells, each of which serves a specific physiological purpose (Taylor S. C. et al., 1999). Acute hypoxia can evoke extremely rapid responses such as selective membrane delimited inhibition of ion channels (Taylor S. C. et al., 1999). PC12 cells represent a well defined, model excitable cell system, which has been used extensively to study the effects of both acute and chronic hypoxia on various cellular processes, including ion channel activity and gene expression (Czyzyk-Krzeska M. F. et al. 1994, Kobayashi S. et al., 1998, Taylor S. C. and Peers C., 1998).

Acute hypoxia evokes catecholamine release from undifferentiated PC12 cells by causing initially membrane depolarization and subsequent Ca²⁺ influx, primarily through N type voltage gated Ca²⁺ channels (Taylor S. C. and Peers C., 1998). Furthermore at any given PO₂, release of NE is greater than release of DA, and a protein kinase dependent pathway(s) seem to be associated with NE but not
DA release during hypoxia (Sakagami H. et al., 1996). These cells survive hypoxia (5% O₂) up to several hours, reminiscent of the large viability differences existing between brain neurons in their sensitivity to hypoxia and anoxia (Siesjo B. K., 1981). Previous studies have shown that PC12 cells maintain ATP levels for several hours in the absence of oxygen if glucose is present (Pereira C. et al., 1998, Caroll J. M., 1992). This suggests that under hypoxia, although the synthesis of ATP in the mitochondria may be already compromised, glycolysis is stimulated, resulting in the maintenance of ATP levels. The stimulation of glycolysis has been proposed to occur in synaptosomes upon inhibition of the respiratory chain with cyanide, and in retinal cells upon chemical ischemia (Pereira C. et al., 1998).

5.5 PC12 Cells In Hypoglycemia

Hypoglycemic brain damage is a major component of stroke and is related to ischemia. Hypoglycemic neuronal cell death has been found to exhibit some elements of necrosis and is largely mediated by excitotoxic activation of glutamate receptors, although hypoglycemic neuronal primary cultures can undergo apoptosis, when glutamate receptors are blocked. This variability may reflect the differences in the level of expression of excitotoxicity in primary cultured neurons when compared to clonal PC12 cells (Tong L and Perez Polo J. R., 1995). In hippocampal neurons initially, hypoglycemia induced calcium current inhibition and a reduction in intracellular free calcium current level with no morphological signs of neuronal damage, while at later times, hypoglycemia induced a large elevation of intracellular Ca with neuronal damage (Tong L and Perez Polo J. R., 1995).

The enhanced susceptibility of the cells in medium lacking glucose could be due to a lack of glucose for glycolysis. Two major pathways are known to participate in elevating intracellular calcium ions in PC12 cells: Ca²⁺ influx through voltage gated action channels such as L-type channels and Ca²⁺ releases from an internal store sensitive to bradykinin or caffeine. Glucose withdrawal induces the elevation of intracellular calcium ions in differentiated PC12 cells that can be blocked by ryanodine but not by nifedipine. It suggests that calcium release from caffeine sensitive stores rather than calcium influx may be a major source for the elevation of intracellular Ca²⁺ induced by glucose withdrawal (Chung J. and Hong J., 1998). In the presence of glucose the ATP levels determined after incubation with mitochondrial respiratory chain inhibitors (hypoxic like conditions) were not significantly different from those determined under control conditions. However when PC12 cells were incubated
for 30 min in a glucose free medium (hypoglycemic like conditions) a significant decrease in ATP levels were observed and this depletion of ATP was greatly potentiated in the presence of mitochondrial respiratory chain inhibitors (Pereira C. et al., 1998).

5.6 Amino Acid Utilization In PC12 Cells

Amino acid requirements are considerably different from cell to cell type. PC12 cells utilize four essential amino acids (valine, methionine, isoleucine, leucine), glutamine and arginine. They produce glycine, alanine and ammonia without significantly affecting threonine, tyrosine, phenylalanine, histidine or lysine (Sakagami H. et al., 1998). Dopamine (Kawase M. et al., 1998), sodium ascorbate (Sakagami H. et al., 1996a) and SBA (Sakagami H. et al., 1996b) stimulated the oxidation of methionine to methionine sulfoxide in HL60 cells, but reduced the consumption of serine, glutamine and arginine by the cells (Sakagami H. et al., 1998). In the absence of cells, these agents stimulated the methionine oxidation to a greater extent thus confirming their pro-oxidant actions in vitro. Oxidative stress might damage the mitochondrial function, such as ATP synthesis and thereby inhibit ATP dependent amino acid transport or utilization (Sakagami H. et al., 1998).

5.7 PC12 Cells And Catecholamines/Eicosanoids

Since PC12 cells are a catecholaminergic cell line, they are vulnerable to the toxic effects of neurotoxins such as 6-OHDA (6 hydroxydopamine) and MPTP (see Abbreviation) which exert their effects on dopamine containing cells in vivo (Walkinshaw G. and Waters C. M., 1994). 6-OHDA is a hydroxylated derivative of dopamine which is selectively toxic to adrenergetic neurons, due to its specificity for the high affinity dopamine uptake system. It is hypothesized that this compound may be formed in vivo by auto-oxidation of dopamine, but direct evidence is lacking (Walkinshaw G. and Waters C. M., 1994). The cytotoxicity of L-DOPA on PC12 cells is most likely due to the action of free radicals formed due to its auto-oxidation. Catalase and superoxide dismutase each partially protected against L-DOPA toxicity in PC12 cells (Basma A. N. et al., 1995).

Free fatty acids released as a result of ischemic insults (Goldberg M. P. and Choi D. W., 1990) are known to have detrimental effects on brain structure and function (Yoshida S. et al., 1980, Bazan
Arachidonic acid, the substrate for eicosanoid production by monooxygenases is the precursor of several compounds: prostaglandins, thromboxanes and leukotrienes (Siesjo B. K., 1981). They play a neuromodulatory role, are vasoactive and their high levels produced in ischemia may damage cells in ways which are yet poorly understood. Hypoxia alone with no reoxygenation slightly increased the basal production and release of eicosanoids, in both naive and differentiated PC12 cells. However upon reoxygenation there was a 2-3 fold increase in the amount of PGE2 in the medium (Raya S. et al., 1993).

6 Role Of Glutamate, Calcium And Free Radicals In Ischemia

CNS injury from ischemia and reperfusion is proposed to occur via multiple interrelated mechanisms including excessive extracellular accumulation of the excitotoxin glutamate, an increase in intracellular Ca\(^{2+}\) and oxidative stress (Shackelford D. A. et al., 1999). These processes contribute to the generation of reactive oxygen species that damage protein, lipid and DNA (Chan D. W. and Lees-Miller S. P., 1996, Simonian N. A. and Coyle J. T., 1996). Depending on the severity of the cellular damage, the cell may activate repair or protective mechanisms or undergo cell death by necrosis or apoptosis (Buja L. M. et al., 1993).

6.1 Extracellular Glutamate

Glutamate is an excitatory neurotransmitter that is widely expressed within the central nervous system. It binds to two distinct families of receptors, the metabotropic and ionotropic receptors. The latter family of glutamate receptors comprises three types, named according to their differential sensitivity to the agonists NMDA, KA and AMPA. The non-NMDA ionotropic glutamate receptors i.e. AMPA and KA receptors have much faster kinetics than the NMDA receptors whereas only the NMDA receptors respond to activation by increasing Ca\(^{2+}\) permeability. However activation of all the ionotropic glutamate receptors leads to an increase in Na\(^+\) and K\(^+\) permeability and the resulting depolarization can secondarily activate voltage-sensitive Ca\(^{2+}\) channels (Love S. et al., 1999).

In ischemia, activation of metabotropic glutamate receptors may also contribute to a rise in intracellular Ca\(^{2+}\), as a result of mobilization of Ca\(^{2+}\) from intracellular stores (Love S. et al., 1999).
Normally, the action of glutamate that is released at synapses is terminated by its uptake from the extracellular space by a family of glutamate transporter proteins. The energy that drives this uptake is derived from the linked transport of Na\(^+\) and K\(^+\) down their respective electrochemical gradient: Na\(^+\) is cotransported with glutamate into the cell (neuronal/glial) and K\(^+\) out of the cell. During ischemia, anaerobic metabolism leads to a fall in pH, and depletion of ATP to slowing and failure of the Na\(^+\)/K\(^+\) pump, resulting in the movement of these ions down their electrochemical gradients across the plasma membrane: Na\(^+\) into the cell and K\(^+\) outwards. This redistribution is initially gradual but after about 2 mins occurs more rapidly as the membranes depolarize. The membrane depolarization and the change in the concentration gradients of Na\(^+\) and K\(^+\) across the plasma membrane reverses the direction of action of the glutamate transporter proteins as a result of which glutamate rapidly accumulates extracellularly until it reaches neurotoxic levels. The accumulation of glutamate is a relatively short lived event, but the consequence is a rise in intracellular Ca\(^{2+}\) largely due to the activation of NMDA receptors (Love S. et al., 1999). There is strong evidence that glutamate, which is known to be neurotoxic, contributes to both global and focal ischemia induced neuronal injury (Buchan A. M., 1990, Choi D. W., 1990). However it is noteworthy that blockade of NMDA receptors alone fails to afford neuroprotection in models of global ischemia. In vivo studies have also shown increases in extracellular glutamate in the brain during transient forebrain ischemia, and following head injury (Beneveniste H. et al., 1984, Faden A. I. et al., 1989). High extracellular glutamate levels then activate ionotropic glutamate receptors, thus inducing an overflow of calcium ions into the neurons (Bickler P. E. and Hansen B. M., 1994, Choi D. W., 1988, Ferreira I. L. et al., 1996) which in turn activates several catabolic processes and causes neuronal damage (Orrenius S. et al., 1994). Moreover, glutamate receptor blockade of the NMDA, and the non-NMDA subtypes, can reduce neuronal damage following focal ischemia (Germano I. M. et al., 1987) and transient forebrain ischemia (Buchan A. M. et al., 1991) respectively. Glutamate receptor blockade can also protect dissociated neuronal cultures from damage induced by combined oxygen and glucose deprivation (Goldberg M. P. and Choi D. W., 1993).

### 6.2 Intracellular Calcium

Calcium has generally been considered to be a major effector of necrotic cell death during ischemia (Kristian T. and Siesjo B. K., 1996), and this is more likely given the fact that Ca\(^{2+}\) very clearly
plays a critical role in the normal cell death caused by ischemia in cell culture systems (Goldberg M. P. and Choi D. W., 1993). Cytosolic Ca\(^{2+}\) could rise as a result of a net entry of Ca\(^{2+}\) across the plasmalemma through ion channels or, transiently, due to liberation of Ca\(^{2+}\) from intracellular stores. In the hippocampal slice, influx of Ca\(^{2+}\) during the first 2.5 min of in vitro ischemia is via NMDA receptors, but these appear to inactivate after this time (Lobner D. and Lipton P., 1993), probably due to the profound ATP decrease and perhaps resulting receptor dephosphorylation. Influx during the next 2.5 min is via L channels (25%), Na\(^+\)/Ca\(^{2+}\) exchange (35%) and via an unidentified pathway (Lobner D. and Lipton P., 1993). Some of the Ca\(^{2+}\) that enters the cell during ischemia is taken up by mitochondria, and this uptake is normally attenuated by the efflux of Ca\(^{2+}\) on 2Na\(^+\)/Ca\(^{2+}\) exchanger, which is activated by the entering Na\(^+\) (Zhang Y. and Lipton P., 1995). Thus the entering Na\(^+\) effectively acts to increase cytosolic Ca\(^{2+}\) during ischemia. There is fairly strong evidence that Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the endoplamic reticulum (ER) contributes to the rise in cytosolic Ca\(^{2+}\). Dantrolene, a specific blocker of CICR, has the same effect as NMDA blockers in ischemic hippocampal slices (Zhang Y. and Lipton P., 1995) and in cultures treated with cyanide (Dubinsky J. M. and Rothman S. M., 1991). Increased cytosolic Ca\(^{2+}\) can cause cellular damage by different mechanisms, activating NOS, calpain, generation of free radicals via phospholipid metabolism or by transforming XD to XO, damaging mitochondria (Dugan L. L. et al., 1995) or by effects on cytoskeletal integrity (Kakiuchi S. and Sobue K., 1983).

6.3 Free Radical Generation During Ischemia And Reperfusion

6.3.1 Reactive Oxygen Species In Ischemia And Reperfusion

Reactive oxygen species (ROS) have been implicated in the tissue injury that follows ischemia and reperfusion (Das D. K., 1994, Hearse D. J. et al., 1973, Meerson Z. F. et al., 1982, Opie L. H. et al., 1989, Zweier J. L. et al., 1987). Studies have suggested that a burst of ROS generation occurs during the first minutes after ischemic tissues are reoxygenated, leading to the conclusion that the return of oxygen to ischemic tissues is a critical event for the generation of ROS (Meerson Z. F. et al., 1982, Zweier J. L. et al., 1987, Ambrosio G. et al., 1993, Vanden Hoek T. L. et al., 1996). Studies in chick cardiomyocytes suggested that increased ROS generation during ischemia was caused by residual oxygen and was associated with increased cell death during reperfusion (Vanden Hoek T. L. et al., 1997). If this is true, ROS generation during ischemia could have very important implications for
ischemia-reperfusion therapies and may also explain why clinical trials with antioxidants given only at reperfusion have failed to show any benefit (Becker L. B. et al., 1999).

Normally the rates of free radical production and elimination are equal, leading to a steady state that is presumably tolerated by the cell. Ischemia and reperfusion create several conditions that could account for the increased net production of free radicals (Freeman B. A. and Crapo J. D., 1982, Halliwell B. and Gutteridge M. C., 1984, Ikeda Y. and Long D. M., 1990).

**Mechanisms Of Free Radical Production During Ischemia**

1. **Xanthine/Hypoxanthine Oxidation**: The breakdown of adenine nucleotide during ischemia leads to accumulation of hypoxanthine within minutes (Hagberg H. et al., 1987) which is then metabolized by xanthine oxidase (XO) or xanthine dehydrogenase (XDH). XO produces free radicals but XDH does not. The reaction for generating superoxide is as follows:

   \[
   \text{Hypoxanthine} + \text{O}_2 \to \text{Superoxide (O}_2^{\cdot}) + \text{Hydrogen peroxide (H}_2\text{O}_2) + \text{Urate}
   \]

   At the moment the evidence favours its importance in global ischemia because changes in ATP and probably Ca^{2+} are larger during the insult. The activity of Xanthine Oxidase (XO) produced by proteolytic cleavage of Xanthine Dehydrogenase (XDH), was increased 5 fold 30 min after 15-min global ischemia in rat, and there was concomitant urate production (Kinuta Y. et al., 1989).

3. Altered Mitochondrial Function: The mitochondria normally generate free radicals at a rapid rate (Boveris A., 1977, Turrens J. F., 1997), but one which is handled by normally functioning cells. When the electron carriers become highly reduced, as much as 2% of the electron flow leads to direct single-electron reduction of oxygen and formation of superoxide (Boveris A., 1977, Freeman B. A. and Crapo J. D., 1982, Dehret J. P., 1993). Accumulation of Ca\(^{2+}\) (Turrens J. F. et al., 1991) and opening of the MTP (Kroemer G. et al., 1998, Marchetti P. et al., 1996, Zarzami N. et al., 1996) are among other factors which very probably cause mitochondrial free radical production.

4. Mechanisms Of NO And Peroxynitrite Generation: Nitric oxide is generated by neuronal or endothelial NOS in an oxygen-dependent reaction that is activated by Ca\(^{2+}\)/Calmodulin in most neurons and endothelial cells (Iadecola C., 1997, Schmidt H. H. et al., 1992). Peroxynitrite is generated by the reaction between superoxide and NO.

\[
\text{Superoxide (O}_2^-\text{) + Nitric Oxide (NO) \rightarrow Peroxynitrite (ONOO')}
\]

It can be protonated to produce the very reactive peroxynitrous acid that dissociates into hydroxyl (OH) and various nitrogen/oxygen species (Beckmann J. S., 1994). The unprotonated form is also very reactive with a half life in biological systems of \(~1-2\text{s}\) and long diffusion distances of \(~100\text{um}\). One or the other of the forms readily crosses the cell membranes so that peroxynitrite, like superoxide, can act in cells other than those in which it is generated (Beckmann J. S., 1994, Vander Vliet A. et al., 1994). NO\(^{-}\) can effectively compete with the very active superoxide dismutase (SOD) for superoxide when it rises to 1-2\text{uM}, as it does in ischemia. Peroxynitrite formation is not expected in the absence of NO\(^{-}\) generation unless superoxide generation is high enough to effectively saturate the competing SOD reaction. This can occur endogenously and does occur when superoxide is artificially generated in cultured PC6 cells (Keller J. N. et al., 1998).

5. Monoamine Accumulation: There is quite strong evidence that H\(_2\)O\(_2\) is produced from accumulated catecholamines (Baker A. J. et al., 1991) via monoamine oxidase (MAO) in the 5 min after 15 min global ischemia in rat (Simonson S. G. et al., 1993). Rapid oxidation of glutathione was blocked by MAO inhibitors, suggesting MAO activation of H\(_2\)O\(_2\) production. This certainly makes
sense given the large release of monoamines during ischemia. Blockade of MAO was not protective indicating that this early production of H₂O₂ was not damaging (Simonson S. G. et al., 1993).

6. Interactions Between Different Species: Superoxide and NO⁻ are usually the first free radicals formed, but superoxide is not very reactive. The formation of ONOO⁻ is likely to mediate much of its toxicity (Lipton P., 1999). Formation of OH⁻ is strongly favoured by the decreased pH that prevails during and after ischemia (Das D. K., 1994, Ikeda Y. and Long D. M., 1990), and also by free iron (Halliwell B. and Gutteridge M. C., 1984).

6.3.2 Reactive Nitrogen Species In Ischemia And Reperfusion

Nitric oxide (NO) is a water and lipid soluble free radical with diverse biological activities including vasodilatation, inhibition of platelet aggregation, inhibition of smooth muscle proliferation, modulation of neurotransmission, promotion of synaptogenesis and synaptic remodeling, an involvement in long-term potentiation and depression (Dawson T. M. and Snyder S. H., 1994).

The members of the nitric oxide synthase (NOS) family catalyse the conversion of L-arginine first to N-hydroxyl-arginine and then to L-citrulline and NO. These reactions are coupled to the donation of five electrons by NADPH. Activation of NOS requires the binding of calmodulin (CaM), FAD, FMN, heme and tetrahydrobiopterin. Neuronal NOS (NOS1) and endothelial NOS (NOS3) bind CaM in a reversible Ca²⁺ dependent manner, because the normal intracellular levels of Ca²⁺ are too low to allow the binding of CaM to these types of NOS, they are active only during periods of transient, agonist induced elevations in intracellular Ca²⁺. The third type of NOS, inducible or NOS2, bind CaM even at very low concentrations of intracellular Ca²⁺ and is therefore constitutively active. Regulation of NOS2 activity is primarily mediated by a wide range of transcriptional inducers (including several cytokines and hypoxia) and inhibitors (Love S., 1999).

The activity of all three isoforms of NOS increases after the initiation of ischemia, NOS1 and 3 within minutes and NOS2 after several hours. The complex balance of protective and destructive effects of NOS activation in brain ischemia has been reviewed extensively. In general, the administration of selective inhibitors of NOS1 or NOS2 results in a reduction of infarct volume and other measures of ischemic damage. Unlike NOS1 and NOS2 knockout mice, the NOS3 knockouts develop larger infarcts than do their wild-type counterparts. Normalization of blood pressure in these
hypertensive rats does not increase infarct volume, but inhibition of residual NOS 1 and 2 activity by an infusion of nitro-L-arginine did. However the overall effects of enhanced NOS 1 and NOS 2 activity after ischemia are detrimental (Love S., 1999).

A fine balance exists between the generation of reactive oxygen and nitrogen species (ROS and RNS) for normal metabolic function and their safe detoxification endogenous by antioxidant pathways. The development of oxidative stress, in which the production of these highly reactive oxidants overwhelms our antioxidant defences, is a feature of many diseases that involve the nervous system. Evidence is increasingly pointing to the involvement of oxidative stress and to it being the primary cause of cytotoxicity and cell death in a wide range of inflammatory, ischemic, metabolic and degenerative neurological diseases.

7 Free Radical Induced Damage – Lipids, Proteins And DNA
7.1 Lipids

The CNS is a rich source of poly-unsaturated fatty acids. Peroxidation of these fatty acids leads to the formation of aldehyde products that impair the function of key metabolic enzymes including Na\(^+\)K\(^+\)-ATPase, Glucose-6-phoshate dehydrogenase, GLT-1 glutamate transporter in astrocytes, and the glucose transporter. A consequence of this is the destabilization of Ca\(^{2+}\) homeostasis, increased levels of extracellular glutamate and a loss of energy metabolism. Inhibitors of lipid peroxidation can partially block the damaging effects of ROS generated by excitotoxicity in vitro. Therefore lipid peroxidation can potentiate neuronal dysfunction and death through a variety of mechanisms including enhanced cytotoxicity (Azbill R. D. et al., 1997).

The thiobarbituric acid reactive substances (TBARS) assay measures the presence of lipid peroxidation products, such as malondialdehyde, which fluoresces in the presence of thiobarbituric acid (Azbill R. D. et al., 1997). Malondialdehyde is one of the major endproducts of the peroxidation of polyunsaturated fatty acids and is thus considered to be a good index of peroxidative mechanisms. In my studies I have used this assay to study free radical induced lipid peroxidation.
7.2 Proteins

The oxidative modification of proteins can also disrupt their secondary and tertiary structures, leading to the exposure of hydrophobic regions normally shielded in the interior of the protein (Davies K. J. and Goldberg A. L., 1987). Increases in hydrophobicity promote the non-covalent aggregation of proteins and decrease their overall water solubility. Amino acid charges are changed shifting the isoelectric point of the protein, another factor contributing to protein aggregation (Ryter S. W. et al., 1990). The aggregation of proteins could be related as well to the ability of OH and aliphatic dialdehydes to form crosslinkages, and to reduce sulphhydryl groups with formation of sulphhydryl bridges. Gross conformational alterations of peroxidized proteins determines their susceptibility to proteolytic degradation. Unlike many other proteolytic substrates, the degradation of oxidized proteins are considered to be a high degree of hydrophobicity and denaturation (Davies K. J., 1990).

7.3 DNA

The mode of cell death following ischemia may be apoptotic or necrotic, although the latter seems to prevail after severe ischemia in the adult human brain. The rapid consumption of NAD+ that results from over activation of poly (ADP-ribose) polymerase (PARP) in response to oxidative damage to DNA probably makes an important contribution to necrosis (Love S., 1999).

The hydroxyl radical mediated reactions with bases and deoxyribose sugars generate a wide range of oxidative modifications, DNA strand breaks, cross links and abasic lesions. The radical induced oxidation of the deoxyribose may facilitate the release of the attached base from the DNA (Teoule R., 1987). The resulting apurinic/apirimidin lesions, if not promptly repaired, interfere with DNA replication, blocking the DNA polymerases (Loeb L. A., 1985). At the same time, DNA polymerase can indiscriminately incorporate deoxyadenosine at the site opposite the AP site in the nascent DNA chain. In this situation DNA peroxidation can be mutagenic. Hydrogen peroxide and other oxidants produce single strand breaks with unusual termini. The character of these termini usually prevents the repair of such strand breaks (Hutchison F., 1985).
8 Role Of Iron In Oxidative Stress

In recent years extensive studies have outlined the role of oxygen free radicals and other oxygen derived reactive species in the disruption of cellular function by the peroxidation of membranes and other cellular components. Transition metal ions play an important role in this non-enzymatic damage to membranes by acting as catalysts in initiating the formation of free radicals. Several metal ions are able to induce peroxidation through the generation of free radicals, although the most important endogenous metal is iron (Subbarao K. V. and Richardson J. S., 1990). Brain iron is a concentration of ~ 12 mM. Most of this is in heme enzymes or bound to ferritin. Iron appears to be delocalized from the proteins in the postischemic phase and possibly during ischemia probably as a result of lowered pH or superoxide induced generation of Fe$^{3+}$. Formation of hydroxyl radical is strongly favored by the decreased pH that prevails after ischemia and also by free iron as shown below in the Fenton reaction (Lipton P., 1999).

\[ \text{O}_2^- + 2\text{H}_2\text{O} \rightarrow +\text{H}_2\text{O}_2 \]
\[ \text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OH}^- + \text{OH}^- \]

Extensive peroxidation of membranes can be initiated by various concentrations of ferrous and ferric ions alone in the absence or oxidizing and reducing agents (Subbarao K. V. and Richardson J. S., 1990). It has been shown in recent studies that a major generator of oxidative stress for neurons in basal medium is iron. Iron can cause oxidative stress by promoting lipid peroxidation as well as DNA and protein damage by catalyzing the formation of hydroxyl radicals. Moreover a number of studies have shown that ferrous iron is toxic to cultured neurons even at relatively low concentrations (Farinelli S. E. et al., 1998). Low concentrations of chelators were protective in trophic factor withdrawal in PC12 and sympathetic neurons perhaps because they bound iron present in basal medium (Farinelli S. E. et al., 1998). The arguments against the importance of the Fenton reaction are that the reactivity of the OH$^-$ may paradoxically render it less harmful than other free radicals. Furthermore the superoxide driven Fenton reaction is slow even in the presence of iron (Lipton P., 1999).
9 Neuroprotective Strategies

Scientific research must be targeted towards identifying at the cellular level, mechanisms of damage and death in neurons following injury or disease. This will make it easier to identify modes of treatment and prevention strategies to overcome the effects of neuronal injury and neurodegenerative disease. Numerous neuroprotective strategies have been identified, and the following is a brief introduction on some of the strategies employed in my model of ischemia. I have studied their effects on acute injury as well as delayed cell death – 24 hrs after the insult.

9.1 NGF

It is becoming increasingly clear that polypeptide growth factors play important roles in the development and maintenance of neural circuits (Greene L. A. and Shooter E. M., 1980, Snider W. D. and Johnson E. M., 1989). Nerve Growth Factor (NGF), the most thoroughly studied neuronal growth factor, promotes the survival and outgrowth of peripheral sensory and sympathetic neurons (Levi-Montalcini R. and Calissano P., 1986). NGF may have a more limited set of neural targets and is believed to be active primarily in cholinergic neurons (Knusel B. et al., 1990). Beyond their roles in normal brain development, GF's may play important roles in the brain's response to injury and neurodegenerative disorders (Hefti F. et al., 1989). NGF and bFGF can prevent the damage of cholinergic neurons associated with brain lesions in animal models of neurodegenerative disorders (Snider W. D. and Johnson E. M., 1989, Hefti F. et al., 1989).

9.2 Effect Of A Decrease In Temperature On Ischemia (Hypothermia)

Hypothermia is so potently neuroprotective that it has been successfully implemented clinically during specific neurosurgical procedures. Hypothermia greatly reduces the extent of brain damage sustained in models of both focal and global cerebral ischemia. In contrast hyperthermia is known to exacerbate ischemic injury (Busto R. et al., 1987). Effects of temperature are manifested for long periods after an insult. Hypothermia is protective when it occurs up to 12 hrs after 2-VO (vessel occlusion), and if temperature is elevated to 39°C even 24 hrs after mild (5-7 mins) 2 V-O, global ischemia damage is strongly enhanced. This indicates that even delayed processes involved in cell death are temperature sensitive (Baena R. C. et al., 1997). Temperature is also important in focal ischemic
Mild hypothermia reduced infarct size by 35% during 12 hr permanent MCA occlusion (Baker C. J. et al., 1992).

Hypothermia has been shown to reduce ischemia induced glutamate release as well as to preserve high energy phosphates during ischemia and thus preserve energy dependent calcium extrusion mechanisms (Newell D. W. et al., 1995). The mechanism underlying the neuroprotective effect of hypothermia was also thought to be related to its energy suppressing action. Recent research on high energy phosphate dynamics at the time of cerebral ischemia has not supported this and the most important mechanism underlying the neuroprotective effect of hypothermia is considered to be excitatory amino acid inhibition based on the glutamate excitotoxicity hypothesis (Murata T. et al., 2000).

9.3 Role Of Anti-Oxidants In Ischemia

SOD and Catalase are two widely used and studied anti-oxidants that can be endogenous or exogenous in origin. SOD dismutates superoxide to hydrogen peroxide and Catalase breaks down hydrogen peroxide to water and oxygen.

Considerable controversy exists in literature regarding the site of action of these two anti-oxidants SOD and Catalase. Previous studies suggested that they blocked the hypoxic enhancement of ROS production (Sylvester J. T., 2001). These large proteins did not enter cells, then ROS released during hypoxia must have exited cells to act at the external reticular surface or traversed the extracellular space before reentry to act at intracellular loci. Recent studies suggest an intracellular site of ROS production during ischemia (Waypa G. B. et al., 2001).

10 Summary Of Introduction

In the introduction I have attempted to give a brief overview of the theoretical aspects of every part of my work. This is not meant to be an exhaustive explanation of all the events occurring during ischemia, but I have tried to cover all aspects which have a bearing on my work and the rationale behind it. I have given an overview of stroke, ischemia and oxygen-glucose deprivation along with some information on in vitro models in use. The cellular and molecular mechanisms are explained at
the level of individual organelles like the mitochondria. I have described in detail all relevant information on PC12 cells including the general features and their use in OGD/OD/GD. Also included briefly is a discussion on the role of glutamate and calcium in ischemia. Free radicals have been discussed in detail, along with a brief discussion on the damage produced to lipids, proteins and DNA. At the end neuroprotective strategies used in my experiments have been discussed including NGF, temperature and anti-oxidants.

11 Hypothesis

Nonlethal short exposures to ischemia/hypoxia are extremely neuroprotective in rats and gerbils. My original hypothesis had this strategy in mind, and I hypothesized that neuronal PC12 cells could be preconditioned and thereby protected against a subsequent episode of lethal ischemia. During the course of these experiments, in the process of identifying a major lethal insult, I tried to use a more physiological medium to expose cells to OGD, accordingly I used culture medium (DMEM). Surprisingly I found a radical difference in cell death when the insult was given in culture medium as compared to BSS. This dramatic difference encouraged me to formulate the following 2 major hypotheses.

1. Survival of neuronal PC12 cells in response to oxygen-glucose deprivation (OGD), glucose deprivation (GD) and oxygen deprivation (OD) will be enhanced in culture medium (DMEM with alternative substrates) compared to 'physiological' - Balanced Salt Solution (BSS).

2. Strategies that target and decrease oxidative stress will improve cell survival in this model of in vitro ischemia.

12 Specific Aims

1. To establish an experimental paradigm to study the response of neuronal PC12 cells relative to duration of OGD/OD and GD.

2. To develop a reliable, reproducible, quick and convenient assay to measure cell death both immediately and 24 hrs after an insult.
3. To identify the mechanism of increased cytotoxicity in neuronal PC12 cells exposed to OGD in culture medium.

4. To develop the appropriate method to measure and visualize ROS during OGD and reperfusion at the level of a single cell using 5'6'CM DCF-DA and confocal microscopy.

5. To develop a second assay using flow cytometry or fluorescence plate reader to measure ROS generation in PC12 cells on a population basis using the same indicator 5'6'CM DCF-DA.

6. To develop a protocol to study lipid peroxidation using the TBARS assay relevant to this model.

7. To develop an experimental paradigm to study preconditioning in neuronal PC12 cells.

8. To study the effects of neuroprotective strategies—NGF, hypothermia and anti-oxidants SOD & Catalase relevant to this model of OGD.
METHODS

1 PC12 Cell Culture

1.1 Growth, Differentiation And Maintenance Of PC12 Cells

PC12 Tet-Off/GFP<sub>gro</sub> cells were grown in 75cm<sup>2</sup> flasks kept in a humidified incubator circulated with 5% CO<sub>2</sub> and 95% air at 37°C (Figure -1). The growth medium contained 85% RPMI (GibcoBRL 11875-093), 10% Horse serum (GibcoBRL16050-122) and 5% Fetal Bovine serum (GibcoBRL16000-044) (Usowicz M. M. et al., 1990) supplemented with 1 ml /100ml of media of Antibiotic-antimycotic mixture (Penicillin, Streptomycin, Amphotericin B) (Usowicz M. M. et al., 1990). Cells were fed on alternate days with complete replacement of medium.

Cells were differentiated for 7 days in NGF supplemented growth media in 75cm<sup>2</sup> flasks. Initially experiments were done with 100ng/ml 2.5S NGF (Harlan Bioproducts-005017), later experiments were done with 25ng/ml 2.5S NGF (Harlan Bioproducts-005017), as we found the new lot of NGF to be more potent (confirmed by the supplier). Feeding was done with complete replacement of the media on alternate days.

1.2 Doxycycline Treatment

For fluorescence microscopy experiments with 5’6’ CMH2DCF, cells were differentiated in NGF containing growth medium supplemented with 10µl/100ml of 100mg/ml Doxycycline stock solution. Fluorescence microscopy was used to confirm the loss of GFP signal in these tet-off PC12 GFP<sub>gro</sub> cells.
Figure -1

A. Undifferentiated PC12 cells

\[ \text{25 ng/ml NGF for 7 days} \]

B. Differentiated (Neuronal) PC12 cells
1.3 Plating Of Cells

PC12 cells were differentiated for 7 days as described above. Plastic (35mm Corning) dishes for regular experiments or glass bottomed (30mm) dishes for microscopy experiments were coated with 25% collagen (see Appendix 4) and allowed to dry overnight. Cells were plated overnight on collagen coated plastic or 48 hours on glass bottomed dishes at a density of 5x10^4 cells (determined optimal from previous experiments) per dish in 2 ml of NGF and serum containing growth medium.

1.4 Experimental Solutions

Matched cultures in 35 mm plastic dishes with the same subculturing history were exposed to the conditions described for the indicated periods of time. All experimental groups were exposed to OGD/OD/GD in two different solutions- DMEM (Dulbecco's Modified Eagle Medium Sigma 5030 excluding Glucose, Glutamine, Sodium Pyruvate and Phenol Red) (see Appendix 1) and BSS (Balanced Salt Solution) (see Appendix 2). Both solutions were made a day prior to the experiments, pH was maintained at 7.3 and osmolarity at 300 mosm/ml. Prior to the start of the experiment, cells were washed twice by flooding the dish with PBS and replenished with 2ml/dish of experimental solution. Experimental solutions were pre-equilibrated for 15 min with 95%N₂/5%CO₂ by bubbling (151). Controls were also washed with PBS and kept in DMEM containing Glucose (5.6 mM) and Glutamine (2 mM) in the incubator. All experimental solutions contained NGF but no serum (Figure -2).
Figure - 2

Methods

Growing medium

DMEM/BSS +/- Glucose
+ NGF
0 Serum

Anoxic chamber (in incubator)

DMEM
+ Glucose
+ NGF
0 Serum

Immediate analysis (in experimental solution)
LDH/PI
Confocal microscopy
Flow-cytometry
TBARS assay

Normoxia (in incubator)

DMEM
+ Glucose
+ NGF
0 Serum

Delayed analysis (medium replaced) analysed at 24 hrs
LDH/PI
1.5 Method Of Inducing OGD/OD/GD

For inducing OGD/OD we used an anoxic chamber (courtesy Dr. James Eubanks), through which a mixture of 95%N₂ and 5%CO₂ flowed continuously at 2.5 lbs/inch². The gas flowed out of the chamber bubbling into a reservoir of water. The chamber was kept throughout the duration of the experiment in the incubator thereby maintaining the temperature at 37°C. Initial experiments were done by bubbling dishes kept in a reservoir of experimental solution but a majority of the cells were lost and hence a continuous flow-thro chamber was used subsequently (Figure 2).

For OGD, cells in DMEM and BSS were kept in the anoxic chamber for the duration of the experiment. For OD cells were kept in the anoxic chamber in DMEM/BSS supplemented with Glucose. For GD cell were in the incubator in DMEM/BSS with no Glucose or Glutamine supplementation.

1.6 Reperfusion

At the end of the duration of OGD/OD/GD all dishes (experimental and controls) were removed from the incubator, sample media aliquots taken, and a gentle complete replacement of medium was done with DMEM containing Glucose (5.6mM) and Glutamine (2mM). All reperfusion medium contained NGF but no serum. Dishes were replaced in the incubator and analyzed at 24hrs of the completion of the insult.

2 Analysis Of Cell Death: LDH Assay/PI Staining

Lactate dehydrogenase (LDH) assay is a colorimetric test for cell damage/death analysis. This assay measures the pyruvate mediated conversion of 2,4-dinitrophenylhydrazine into a visible hydrazone precipitate. Initial experiments indicated a good correlation between LDH value and PI counts, hence LDH values have been considered to represent cell death in my model of OGD/OD/GD.
2.1 Immediate LDH Measurements

At the end of the insult, an aliquot of medium (extracellular LDH) from each dish was taken centrifuged and 100μl loaded into a 96 well flat bottomed plate (Nunc). Total intracellular LDH was determined by lysing cells with 1% TritonX detergent, samples collected, centrifuged and loaded into the 96 well plate. LDH reagent (Roche Diagnostics) was then added, 15 mins later the optical density was read with dual wavelength 490/630 using a multiwavelength ELISA plate reader in both medium and lysis buffer. LDH efflux was expressed as a percentage of the maximal LDH release (where maximal LDH is the sum of extracellular plus intracellular values), i.e. percentage extracellular LDH/maximal LDH. The basal activity present in culture medium alone was routinely subtracted from that in the supernatant.

2.2 Delayed LDH Measurements

24 hours after the completion of the insult, the same process as described above was repeated and the LDH efflux determined for experimental and control groups.

2.3 PI Staining

Propidium Iodide (PI, Molecular Probes) stock solution was made as 1mg/ml in water, and stored at 4°C in the dark. 4μl/2ml was used (Figure-3). The % of dead cells per field was quantified by counting the number of PI positive (red) nuclei; live cultures were viewed on the confocal microscope with the Texas red filter block. As the floating cells could not be counted, and live counts were needed for immediate cell death analysis causing time constraints, this method of analyzing cell death was subsequently abandoned (Figure -3).
Figure 3: Neuronal PC12 Cells With PI + Nuclei And GFP+ Mitochondria

This Figure 3 shows simultaneous dual channel imaging using the green signal for GFP labeled mitochondria and the red signal of PI stained nuclei in differentiated PC12 cells.

Figure 3A and 3B represent fluorescent and phase images of the same cells using confocal microscopy (20X objective). In Figure 3A small arrow points to the GFP labeled mitochondria and the large arrow to the PI positive nuclei.

Figure 3C shows PI labeled nuclei seen using confocal microscopy (20X objective) with texas red filter block.
Figure - 3

A. PI positive nuclei

B. Parallel phase GFP+ mitochondria image (20X objective)

C. PI positive nuclei
3 Confocal Microscopy, Fluorescence Analysis And Image Processing

3.1 Confocal Microscopy

The confocal imaging system used for studying GFP labeled mitochondrial morphology, PI staining and 5’6’CM DCF-DA experiments consisted of an MRC-600 confocal laser scanning microscope (Bio-Rad Microsciences Ltd. UK). Phase contrast microscopy was used to acquire non-confocal transmission images during the experiments. The 20X (Nikon Ph2 DL 20/0.4 160/1.2) phase objective and the Fluor 20X objective (Nikon 0.75 160/0.17) was used for fluorescence microscopy. GFP labeled mitochondrial morphology was studied using the 60X plan apo oil immersion objective.

3.2 Mitochondrial Morphology Study

To study the mitochondrial morphology, GFP labeled mitochondria were studied throughout the duration of OGD and the first 2 hrs of reperfusion. Cells were plated in glass bottomed dishes. Pre-insult pictures were taken in KRH solution (see Appendix 3) and are referred to as t=0 pictures. Then cells were washed and the medium replaced with the experimental solution containing PI. In every experiment at least 2 dishes were prepared for each treatment group. The experimental dishes were then sealed first with parafilm and then with sticky tape. The dishes were then kept in the anoxic chamber within the incubator. At 1 hour intervals, dishes were removed from the chamber and visualized using the 60X oil immersion objective. Images were acquired using the DOS based SOM and were stored on the network.

3.3 Study Of ROS Generation Using 5’6’CMH2DCF-DA

ROS generation in neuronal PC12 cells was assessed using the probe 5’6’chloromethyl dichlorodihydrofluorescein diacetate (5’6’CMH2DCF-DA, C-6827, Molecular probes). Cells used were pre treated with doxycycline as mentioned above. Stock solution of 5’6’CMH2DCF-DA was made in DMSO, 25μl of DMSO was added to 50μg of solute giving a stock solution of 4 mM, final concentration used for the experiments was 10μM, this concentration was developed from prior trials and errors experiences. Cells were loaded in KRH buffer (see Appendix 3) for 45 mins, pre-insult
pictures representing t=0 were then taken using the SOM software program. KRH was then replaced with the experimental solution as mentioned above and OGD started. When doing simultaneous PI imaging, PI was added to the experimental solution.

PC12 cells are known to have an efficient multi-drug transporter in the cell membrane and control cells were found to be losing the dye during the course of my experiments. During reperfusion the signal appeared to first increase and then decline in the experimental cells. I used Probenecid (1 mM) to minimize dye leakage as suggested by Molecular probes. Probenecid was not effective and consequently the dye was left in continuously throughout the experiment in the same loading concentration in the extracellular medium. This did not prevent dye leakage as is discussed in Results, but decreased leakage to some extent. The diacetate form enters cells where esterases cleave the acetate group, tending to trap the nonfluorescent DCFH intracellularly. This indicator dye is oxidized by cellular hydrogen peroxide, hydroxyl radical and various free radical products lying downstream from hydrogen peroxide. It is relatively insensitive to superoxide (Royall J. A. and Ischiripouls H., 1993). In the presence of H$_2$O$_2$, this probe is oxidized to DCF, which was quantified using fluorescence imaging (ex: 488 nm, em: 535 nm) and fluor20X objective. Care was taken to image cells using only a single scan of the laser to minimize photo-oxidation, as it was found in the course of experiments that even a second scan enhanced photo-oxidation considerably.

Image acquisition was done at a resolution of 384x256 pixels, with 488 nm excitation and 580 nm emission, neutral density 3 and constant black levels and gain settings within an experiment. All images were taken and saved on the network. When PI images were taken, dual channel images were taken, with the 2nd channel using texas red filter block at em: 690 nm.

3.4 Image Analysis

In DCF-DA loaded cells, changes in intracellular fluorescence both pre and post treatment were measured at 1 hr interval during OGD and at 30 mins interval during reperfusion. Images were analysed using image analysis software (DOS based SOM). Average fluorescence intensity of every cell in the field was then determined in a constant area, 20 cells representative of the field were counted at each time point for each of the experimental groups and controls. All fluorescence measurements were expressed as a percentage of control at each time point.
3.5 Image Processing

All images were processed using confocal assistant and adobe photoshop. To facilitate contrast and enhance viewing, background was darkened in fluorescence images and lightened in phase images. To clearly visualize the PI signal which is seen through the GFP channel, red coloring of PI+ nuclei, determined by merged images was done.

4 Flow Cytometric Evaluation Of ROS Generation

Flow cytometry was done to study ROS generation in a population of neuronal PC12 cells immediately after OGD. To be able to compare results, the same indicator 5′6′-CMH2DCF-DA was used in the same final concentration of 10μM. Cells were loaded as described above, and the dye was left in the experimental solution throughout the insult. Prior to completing the insult, the FACScan (Becton Dickinson BD) machine was started and set accordingly to standard settings of the dye to be studied prior to use in every experiment. Cells were rapidly then taken to the FACScan machine in the anoxic chamber with its two ends sealed. The flow cytometer allows two cell parameters to be analyzed: forward-angle light scatter (FSC) and side-angle light scatter (SSC) to be determined by an argon ion laser beam. Flow cytometric evaluation was then done using the ex:488 and em:580. In between samples water was run to wash out any cells sticking to the tubing which can likely cause contamination of results. All neuronal experiments were done in a neuronal population that was selected from PI negative cells with a specific 'gate'. This gate was defined by sorting analysis according to the relation FSC/SSC. Fluorescence distribution was then determined in 10,000 cells in each experimental group and a histogram generated. The change in the shape of the curve and the direction of movement of the curve reflect the change in fluorescence intensity. After the first cycle, a second cycle was run in every experiment, there was no immediate change in fluorescence values. All flow cytometry experiments were done only after the completion of an episode of OGD.

5 Thiobarbituric Acid Reactive Substances Assay (TBARS)

The determination of malondialdehyde (MDA) has attracted wide-spread interest as a means to assess lipid peroxidation in biological materials. The relative levels of malondialdehyde , an indicator of lipid peroxidation were measured using the thiobarbituric acid reactive substances (TBARS) assay.
Cells were exposed to OGD for 5 hrs, cells were then harvested and centrifuged at 300g for 5 mins. Equal volumes 200µl of digitonin release buffer and lysis release buffer both containing protease inhibitors were then added to the pellet triturated and left at room temperature for 1 minute. Protein concentrations for each group was then determined using the biorad protein assay. Equal volume of 20% Tricholoroacetic acid was then added to the homogenates. The mixture immediately takes on a milky appearance and was centrifuged at 12,000g x 3 mins. The supernatant obtained was then added to equal volume of the TBARS reagent (0.335% 2-thiobarbituric acid in 50% glacial acetic acid, all reagents were obtained from Sigma Aldrich Chemicals) and the sample incubated at 100°C for 30 mins. This assay is based on the reaction of malondialdehyde with TBA to form a pink complex with an absorption maximum at 532-535 nm. Heating the sample at a pH of 3 or below is necessary for complex formation and for release of MDA from bound forms. The samples were then cooled to room temperature, showing an orange colour. These samples were then centrifuged for 12,000g x 3 mins, and the absorbance read at 530 nm. TBARS reagent served as blanks. The values of TBARS fluorescence were compared between experimental and controls and expressed on a per mg protein basis.

6 Statistical Assays

All the graphs and statistics used in my work have been done using the SigmaPlot and SigmaStat software programs. The data obtained from each experiment was pooled and analysed as a collection of 3-6 individual values obtained for each experimental and control condition. Average experimental values and standard errors of mean were then calculated. All experimental data were compared with cells obtained from the same culture and exposed to the insult in parallel. At each time point both immediately after and 24 hrs later, average values, numbers of samples and standard errors of mean from each experiment were compared using the One Way ANOVA test. Multiple comparisions within each group were then calculated using the Tukey's test, for which p value is set at 0.05 reflecting 95 % confidence limits.
RESULTS

ESTABLISHING THE MODEL TO STUDY OGD/GD/OD USING NEURONAL PC12 CELLS

1 General Morphological Features Of Differentiated PC12 Cells

Exposure of rat Pheochromocytoma PC12 cells to nerve growth factor (NGF) results in the differentiation from immature adrenosympathetic precursor-like cells to cells that resemble mature sympathetic neurons (Figure 1). Differentiated PC12 cells stop dividing, grow extensive neurites and become electrically excitable. I am using a stable PC12 cell line which expresses an inducible mitochondrially targeted GFP fusion protein – PC12 Tet-Off/GFP$_{\text{mito}}$ (Wadia thesis in progress) (Figure 4). Cells were differentiated in 25 ng/ml NGF 2.5S for 7 days, however initial experiments were done in 100 ng/ml NGF 2.5S. The original lot of NGF was less potent than the present one (confirmed by Harlan Bioproducts), accordingly from my cytotoxicity experiments a new dose of NGF was decided at 25 ng/ml (which is $\frac{1}{4}$ the old concentration) (see Methods, section 1). I have indicated where appropriate the ‘OLD’ LOT NGF, when the older lot of NGF was used.
Figure - 4: PC12 GFP$_{(Mio)}$ Cell Line

This Figure 4 shows the PC12 GFP$_{(Mio)}$ cell line that has been used for all of the experiments. Figure 4A - shows the GFP labeled mitochondria using confocal microscopy with 20X objective, the mitochondria can be seen perinuclearly and along the neuritic processes. The arrow points to the mitochondria in the cytoplasm.

Figure 4B - shows a merged phase and fluorescent image of a differentiated PC12 cell with the 60X oil immersion objective imaged using confocal microscopy. The mitochondria are well visualized with a stringy appearance distributed around the nucleus, distributed throughout the cytoplasm. The yellow arrow shows the outer edge of the cell membrane as in phase microscopy, whereas the red arrow shows the overlying mitochondria from the fluorescent image.
Figure - 4

A. GFP labeled mitochondria in neuronal PC12 cells (20X objective)

B. Merged phase and GFP labeled mitochondrial image (60X objective)
2 Culture Medium Increases OGD Induced Cytotoxicity

In an attempt to simulate ischemia in vivo, I have combined oxygen and glucose deprivation in culture medium (DMEM-O-G) and compared with the more typically used balanced salt solution (BSS-O-G). Controls were cells maintained in DMEM with Glucose and L-Glutamine (DMEM+O+G), in the incubator for the same duration of time. All experiments were done in the absence of serum, but in the presence of NGF throughout, to avoid the effects of trophic withdrawal. As mentioned above, initial experiments were done with an older lot of NGF, cells were differentiated in 100ng/ml NGF for 7 days. Data represent mean percentages of cell death from 3 or more different experiments. Number of samples for each group in each experiment, at both time points varied from 3-6. Actual numbers of cells were maintained for all experiments at 5x10⁴, which had been determined as optimal for the LDH assay from preliminary experiments. When the mean percentages of cell death at each time point were subjected to statistical comparison using the one way ANOVA test, a significant difference within groups at all time points both immediate and delayed was found. Multiple pairwise comparisons using Tukey's test were then performed. These experiments were done in 'OLD' LOT NGF. (Figure – 5)

LDH analysis immediately after a 1 hr insult (Figure 5A) was significantly (p=0.044, ANOVA) different within the group, and between experimentals and controls (p>0.05, Tukey's test). Changes in mean cell death were also significant at 2, 3, and 5hrs of OGD (p<0.001, ANOVA), cells in the DMEM group showed significantly (p<0.05, Tukey's test) greater, immediate mean cell death than cells in BSS or controls. 5 hrs of OGD caused complete cell death in DMEM group as compared to only 13.49±0.20% mean cell death in the BSS group. (*) represents significant difference (p<0.05, Tukey's test) from controls, and (**) represents significant difference (p<0.05, Tukey's test) from the corresponding other experimental group within the same duration of time (Figure 5A)
Figure 5: Effect of OGD on Neuronal Cell Death

The graphs Figure 5A and 5B illustrate the percentage of maximal LDH release in neuronal PC12 cells exposed to OGD immediately after the insult (A) and 24 hrs after the insult (B). X axis represents the duration in hours of the OGD and Y axis represents the % of maximal LDH release. All cells used were differentiated for 7 days in NGF2.5S (100ng/ml, 'OLD LOT NGF'). At all time points shown 3 groups of cells were analysed as control, BSS and DMEM as mentioned in METHODS. All the cells in each experiment were derived from the same subculture. Data are represented as mean values from at least 3 experiments (3 dishes in each group) with error bars indicating SEM. The p values were derived using One Way ANOVA test on each group at the respective time points mentioned, both for immediate and delayed cell death. Asterisks (*) denote a statistically significant difference (p<0.05, Tukey's test) between experimental and control groups, whereas double asterisks (**) denote significant difference between experimental groups.
Figure - 5
A. Effect of OGD on Immediate Neuronal Cell Death ('OLD LOT' NGF)

B. Effect of OGD on Delayed Neuronal Cell Death ('OLD' LOT NGF)
Figure - 6: Comparison Of The Effects Of OGD In DMEM And BSS

This figure 6 gives a glimpse of a comparison of the changes in cells in BSS (Figure 6B) and in cells in DMEM (Figure 6C) as compared to controls which represent the normal cellular and mitochondrial morphology (Figure 6A) using dual channel imaging with confocal microscopy (60X objective).

Cells in BSS (Figure 6B) are not different from controls, large arrow shows the healthy mitochondria, small arrow shows the PI labeled nuclei.

But cells in DMEM (Figure 6C) show swelling and blebbing of the plasma membrane, mitochondria are condensed and seem to have lost their stringy appearance. Mitochondria along the neuritic processes are not seen, suggesting the loss of neurites with cell swelling. Some PI positive nuclei are also seen. Large arrow shows the swollen and unhealthy looking mitochondria, small arrow shows the blebbing of the plasma membrane. Note that the cell is still alive with no PI staining of the nuclei.
Figure - 6

A. Healthy mitochondria in a control PC12 cell

B. Immediately after OGD in BSS

C. Immediately after OGD in DMEM
Cell death analysis 24 hrs after the insult reflects delayed cell death, this revealed significant differences (p<0.001, ANOVA) in cell death at each time point (Figure 5B). Cells in the DMEM group showed increasing cell death with duration of OGD, and at every time point showed significantly greater (p<0.05, Tukey’s test) delayed mean cell death than cells in BSS or controls.

Cells exposed to OGD in DMEM (Figure 6C) showed rapid deterioration in morphology (see section 7, results), corresponding with increased cell death, as compared to cells in BSS (Figure 6B) which were not different from Controls (Figure 6A).

3 Oxygen Deprivation Does Not Increase Cell Death

The next question was how much is the contribution of oxygen deprivation alone to neuronal cell death? PC12 cells are an oxygen sensitive cell line and represent a well defined system extensively used to study the effects of acute and chronic hypoxia on various cellular processes. When the mean percentages of cell death were compared using the one way ANOVA test, significant difference (p=0.002) was seen immediately after a 1 hr insult, multiple pairwise comparison within the group revealed a significant (p<0.05, Tukey’s test, **) increase in immediate mean cell death in DMEM as compared to BSS (Figure 7A). These experiments were done in 'OLD' LOT NGF.

When delayed mean cell death (Figure 7B), was statistically compared at each time point using the one way ANOVA test, there was no significant difference at 1hr (p=0.170), 2hrs (0.066) or even at 3hrs (p=0.072). These findings confirm previous studies showing that neuronal PC12 cells can survive in the absence of oxygen when glucose is present, presumably because they are known to rely heavily on glycolysis, and can thus generate ATP in the absence of oxygen.
Figure - 7: Oxygen Deprivation Does Not Increase Cell Death

The graphs Figure 7A and Figure 7B illustrate the percentage of maximal LDH release in neuronal PC12 cells exposed to increasing durations of Oxygen deprivation alone in the presence of glucose immediately after OD (A) and 24 hrs later (B). All cells used were differentiated in NGF2.5S (100ng/ml, 'OLD' LOT NGF) and derived from the same subculture for each experiment. At each time point cells were exposed to OD in DMEM and BSS containing glucose and compared with controls. Data are means from at least 3 different experiments with error bars indicating SEM, X axis represents the duration of OD in hours, Y axis represents the percentage of maximal LDH release. Statistical significance was derived at each time point using one way ANOVA test. Multiple pairwise comparisons were done using Tukey's test. Asterisks (*) indicates significance at p<0.05 on the Tukey's test between experimental groups and controls, (**) indicates significance (p<0.05) between the experimental groups DMEM and BSS.
Figure - 7

A. Effect of OD on Immediate Neuronal Cell Death ('OLD' LOT - NGF)

B. Effect of OD on Delayed Neuronal Cell Death ('OLD' LOT - NGF)
4 Glucose Deprivation Increases Cytotoxicity

Cell death in DMEM dramatically increased with glucose deprivation in contrast to oxygen deprivation over the same duration of exposure. DMEM used for the experiments lacks glucose, glutamine and pyruvate (alternative energy substrates). Cells in this medium were compared with cells exposed to glucose deprivation in BSS. Glucose deprivation is known to deplete ATP levels in PC12 cells, transient oxygen deprivation alone does not. The mean percentages of cell death were compared within the immediate group using the one way ANOVA test, there was no significant difference (p=0.947) at 1 hr, but a significant (p<0.001) difference was found at 2 and 3 hrs of glucose deprivation. Multiple pairwise comparison using the Tukey’s test showed a significant (p<0.05, Tukey’s test) increase in mean cell death at 3 hrs of GD in the DMEM group (51.07±1.49%) as compared to cells in BSS (9.95±0.63%) and controls (9.73±0.41%) (Figure 8A). These experiments were done in ‘OLD’ LOT NGF.

Delayed mean cell death is also significantly different (p<0.001, ANOVA) at each time point, cells in the DMEM group consistently show significantly greater (p<0.05, Tukey’s test) mean cell death when compared to BSS (***) or controls (*) (Figure 8B). DMEM increases the sensitivity of differentiated PC12 cells to glucose deprivation increasing cell death. After 3 hrs of glucose deprivation, delayed mean cell death is 93.03±3.19% in the DMEM group, significantly greater (p<0.05, Tukey's test) as compared to 15.06±0.70% in the BSS group as well as controls at 15.09±0.97%.
**Figure 8: Glucose Deprivation Significantly Increases Cell Death**

The graphs Figure 8A and Figure 8B illustrate the percentage of maximal LDH release in neuronal PC12 cells exposed to increasing durations of glucose deprivation alone in the presence of oxygen immediately after GD (A) and 24 hrs later (B). All cells used were differentiated in NGF2.5S (100ng/ml, 'OLD' LOT NGF) and derived from the same subculture for each experiment. At each time point cells were exposed to GD in DMEM and BSS lacking glucose and glutamine. Controls were in DMEM containing glucose and glutamine. Data are means from at least 3 different experiments with error bars indicating SEM, X axis represents the duration of GD in hours, Y axis represents the percentage of maximal LDH release. Statistical significance was derived at each time point using one way ANOVA test. Multiple pairwise comparisons were done using Tukey's test. Asterisks (*) indicates significance at p<0.05 on the Tukey's test between experimental groups and controls, (**) indicates the significance (p<0.05) between the experimental groups DMEM and BSS.
Figure 8

A. Effect of GD on Immediate Neuronal Cell Death (old LOT - NGF)

B. Effect of GD on Delayed Neuronal Cell Death (old LOT - NGF)
5 Undifferentiated PC12 Cells Respond Similarly To OGD Induced Cell Death

PC12 cells are an oxygen sensitive cell line, and both undifferentiated as well as differentiated PC12 cells are extensively used in ischemia/hypoxia and hypoglycemia related studies. To find out how undifferentiated PC12 cells respond to OGD, LDH assay was used to analyze cell death immediately after (Figure 9A) and 24 hrs following (Figure 9B) 2 and 4 hrs of OGD. At each time point, mean percentages of cell death were compared within the immediate group and within the delayed group using the one way ANOVA test and when the p value was significant, multiple comparisons were made using the Tukey’s test. Immediately after 2 hrs of OGD, there was no significant difference (p=0.584, ANOVA) but after 4 hrs of OGD, mean cell death was significant (P<0.001, ANOVA), cells in the DMEM group showed significantly greater (p<0.05, Tukey’s test) mean cell death as compared to cells in BSS (**) and controls (*)(Figure 9A).

Delayed mean cell death was significantly greater (p<0.001, ANOVA) at both 2 hrs and 4 hrs (Figure 9B). Throughout the durations of OGD studied, delayed mean cell death was significantly higher (p<0.05, Tukey’s test) in the DMEM group as compared to BSS (**) and controls (*). Surprisingly controls showed considerable mean delayed cell death (nearly 34%) probably reflecting the serum dependence of undifferentiated PC12 cells exposed to 24 hrs of serum deprivation.
Figure 9: Undifferentiated Cells Respond Similarly To OGD

The graphs Figure 9A and Figure 9B illustrate the percentage of maximal LDH release in undifferentiated PC12 cells exposed to increasing durations of oxygen and glucose deprivation (OGD) immediately after OGD (A) and 24 hrs later (B). All cells used were derived from the same subculture. At each time point cells were exposed to OGD in DMEM and BSS lacking glucose and glutamine. Controls were in DMEM containing glucose and glutamine. In case of delayed cell death, cells were in 0 serum for 24 hrs prior to analysis. Data are means from at least 2 different experiments with error bars indicating SEM, X axis represents the duration of OGD in hours, Y axis represents the percentage of maximal LDH release. Statistical significance was derived at each time point using one way ANOVA test. Multiple pairwise comparisons were done using Tukey’s test. Asterisks (*) indicates significance at p<0.05 on the Tukey’s test between experimental groups and controls, (**) indicates the significance (p<0.05) between the experimental groups DMEM and BSS.
Figure - 9

A. Effect of OGD on Immediate Cell Death in Undifferentiated PC12 Cells

![Diagram showing effect of OGD on immediate cell death in PC12 cells.]

B. Effect of OGD on Delayed Cell Death in Undifferentiated PC12 Cells

![Diagram showing effect of OGD on delayed cell death in PC12 cells.]

Note: The diagrams illustrate the percentage of maximal LDH release over different durations of oxygen-glucose deprivation (OGD) for control, BSS, and DMEM conditions.
6 Effect Of Oxygen-Glucose Deprivation On Neuronal Cell Death (NGF 25ng/ml)

Later experiments used a new lot of NGF; according to the supplier Harlan Bioproducts, it was 2.5 times more potent than older lots of NGF. My OGD experiments suggested it was up to 4 times more stronger than normal as assayed using cytotoxicity. Accordingly a new OGD insult paradigm had to be developed for further experiments, using NGF at 25ng/ml concentration (which is ¼ the concentration used in earlier experiments – 100ng/ml). 2 hrs (p=0.640) and 3 hrs (p=0.319) of OGD did not cause any significant increase in immediate mean cell death, but 4, 5 and 6 hrs of OGD showed statistically significant (p<0.001, ANOVA) difference in immediate cell death. At the later time points of 4, 5 and 6 hrs, cells in the DMEM group showed significantly (p<0.05, Tukey’s test) greater immediate mean cell death as compared to BSS (**) and Controls (*). 5 hrs of OGD in 25 ng/ml NGF caused 29.09±2.12% immediate mean cell death in the DMEM group which was significantly greater (p<0.05, Tukey’s test) than 12.28±1.52% in the BSS group (Figure 10A) (Table 1).

When mean percentages of delayed cell death were compared, statistically significant (p<0.001, ANOVA) differences were found throughout. Multiple pairwise comparisons of delayed mean cell death revealed that cells in DMEM consistently showed significantly (p<0.05, Tukey’s test) increased cell death as compared to BSS (**) and Controls (*). 5 hrs of OGD increased delayed mean cell death in the DMEM group to 47.76±2.94% as compared to BSS at 17.37±0.57%(Figure 10B). Delayed mean cell death in BSS significantly increased (p<0.05, Tukey’s test) only at 6 hrs of OGD to 48.30±3.19% as compared to controls at 14.83±1.91% (Table 1).

Henceforth this insult duration of 5 hrs in 25 ng/ml NGF (new lot) has been taken as the model paradigm of OGD induced cell death for all further experiments.
Figure 10: Response Of OGD On Neuronal Cell Death In 25 ng/ml NGF

The graphs Figure 10A and Figure 10B illustrate the percentage of maximal LDH release in differentiated PC12 cells exposed to increasing durations of oxygen and glucose deprivation (OGD) immediately after OGD (A) and 24 hrs later (B). All cells used were differentiated in NGF2.5S (25 ng/ml) and derived from the same subculture. At each time point cells were exposed to OGD in DMEM and BSS lacking glucose and glutamine. Controls were in DMEM containing glucose and glutamine. All experiments were done in the presence of NGF but in 0 serum. Data are means from at least 3 different experiments with error bars indicating SEM, X axis represents the duration of OGD in hours, Y axis represents the percentage of maximal LDH release. Statistical significance was derived at each time point using one way ANOVA test. Multiple pairwise comparisons were done using Tukey's test. Asterisks (*) indicates significance at p<0.05 on the Tukey's test between experimental groups and controls, (**) indicates the significance (p<0.05, Tukey's test) between the experimental groups DMEM and BSS.
Figure 10

B. Effect of OGD on Delayed Neuronal Cell Death

Duration of Oxygen-Glucose Deprivation (OGD)

2 hrs 3 hrs 4 hrs 5 hrs 6 hrs

% of Maximal LDH Release

Control

DMEM

BSS

A. Effect of OGD on Immediate Neuronal Cell Death

Duration of Oxygen-Glucose Deprivation (OGD)

2 hrs 3 hrs 4 hrs 5 hrs 6 hrs

% of Maximal LDH Release

Control

DMEM

BSS
TABLE – 1

Effect of 5 hrs of OGD on neuronal cell death (model paradigm)

<table>
<thead>
<tr>
<th>Treatment group (5 hrs)</th>
<th>Sample size (n) = no. of dishes</th>
<th>% Immediate cell death mean±sem</th>
<th>ANOVA test Significance (p)</th>
<th>Tukey’s test Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>11</td>
<td>10.17±0.79</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BSS</td>
<td>12</td>
<td>12.28±1.52</td>
<td></td>
<td>p&gt;0.05/C</td>
</tr>
<tr>
<td>DMEM</td>
<td>13</td>
<td>29.09±2.12</td>
<td></td>
<td>p&lt;0.05/C (*) , BSS (**)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment group (5 hrs)</th>
<th>Sample size (n) = no. of dishes</th>
<th>% Delayed cell death mean±sem</th>
<th>ANOVA test Significance (p)</th>
<th>Tukey’s test Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>11</td>
<td>15.28±0.49</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BSS</td>
<td>11</td>
<td>17.37±0.57</td>
<td>p&gt;0.05/C</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>10</td>
<td>47.76±2.94</td>
<td>P&lt;0.05/C (*) , BSS (**)</td>
<td></td>
</tr>
</tbody>
</table>
7 Morphological Changes During OGD in Neuronal Cells

Necrosis is characterized by cellular swelling due to loss of ionic homeostasis, damage to organelles and finally cell lysis. Necrosis is the primary mode of cell death in the ischemic core. The rapid nature of cell lysis in the DMEM group suggests that death is primarily necrotic, this was supported by Hoechst staining (preliminary data) which did not reveal any significant apoptotic component in both immediate and delayed cell death. Phase images at 20X (Figure 11) compare the changes in cellular morphology of cells exposed to OGD from t=0 (Figure 11A) and at 1 hr and 5 hrs of OGD in controls, BSS and DMEM. In the DMEM group, cellular morphology changes rapidly within 1 hr (Figure 11D), cells get swollen, rounded, neuritic processes disappear early on (Figure 11D,G). Cells in BSS have a relatively well preserved morphology with intact processes well preserved right till the end of the insult (Figure 11C,F) as compared to controls (Figure 11B,E).

8 Morphological Changes During Early Reperfusion

Immediately after reperfusion cellular morphology does not change significantly. At 30 mins of reperfusion cells in DMEM (Figure 12C) continue to show rounding of cells with loss of neuritic processes. Cells in BSS (Figure 12B) show some swelling of a few cells and loss of processes probably coinciding with the burst in ROS – as discussed in Results (section 17). Control cell morphology is well preserved (Figure 12A). There are no significant changes in cell morphology in DMEM (Figure 12F) at 60 mins of reperfusion, but cells in BSS already begin to show some recovery of morphology (Figure 12 E). Remarkably at 2 hrs of reperfusion, cells in DMEM begin to show neuritic processes and cell bodies can be distinctly identified (Figure 12 I).
Figure 11: Morphological Changes During OGD

Figure 11 is a series of phase contrast microscopy images with 20 X objective, comparing cells in BSS, DMEM and Controls throughout the duration of OGD.

Figure 11A shows a representative sample of cells prior to the start of the insult. Cells were differentiated in NGF (25 ng/ml) for 7 days. Cell bodies are clearly visualized and neuritic processes are branching out. These features are preserved in control cells throughout the duration of OGD.

Figures 11C and 11F show the morphological changes in cells in BSS at 1 hr and at the end of OGD (5 hrs) respectively. Arrows in Figure 11C and 11F point to the recovering neuritic processes.

Figures 11D and 11G show the morphological changes during OGD in cells in DMEM at 1hr and at 5 hrs. Arrows point to the rounded cell bodies.
Figure - 11

OGD-1 hr

A. Phase image (20X objective) cells at t=0

B. Controls

OGD-5 hrs

C. Cells in BSS

D. Cells in DMEM

E. Controls

F. Cells in BSS

G. Cells in DMEM
Figure 12: Morphological Changes In Early Reperfusion

Figure 12 is a composite figure showing the changes in cellular morphology at early time points in reperfusion using phase contrast microscopy (20X objective) following 5 hrs of OGD.

Figure 12 A, B, C represent the morphological changes at 30 mins of reperfusion in controls, BSS and DMEM. Cells in DMEM (Figure 12C) show rounded appearance with loss of neuritic processes which is very similar to their morphology throughout OGD. But cells in BSS (Figure 12B) at 30 mins of reperfusion show a rapid deterioration in morphology, with rounding up of cell bodies (arrow) and loss of neurites.

Figure 12D, E, F show the changes at 60 mins of reperfusion, cells in BSS (Figure 12E) are already in the process of recovery with cell processes seen. However cells in DMEM continue to show a rounded morphology with no processes (Figure 12F).

Figure 12G, H, I show the morphological changes at 2 hrs of reperfusion. Remarkably cells in DMEM (Figure 12I) are now in the recovery stage with neuritic processes and normal cell bodies seen in some cells (Figure 12I arrow point to cell with normal looking morphology).
Figure - 12

30 mins repf.

A. Controls

B. Cells in BSS

C. Cells in DMEM

60 mins repf.

D. Controls

E. Cells in BSS

F. Cells in DMEM

2 hrs repf.

G. Controls

H. Cells in BSS

I. Cells in DMEM
9 Mitochondrial Morphology During OGD

Mitochondria are assuming an increasingly important role in hypotheses about necrotic and apoptotic cell death in ischemia. Mitochondria undergo a transient swelling for a few hours after any form of ischemia. The origin has not been studied but may be the reversible opening of the MTP due to free radical generation (Lipton P., 1999). I have used PC12 GFP \(_{h_0}\) cells (Wadia thesis in progress) to study the mitochondrial morphology throughout 5 hrs of OGD using confocal microscopy with 60X oil immersion objective. At the start of the experiments, GFP labeled mitochondria are seen as stringy looking distributed throughout the cytoplasm and along the processes (Figure 13A). But within 1 hr cells in the DMEM group show early condensation and swelling of the mitochondria (Figure 13D) as compared to cells in BSS (Figure 13C) or to controls (Figure 13B). At the end of 5 hrs of OGD, the mitochondria of cells in DMEM are edematous, rounded up and condensed perinuclearly (Figure 14C) as compared to those in BSS (Figure 14B) and to controls (Figure 14A). The addition of PI during the insult showed the increased cell death in the DMEM group, it also identified cells with severe morphological changes but yet alive (Figure 14C) thereby confirming the possibility of reversibility of effects of OGD.

10 Mitochondrial Morphology During Early Reperfusion

At the end of the insult, reperfusion was done by complete replacement of medium with glucose containing solutions. At 30 mins of reperfusion, mitochondrial morphology of cells in BSS (Figure 15B) is condensed and swollen as compared to controls (Figure 15A), but cellular morphology is relatively well preserved. However the mitochondrial morphology of cells in DMEM shows condensation perinuclearly with loss of stringy appearance (Figure 15C). These changes are also seen at 60 mins after reperfusion (Figure 15D,E,F).
Figure 13: Mitochondrial Morphology During Early OGD

Figure 13 represents the changes in mitochondrial morphology during the early phase of OGD. Confocal images were taken with 60X oil immersion objective using dual channel imaging for simultaneous GFP and PI imaging. To improve picture visualization, PI stained nuclei were colored red as mentioned in Methods.

Figure 13A shows a representative sample of cells prior to the start of the experiment. In a single cell, mitochondria is visualized in the upper image (arrow), well spread out in the cytoplasm. The center dark region represents the nucleus. Healthy mitochondria are also seen along the processes. The lower image shows a cluster of GFP labeled cells.

Figure 13B represents the control cells at one hour under normoxic conditions in the presence of glucose, mitochondrial morphology is seen and is unaffected. Figure 13C shows cells in BSS at one hour of OGD (arrow points to the mitochondria) show no difference in morphology as compared to controls.

Figure 13D shows cells in DMEM at 1 hour of the start of OGD, cells show blebbing of the plasma membrane, with loss of neuritic processes. Mitochondria are swollen and condensed with distribution located perinuclearly. Some PI+ cells are also seen (red nuclei). Strikingly some cells with blebbing and swollen mitochondria are not PI+, indicating the presence of intact cell membranes.
Figure - 13

A. GFP+ Neuronal PC12 cell (60X objective)

B. Control cells

C. Cells in BSS

D. Cells in DMEM
Figure 14: Mitochondrial Morphology At The End Of OGD

Figure 14 shows a comparison of mitochondrial morphology at the end of 5 hrs of OGD using confocal microscopy (60X objective) with dual channel imaging.

Figure 14A shows controls under normoxic conditions in the presence of glucose. Arrow shows healthy mitochondria in the cell bodies. The adjacent image also shows a single PI+ nuclei.

Figure 14B shows cells exposed to OGD in BSS, arrow show the mitochondria which show some swelling and condensation towards the end of the insult.

Figure 14C shows cells exposed to OGD in DMEM, showing GFP labeled mitochondria along with PI+ nuclei. Some cells show extensive plasma membrane blebbing, but no PI labeling of nuclei. Arrows show mitochondria in the left image, arrow in the right image shows blebbing of the plasma membrane.
Figure - 14

A. Controls at 5 hrs of OGD

B. Cells in BSS at 5 hrs of OGD

C. Cells in DMEM at 5 hrs of OGD
Figure 15: Mitochondrial Morphology In Early Reperfusion

Figure 15 represents changes in mitochondrial morphology during the early phase of reperfusion studied using confocal microscopy with (60X objective) dual channel imaging following 5 hrs of OGD.

Figure 15 A, 15D represents the controls under normoxic conditions with glucose. At 30 and 60 mins of reperfusion, there is no change in mitochondrial morphology.

Figure 15 B and 15E represent changes in mitochondrial morphology at 30 and 60 mins of reperfusion. At 30 mins, cells in BSS show condensation and swollen appearance of mitochondria, coinciding with the burst in ROS (see results in section 24). At 1 hr there is a significant recovery in mitochondrial and cellular morphology.

Figure 15C shows a parallel phase contrast and GFP+ image of the same cells at 30 mins of reperfusion after 5 hrs of OGD in DMEM. Phase contrast microscopy shows rounding of cells with loss of processes. The fluorescent image shows the mitochondria looking swollen and condensed.
Figure - 15

A. Controls 30 min repf.

B. Cells in BSS at 30 min repf.

C. Cells in DMEM at 30 mins of repf.

D. Controls 60 min repf.

E. Cells in BSS at 60 mn repf.

F. Cells in DMEM at 60 mins of repf.
11 Comparison Of The Effects Of OGD/OD/GD for 5 Hours

On dissecting the insult, into the individual components of oxygen and glucose deprivation, cells were not significantly affected by oxygen deprivation alone but glucose deprivation did significantly increase cell death (Figure 16). Mean percentages of cell death within the immediate group and delayed group were compared using the one way ANOVA and found to be significant (p<0.001). Multiple pairwise comparisons were then done using the Tukey’s test. Glucose deprivation in DMEM significantly increased (p<0.05, Tukey’s test) immediate mean cell death (52.73±2.43%) as compared to oxygen deprivation (8.67±0.15%), or combined oxygen and glucose deprivation (29.09±2.12%). Glucose deprivation in DMEM in the presence of oxygen is significantly more toxic than in the absence of oxygen in neuronal PC12 cells. Glucose deprivation in BSS (14.04±0.39%) also significantly (p<0.05, Tukey’s test) increases immediate mean cell death as compared to oxygen deprivation (6.72±0.16%) or oxygen and glucose deprivation (9.24±0.42%). Indeed the absence of glucose (GD) alone, increases immediate mean cell death in cells in DMEM and BSS as compared to OGD.

In case of delayed cell death, cells exposed in BSS to OGD show significantly (p<0.05, Tukey’s test) increased cell death as compared to OD/GD (Figure 16B). Delayed mean cell death in DMEM following GD is 40.04±1.37% as compared to 47.96±2.94% in OGD.
Figure 16: Comparison Of The Effects Of OGD/OD/GD For 5 Hrs

These bar graphs Figure 16A and 16B illustrate the difference in cell death in response to OGD/OD/GD for the same duration of time i.e. 5 hrs, with effects on immediate cell death represented in 16A and delayed cell death represented in 16B.

All cells used were differentiated in NGF2.5S (25 ng/ml) and derived from the same subculture. At each time point cells were exposed to OGD in DMEM and BSS lacking glucose and glutamine. Controls were in DMEM containing glucose and glutamine. All experiments were done in the presence of NGF but in 0 serum. Data are means from at least 3 different experiments with error bars indicating SEM, X axis represents the duration of OGD in hours, Y axis represents the percentage of maximal LDH release. Statistical significance was derived at each time point using one way ANOVA test. Multiple pairwise comparisons were done using Tukey's test. Asterisks (*) indicates significance at p<0.05 on the Tukey's test between experimental groups (-O/-G), (**) indicates the significance (p<0.05, Tukey's test) between the experimental groups and DMEM/BSS (-O-G).
Figure - 16
A. Comparison of the Effect of OGD/OD/GD for 5 hrs on Immediate Neuronal Cell Death

B. Comparison of the Effect of OGD/OD/GD for 5 hrs on Delayed Neuronal Cell Death
12 Effect Of L-Glutamine On OGD-Induced Cell Death

How would adding a metabolic substrate (not glucose) during OGD affect the outcome? During glucose deprivation PC12 cells can consume other energy sources like L-Glutamine or lactate and thereby maintain ATP levels. Statistical comparisons done on immediate and delayed groups using the one way ANOVA test were significant (p<0.001), multiple pairwise comparisons were then done using the Tukey's test. L-Glutamine significantly (p<0.05, Tukey's test) decreases immediate mean cell death in the DMEM group (19.83±1.33%) as compared to 28.42±2.14% in the absence of glutamine during OGD (Figure 17A).

Delayed mean cell death (Figure 17B) is significantly (p<0.05, Tukey's test) decreased in both DMEM (14.13±0.72%) and BSS (8.36±0.28%) in the presence of L-Glutamine.
Figure 17: Effect Of L-Glutamine Supplementation During OGD

These bar graphs Figure 17A and 76B illustrate the difference in cell death in response to addition of a substrate, L-Glutamine during OGD for 5 hrs, with effects on immediate cell death represented in 17A and delayed cell death represented in 17B.

All cells for each experiment were differentiated in NGF2.5S (25 ng/ml) and derived from the same subculture. At each time point cells were exposed to OGD in DMEM and BSS lacking glucose and glutamine and compared with cells in BSS and DMEM lacking glucose but supplemented with 2 mM L-Glutamine (regular concentration in media). Controls were in DMEM containing glucose and glutamine. All experiments were done in the presence of NGF but in 0 serum. Data are means from at least 2 different experiments with error bars indicating SEM, all the data has been derived from the same subcultures. X axis represents the duration of OGD in hours, Y axis represents the percentage of maximal LDH release. Statistical significance was derived at each time point using one way ANOVA test. Multiple pairwise comparisons were done using Tukey’s test. Asterisks (*) indicates significance at p<0.05 on the Tukey’s test between experimental groups and (+/-) Glutamine.
Figure - 17

A. Effect of L-Glutamine During OGD on Immediate Neuronal Cell Death

B. Effect of L-Glutamine During OGD on Delayed Neuronal Cell Death
13 Effect Of 2-DG On OGD Induced Cell Death

The next question that followed was- if adding a metabolic substrate does the above, then what would happen with complete substrate deprivation? Glucose deprivation alone does not result in complete inhibition of glycolysis, because glucose-6-phosphate can be provided from cellular glycogen stores and by gluconeogenesis. 2-Deoxy-D-Glucose (2DG) is a competitive inhibitor of glycolysis, a concentration of 10 mM was decided based on literature reviews. Although gluconeogenesis does not occur in neurons, it is known to occur in PC12 cells. These cells can utilize amino acids in the absence of glucose. Mean percentages of cell death within the immediate group and within the delayed group were found to be significant (p<0.001, ANOVA), multiple comparisons were done using the Tukey’s test. In the presence of 2DG, 5 hrs of OGD increased immediate mean cell death in the BSS group (23.29±1.55%) which was similar to mean cell death in DMEM (27.5±4.5%) (Figure 18A). Therefore glycolytic inhibition causes similar levels of immediate mean cell death in both BSS and DMEM exposed to 5 hrs of OGD.

Reperfusion was done in 20 mM glucose to reverse the effects of 2DG, a competitive inhibitor of glycolysis. Accordingly delayed mean cell death in the groups are compared on reperfusion with 20 mM glucose (Figure 18B). Differences in delayed cell death were statistically significant (p<0.001, ANOVA test). Delayed mean cell death in DMEM+2DG (59.38±5.98%) was significantly greater (p<0.05, Tukey’s test) than in DMEM alone (43.34±2.43%). Similarly delayed mean cell death in BSS+2DG (53.02±4.5%) was significantly greater (p<0.05, Tukey’s test) than in BSS (19.80±1.41%) alone. But more importantly delayed mean cell death in BSS+2DG (53.02±4.5%) was similar to DMEM+2DG (59.38±5.98%) (Table 2). In the presence of 2DG, cell death is almost of the same level in BSS and DMEM.
Figure – 18: Effect Of Glycolytic Inhibition During OGD Using 2 Deoxy-D-glucose

These bar graphs Figure 18A and 18B illustrate the difference in cell death in response to inhibition of glycolysis during OGD for 5 hrs, with effects on immediate cell death represented in 18A and delayed cell death represented in 18B.

All cells for each experiment were differentiated in NGF2.5S (25 ng/ml) and derived from the same subculture. At each time point cells were exposed to OGD in DMEM and BSS and compared with cells in BSS, DMEM and controls with 10 mM 2 Deoxy-D-Glucose (2 DDG). As 2 DDG is a competitive inhibitor of glycolysis, reperfusion was done in 20 mM glucose. Therefore all delayed cell death reflects reperfusion in 20 mM glucose in both groups. All experiments were done in the presence of NGF but in 0 serum. Data are means from at least 2 different experiments with error bars indicating SEM, all the data has been derived from the same subcultures. X axis represents the duration of OGD in hours, Y axis represents the percentage of maximal LDH release. Statistical significance was derived at each time point using one way ANOVA test. Multiple pairwise comparisons were done using Tukey’s test. Asterisks (*) indicates significance at p<0.05 on the Tukey’s test between experimental groups +/- 2 Deoxy-D-Glucose.
Figure - 18

A. Effect of 2Deoxy-D-Glucose (2DDG) During OGD on Immediate Neuronal Cell Death

![Graph showing the effect of 2Deoxy-D-Glucose (2DDG) on immediate neuronal cell death.](image)

B. Effect of 2Deoxy-D-Glucose (2DDG) During OGD on Delayed Neuronal Cell Death

![Graph showing the effect of 2Deoxy-D-Glucose (2DDG) on delayed neuronal cell death.](image)
TABLE – 2

Effect of 2 Deoxy-D-Glucose (2DDG) on OGD induced cell death

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Sample size (n) = no. of dishes</th>
<th>% Immediate cell death mean±sem</th>
<th>ANOVA test Signific. (p)</th>
<th>Tukey’s test Significance (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7</td>
<td>11.17±1.06</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BSS</td>
<td>9</td>
<td>13.66±1.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>10</td>
<td>25.58±1.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls+2DDG</td>
<td>10</td>
<td>11.50±1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSS+2DDG</td>
<td>9</td>
<td>23.29±1.55</td>
<td>P&lt;0.05/C+2DDG(*)</td>
<td></td>
</tr>
<tr>
<td>DMEM+2DDG</td>
<td>11</td>
<td>27.50±4.51</td>
<td>P&lt;0.05/C+2DDG(*)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Sample size (n) = no. of dishes</th>
<th>% Delayed cell death mean±sem</th>
<th>ANOVA test Signific. (p)</th>
<th>Tukey’s test Significance (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7</td>
<td>15.36±0.65</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BSS</td>
<td>6</td>
<td>19.80±1.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>6</td>
<td>43.34±2.43</td>
<td>p&lt;0.05/C(*), BSS(**)</td>
<td></td>
</tr>
<tr>
<td>Controls+2DDG</td>
<td>6</td>
<td>15.52±0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSS+2DDG</td>
<td>8</td>
<td>53.02±4.55</td>
<td>p&lt;0.05/BSS(**)C+(*)</td>
<td></td>
</tr>
<tr>
<td>DMEM+2DDG</td>
<td>8</td>
<td>59.39±5.98</td>
<td>p&lt;0.05/C+2DDG(*)</td>
<td></td>
</tr>
</tbody>
</table>
14 Ferric Nitrate as A Source Of Oxidative Stress

(Personal Communication With Dr. Lloyd A. Greene)

In the process of identifying a cause of cytotoxicity in the DMEM group, personal communication with Dr. Lloyd A. Greene, sparked an interest in the possible role of Iron as a pro-oxidant and ultimately leading to cell death. As DMEM has Ferric Nitrate (Fe$_3$NO$_9$.9H$_2$O), I added the same to BSS in the same concentration of 0.0001g/L. Mean percentages of cell death within the immediate group and within the delayed group were statistically significant (p<0.001) when compared using the one way ANOVA, multiple comparisons were done using the Tukey's test. Immediate mean cell death after 5 hrs of OGD in the BSS group with iron was 10.74±0.70% as compared to the BSS group without iron (8.13±0.47%) and was significantly (p<0.05, Tukey's test) lesser than mean cell death in the DMEM (40.79±2.13%) group (Figure 19A).

Similarly delayed mean cell death in the presence of iron in the BSS (20.62±1.36%) group was not significantly (p>0.05) different from mean cell death in BSS alone (17.58±1.40%) but was significantly (p<0.05) lesser than mean cell death in DMEM (57.06±2.99%) (Figure 19B).

Contrary to my expectations the addition of Ferric Nitrate to BSS did not significantly increase cell death.
**Figure 19: Effect Of Addition Of Iron To BSS During OGD**

These bar graphs Figure 19A and 19B illustrate the difference in cell death in response to addition of iron to BSS during OGD for 5 hrs and 6 hrs, with effects on immediate cell death represented in 19A and delayed cell death represented in 19B.

All cells for each experiment were differentiated in NGF2.5S (25 ng/ml) and derived from the same subculture. At each time point cells were exposed to OGD in DMEM and BSS and compared with cells in BSS containing iron (Ferric Nitrate 0.0001g/L). DMEM has iron, it is a pro-oxidant and a known cause of cytotoxicity, therefore added iron to BSS in exactly the same concentration. Cells were exposed to OGD for 5 hours and further for 6 hrs to identify any increase in cell death in BSS, caused by the presence of iron. All experiments were done in the presence of NGF but in 0 serum. Data are means from at least 2 different experiments with error bars indicating SEM, all the data has been derived from the same subcultures. X axis represents the duration of OGD in hours, Y axis represents the percentage of maximal LDH release. Statistical significance was derived at each time point using one way ANOVA test. Multiple pairwise comparisons were done using Tukey’s test. Asterisks (*) indicates significance at p<0.05 on the Tukey’s test between experimental groups at each time point.
Figure 19

A. Effect of Addition of Iron to BSS During OGD on Immediate Neuronal Cell Death

B. Effect of Addition of Iron to BSS During OGD on Delayed Neuronal Cell Death
OXIDATIVE STRESS DURING OGD AND REPERFUSION

15 Confocal Microscopic Measurement Of ROS During OGD

Confocal microscopy using the ROS indicator 5'6'CMH2DCF (10μM) was used to study free radical generation throughout the duration of OGD. 5'6'CMH2DCF-DA is generally assumed to be specific for hydrogen peroxide, although it is known to react with other species like hydroxyl radical and nitric oxide as well. The fluorescent indicator (10μM) was left in the medium throughout the insult, in an effort to minimize dye leakage. PC12 cells are known to have a multidrug transporter system on the plasma membrane, which can cause dye leakage, controls cells showed leakage of the dye although experimental groups did not, indicating perhaps that the transporter might be ATP dependent. Cells were analyzed under ischemic conditions at 1 hr time intervals under temperature controlled conditions (37°C) for up to 5 hrs of OGD. In between time points, dishes were replaced in the incubator (Figure 20). Data are means of 20 different cells, from random fields in at least 2 dishes, represented as a percentage of control fluorescence. When the mean fluorescence values between DMEM and BSS groups were compared using the unpaired t test, at every time point assessed, the difference was highly significant (p<0.001) (Figure 21). Considerable controversy exists in literature regarding an increase or a decrease in ROS during OGD (see Discussion section 9). In my model of ischemia, cells in the DMEM group show an immediate, significant (p<0.001) and persistent rise in free radicals throughout OGD. In contrast cells in BSS are however not significantly different from controls throughout the duration of OGD.
**Figure - 20: Changes In ROS Generation During OGD**

Figure 20 is a series of images showing the generation of ROS during OGD with the indicator 5’6’CMH2DCF-DA using confocal microscopy with 20Fluor objective. All cells were treated with doxycycline and NGF (25 ng/ml) for 7 days to turn off the GFP signal (see Methods). Differentiated PC12 cells were plated overnight on glass bottomed collagen coated dishes. Loading was done with the indicator dye for 45 mins in KRH, images were taken just prior to the start of the experiment and represented t=0. Cells were subjected to OGD as described in Methods. The first image was taken at 1 hr after the start of OGD. I have not looked at earlier time points. Images are from at least 2 dishes for each condition. Instrument settings were kept constant throughout the duration of the experiment.

Figure 20A is an image of a representative sample of cells prior to the start of the experiment. Cells were loaded uniformly with the ROS indicator 5’6’CMH2DCF-DA.

Figure 20B, 20C and 20D show the changes in fluorescence signal in controls, BSS and DMEM at 1 hr of OGD. Arrow in Figure 20D points to saturated fluorescent signal intensity in cells in DMEM.

Figure 20E, 20F and 20G show the changes in fluorescence signal at the end of OGD of 5 hrs duration. Arrow indicate the fluorescent signal from cells showing well preserved morphology in controls (Figure 20E) and BSS (Figure 20F). Saturated fluorescent signal in seen in rounded cells in DMEM (Figure 20G).
Figure 20

1 hr of OGD

B. Controls

E. Controls

A. DCF loaded cells at t=0

C. Cells in BSS

D. Cells in DMEM

F. Cells in BSS

G. Cells in DMEM
Figure - 21: Fluorescent Measurements Of ROS During OGD

This figure 21 shows a comparison of the changes in fluorescent measurements of the ROS indicator 5′6′CMH2DCF-DA between cells in DMEM and BSS at 1 hr intervals throughout the duration of OGD.

These line plots illustrate the continuum of changes in fluorescent measurements expressed as a percentage of control values during OGD at 1 hr intervals. X axis represents the time points at 1 hr intervals studied during OGD, Y axis denotes the fluorescence measurements expressed as a percentage of control values. Data are representative of mean fluorescence values obtained from 20 random cells in 2 dishes in each group, from at least 2 experiments. Error bars indicate SEM, * indicates statistical significance and p values were calculated using Unpaired t test at each time point. As can be seen from the previous images in Figure 20, the fluorescent signal in the DMEM was saturated as early as 1 hr after the start of OGD. The increase in fluorescence signal in cells in DMEM was immediate, significant and persistent throughout OGD.
Figure - 21

Fluorescence Measurements of ROS with 5'6' CMH2DCF-DA During OGD

% of Control Fluorescence

0 1hr 2hr 3hr 4hr 5hr

Duration of OGD - 5 hrs
Flow Cytometry Confirms The Increase In Free Radical Generation

The use of Flow cytometric techniques in neuronal populations has several advantages over standard spectrofluorometric techniques in cell culture. This technique can be used to evaluate simultaneously and in a short duration of time multiple factors like ROS production, intracellular calcium and mitochondrial membrane potential. It can analyze a large number of cells and allows the exclusion of nonviable cells from the study. As in every technique there are a few disadvantages such as experiments involving long exposure times or repeated examination of samples.

Using the same indicator as above 5'6'CMH2DCF-DA (10µM), flow cytometry was done immediately after the termination of OGD, in the ischemic medium using differentiated PC12 cells. The indicator dye was left in the medium throughout the duration of the insult, in the same concentration. As is seen in Figure 22A, cells in DMEM show increased generation of free radicals with a shift in the curve towards the right, as compared to BSS which is similar to controls. Hydrogen peroxide was used as a positive control, it moved the curve further to the right and confirmed the activity of the dye used (Figure 22B).
Figure – 22: Flow Cytometric Measurements Of ROS At The End Of OGD

Figure 22A and 22B represent histograms of fluorescence measurements of ROS using the indicator 5'6'CMH2DCF-DA. Measurements were made using the FACScan at the end of OGD. All cells used were treated with doxycycline for 7 days to turn off the GFP signal (see Methods). In graph 22A, blue color represents controls, green color BSS and red color DMEM. Graph 22B demonstrates the effect of adding hydrogen peroxide to the cells represented in black color, thereby confirming the activity of the dye and the reliability of the instrument settings.
Figure - 22
Flow Cytometric Measurements of ROS with 5’6’CMH2DCF-DA after OGD

Response of 5’6’CMH2DCF-DA To Positive Control H2O2
17 Generation of ROS During Early Reperfusion

Reperfusion was studied at 30 mins, 60 mins and 2 hrs following the termination of OGD (5 hrs) using the ROS indicator 5,6-CMHR2DCF (10μM). Reperfusion medium contained the indicator in the same concentration. Confocal microscopy was used to measure fluorescence during the early phase of reperfusion (Figure 23). Data are means of 20 different cells, from random fields in at least 2 dishes, represented as a percentage of control fluorescence (Figure 24). When the mean fluorescence values between DMEM and BSS groups were compared using the unpaired t test, at time 30 mins, and 60 mins, the difference was significant (p<0.001). 30 mins after the cessation of the insult, there is a rapid, short-lived, significant (p<0.001) burst in free radical generation in cells in BSS (Figure 23B). Cells in DMEM also showed a small but significant (p=0.009) burst in ROS (Figure 23C) compared to signal intensities at the end of OGD. But at 2 hrs of reperfusion, fluorescence values in both experimental groups and controls declined radically and were not significantly different (p = 0.766). This massive loss of fluorescence was plausibly a consequence of dye leakage (Figure 23G, H, I discussed in detail in Methods section 3.3).
Figure – 23: Changes In ROS During Early Reperfusion

Figure 23 is a series of images showing the generation of ROS during early reperfusion following 5 hrs of OGD with the indicator 5'6'CMH2DCF-DA using confocal microscopy with 20X Fluor objective. All cells were treated with doxycycline and NGF (25 ng/ml) for 7 days to turn off the GFP signal (see Methods). Cells were plated overnight on glass bottomed collagen coated dishes. Cells were subjected to OGD as described in Methods, reperfusion was done by complete change of medium, cells were left in the incubator under normoxic conditions in the presence of glucose. Images are from at least 2 dishes for each condition. Instrument settings were kept constant throughout the duration of the experiment. The first image was taken at 30 mins of reperfusion.

Figure 23A, B, C show the changes in fluorescence of the ROS indicator 5'6'CMH2DCF-DA at 30 mins of reperfusion in controls (arrow points to fading signal from cells), BSS and DMEM. An increase in fluorescence intensity is seen in cells in BSS at 30 mins of reperfusion (Figure 23B).

Figure 23D, E, F show the changes in fluorescence of the ROS indicator 5'6'CMH2DCF-DA at 60 mins of reperfusion in controls, BSS and DMEM.

Figure 23G, H, I show the changes in fluorescence in controls (arrow points to cell with loss of fluorescence signal), BSS and DMEM at 2 hrs of reperfusion. Note the rapid fall in fluorescence in all groups suggestive of dye leakage (discussed in Methods).
Figure - 23

30 mins repf. 60 mins repf. 2 hrs repf

A. Controls D. Controls G. Controls

B. Cells in BSS E. Cells in BSS H. Cells in BSS

C. Cells in DMEM F. Cells in DMEM I. Cells in DMEM
This figure 24 shows a comparison of the changes in fluorescent measurements of the ROS indicator 5′6′CMH2DCF-DA between cells in DMEM and BSS at 30 mins, 60 mins and 2 hrs of reperfusion.

These line plots illustrate the changes in fluorescent measurements expressed as a percentage of control values during early reperfusion at 30 mins, 60 mins and 2 hrs. X axis represents the time points at 30 mins, 60 mins and 2 hrs. Y axis denotes the fluorescence measurements expressed as a percentage of control values. Data are representative of mean fluorescence values obtained from 20 random cells in 2 dishes in each group, from at least 2 experiments. Error bars indicate SEM, * indicates statistical significance and p values were calculated using Unpaired t test at each time point. As can be seen from the previous images in Figure 23, there is a significant but transient burst in ROS in cells in BSS at 30 mins of reperfusion, but by 2 hrs all the 3 groups show similar fluorescence perhaps indicating dye leakage (see Methods section 3.3).
Figure - 24

Fluorescence Measurements of ROS with 5'-6'-CMH2DCF-DA During Reperfusion

% of Control Fluorescence

Duration of Reperfusion

DMEM

BSS
18 Lipid Peroxidation after OGD

Free radicals are short lived, hence formation of lipid, protein or DNA breakdown products is often considered a marker for oxidative damage. The Thiobarbituric acid reactive substance (TBARS) test is a widely used colorimetric assay and reflects malondialdehyde (MDA) concentration. Data are expressed as a percentage of control values per mg of protein (Figure 25). The TBARS test was done immediately after the termination of 5 hrs of OGD. Statistical comparison between the two groups was done using the unpaired t test. Levels of lipid peroxidation assayed using the TBARS test, were found to be significantly (p<0.001) higher in the DMEM group (107.66±2.21%) as compared to levels of lipid peroxidation in the BSS group (50.89±5.18%).

19 Effect of Anti-Oxidants On OGD Induced Cell Death

Exogenously administered SOD (500ng/ml) and Catalase (4000u/ml) during OGD and reperfusion were found to significantly decrease both immediate and delayed cell death in the DMEM group. Mean percentages of cell death within the immediate group and within the delayed group were compared using the one way ANOVA, differences were statistically significant (p<0.001). Multiple pairwise comparisons were done using the Tukey’s test. The addition of SOD and Catalase decreased immediate mean cell death significantly (p<0.05, Tukey’s test) in DMEM from 25.58±1.25% to 10.90±2.00% (Figure 26A).

Reperfusion medium containing SOD and Catalase decreased delayed mean cell death in the DMEM group (17.23±2.4%) significantly (p<0.05, Tukey’s test) as compared to 41.56±1.76% (Figure 26B). This probably reflects the role oxidative stress plays in OGD induced cell death in the DMEM group, hence cytoprotection by anti-oxidants. The data are summarized in Table 3.
Figure – 25: Lipid Peroxidation At 5 hrs Of OGD (TBARS Assay)

This figure 25 represents the levels of lipid peroxidation immediately after 5 hrs of OGD. Levels of lipid peroxidation were determined using the thiobarbituric acid reactive substances assay (TBARS) Data represent average values from 2 different experiments expressed as a percentage of control, within each experiment cells were derived from the same subculture. X axis represents duration of OGD – 5 hrs. Y axis represents percentage of control lipid peroxidation. Statistical significance was determined between experimental groups using the unpaired t test (p<0.001) and represented by asterisks (*).
Figure - 25

Levels of Lipid Peroxidation Using the TBARS Assay

Duration of OGD - 5 hrs
Figure – 26: Effect Of Anti-oxidants During OGD And Reperfusion

These graphs Figure 26A and 26B illustrate the neuroprotective effect of SOD (500ng/ml) and Catalase 4000u/ml during OGD and reperfusion on immediate (A) and delayed (B) neuronal cell death.

Neuronal cells were subjected to OGD and reperfusion in the presence of SOD and Catalase and compared with cell death in the absence of SOD and catalase. Data represent means and SEM from at least 2 experiments, all cells in each experiment were derived from the same subculture. The sample size of each group in every experiment varied from 3-6 for immediate cell death and from 3-4 for delayed cell death. Number of cells plated for each experiment was maintained constant at 5x10^4. Statistical significance was calculated using the one way ANOVA test for immediate (p<0.001) and delayed (p<0.001) time points, and multiple comparisons done using the Tukey’s test.

The presence of SOD & Catalase during OGD and Reperfusion significantly decreases cell death in the DMEM group by 75% and in the BSS group by 31%. Cell death is significantly decreased in controls as well.
Figure - 26

A. Effect of SOD & Catalase During OGD on Immediate Neuronal Cell Death

% of Maximal LDH Release

- SOD/Catalase  + SOD/Catalase
Duration of OGD - 5 hrs

B. Effect of SOD & Catalase During Reperfusion on Delayed Neuronal Cell Death

% of Maximal LDH Release

- SOD/Catalase  + SOD/Catalase
Duration of OGD - 5 hrs
### TABLE – 3

Effect of anti-oxidants SOD and Catalase on OGD induced cell death

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Samplesize (n) = no. of dishes</th>
<th>% Immediate cell death</th>
<th>ANOVA test significance(p) p&lt;0.001</th>
<th>Tukey’s test Significance(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7</td>
<td>11.17±1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSS</td>
<td>9</td>
<td>13.66±1.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>10</td>
<td>25.58±1.25</td>
<td>p&lt;0.05/DM+,BSS,C</td>
<td></td>
</tr>
<tr>
<td>Controls+SOD/Cat</td>
<td>8</td>
<td>7.82±1.48</td>
<td>p&lt;0.05/DM</td>
<td></td>
</tr>
<tr>
<td>BSS+SOD/Cat</td>
<td>7</td>
<td>10.18±1.46</td>
<td>p&lt;0.05/DM,p&gt;0.05/BSS</td>
<td></td>
</tr>
<tr>
<td>DMEM+SOD/Cat</td>
<td>7</td>
<td>10.90±2.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Samplesize (n) = no. of dishes</th>
<th>% Immediate cell death</th>
<th>ANOVA test significance(p) p&lt;0.001</th>
<th>Tukey’s test Significance(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7</td>
<td>15.36±0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSS</td>
<td>7</td>
<td>17.25±0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>6</td>
<td>41.56±1.76</td>
<td>P&lt;0.05/BSS,C</td>
<td></td>
</tr>
<tr>
<td>Controls+SOD/Cat</td>
<td>8</td>
<td>12.05±0.96</td>
<td>P&lt;0.05/DM</td>
<td></td>
</tr>
<tr>
<td>BSS+SOD/Cat</td>
<td>8</td>
<td>15.19±1.25</td>
<td>p&gt;0.05/BSS,p&lt;0.05/DM</td>
<td></td>
</tr>
<tr>
<td>DMEM+SOD/Cat</td>
<td>7</td>
<td>17.23±2.39</td>
<td>P&lt;0.05/DM</td>
<td></td>
</tr>
</tbody>
</table>
Inhibition Of Nitric Oxide During OGD And Reperfusion Increases Cytotoxicity

Differentiation of PC12 cells with NGF for 8 days, induces neuronal NOS (nNOS), undifferentiated cells do not express nNOS (see Introduction). Nitric oxide (NO) is synthesized by NOS by oxidation of L-arginine to L-citrulline. L-NAME is an irreversible inhibitor of constitutive NOS and a reversible inhibitor of inducible NOS. Cells were preincubated with L-NAME for 1 hr before each experiment, and L-NAME was left throughout the 24 hrs of reperfusion phase. Immediate cell death was compared between controls, BSS and DMEM using the one way ANOVA test, differences were statistically significant (p<0.001). Multiple pairwise comparisons were done within each group using the Tukey's test. L-NAME (1mM) significantly (p<0.05) decreased immediate cell death only in the control group. Experiments were also done with a higher dose of L-NAME (10 mM), as it is a competitive inhibitor of NOS, and DMEM contains 84 mg/L of L-Arginine, the substrate for NOS. However the addition of 10 mM L-NAME significantly (p<0.05, Tukey's test) increased immediate cell death in all groups (Figure 27A).

Delayed cell death (Figure 27B) in the presence of L-NAME (0, 1 mM and 10 mM), was compared within controls, BSS and DMEM using the one way ANOVA test, differences were statistically significant (p<0.001). L-NAME at 1 mM significantly (p<0.05, Tukey's test) decreased delayed cell death in the control group, increased (p<0.05, Tukey's test) cell death in DMEM and (p>0.05 Tukey's test) in BSS. L-NAME 10 mM significantly (p<0.05, Tukey's test) increased cell death in controls, BSS and DMEM as compared to cells in 1 mM L-NAME or 0 L-NAME.
These graphs Figure 27A and 276B illustrate the effects of inhibition of nitric oxide using L-NAME, an irreversible competitive inhibitor of nitric oxide synthase (NOS). Cells were pre-incubated with L-NAME for 1 hr before the start of OGD in the concentration of 1mM (decided from literature review) and 10 mM (as DMEM contains arginine, a substrate of NOS).

Neuronal cells were subjected to OGD and reperfusion in the presence of 1mM and 10mM L-NAME and compared with cell death in the absence of L-NAME. Data represent means and SEM from at least 2 experiments, all cells in each experiment were derived from the same subculture. The sample size of each group in every experiment varied from 3-6 for immediate cell death and from 3-4 for delayed cell death. Number of cells plated for each experiment was maintained constant at 5x10^4. Statistical significance was calculated using the one way ANOVA test for immediate and delayed time points, and multiple comparisons done using the Tukey's test. (*) indicate significance (p<0.05, Tukey's test) between each group (0/1/10 mM L-NAME) at both immediate and delayed time point.

The presence of L-NAME at 1 mM and 10 mM during OGD and reperfusion in DMEM or BSS is not protective to neuronal PC12 cells.
Figure - 27

A. Effect of NOS inhibition with L-NAME on OGD induced immediate neuronal cell death

B. Effect of NOS inhibition with L-NAME on OGD induced delayed neuronal cell death
21 Ischemic Preconditioning (IPC) Using BSS On OGD Induced Cell Death

In both heart and brain, ischemic preconditioning (IPC) depends on the intensity of ischemia and the latency between insults. Neuronal PC12 cells (NGF 25 ng/ml) were subjected to IPC in BSS for 30, 60 and 90 mins, 24 hrs later and 48 hrs later, cells were exposed to a major insult of OGD for 6 hrs in BSS. Prior experiments had determined OGD in BSS for 30, 60 and 90 mins as sublethal and therefore these duration of OGD in BSS were tried for inducing IPC. Controls represent cells given only a media change, no preconditioning. Immediate mean cell death (Figure 28A) at an interval of 24 hrs was found to be significantly lesser (p<0.05, Tukey's test) in the IPC (30, 60 and 90 mins) group as compared to controls (No IPC). At 48 hrs interval immediate mean cell death was significantly lesser only in the IPC 30 mins group (28.33±0.58%) as compared to controls (No IPC, 38.54±2.38%).

Delayed cell death (Figure 28B) was significantly (p=0.003, ANOVA) different only at the 24 hr interval group not at the 48 hr interval group (p=0.761, ANOVA). At 24 hrs interval delayed mean cell death at IPC 30 mins was 22.10±0.53%, IPC 60 was 25.17±1.25%, IPC 90 mins was 26.39±0.61% as compared to controls (Non IPC) at 24.26±0.17%.

The above paradigm of IPC using BSS is not protective in neuronal PC12 cells exposed to OGD for 6 hrs in BSS.

22 Hypoxic (HPC)/Ischemic Preconditioning (IPC) Using DMEM

Several reports have documented that tolerance to lethal ischemia can be induced by a preceding period of hypoxia or ischemia. These experiments were done in 100ng/ml NGF ("OLD LOT NGF"). Cells were subjected to IPC/HPC for 15 mins in DMEM, and 4 hrs, 24 hrs and 48 hrs later cells were exposed to a major insult of OGD for 90 mins in DMEM. The duration of 15 mins under OGD/OD in DMEM had been determined to be sublethal from previous experiments and was therefore used to induce preconditioning. Controls were cells subjected only to a media change, no preconditioning. Statistical significance values were derived using the one way ANOVA test, (p=0.086)
at 4 hrs, at 24 hrs (p=0.039), at 48 hrs (p<0.001). At all time points studied, cell death was greater in the preconditioned groups as compared to the controls (NO IPC/HPC) (Figure 29A).

Delayed cell death was compared at each time point using the one way ANOVA test, at each time point studied there was no statistically significant difference in cell death between preconditioned and controls (No IPC/HPC) at 4 hr (p=0.082), 24 hrs (p=0.498) and 48 hrs (0.773) interval (Figure 29 B).

These findings suggest that neither ischemic nor hypoxic preconditioning in DMEM can protect neuronal PC12 cells from OGD in DMEM using the above paradigm.
Figure – 28: Ischemic Preconditioning Using BSS

Figure 28 shows the effects of ischemic preconditioning (IPC) using BSS for both the sublethal insult and the lethal insult. Cell death immediately after the insult is represented in Figure 28A and delayed cell death is represented in Figure 28B.

Cells were subjected to IPC in BSS for 30 mins, 60 mins and 90 mins. OGD in BSS for 6 hrs represented the major lethal insult. The intervals between IPC and major insult studied were 24 hrs and 48 hrs, with cell death assessed at immediate and delayed time points. Data represent means and SEM from at least 3 experiments, all cells in each experiment were derived from the same subculture. The sample size of each group in every experiment varied from 3-6 for immediate cell death and from 3-4 for delayed cell death. Number of cells plated for each experiment was maintained constant at 5x10^4. Statistical significance was calculated using the one way ANOVA test for immediate and delayed time points, and multiple comparisons done using the Tukey’s test. X axis shows critical time intervals studied i.e. 24 hrs and 48 hrs. Y axis shows the percentage of maximal LDH release. Controls in this case represent cells which have not been preconditioned. (*) indicates significance (p<0.05, Tukey’s test) between experimental and control groups.

This paradigm of ischemic preconditioning using BSS is not protective in my model of OGD in BSS for 6 hrs.
**Figure - 28**

A. Effect of IPC on OGD (BSS) Induced Immediate Neuronal Cell Death

B. Effect of IPC on OGD (BSS) Induced Delayed Neuronal Cell Death
Figure 29: Ischemic/Hypoxic Preconditioning In DMEM

Figure 29 shows the effects of ischemic preconditioning (IPC) and hypoxic preconditioning (HPC) using DMEM for both the sublethal insult and the lethal insult. Cell death immediately after the insult is represented in Figure 29A and delayed cell death is represented in Figure 29B. These experiments were done in 'OLD' LOT NGF.

Cells were subjected to IPC and HPC in DMEM for 15 mins. OGD in DMEM for 90 mins represented the major lethal insult. The intervals between IPC/HPC and major insult studied were 4 hrs, 24 hrs and 48 hrs, with cell death assessed at immediate and delayed time points. Data represent means and SEM from at least 3 experiments, all cells in each experiment were derived from the same subculture. The sample size of each group in every experiment varied from 3-6 for immediate cell death and from 3-4 for delayed cell death. Number of cells plated for each experiment was maintained constant at 5x10⁴. Statistical significance was calculated using the one way ANOVA test for immediate and delayed time points, and multiple comparisons done using the Tukey's test. X axis shows critical time intervals studied i.e. 4 hrs, 24 hrs and 48 hrs. Y axis shows the percentage of maximal LDH release. Controls in this case represent cells which have not been preconditioned.

This ischemic (IPC) and hypoxic (HPC) preconditioning paradigm used in my model of OGD for 90 minutes is not protective.
Figure - 29

A. Effect of IPC/HPC on OGD (DMEM) Induced Immediate Neuronal Cell Death ('OLD' LOT NGF)

Critical Interval 4/24/48 hrs, OGD - 90 mins in DMEM

B. Effect of IPC/HPC on OGD (DMEM) Induced Delayed Neuronal Cell Death ('OLD' LOT NGF)

Critical Interval 4/24/48 hrs, OGD - 90 mins in DMEM
23 Response Of Cells In 2X NGF To OGD Induced Neuronal Cell Death

Cells were differentiated in 2X NGF, new lot (50ng/ml) for 7 days and then exposed to 3 and 5 hrs of OGD. Immediate mean cell death (Figure 30A) was not significantly different (p=0.068) between the 3 groups following a 3 hr insult, but was significant (p<0.001) at 5 hrs of OGD. Multiple pairwise comparisons done using the Tukey’s test showed significantly (p<0.05) increased cell death (11.83±0.38%) in the DMEM group. (*) indicates significant difference (p<0.05, Tukey’s test) between experimental group and controls, (**) indicates significant difference (p<0.05, Tukey’s test) between the experimental groups.

Delayed mean cell death was significantly different (p<0.001) after both 3 and 5 hrs of OGD. Tukey’s test revealed a significant (p<0.05) increase in cell death (33.24±2.36%) in the DMEM group (Figure 30B) after 5 hrs of OGD.

24 Response Of Cells In 4X NGF To OGD Induced Neuronal Cell Death

Cells were differentiated in 4X NGF new lot (100ng/ml) for 7 days and then exposed to 2 hrs and 5 hrs of OGD. Statistical comparison within the immediate group at each time point revealed a significant difference at 2 hrs (p=0.027) and at 5 hrs (p<0.001). Multiple pairwise comparisons using the Tukey’s test, showed a significantly (p<0.05) increased cell death in both DMEM and BSS compared to controls (Figure 31A). (*) indicates significant difference (p<0.05, Tukey’s test) between experimental group and controls, (**) indicates significant difference (p<0.05, Tukey’s test) between the experimental groups.

Statistical comparison of delayed cell death using the one way ANOVA test, showed no significant (p=0.092) difference at 2 hrs, but 5 hrs of OGD was significant (p<0.001) (Figure 31B). Multiple comparisons using the Tukey’s test showed a significantly (p<0.05) increased cell death in both DMEM (21.23±0.66%) and BSS (16.45±1.30%) as compared to controls (11.88±0.25%) (Table 4).
Figure - 30: Effect Of 2X NGF On OGD Induced Cell Death

Figure 30 represents the effects of doubling (2X) the concentration of NGF on OGD induced cell death. OGD induced cell death immediately after the insult is shown in Figure 30A and delayed cell death is shown in Figure 30B.

PC12 cells were differentiated in 50ng/ml (new lot) NGF for 7 days. Neuronal cells were then subjected to OGD in DMEM and BSS for 3 hrs and 5 hrs respectively. Data represent means and SEM from at least 2 experiments, all cells in each experiment were derived from the same subculture. The sample size of each group in every experiment were 3 dishes for each immediate and delayed cell death. Number of cells plated for each experiment was maintained constant at 5x10⁴ Statistical significance was calculated using the one way ANOVA test for immediate and delayed time points, and multiple comparisons done using the Tukey’s test. (*) indicates significant difference (p<0.05, Tukey’s test) between experimental group and controls, (**) indicates significant difference (p<0.05, Tukey’s test) between the experimental groups.

X axis shows the duration of OGD i.e. 3 and 5 hrs, Y axis shows the percentage of maximal LDH release.
Figure - 30

A. Effect of 2X NGF on OGD Induced Immediate Neuronal Cell Death

B. Effect of 2X NGF on OGD Induced Delayed Neuronal Cell Death
**Figure 31: Effect Of 4X NGF On OGD Induced Cell Death**

Figure 31 represents the effects of quadrupling (4X) the concentration of NGF on OGD induced cell death. OGD induced cell death immediately after the insult is shown in Figure 31A and delayed cell death is shown in Figure 31B.

PC12 cells were differentiated in 100ng/ml (new lot) NGF for 7 days. Neuronal cells were then subjected to OGD in DMEM and BSS for 2 hrs and 5 hrs respectively. Data represent means and SEM from at least 3 experiments, all cells in each experiment were derived from the same subculture. The sample size of each group in every experiment varied between 3 and 4 dishes for each immediate and delayed cell death. Number of cells plated for each experiment was maintained constant at 5x10^4. Statistical significance was calculated using the one way ANOVA test for immediate and delayed time points, and multiple comparisons done using the Tukey’s test. (*) indicates significant difference (p<0.05, Tukey’s test) between experimental group and controls, (**) indicates significant difference (p<0.05, Tukey’s test) between the experimental groups.

X axis shows the duration of OGD i.e. 2 and 5 hrs, Y axis shows the percentage of maximal LDH release.
## Table 4

**Effect of 4X NGF (25ng/ml) on OGD induced cell death**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Sample size (n) = no. of dishes</th>
<th>% Immediate cell death mean ± sem</th>
<th>ANOVA test Significance (p)</th>
<th>Tukey’s test Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (2 hrs)</td>
<td>11</td>
<td>6.86±0.12</td>
<td>P=0.027</td>
<td></td>
</tr>
<tr>
<td>BSS (2 hrs)</td>
<td>11</td>
<td>10.02±1.38</td>
<td>P&lt;0.05/C(*)</td>
<td></td>
</tr>
<tr>
<td>DMEM (2 hrs)</td>
<td>11</td>
<td>9.26±0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (5 hrs)</td>
<td>8</td>
<td>7.22±0.49</td>
<td>P&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BSS (5 hrs)</td>
<td>8</td>
<td>10.63±0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM (5 hrs)</td>
<td>8</td>
<td>13.80±0.82</td>
<td>P&lt;0.05/C (*), BSS(++)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Sample size (n) = no. of dishes</th>
<th>% Delayed cell death mean ± sem</th>
<th>ANOVA test Significance (p)</th>
<th>Tukey’s test Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (2 hrs)</td>
<td>11</td>
<td>13.28±0.52</td>
<td>P=0.092</td>
<td></td>
</tr>
<tr>
<td>BSS (2 hrs)</td>
<td>12</td>
<td>12.34±0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM (2 hrs)</td>
<td>12</td>
<td>13.88±0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (5 hrs)</td>
<td>7</td>
<td>11.88±0.25</td>
<td>P&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>BSS (5 hrs)</td>
<td>8</td>
<td>16.45±1.30</td>
<td>P&lt;0.05/C(*)</td>
<td></td>
</tr>
<tr>
<td>DMEM (5 hrs)</td>
<td>8</td>
<td>21.23±0.66</td>
<td>P&lt;0.05/C(*), BSS(++)</td>
<td></td>
</tr>
</tbody>
</table>
25 NGF Is Neuroprotective In OGD In A Dose Dependent Manner

The effects of 5 hrs of OGD on cell death, was studied in cells differentiated for 7 days in 1X(25ng/ml), 2X(50ng/ml) and 4X(100ng/ml) NGF(new lot). Statistical comparisons were done from within each group using the one way ANOVA test, both immediate and delayed cell death showed significant (p<0.001) differences within the groups. Increasing the concentration of NGF significantly (p<0.05) decreased immediate mean cell death in the DMEM group. Immediate mean cell death in 1X NGF in DMEM was 29.09±2.12%, doubling NGF to 2X decreased cell death to 11.83±0.38%. Cell death in BSS as well as controls also decreased (Figure 32A).

Delayed mean cell death (Figure 32B) was significantly (p<0.05) decreased in control as well as experimental groups. Cell death in DMEM at 1X NGF (47.76±2.94%), decreases in 2X NGF to (33.24±2.36%) and decreases further in 4X NGF (21.23±0.66%).

NGF protects neuronal PC12 cells from OGD induced cell death in a dose dependent manner.

26 Role Of NGF Induced Protection Against Serum Deprivation

Differentiation in NGF improves survival of PC12 cells under conditions of serum deprivation. I have compared cell death in undifferentiated and differentiated cells exposed to normoxic conditions in culture medium lacking serum for 24 hrs. Differentiated cells in addition were in the presence of 25 ng/ml of NGF throughout. Mean percentages of cell death were significant(p<0.001) using the one way ANOVA test. (*) indicates significance (p<0.05, Tukey's test) between groups. Cell death in undifferentiated PC12 cells (33.49±0.90%) was significantly greater than cell death in differentiated cells 1X NGF (15.28± 0.49%) and 4X NGF (11.88±0.25%) following 24 hrs of serum deprivation (Figure 33). This probably reflects the serum dependence of undifferentiated cells and the neuroprotective effect of NGF against serum deprivation in case of differentiated cells. Serum deprivation is known to increase ROS generation as serum has anti-oxidants. NGF increases endogenous anti-oxidants SOD, catalase and GSH thereby protecting against ROS and thereby serum deprivation.
Figure – 32: Dose Dependent Protection Of NGF Against OGD

This Figure 32 represents a composite graph showing the dose dependent protective effect of NGF on OGD for 5 hrs. Immediate cell death is represented in Figure 32A and delayed cell death in Figure 32B.

Data used in this graph has been obtained from Figure 30 and Figure 31, and it shows a comparison of increasing doses of NGF from OGD induced cell death. Statistical significance was calculated using the one way ANOVA test for immediate and delayed time points, and multiple comparisons done using the Tukey's test, (*) indicates significance p<0.05, Tukey's test. X axis shows the duration of OGD i.e. 5 hrs, Y axis shows the percentage of maximal LDH release.
Figure - 32

A. Comparative Effect of 1X/2X/4X NGF on OGD Induced Immediate Cell Death

B. Comparative Effect of 1X/2X/4X NGF on OGD Induced Delayed Neuronal Cell Death
Figure 33: Protective Effect Of NGF On Serum Withdrawal Induced Cell Death

Figure 33 shows a comparison of cell death following 24 hrs of serum deprivation in undifferentiated cells and differentiated cells (1X and 4X NGF).

Undifferentiated and differentiated cells (1X and 4X NGF) were kept under normoxic conditions in the presence of glucose for 24 hrs. Cytotoxicity was then determined using the LDH assay. Data represent means of at least 3 experiments. Statistical significance was calculated using the one way ANOVA test for immediate and delayed time points, and multiple comparisons done using the Tukey's test, (*) indicates significance p<0.05, Tukey's test. X axis shows the duration of serum deprivation for 24 hrs, Y axis shows the percentage of maximal LDH release.
Figure - 33

Effect of NGF on Serum Deprivation (24 hrs) Induced Cell Death

% of Maximal LDH Release

Duration of Serum Deprivation - 24 hrs
27 Hypothermia (24°C) During OGD Is Neuroprotective

Hypothermia can protect during ischemia by preserving high energy phosphates and excitatory amino acid inhibition. It can also decrease intracellular calcium and free radical generation. Closed incubator room temperature was maintained at 24°C and confirmed with a thermometer and no exchange with outside air was allowed during the 1 hr duration of the experiment. These experiments were done in 100ng/ml NGF ('OLD' LOT). Statistical comparisons on immediate cell death using the one way ANOVA test, showed a significant (p=0.018) difference within the group, Tukey's test was significantly (p>0.05) different on decreasing the temperature in both groups (Figure 34A). Cell death in DMEM at 37°C was 12.04±1.38%, it decreased to 9.72±1.04% at 24°C.

Delayed cell death was significantly different (p<0.001, ANOVA), among the groups, Tukey's test revealed that hypothermia at 24°C causes a significant (p<0.05, * , Tukey's test) decrease in cell death in the DMEM group from 31.11±0.94% to 21.15±1.26% (Figure 34B).

28 Hypothermia (24°C) During OD Is Neuroprotective

Cells were exposed to OD alone, in the presence of glucose at 24°C and at 37°C. Statistical comparisons done from within the immediate group was significant (p<0.001) (Figure 35A). These experiments were done in 100ng/ml NGF ('OLD' LOT NGF).

One way ANOVA test was significant (p=0.005) in the delayed group, cell death decreased in the DMEM group from 14.10±1.72% to 10.90±1.05% under hypothermic conditions.
Figure - 34: Effect Of Hypothermia On OGD Induced Cell Death

This Figure 34 shows the effects of hypothermia (24°C) during OGD on immediate (Figure 34A) and delayed (Figure 34B) cell death.

Cells were differentiated in 100ng/ml NGF (OLD LOT) and subjected to 1 hour of OGD at 37°C and 24°C in BSS and DMEM. During reperfusion all cells were kept at 37°C. Data represent means of at least 2 experiments, all cells in each experiment were from the same culture. Statistical comparisons for immediate and delayed groups were done using the one way ANOVA test and multiple comparisons done using the Tukey's test. X axis shows the duration of OGD for 1 hour, Y axis shows the percentage of maximal LDH release.
Figure - 34

A. Effect of Hypothermia on OGD Induced Neuronal Cell Death ('OLD' LOT NGF)

<table>
<thead>
<tr>
<th>% of Maximal LDH Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>BSS</td>
</tr>
<tr>
<td>DMEM</td>
</tr>
</tbody>
</table>

37°C  24°C
Duration of OGD - 1 hr

B. Effect of Hypothermia on OGD Induced Neuronal Cell Death ('OLD' LOT NGF)

<table>
<thead>
<tr>
<th>% of Maximal LDH Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>BSS</td>
</tr>
<tr>
<td>DMEM</td>
</tr>
</tbody>
</table>

37°C  24°C
Duration of OGD - 1 hr
Figure 35: Protective Effect Of Hypothermia In OD

This Figure 35 shows the effects of hypothermia (24°C) during OD on immediate (Figure 35A) and delayed (Figure 35B) cell death.

Cells were differentiated in 100ng/ml NGF ('OLD LOT) and subjected to 1 hour of OD at 37°C and 24°C in BSS and DMEM. During reperfusion all cells were kept at 37°C. Data represent means of at least 2 experiments, all cells in each experiment were from the same culture. Statistical comparisons for immediate and delayed groups were done using the one way ANOVA test and multiple comparisons done using the Tukey's test. X axis shows the duration of OD for 1 hour, Y axis shows the percentage of maximal LDH release.
Figure - 35

A. Effect of Hypothermia on OD Induced Immediate Neuronal Cell Death ('OLD' LOT NGF)

B. Effect of Hypothermia on OD Induced Delayed Neuronal Cell Death ('OLD' LOT NGF)
Summary Of Results

1. Undifferentiated and differentiated PC12 cells show a significant increase in cell death with increasing duration of OGD in culture medium (DMEM) as compared to balanced salt solution (BSS).

2. Oxygen deprivation alone does not significantly increase cell death in neuronal PC12 cells.

3. Culture medium (DMEM) increases significantly the sensitivity of neuronal PC12 cells to substrate deprivation, balanced salt solution (BSS) does not.

4. Rapid cellular morphological changes ultimately leading to cell lysis, along with a rapid rise in LDH levels suggest a major contribution of necrosis to cell death following OGD in culture medium (DMEM).

5. Complete glycolytic inhibition using 2 Deoxy-D-Glucose (2DG), causes a similar level of cell death in both culture medium (DMEM) and balanced salt solution (BSS).

6. L-Glutamine supplementation during OGD protects neuronal PC12 cells from oxygen-glucose deprivation (OGD) induced cell death.

7. The presence of iron in culture medium (DMEM), alone is not sufficient to increase cell death.

8. Confocal microscopy indicates two different patterns in oxidative stress during OGD. There is a rise in free radical generation in the culture medium (DMEM) group, but no change in cells in balanced salt solution (BSS).

9. Confocal microscopy also shows a significant burst in free radical generation during the early phase of reperfusion.

10. Flow cytometry confirms the findings of increased free radical generation in a population of cells in culture medium (DMEM) as compared to controls or cells in balanced salt solution (BSS).

11. The presence of anti-oxidants SOD and Catalase during OGD and reperfusion significantly decreases cell death in neuronal PC12 cells exposed to OGD in culture medium (DMEM).

12. NOS inhibitor L-NAME is not protective against OGD in neuronal PC12 cells.

13. Ischemic (IPC) or hypoxic (HPC) preconditioning in balanced salt solution (BSS) or in culture medium (DMEM) does not improve survival following OGD in neuronal PC12 cells.

14. Differentiation in NGF protects PC12 cells from oxygen-glucose deprivation and serum deprivation induced cell death in a dose dependent manner.

15. Hypothermia decreases cell death following oxygen-glucose deprivation (OGD) or oxygen deprivation (OD) in neuronal PC12 cells.
**Figure – 36: Summary Of Results**

<table>
<thead>
<tr>
<th>Establishing Model Of OGD/OD/GD</th>
<th>Role of Oxidative Stress</th>
<th>Neuroprotective Strategies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.</strong> DMEM increases cell death – OGD/GD in Diff/undiff. PC12 cells</td>
<td><strong>1.</strong> Confocal microscopy and Flow-cytometry demonstrate an <em>increase in ROS</em> in DMEM</td>
<td><strong>1.</strong> NGF protects against OGD and serum deprivation</td>
</tr>
<tr>
<td><strong>2.</strong> OD does not increase cell death</td>
<td><strong>2.</strong> Lipid peroxidation is increased in cells in DMEM</td>
<td><strong>2.</strong> Hypothermia is neuroprotective in OGD and OD</td>
</tr>
<tr>
<td><strong>3.</strong> 2DDG equalizes cell death in both groups</td>
<td><strong>3.</strong> <em>Anti-oxidants</em> are protective in cells exposed to OGD in DMEM</td>
<td><strong>3.</strong> <em>Anti-oxidants</em> in DMEM protect in OGD</td>
</tr>
<tr>
<td><strong>4.</strong> Glutamine—a energy substrate decreases cell death</td>
<td></td>
<td><strong>4.</strong> Addition of energy substrate Glutamine decreases cell death</td>
</tr>
<tr>
<td><strong>5.</strong> Iron does not increase cell death in BSS</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>6.</strong> Rapid morphological changes s/o Necrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>7.</strong> Early changes seen in Mitochondrial morphology</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

ESTABLISHING A MODEL TO STUDY OGD/OD/GD USING NEURONAL PC12 CELLS

In vivo models of ischemia have provided valuable information regarding tissue damage, changes in regional blood flow, fluctuations in ionic concentrations, etc. However in order to understand the specific nature of ischemic injury and develop better treatment and prevention strategies, it is necessary to understand the cellular and molecular mechanisms of damage. In vitro systems offer the unique ability to precisely control the environmental conditions outside the cell. Correlation between cause and effect is much easier to establish in such systems, in the absence of the many overlying in vivo homeostatic influences, including blood flow and the presence of non-neuronal cells. These studies are often conducted to aid in the interpretation of observations obtained in vivo, whereas at other times, they provide the very foundation upon which in vivo experiments are based (Tymianski M. et al. 1993).

Clonal cell lines which express neuronal properties are useful model systems for studying the nervous system at the single cell and molecular levels. Such lines have been established from human and murine neuroblastomas (Kolber A. R. et al., 1974, McMorris F. A. et al., 1973), rat central nervous system tumors (Schubert D. et al., 1974) and rat Pheochromocytoma (Greene L. A. and Tischler A. S., 1976). Clonal rat Pheochromocytoma (PC12) cell, which are derived from normal medullar chromaffin cells, are widely used as a model system for sympathetic ganglion like neurons. The rat Pheochromocytoma cell line PC12 represents a useful catecholaminergic system. After treatment with NGF for 7-14 days, PC12 cells develop features of mature sympathetic neurons i.e. stop dividing, develop neurite outgrowths, electrical excitation and express tetanus toxin binding sites. PC12 cells provide an attractive culture system in which the underlying mechanisms associated with cellular response to oxygen deprivation may be explored (Caroll J. M. et al., 1992).

To summarize my results: My initial goal was to develop a model to study the cellular and molecular mechanisms of damage in oxygen-glucose/oxygen and glucose deprivation using
differentiated PC12 cells. Using an anoxic chamber and glucose deficient media, I evaluated cytotoxicity under conditions of substrate deprivation (BSS) and partial substrate availability (DMEM). My experiments indicated that a primary cause of cytotoxicity in DMEM was oxidative stress. Preliminary experiments with the intracellular Ca^{2+} indicator, Fio-4 also showed a parallel increase in Ca^{2+} during OGD in cells in DMEM. NGF, hypothermia, anti-oxidants and L-Glutamine have been found to be neuroprotective in this model. My studies used a stable PC12 cell line which expresses an inducible mitochondrially targeted GFP fusion protein – PC12 GFP_{mit} (Wadia thesis in progress), enabling me to study simultaneously both cellular and mitochondrial morphology during OGD and reperfusion. This is the first study of live mitochondrial morphological changes, during OGD in differentiated PC12 cells. Finally, unlike primary neuronal cultures and cardiomyocytes which can be preconditioned and partially protected from a subsequent ischemic insult, differentiated PC 12 cells could not be preconditioned with the paradigms I tested.

1. **PC12 Cells In Ischemia (OGD)**

Differentiation with NGF induces a neuronal phenotype, cells cease division, acquire electrical excitability, extend long branching neurites and ultimately develop many characteristics of mature sympathetic neurons (Thoenen H. and Barde Y. A., 1980). Apart from the obvious advantages of using a cell line, PC12 cells are convenient to grow without special requirements like a glial layer, their availability in large numbers and relatively good survival under serum deprived conditions compelled me to use this cell line to establish my model for ischemia (OGD), anoxia (OD) and hypoglycemia (GD). The absence of NMDA receptors and presence of mGluR group I has enabled me to study the effects of OGD in the absence of NMDA receptors. This model can also be used to provide valuable information on the role of mGluRs in OGD/OD/GD. Although there are several advantages in using this cell line, as in every model, there are a few disadvantages, it is a neoplastic cell line and cells are transformed some caution should be exercised before directly extrapolating this data to primary neurons.

PC12 cells represent an excellent model system to analyze ischemic insults to sensory and /or sympathetic neurons, NGF target cells (Raya S. A. et al., 1993). Such ischemic experiments with sensory or sympathetic ganglia in vitro which depend on NGF for their survival (Raya S. A. et al., 1993) are difficult to perform due to ganglia death in the absence of NGF. PC12 cells differentially
express group I mGluRs (Kane M. D. et al., 1998). Activation of the metabotropic glutamate receptors may also contribute to a rise in intracellular Ca\(^{2+}\) in ischemia, as a result of mobilization of Ca\(^{2+}\) from the intracellular stores (Love S., 1999). A controversy exists on whether PC12 cells have NMDA receptor, the general notion is that they do not have NMDA receptors. NMDA receptor mRNA has been reported in undifferentiated and differentiated PC12 cells (Lopez-Guajardo A. et al., 1993), although no functional receptors or detectable NMDA proteins were found. Studies by Schubert et al., (1992) using patch-clamp techniques demonstrated NMDA and glutamate induced responses in PC12 cells. PC12 cells can derive much of their energy in the form of ATP from glycolysis, therefore inhibition of oxidative phosphorylation alone does not necessarily result in depletion of ATP stores because the glycolytic pathway is still intact. Thus both glycolysis and oxidative phosphorylation must be inhibited for total depletion of ATP stores (Basma A. N. et al., 1992).

I am simulating ischemia, by combining both hypoxia and glucose deprivation in culture medium using differentiated PC12 cells. These conditions might mimic certain circumstances of incomplete brain ischemia (Siesjo B. K., 1981) when partial substrate deprivation occurs as in haemorrhagic stroke. This work also represents the response of dopaminergic cells to ischemia which can occur in global ischemia. Essentially I have compared cell death in cells exposed to OGD/OD/GD in culture medium (DMEM) where alternative substrates are available (see Appendix 1), with balanced salt solution (BSS) lacking any substrate (see Appendix 2). DMEM was chosen as I needed a medium that was formulated lacking glucose, glutamine and sodium pyruvate, at the same time containing all the other necessary nutrients to support cells in the absence of serum. Previously there have been few other studies using DMEM as the experimental solution, and physical methods of hypoxia, to induce OGD/OD in PC12 cells (Abu-Raya S. et al., 1999, Kumar G. K. et al., 1998, Raya S. A. et al., 1993), these were primarily to study eicosanoid and catecholamine release. Most studies of hypoxia in PC12 cells use chemical hypoxia (cyanide, rotenone, antimycin A) in BSS, which affects the mitochondrial respiratory complexes and thereby free radical generation (Pereira C. et al., 1998, Caroll J. M. et al., 1992). To avoid the influence of these factors on ROS generation during OGD, I have confined my studies to only physical methods to induce anoxia/ischemia. Typically BSS is the choice as an experimental medium to induce OGD in all cells including PC12 (Pereira C. et al., 1998, Caroll J. M. et al., 1992), primary neuronal cultures (Goldberg M. P. and Choi D. W., 1993) and cardiomyocytes (Becker L. B. et al., 1999).
To date there have been no studies in undifferentiated/differentiated PC12 cells examining the mechanisms of increased cell death during OGD/GD in the more complex culture medium compared to BSS. Cell injury was quantitatively assessed by measuring lactate dehydrogenase (LDH) released from damaged cells into the extracellular medium during the insult and 24 hrs later (Goldberg M. P. and Choi D. W., 1993). My preliminary experiments comparing between LDH assay and PI staining, showed excellent correlation between the two modes of cell death analysis validating the use of the LDH assay in my model. The analysis of live cultures was found to cause time constraints, also the presence of floating cells- 'floaters' made it impossible to use PI counts, therefore necessitating the use of the LDH assay as a measure of cell death and damage for further experiments. In this study, as the duration of OGD increases, cell death in the DMEM group steadily increases; the degree of cell death in BSS was similar to that observed in other studies using differentiated PC12 cells (Abu-Raya S. et al., 1999) and immediate cell death is not significantly different from controls until 6 hrs of OGD. Previous studies using cortical cells in culture, show a substantial LDH release 2 hrs after the conclusion of OGD, maximal release is seen 12-16 hrs later. The LDH signal measured 1 day after OGD exposure is supposed to reflect the degree of overall neuronal injury (Goldberg M. P. and Choi D. W., 1993). Consequently, I have assessed in all my experiments the effects of OGD/OD/GD at two time points – immediate (to reflect acute injury) and delayed (24hrs later to assess overall injury).

**What are the metabolic changes during OGD in PC12 cells?** Undifferentiated (Basma A. N. et al., 1992) and differentiated (Morelli A. et al., 1986) PC12 cells have a high rate of glycolysis accompanied by a large production of lactate and a low use of glucose carbon through the Krebbs cycle. Normally pyruvate formed from glucose in the glycolytic pathway is oxidatively metabolized. However under anaerobic conditions, the ratio of NADH/NAD+ is increased and pyruvate is converted to lactate. Lactate can be used by the cells, if glucose is unavailable (Basma A. N. et al., 1992). PC12 cells rely heavily on glycolysis for their energy needs, mitochondrial respiration is also crucial for cell survival. It is likely that the increase in NADH levels and decrease in NAD+ content after inhibition of mitochondrial respiration leads to the impairment of enzymatic reactions that require NAD+ ultimately resulting in cell death. The increase in lactate production could also partly explain the toxicity by increasing the acidity of the medium and this may affect cell survival (Basma A. N. et al., 1992). Cell death here is unlikely to be a receptor dependent phenomenon as the cells were the same in both groups (DMEM and BSS) and obtained from the same culture. Both the increase in
lactate or a receptor dependent phenomenon alone, cannot be responsible for the increase in cell death during ischemia in culture medium (DMEM).

2. **PC12 Cells In Hypoxia (OD)**

Reduction of available oxygen is a key feature of ischemia. Hypoxia is known to exert a diverse range of responses in cells, each of which serves a specific physiological purpose (Taylor S. C. et al., 1999). Acute hypoxia (oxygen deprivation in the presence of glucose) can evoke extremely rapid responses such as selective membrane delimited inhibition of ion channels (Czyzyk-Krzeska M. F. et al., 1994). Acute hypoxia evokes catecholamine release from PC12 cells by causing membrane depolarization and subsequent Ca\(^{2+}\) influx, primarily through N type voltage gated Ca\(^{2+}\) channels (Taylor S. C. et al., 1998). Furthermore at any given PO\(_2\) release of NE is greater than release of DA, and a protein kinase dependent pathway(s) seem to be associated with NE but not DA release during hypoxia (Taylor S. C. et al., 1998). Effects of acute and chronic hypoxia on various cellular processes, including ion channel activity and gene expression (Taylor S. C. et al., 1998, Czyzyk-Krzeska M. F. et al., 1994, Kobayashi S. et al., 1998) have been studied in PC12 cells. These cells survive hypoxia (5% O\(_2\)) up to several hours, reminiscent of the large viability differences existing between brain neurons in their sensitivity to hypoxia and anoxia (Siesjo B. K., 1981). Other in vitro studies have also suggested that an increased availability of extracellular glucose may reduce the impact of hypoxia on central neurons. In hippocampal slice, recovery from hypoxia induced synaptic transmission failure depends directly on the concentration of extracellular glucose (Goldberg M. P. and Choi D. W., 1993).

My results are consistent with previous studies confirming that hypoxia is less detrimental to PC12 cells than ischemia (Pereira C. et al., 1998). In NGF-differentiated PC12 cells there is a marked increase of glucose utilization via the oxidative pathway of the Krebs cycle (Morelli A. et al., 1986) as for brain neurons. PC12 cells have multiple pathways that are capable of maintaining ATP production. They can maintain ATP concentrations in the absence of oxygen if glucose is present (Pereira C. et al., 1998, Caroll J. M. et al., 1992). This suggests that under hypoxia, although the synthesis of ATP in the mitochondria may be already compromised glycolysis is stimulated, resulting in the maintenance of ATP levels. The stimulation of glycolysis has been proposed to occur in synaptosomes upon inhibition of the respiratory chain with cyanide, and in retinal cells upon chemical ischemia (Pereira C. et al., 1998). This does not however occur in primary neurons which are totally
dependent on glucose/lactate/pyruvate for their ATP needs, and are incapable of using any other substrates. In the presence of glucose the ATP levels determined after incubation with mitochondrial respiratory chain inhibitors (hypoxic like conditions) in PC12 cells were not significantly different from those determined under control conditions (Pereira C. et al., 1998).

3. PC12 Cells In Hypoglycemia (GD)

Hypoglycemic brain damage is a major component of stroke and is related to ischemia. Hypoglycemic neuronal cell death has been found to exhibit some elements of necrosis and is largely mediated by excitotoxic activation of glutamate receptors although hypoglycemic neuronal primary cultures can undergo apoptosis when glutamate receptors are blocked (Tong L. and Perez-Polo R., 1995). This variability may reflect differences in the level of expression of excitotoxicity in primary cultured neurons when compared to clonal PC12 cells.

One mechanism of toxicity in hypoglycemia is elevation of intracellular Ca²⁺ (Tong L. and Perez-Polo R., 1995). Two major pathways are known to participate in elevating intracellular calcium ions in naïve PC12 cells: calcium influx through voltage gated cation channels such as L-type channels and calcium releases from an internal store sensitive to bradykinin or caffeine. Glucose withdrawal induces the elevation of intracellular calcium ions in PC12 cells that can be blocked by ryanodine but not by nifedipine. It suggests that calcium release from caffeine sensitive stores rather than calcium influx may be a major source for the elevation of intracellular calcium induced by glucose withdrawal (Chung J. and Hong J., 1998).

One possible alternative substrate for neuronal cells is lactate, if glucose is unavailable (Basma A. N. et al., 1992). Amino acid requirements are considerably different from cell to cell, PC12 cells can use amino acids as substrates. These cells are known to utilize four essential amino acids (valine, methionine, isoleucine, leucine), glutamine and arginine (Sakagami H. et al., 1998).

Previous studies (all in BSS Pereira C. et al., 1998, Caroll J. M. et al., 1992) show significantly increased cell death under ischemic and hypoglycemic conditions compared to hypoxic conditions in PC12 cells, indicating that glycolysis plays a very important role in regulating the energy status of PC12 cells (Pereira C. et al., 1998). My study is the first to show massive cell death after glucose deprivation
in DMEM (lacking glucose, glutamine and sodium pyruvate essentially causing major substrate deprivation, see Appendix 1) compared to cell death in BSS (contains only inorganic salts) (see Appendix 2). If the enhanced susceptibility of the cells in medium lacking glucose is due to a lack of glucose for glycolysis, it should have been the same in both groups. Glucose deprivation in culture medium (DMEM) which also contains valine, methionine, isoleucine and leucine, all of which are alternative substrates for PC12 cells, should have been beneficial, instead of increasing cytotoxicity. However contrary to my expectations glucose deprivation in culture medium significantly increased cell death in neuronal PC12 cells as compared to glucose deprivation in BSS.

4. Undifferentiated PC12 Cells And OGD

Undifferentiated and differentiated PC12 cells have been used in neuroscience as a model for neuronal cell death caused by serum or NGF deprivation, toxins, ischemia, hypoxia (Taylor S. C. et al., 1997, Crzyzyk-krzeska M. F. et al., 1994, Kobayashi M. et al., 1998, Taylor S. C. et al., 1998, Pereira C. et al., 1998, Abu-Raya S. et al., 1999). Catecholamines are released during hypoxia from undifferentiated PC12 cells in a Ca²⁺ dependent manner and involve activation of voltage dependent Ca²⁺ channels (Taylor S. C. et al., 1998, Siesjo B. K., 1981). Furthermore at any given PO₂, release of NE is greater than release of DA, and a protein kinase dependent pathway(s) seems to be associated with NE but not DA release during hypoxia (Kumar G. K. et al., 1998, Siesjo B. K., 1981).

In my model, undifferentiated PC12 cells show a similar pattern of increased cell death in culture medium (DMEM) in their response to OGD. Delayed cell death was however increased in the control group reflecting probably the dependence on serum for survival in undifferentiated PC12 cells. This similarity in the pattern of cell death in response to OGD in undifferentiated and differentiated PC12 cells in DMEM and BSS, suggests that the cause of increased cell death in DMEM is not due to NGF mediated differentiation. It is therefore likely that one of the possible factors responsible for increased cell death during OGD is likely to be inherent within the PC12 cell line. I have explained this in detail at the end, in the discussion of my model.
5. **Cellular And Mitochondrial Morphology During OGD And Reperfusion**

Necrosis is characterized by cellular swelling due to loss of ionic homeostasis, damage to organelles and finally cell lysis. The rapid nature of cell lysis in DMEM following OGD/GD suggests that death is primarily necrotic. Cells exposed to OGD in DMEM when viewed using phase optics showed immediate (within 30 mins) deterioration in morphology, with progressive neuronal swelling and disappearance of neurite processes. Cells became markedly swollen and plasma membrane blebbing was seen. However, cells exposed to OGD in BSS seemed to remain unaffected by OGD, morphology was very well preserved, neurite processes were unaffected. Cells were not different from controls throughout the duration of OGD. Towards the end of 5 hrs of OGD in BSS, some cells began to show swelling and loss of processes, but however changes seemed minimal as compared to cells exposed to OGD/GD in DMEM. During reperfusion, cells slowly return to their pre-insult appearance, and by 2 hrs begin to regain their normal morphology.

My experiments used a stable PC12 cell line which expresses an inducible mitochondrially targeted GFP fusion protein – PC12 GFP$_{(\text{mit})}$ consequently I had the advantage of studying the GFP-labeled mitochondria. Mitochondria are assuming an increasingly important role in hypotheses regarding necrotic and apoptotic cell death in ischemia (Eskes R. B. et al., 1998, Reed J. C., 1997). Mitochondria undergo a transient swelling for a few hours after any form of ischemia. The origin has not been studied but may be the reversible opening of the MTP due to free radical generation (Lipton P., 1999). Confocal microscopy at 20X and 60X magnification showed the rapid changes in cellular morphology which appeared in the DMEM group soon after the start of the insult. In response to OGD, the mitochondria changed from an elongated string-like structure to a condensed and swollen appearance, concentrated centrally. The cells were rounded up and showed plasma membrane blebbing. Cells in BSS are not markedly different from controls. Remarkably by 2 hrs of reperfusion, mitochondrial and cellular morphology seems to start recovering in surviving cells in both groups. This is the first study of live mitochondrial morphological changes during OGD and reperfusion in differentiated PC12 cells.

6. **Glycolytic Inhibition With 2- Deoxy-D-Glucose (2DDG)**

Glucose deprivation alone does not result in complete inhibition of glycolysis, because glucose-6-phosphate can be provided from cellular glycogen stores and by gluconeogenesis. Lactate can be used
by primary neuronal cells if glucose is unavailable (Basma A. N. et al., 1992). PC12 cells are known to utilize four essential amino acids (valine, methionine, isoleucine, leucine), glutamine and arginine (Sakagami H. et al., 1998).

The addition of 2DDG inhibits glycolytic production of ATP because its metabolite 2 Deoxyglucose-6-phosphate (2dG6P) competitively inhibits phosphoglucoisomerase. Metabolism of 2DDG to 2dG6P also depletes ATP, since a high energy phosphate from ATP is transferred to a metabolite (2dG6P) that cannot be metabolized further (Johnson M. E. et al., 1994). 2dG6P accumulates intracellularly, progressively blocking glycolysis and reducing glycogenolysis by inhibition of glycogen phosphorylase (Rose R. C. et al., 1998). This approach does not inhibit glycolysis in isolation, it reduces pyruvate the primary substrate for oxidative metabolism at the same time.

In my studies the addition of 2 DDG to both experimental solutions, increased immediate cell death in BSS to the high levels seen normally in DMEM, almost equalizing cell death in both groups. There was no change in immediate cell death with the addition of 2DDG to DMEM, this means that alternative glycolytic substrates (present in DMEM) and glycogen (stores within cells) were not being utilized to generate ATP in the first place, something prevented the use of the substrates to generate ATP. But cell death in the BSS group was increased after the addition of 2DDG, because most probably the cells were (in absence of 2DDG) utilizing the rich glycogen stores (PC12 and neoplastic cell lines are rich in glycogen stores) and generating ATP, thus increasing their survival during OGD/GD.

Reperfusion was done in 20 mM glucose to reverse the effects of 2DDG, a competitive inhibitor of glycolysis. Delayed cell death was increased in both groups and again was almost equal, suggesting that complete substrate deprivation increases and equalizes cell death in both experimental groups. Another important implication of this experiment is that, if cell death in the DMEM group does not increase after the addition of 2DDG, than something else present in DMEM is already acting to prevent glycogenolysis and gluconeogenesis (see Discussion of my model Figure 39).
7. L-Glutamine Decreases OGD Induced Cell Death

If the above described effects are seen on complete deprivation of glycolytic substrates, then what happens on addition of a metabolic substrate? I therefore studied the effect of addition of L-Glutamine (2 mM) (same concentration as regular media) on OGD induced cell death. Upon glucose deprivation, the inhibition of glycolysis may stimulate mitochondria to maintain ATP levels above a ‘critical threshold’, such that the activity of the mitochondrial dehydrogenases is not compromised (Pereira S C. et al., 1998). This may also be due to the presence of other energy sources such as glutamine, that can be used to generate ATP or even the use of lactate as a ready energy source after glucose depletion (Pereira S C. et al., 1998). PC12 cells are known to utilize four essential amino acids (valine, methionine, isoleucine, leucine), glutamine and arginine (Sakagami H. et al., 1998). The use of other energy sources by glycolysis to produce and to maintain ATP levels above a ‘critical threshold’ has been shown during hypoglycemia in PC12 cells in the presence of iodoacetic acid, an inhibitor of the glycolytic pathway (Pereira S C. et al., 1998). Recent studies also have reported that glycolysis intermediates prevent hepatocyte injury induced by chemical hypoxia, by enhancing the production of glycolytic ATP (Pereira S C. et al., 1998). The increase in the rate of anaerobic glycolysis, to preserve tissue ATP, also can occur in ischemic preconditioning, by the mediation of glucose uptake or by stimulation of key enzymes of the glycolytic pathway (Pereira S C. et al., 1998).

In my model the presence of L-Glutamine during OGD, decreased cell death in both experimental groups presumably by providing an alternative substrate for PC12 cells, thereby maintaining ATP synthesis and cellular functions.

8 Ferric Nitrate Is Not A Significant Cause Of Cell Death In DMEM

In the process of trying to identify the source of oxidative stress/cell death in my model, an important clue in this direction was obtained from Dr. Lloyd A. Greene regarding iron being a potential pro-oxidant in culture medium. DMEM contains 0.0001g/L Ferric nitrate nonahydrate (Fe₃O₆.9H₂O). Accordingly a sub-hypothesis was that, if iron alone is responsible for the cytotoxicity in DMEM then addition of iron to BSS should equalize cell death in both groups. To determine if the presence of iron alone is sufficient to cause neuronal cell death, Ferric nitrate nonahydrate (Fe₃O₆.9H₂O) was added to BSS in exactly the same concentration as was present in culture medium
(DMEM). Completely contrary to my expectations the addition of iron made no significant difference to cytotoxicity in the BSS group.

In recent years extensive studies have outlined the role of oxygen free radicals and other oxygen derived reactive species in the disruption of cellular function by the peroxidation of membranes and other cellular components. The major source endogenous sources of intracellular reactive oxygenated free radicals are oxidase enzymes and electron transport systems. The transfer of electrons between free ions of metals such as copper or iron and oxygen-containing molecules can also initiate the formation of oxygen radicals. Although several metal ions are able to induce peroxidation through the generation of free radicals, the most important endogenous metal is iron (Subbarao K. V. and Richardson J. S., 1990).

Formation of hydroxyl radical is strongly favored by the decreased pH that prevails after ischemia and also by free iron as shown below in the Fenton reaction (Lipton P., 1999).

\[
\begin{align*}
O^2- + 2H_2O & \rightarrow H_2O_2 \\
Fe^{2+} + H_2O_2 & \rightarrow Fe^{3+} + OH^- + OH^-
\end{align*}
\]

Either the oxidation of free ferrous ions or the reduction of free ferric ions will generate reactive radicals and initiate peroxidative reactions. Extensive peroxidation of membranes can be initiated by various concentrations of ferrous and ferric ions alone in the absence of oxidizing and reducing agents (Subbarao K. V. and Richardson J. S., 1990). Recent studies indicate that a major generator of oxidative stress for neurons in basal medium is iron. Iron can cause oxidative stress by promoting lipid peroxidation as well as DNA and protein damage by catalyzing the formation of hydroxyl radicals. Moreover a number of studies have shown that ferrous iron is toxic to cultured neurons even at relatively low concentrations (Farinelli S. E. et al., 1998). The arguments against the importance of the Fenton reaction are that the reactivity of the OH. may paradoxically render it less harmful than other free radicals. Furthermore the superoxide driven Fenton reaction is slow even in the presence of iron (Lipton P., 1999). Low concentrations of chelators were likely protective in
trophic factor withdrawal in PC12 and sympathetic neurons because they bind iron present in basal medium (Farinelli S. E. et al., 1998).

Contrary to previous studies highlighting the role of ferrous sulphate as a pro-oxidant and cytotoxic agent in culture medium (Farinelli S. E. et al., 1998), my studies indicate that the addition of ferric nitrate to BSS failed to increase cell death significantly. It is possible that the highly reactive hydroxyl radical by combining with other molecules renders it less harmful (Lipton P., 1999) in this model of ischemia. My results indicate that the differences in cell death between culture medium and BSS during OGD, were not solely caused by iron. Plausibly iron may not be the only source of oxidative stress in this model of ischemia.

**OXIDATIVE STRESS DURING OGD AND REPERFUSION**

Reactive oxygen species (ROS) have been implicated in the tissue injury that follows ischemia and reperfusion (Das D. K., 1994, Hearse D. J. et al., 1973, Meerson Z. F. et al., 1982, Opie L. H. et al., 1989, Zweier J. L. et al., 1987). Studies have suggested that a burst of ROS generation occurs during the first minutes after ischemic tissues are re-oxygenated, leading to the conclusion that the return of O2 to ischemic tissues is a critical event for the generation of ROS (Meerson Z. F. et al., 1982, Zweier J. L. et al., 1987, Ambrosio G. et al., 1993, Vanden Hoek T. L. et al., 1996).

**9 Free Radical Generation During OGD And Reperfusion**

Considerable controversy (Sylvester J. T., 2001, Waypa G. B. et al., 2001, Becker L. B. et al. 1999) exists on whether free radicals are increased or decreased during OGD. The traditional view has been that if sufficiently severe, hypoxia slows mitochondrial electron transport, leading to accumulation of reducing equivalents, decreased ROS production and a shift of cytosolic redox state toward reduction (Sylvester J. T., 2001). In hypoxia/ischemia, glycolysis is enhanced and nicotinamide adenine dinucleotide which acts as an electron donating molecule is produced and enters the mitochondrial electron transport chain. Electrons cannot be transferred to oxygen because of the lack of oxygen and therefore accumulate in the mitochondria, from which they then leak in greater quantities. At the time of re-oxygenation, abundant oxygen becomes available for these electrons to react with, resulting in
the production of large quantities of free radicals, which are then thought to at once cause neuronal damage (Murata T. et al., 2000).

In my model, there is both a massive increase in free radicals in cells in DMEM throughout the duration of OGD, whereas cells in BSS are not very different from controls. If this is true, ROS generation during ischemia could have important implications for ischemia-reperfusion therapies and may explain why clinical trials of antioxidants given only at reperfusion have failed to show benefit (Becker L. B. et al., 1999). A study in chick cardiomyocytes showed an increase in ROS generation during OGD in BSS, a probable cause suggested was the presence of residual oxygen during ischemia, giving rise to the possibility that ROS generation may also occur before reperfusion (Becker L. B. et al., 1999). Although ischemia is associated with lowered oxygen levels, it is not normally associated with anoxia. Moreover ischemia must be accompanied by progressive tissue hypoxia creating an opportunity for significant ROS generation. Animal studies of tissue ischemia rarely show PO\textsubscript{2} levels < 4 Torr, therefore sufficient oxygen is still available to create superoxide during ischemia (Becker L. B. et al., 1999).

Multiple sources of oxidant generation could function during ischemia, although a likely source is the mitochondria. Mitochondria have been reported to generate superoxide and may release these radicals into the extramitochondrial space. Under normal physiological conditions it is estimated that 2-5% of O\textsubscript{2} utilized by intact mitochondria is reduced by electrons that escape the electron carriers of the respiratory chain. However during ischemia-reperfusion highly redox reduced electron carriers could directly transfer electrons to the residual molecular oxygen producing superoxide (Becker L. B. et al., 1999). A recent study presents a compelling case that the mitochondria function as oxygen sensors by increasing the release of ROS during hypoxia (Waypa G. B., 2001). In the simplest model, electrons accumulating during hypoxia upstream from the cytochrome oxidase would react nonenzymatically with oxygen to form superoxide, but it is difficult to understand how superoxide production would increase, because increased availability of electrons would be offset by decreased availability of oxygen (Sylvestre J. T., 2001). In Hep3B cells hypoxia increased mitochondrial production of ROS even when the proximal portion of the electron transport chain was fully reduced by antimycin A. Thus hypoxic enhancement of ROS production seems to occur at sites upstream from ubisemiquinone (site of antimycin A) because of factors other than electron availability, such as superoxide dependent facilitation of electron transfer to oxygen or enhanced egress of superoxide.
Two possible mechanisms can explain how mitochondrial enzymes sustain a continuous production of hydrogen peroxide even after most of the oxygen has been consumed. The first reason is that submicromolar concentrations of oxygen that remain in the medium after succinate respiration still react with reduced low potential groups, e.g. iron-sulfur clusters or semiquinones, thereby producing nanomolar quantities of superoxide and hydrogen peroxide. The second reason is that the abundant reduced iron in mitochondria catalyses self-sustaining Fenton reactions with hydrogen peroxide formed by substrate oxidation (Esposti M. D. et al., 1998). Partial ubiquinone oxidation resulting from blockade of complex I can lead to superoxide formation in submitochondrial particles (Lotharius J. et al., 1999).

Glycolysis is a major metabolic pathway to provide substrate to the citric acid cycle of which activity is essential for the normal proceeding of the mitochondrial respiratory chain. It has been reported that hexokinase in RBC's is inactivated by oxygen radicals. The inhibition of glycolysis may be an important factor for oxygen radical induced depletion of intracellular ATP particularly in neuronal cells because nerve cells exclusively utilize glucose as the energy source (Kang D. et al., 1997). Oxidative inactivation of key components of both the electron transport chain and the glycolytic pathway might contribute to a subsequent 'metabolic death'. Glyceraldehyde-3-phosphate dehydrogenase can undergo reversible oxidative inactivation by hydrogen peroxide (Lotharius J. et al., 1999).

Green and Reed (1998) have proposed a model by which mitochondria mediate both apoptotic and nonapoptotic cell death via several mechanisms, including impairment of electron transport and mitochondrial membrane potential, release of proteins that activate caspases and alterations in cellular redox potential. Thus mitochondria represent both a target for cell death processes and a source of cytotoxic oxygen radicals. Blockage of complex I activity would lead to decreased ATP levels, alterations in membrane permeability and calcium influx akin to excitotoxic cell death processes. Such a model would predict an early loss in mitochondrial potential, superoxide generation and a subsequent cellular energy drain. Although mitochondria are the major intracellular source of ROS, they lack catalase and depend on GSH and SOD to decompose the superoxide radicals that are constantly generated during cell respiration. A decrease in mitochondrial GSH therefore diminishes
the capacity of cells to compensate oxidative stress and could contribute to the pathogenesis of neurodegenerative disorders in which oxidative stress is considered a possible pathogenic factor (Seyfried J. et al., 1999).

In addition to confocal microscopy to study individual cells, flow cytometric evaluation of a population of cells was done, and my findings of increased oxidative stress in cells exposed to OGD in DMEM confirmed. Flow cytometry allowed me to evaluate simultaneously using the same indicator and in a short period of time ROS production, in a large number of individual cells. Furthermore flow cytometry allowed removal of nonviable cells from the analysis.

10 Free Radical Induced Damage To Lipid

Elevation of free radicals can lead to oxidative damage to lipids, proteins and DNA. Resultant changes can affect membrane integrity, ionic homeostasis and receptor function. Because of the difficulty in directly measuring short-lived reactive free radicals, formation of lipid, protein or DNA breakdown products is often used as a marker for oxidative damage (Azbill R. D. et al., 1997). To assess oxidative stress thiobarbituric acid reactive substances, a reflection of malondialdehyde (MDA) concentration have been widely used. The peroxidation of polyunsaturated fatty acids, amino acids, carbohydrates or nucleic acids all result in the formation of malondialdehyde (MDA), the major thiobarbituric acid reactive product (Subbarao K. V. and Richardson J. S., 1990). Although the TBARS test is the most widely used for the determination of MDA, there are several sources of error in using this test. The MDA content may be underestimated as a result of adsorption of the TBA complex onto protein precipitate. Other aldehydes if present can react with TBA to produce a colored complex. MDA can be formed during the procedure itself by oxidation of PUFA or decomposition of lipids (Draper H. H. and Hadley M., 1990).

Cyanide causes a rapid increase in ROS generation in PC12 cells, but lipid peroxidation occurs at a later time (Kanthasamy A. G. et al., 1997). Lipid peroxidation following cyanide exposure has been studied in PC12 cells. 4-Hydroxynonenal (HNE) an aldehydic product of lipid peroxidation, is cytotoxic to non-neuronal cells at concentrations reached when cells are exposed to various oxidative insults. HNE forms covalent cross-links with proteins via Michael addition to lysine, cysteine and histidine residues, it is normally detoxified by GSH. HNE generated in response to oxidative insults
(FeSO₄) in PC12 cells can induce neuronal apoptosis at pathophysiologically relevant concentrations (Kruman I. et al., 1997).

This model shows completely contrasting results during OGD in DMEM and BSS, there is a significant increase in levels of MDA at the end of OGD only in the DMEM group, it is significantly decreased in cells exposed to OGD in BSS. This finding correlates with my earlier demonstration of massive oxidative stress in cells in DMEM during OGD. Lipid peroxidation in previous models of ischemia and injury show increased levels only after reperfusion (Kanthasamy A. G. et al., 1997, Azbill R. D. et al., 1997, Kruman I. et al., 1997), correlating with a burst in free radical generation during reperfusion. However in my model of ischemia, the massive oxidative stress in cells in DMEM, increases lipid peroxidation in the acute phase of the insult itself.

11 Role Of Anti-Oxidants During OGD And Reperfusion

My results indicated oxidative stress to be the cause of increased cell death during OGD in DMEM, I therefore hypothesized that anti-oxidants by reducing oxidative stress would be neuroprotective for cells exposed to OGD in DMEM. SOD dismutates superoxide to hydrogen peroxide and Catalase breaks down hydrogen peroxide to water and oxygen. In my model anti-oxidants SOD and Catalase significantly decreased cell death in cells in DMEM, confirming the role of oxidative stress in causing cell death. The unanswered question remains as to why an efficient enzyme as endogenous SOD does not completely metabolize the extra superoxide generated during ischemia?

It seems possible that ischemia itself may lead to inhibition of endogenous SOD (Becker L. B. et al., 1999), another possibility is endogenous anti-oxidants might be overwhelmed with the massive oxidative stress seen during OGD in DMEM. It also suggests a probable extracellular source of free radical generation as both SOD and Catalase do not normally cross the cell membrane.

The possible sites of action of SOD and Catalase can be both intra and/or extracellular –

1. Although recent studies suggest an intracellular site of ROS generation during ischemia (Waypa G. B. et al., 2001), previous studies demonstrated that exogenous SOD or Catalase blocked the hypoxic enhancement of ROS production (Sylvester J. T., 2001). If these large proteins did not
enter cells, then ROS released during hypoxia must have exited cells to act at the external reticular surface or traversed the extracellular space before reentry to act at intracellular loci.

2. Alternatively, SOD and Catalase may have entered cells by endocytosis which occurs in smooth muscle and may be enhanced by ROS (Waypa G. B. et al., 2001).

3. Pretreatment with SOD/catalase could deplete superoxide and protect neurons by prevention of peroxynitrite formation by scavenging superoxide (Bonfoco E. et al., 1995).

4. The enzyme extracellular SOD (ECSOD) exists only in low concentrations in extracellular fluids and is not thought to function as a bulk scavenger of superoxide. Biochemical data suggest that ECSOD binds to heparan sulfate proteoglycans on endothelial cells, where it has been speculated to serve as a 'protective coat'. Because superoxide is known to inactivate NO, induced vasorelaxation, one possible function for ECSOD may be to protect NO released from cells from superoxide mediated inactivation (Oury T. D. et al., 1992).

5. The use of exogenously administered Catalase as an intracellular anti-oxidant has been reported (Mills E. M. et al., 1996).

6. Catalase an enzyme involved in the breakdown of hydrogen peroxide is a large molecule which is unlikely to penetrate the cell membrane, therefore the toxicity must be extracellular (Woodgate A. et al., 1999).

In my model of OGD, anti-oxidants SOD and Catalase significantly decreased cell death in cells in DMEM, confirming the role of oxidative stress in causing cell death in this group. It also suggests a probable extracellular source of free radical generation as both SOD and Catalase do not normally cross the cell membrane.

12  Role Of Nitric Oxide (NO) Generation In OGD And Reperfusion

NO may possess either neurodestructive and neuroprotective properties depending upon its oxidation-reduction status with NO being neurodestructive and NO+ being neuroprotective (Dawson T. M. et al., 1994). Nitric oxide (NO) is formed directly from the guanidino nitrogen of L-arginine by NOS through a process that consumes five electrons and results in the formation of L-citrulline. NOS neurons are peculiar in that they are resistant to NMDA toxicity but exquisitely sensitive to AMPA/KA neurotoxicity. NOS neurons release NO to kill non-NOS neurons.
The possible mechanisms involved in resistance of NOS neurons to NO toxicity are

1. Perhaps the diaphorase activity of NOS protects the cell against NO as induction of a related diaphorase enzyme renders a non-NOS neuronal cell line resistant to glutamate toxicity (Dawson T. M. et al., 1994).

2. NOS neurons within the striatum are enriched in MnSOD, which prevents the local formation of peroxynitrite rendering the NOS neurons resistant to the toxicity of NO.

3. Phosphorylation of endothelial NOS translocates it from membrane to cytosol, since phosphorylated NOS is catalytically inactive, NO will not be generated within the cytoplasm. Instead, catalytically active nonphosphorylated NOS is restricted to the plasma membrane, where it presumably generates NO that is released into the extracellular space. Thus it is conceivable that within neurons, the active form of NOS occurs at the plasma membrane, where it releases NO to the outside, and no NO would be produced in the interior of NOS cells, rendering them resistant to damage (Dawson T. M. et al., 1994).

PC12 cells differentiated with NGF but not undifferentiated express nNOS. After 3-10 days with NGF, positive staining with NOS was observed. Interestingly PC12 cells treated with NGF for more than 3 days lost the sensitivity to NO donor. Thus the induction of NOS in PC12 cells took place in parallel to the acquisition of NO resistance. However the activity of NOS is not responsible for the NO resistance of PC12 cells, because treatment with NOS inhibitors did not affect the resistance to NO toxicity in PC12 cells (Wada K. et al., 1996). Release of both Ach and dopamine from NGF treated PC12 cells is blocked by NOS inhibitors and reversed by excess L-arginine (Dawson T. M. et al., 1994). The exact mechanism whereby NO enhances neurotransmitter release is unclear, but may involve phosphorylation of synaptic vesicle proteins through activation of guanyl cyclase (Dawson T. M. et al., 1994). Inhibition of endogenous NO generation by L-NAME did not protect PC12 cells or sympathetic neurons from Beta-Amyloid however concurrent treatment with the exogenous NO generator SNAP led to complete protection in these neurons (Troy C. M. et al., 2000). Additionally cyanide, which is known to inhibit the heme portion of NOS also failed to attenuate the ROS signal (Becker L. B. et al., 1999). CGC strongly express nitric oxide synthase and are assumed to have developed mechanisms to prevent direct NO toxicity (Leist M. et al., 1998) whether such a mechanism is also present in PC12 cells is unknown.
During aglycemia in rat hippocampal slices, lowering pH decreases the activity of neuronal NOS. The lowering of NO production probably occurs because NOS requires molecular O₂ to generate NO. NO is mainly produced in the CA1 area in hippocampal ischemia, however it appears to be generated during reperfusion rather than in ischemia when the oxygen concentration is extremely low (Kojima H. et al., 2001). It has been shown that NOS 1 like NOS 2 and 3 can generate superoxide and NO, even in absence of substrate. NOS inhibitors can enhance NOS 1 derived superoxide even in the presence of saturating concentrations of L-arginine (Pou S. et al., 1999). NO inhibits apoptotic death in PC12 cells primarily through a cGMP-dependent prevention of caspase-3 activation and mitochondrial cytochrome. Low doses of nitroarginine reduce infarct volume, but high doses of NOS inhibitors exacerbate damage following occlusion of the middle cerebral artery presumably by decreasing cerebral blood flow (Dawson T. M. et al., 1994).

I have studied the effects of NOS inhibitor L-NAME, in this model of ischemia as in differentiated PC12 cells NOS is known to be induced. In various models of ischemia NO is known to produced during OGD and reperfusion, in vivo models also report the neuroprotective effects of NO inhibition. However my results indicate that NOS inhibition using L-NAME was not protective. L-NAME at 1 mM was not protective during OGD and reperfusion in DMEM, BSS or Controls. I used a higher concentration of L-NAME at 10 mM to overcome the competitive effects of arginine present in DMEM on NOS. L-NAME 10 mM was toxic to the cells increasing cell death in DMEM, BSS as well as controls.

Why is NOS inhibition not protective in this model? One possibility is that the effects of L-NAME, which blocks the release of ACh and DA from PC12 cells, may be reversed due to arginine, a competitive substrate for NOS present in DMEM (see Appendix 1). This however does not explain the increase in cell death for cells in BSS which lacks arginine. Whether build-up of arginine within the cell after NOS inhibition could be toxic is not known. NOS inhibitors are known to generate superoxide even in the presence of saturating concentrations of substrates (Love S. et al., 1999), this could also be relevant to this model. It is also possible that there is no role of NO in this model of ischemia. Lowered intracellular pH decreases the activity of NOS, NADPH and molecular O₂ are required for NOS activity, hence it is less likely to have a role under ischemic conditions, when both NADPH and molecular O₂ is also not available. L-NAME did not protect PC12 cells from Beta amyloid toxicity, where one of the mechanisms involved oxidative stress, cyanide which inhibits the
heme moiety of NOS does not decrease ROS generation during OGD in PC12 cells. Another aspect to be considered is L-NAME is an irreversible inhibitor of neuronal NOS (constitutive) and a reversible inhibitor of inducible NOS. Although I do not think this can affect my model of ischemia as cells were preincubated in L-NAME (1/10 mM) and L-NAME was present throughout OGD and reperfusion.

**NEUROPROTECTIVE STRATEGIES - PRECONDITIONING, NGF, HYPOTHERMIA**

13 Ischemic (IPC) And Hypoxic (HPC) Preconditioning

Brief episodes of brain ischemia define a patient population at risk for stroke. These brief events (TIAs) warrant urgent evaluation and treatment. It has therefore been suggested that transient ischemia might induce tolerance and protect the brain from subsequent ischemia. The only 2 organs in which tolerance is possible and has been studied is in the heart and the brain. The mechanisms involved in tolerance are however different in both of these organs. An understanding of the mechanisms involved in preconditioning may define novel therapeutic strategies for brain protection. Some of the mechanisms involved in the protective response of preconditioning are new protein synthesis (Nakata N. et al., 1993), overexpression of MnSOD (Ohtsuki T. et al., 1992), induction of heat shock proteins (Nakata N. et al., 1993), and upregulation of Bcl2 (Kane D. J. et al., 1995). In the myocardium intracellular ROS generation is known to induce preconditioning (Becker L. B. et al., 1999). Adenosine (A1) receptors in myocytes have a role in preconditioning, as PC12 cells express only A2 receptors, it is possible they cannot be preconditioned. Considering a literature review, wherein no previous information is available on the role of preconditioning in PC12 cells. I tried to precondition neuronal PC12 cells. The mechanisms involved in tolerance are however different in both of these organs. My results indicate that neuronal PC12 cells cannot be preconditioned against a future OGD insult using either BSS or DMEM in the paradigm mentioned.

I have studied preconditioning using both BSS and DMEM for the initial insult and subsequently the same experimental solution for the major insult. Although traditionally brain ischemic tolerance is said to develop 24-48 hrs after the preconditioning insult, whereas heart preconditioning is seen at 4 hrs, I have tried a regimen interval of 4 hrs, 24 hrs and 48 hrs. Although ischemic exposure is capable of inducing tolerance, some studies have suggested the role of other injury mechanisms ex. Hypoxic
preconditioning (Gidday J. M. et al., 1994), accordingly my experiment also involved hypoxic injury in both groups as the preconditioning stimuli.

My results show no protection against a future OGD insult using the paradigms mentioned in either BSS or in DMEM. The protective effect of preconditioning is inhibited in the presence of NMDA antagonists, suggesting a role of NMDA receptor in preconditioning (Kato H. et al., 1992). PC12 cells do not have NMDA receptors, although NMDA receptor mRNA is seen in these cells (detailed in Introduction section 5). Ischemia induces the release of adenosine resulting in the opening of KATP channels via A1 receptor binding (Heurteaux C. et al., 1995). PC12 cells do not have A1 receptors. It is therefore possible that PC12 cells cannot be preconditioned.

14 Role Of NGF In Protection Against OGD And Serum Withdrawal

In my studies, NGF protected differentiated PC12 cells from OGD induced both immediate and delayed cell death in a dose dependent manner. Cells were differentiated in NGF at 25 ng/ml and all experiments were done in the presence of NGF throughout to avoid the toxic effects of trophic withdrawal. NGF at 2X (50 ng/ml) and at 4X (100 ng/ml) improved cell survival in a dose dependent manner. Cell death in the control group was also decreased in parallel confirming it’s role in protection from serum deprivation.

Nerve Growth Factor (NGF) the most thoroughly studied neuronal growth factor, promotes the survival and outgrowth of peripheral sensory and sympathetic neurons (Levi-Montalcini R. et al., 1986). NGF may have a more limited set of neural targets and is believed to be active primarily in cholinergic neurons (Knusel B. et al., 1990). Beyond their roles in normal brain development, GF’s may play important roles in the brain’s response to injury and neurodegenerative disorders (Hefti F. et al., 1989). NGF and bFGF can prevent the damage of cholinergic neurons associated with brain lesions in animal models of neurodegenerative disorders (Snider W. D. et al. 1989, Hefti F. et al., 1989). Pretreatment of both rat hippocampal and human cortical cultures for 24 hr with NGF or bFGF at 10 ng/ml resulted in nearly complete protection against hypoglycemia induced neuronal damage. NGF and bFGF were found to protect against neuronal death induced by glucose deprivation in glia-depleted cultures (Cheng B. and Mattson M. P., 1991). NGF produced a small but significant increase in viability of severely hypoxic PC12 cells. This sparing effect may be more pronounced with
mild hypoxia. NGF did not alter basal $Ca^{2+}$ or hypoxia induced changes in $Ca^{2+}$ which suggest NGF's effects on viability is not mediated via calcium dependent events (Caroll J. M. et al., 1992).

The possible mechanisms by which NGF is neuroprotective are –


2. Although details of changes in the expression of different types of NGF and FGF receptor proteins during development and in response to injury remain to be determined, it might be the case that neurons in cell culture express NGF receptors that they would not normally exhibit under normal conditions in vivo.

3. The protective effects of NGF and bFGF against environmental insults may also be mediated by actions on protein expression therefore requiring pretreatment for 24 hrs preceding the insult (Cheng B. and Mattson M. P., 1991).

4. One mechanism of protection against glutamate toxicity may involve a reduction in excitatory amino acid receptor expression.

5. In addition NGF increased the expression of the calcium binding protein calbindin in rat brain in vivo, calbindin is believed to play a role in protecting neurons against excitotoxic insults (Masiakowski M. et al., 1988). NGF also induced the expression of genes for two other calcium binding proteins in PC12 cells (Conrad P. W. et al., 2001). NGF produced a small but significant increase in viability of severely hypoxic PC12 cells. This sparing effect may be more pronounced with mild hypoxia. NGF did not alter basal intracellular $Ca^{2+}$ or hypoxia induced changes in $Ca^{2+}$ which suggest NGF's effects on viability is not mediated via calcium dependent events (Caroll J. M. et al., 1992).

6. NGF did however double the low ATP in the hypoxic cells, which is consistent with results that demonstrate that NGF affects oxidative metabolism. The effects of NGF on ATP levels likely reflect a shift in the relative rates of production and use, with decreased use the likely factor (Caroll J. M. et al., 1992).

7. NGF protects PC12 cells from exogenously administered hydrogen peroxide by inducing catalase intracellularly (Jackson G. R. et al., 1990). Neurotrophins and growth factors also protect
neurons against excitatory amino acids but do not affect the expression of the NMDA receptor or binding of glutamate. Instead they cause a rise in catalase activity and glutathione biosynthesis thus providing improved defense against oxidative stresses (Gassen M. et al., 1998). NGF has been found to be protective against H$_2$O$_2$ induced oxidative stress likely due at least in part to increases in the activities of both enzymes Glutathione peroxidase (GSHPx) and glutamyl cysteine synthetase (GCS), the rate limiting enzyme in de novo GSH synthesis (Newell D. W. and Barth A., 1995).

8. NGF increases the uptake of L-cysteine or L-cystine which in turn resulted in increased concentrations of GSH. L-cysteine in the free thiol form also acted as an antioxidant and a minor protecting agent from oxidative stress in the PC12 cell system (Schubert D. et al., 1992).

In my model of OGD, NGF protected cells from OGD induced cell death in a dose dependent manner. Presumably differentiating cells in NGF for 7 days, increases the expression of intracellular catalase an important defence against H$_2$O$_2$ mediated cytotoxicity. Differentiation in NGF also increases the uptake of L-cysteine which increases intracellular GSH levels another important antioxidant defence system. NGF has been shown to induce the expression of genes for at least two calcium binding proteins in PC12 cells, this is a probability in this model too. Preliminary experiments with Flo-4 did indicate a simultaneous rise in Ca$^{2+}$ intracellularly along with the rise in free radical generation. Cells were differentiated for 7 days in NGF, NGF is known to affect calcium channel expression, electrical excitability and protein expression all of which can be involved in its neuroprotective effects. The protective role of NGF against serum deprivation induced cell death is also shown in my studies, control cells in differentiated PC12 cells show significantly reduced cell death as compared to undifferentiated PC12 cells exposed to 24 hrs of serum deprivation. Serum deprivation is known to increase cell death by ROS generation, again NGF induced increase in antioxidants may possibly protect differentiated PC12 cells from serum deprivation.

15 Hypothermia Is Protective Against OGD/OD In This Model

Hypothermia is so potently neuroprotective that it has been successfully implemented clinically during specific neurosurgical procedures. Hypothermia greatly reduces the extent of brain damage sustained in models of both focal and global cerebral ischemia. In contrast hyperthermia is known to exacerbate ischemic injury (Busto R. et al., 1987).
Hypothermia has been shown to reduce ischemia induced glutamate release as well as to preserve high energy phosphates during ischemia and thus preserve energy dependent calcium extrusion mechanisms (Newell D. W. et al., 1995). The following are the possible mechanisms of neuroprotection by hypothermia –

1. One of the mechanisms underlying the neuroprotective effect of hypothermia was thought to be related to its energy suppressing action. However recent research on high energy phosphate dynamics at the time of cerebral ischemia has not supported this (Murata T. et al., 2000).
2. Another important mechanism underlying the neuroprotective effect of hypothermia is considered to be excitatory amino acid inhibition based on the glutamate excitotoxicity hypothesis (Murata T. et al., 2000). In 5 mins gerbil hippocampal ischemia, hypothermia at both 34°C and 30°C significantly inhibited the H2O2 production during ischemia and reperfusion (Lei B. et al., 1997).
3. Hypothermia is also proposed to decrease the rise in intracellular Ca2+ during ischemia (Newell D. W. et al., 1995).
4. A decrease in temperature may also cause a decrease in free radical generation or release of neurotransmitters, although the mechanisms are not known.

In my model of ischemia (OGD) and anoxia (OGD), hypothermia at 24°C during OGD/OD consistently protected cells and decreased cell death. These results suggest that at least one of the major processes involved in ischemic/hypoxic damage in this model is extremely sensitive to temperature. Another important implication of this result is that temperature differences during the insult have long lasting influences and delayed cell death assayed at 24 hrs is significantly affected.

16 Catecholamine Oxidation Causing Free Radical Generation And Damage


Catecholamines have been shown to be toxic both in vivo and in vitro via mechanisms of oxidative stress. DA can be autooxidized (Figure 37) or enzymatically oxidized (Figure 38) by monoamine oxidase, prostaglandin H synthase, lipoxygenase, tyrosinase and xanthine oxidase resulting in the formation of ROS and/or quinone intermediates which in turn can lead to lipid peroxidation, DNA damage and mitochondrial impairment (Jones D. C. et al., 2000). DA has both extra and intra cellular mechanisms of toxicity and considerable controversy exists regarding its site of action (Jones D. C. et al., 2000). The possible mechanisms of toxicity of dopamine are:

1. DA may auto-oxidize to form superoxide and hydrogen peroxide which can then form the toxic hydroxyl radical in the presence of transition metals. Also catecholamines can undergo free radical exchange reactions and thus would react with superoxide to form a semiquinone radical (Hyslop P. A. et al., 1995)(Figure 37).

2. DA oxidation also results in the formation of DA quinone, which readily participates in nucleophilic addition reactions with sulfhydryl groups on free cysteine, glutathione or cysteine found in protein.

2.1 The reaction between DA quinone and cysteine results in the formation of 5-cysteinyl DA. Because cysteinyl residues are often found at the active sites of proteins, the covalent addition of the catechol, moiety to cysteine may inhibit protein function and possibly lead to cellular damage and/or cell death.

2.2 DA quinone is able to react with the sulfhydryl group of cysteine in glutathione, which may decrease levels of this important antioxidant (La Voie M. J. et al., 1994). GSH is important for maintaining appropriate protein thiols and minimizing oxidative damage due to DA. DA decreases the intracellular GSH pool creating an imbalance between DA oxidation and the anti-oxidant system (DA).
2.3 DA quinone can act as an oxidant producing toxic hydroxy radicals (Jones D. C. et al., 2000)

3. It has been well demonstrated that DA can oxidize and form cysteiny1-DA via an autoxidation mechanism that is facilitated by the presence of transition metals, such as iron.

4. Pro-oxidants may also be formed in vivo that can react with DA and increase the formation of protein cysteiny1-DA. Increased extracellular glutamate can lead to ROS generation such as superoxide (La Voie M. J. et al., 1994) which can then react with DA.

5. DA may also be oxidized via enzymatic mechanisms, prostaglandin H synthase, lipoxygenase, tyrosinase and xanthine oxidase have the ability to oxidize DA and increase the formation of protein-cysteiny1 DA (Figure 38).
**Figure - 37**

**Auto-oxidation of Dopamine (DA)**

- **Strong pro-oxidant** → **Toxic OH⁻**
- **Dopamine quinones** → **Nucleophilic Fe-S centers Glutathione**
- **Depletes GSH** (Intracellular anti-oxidant)
- **Free cysteine**
- **Cysteinyl-DA**
- **Protein damage**
- **O₂⁻** + **H₂O₂**
- **OH⁻**
- **Fe**
- **Auto oxidation**
Enzymatic oxidation of Dopamine (DA)

Mono-amine oxidase (MAO)
PGH synthase
Lipoxygenase
Tyrosinase
Xanthine oxidase (XO)

Dopamine

DOPAL
DOPET

Toxic Cysteinyln DA

(i) Mitochondrial complex I/II inhibition increases DOPAL & DOPET

(ii) Metabolic stress increases toxic DOPAL and DOPET

(iii) DOPAL and DOPET appear rapidly after complex I inhibition

IN PC12 CELLS (see text)
6. DOPAL (3,4-Dihydroxyphenylacetaldehyde) has been reported to be a toxic metabolite formed by the oxidative-deamination of dopamine, catalysed by monoamine oxidase. This aldehyde is either oxidized to DOPAC (3,4-dihydroxyphenylacetic acid) by aldehyde dehydrogenase, a NAD-dependent enzyme or reduced to DOPET (3,4-dihydroxyphenylethanolamine) by aldehyde or aldose reductase. Inhibitors of mitochondrial complexes I and II in PC12 cells, increased levels of DOPAL and DOPET. NGF markedly potentiated DOPAL and DOPET accumulation in response to metabolic stress (200). The rapid appearance of DOPAL and DOPET after complex I inhibition may be a useful early index of oxidative stress in DA-forming neurons and may also explain the vulnerability of the dopaminergic system to complex I inhibition (Lamensdorf I. et al., 2000).

7. Initiated by DA oxidation and enhanced by cyanide induced ROS generation and /or energy impairment, excess ROS may inhibit mitochondrial metabolism and energy inhibition may increase ROS generation, consequently entering into a vicious cycle of toxic events (Jones D. C. et al., 2000).

8. DA stimulated the oxidation of methionine to methionine sulfoxide in HL60 cells. In a cell free system DA stimulated the methionine oxidation to a greater extent confirming it's pro-oxidant actions in vitro (Sakagami H. et al., 1998, Kawase M. et al., 1998).

Following 30 mins of cerebral ischemia in rats there is a highly reproducible damage to striatal medium sized neurons. During the ischemic episode massive release of dopamine occurs. Depletion of striatal dopamine protects these neurons against ischemia induced damage. The mechanism by which dopamine participates in striatal cell death is unknown, but it has been speculated that catecholamine oxidation both enzymatically (by monoamine oxidase) and non-enzymatically (by molecular oxygen) generates H₂O₂, superoxide respectively and this process once accelerated results in the rapid auto-oxidation of this class of neurotransmitter. H₂O₂ exerts injurious effects on tissues by a number of different mechanisms such as perturbing intracellular calcium homeostasis, decreasing intracellular ATP by directly inhibiting glycolysis and mitochondrial ADP phosphorylation, perturbing cytoskeleton components and inhibiting neuronal cell-matrix adhesion (Hyslop P. A. et al., 1995). 6-hydroxydopamine (6-OHDA) is a widely used neurotoxin to create animal models of Parkinson's disease. It's toxicity is dependent mainly on its non-enzymatic degradation. In a cell free system, when
added to culture medium, it is completely auto-oxidized to quinones within 3.5 hrs (half auto-oxidation time of approximately 10 mins) (Blum D. et al., 2000). DA and 6-OHDA toxicity has been shown to be directly correlated to their auto-oxidation rate (Blum D. et al., 2000). The toxicity of 6-OHDA on PC12 cells is proposed to be induced by extracellular generation of hydrogen peroxide through auto-oxidation of the toxin (Blum D. et al., 2000).

17 Proposed Mechanisms Of Cytotoxicity In My Model of Ischemia and Reperfusion

Summary of what I do know about this model of OGD/GD in neuronal PC12 cells

1. Undifferentiated and differentiated PC12 cells show a similar pattern of increased cell death when exposed to OGD in culture medium (DMEM) as compared to BSS.
2. Cellular and mitochondrial morphological changes are rapid and occur early (within 30 mins) of the start of the insult and are persistent in cells in DMEM throughout the duration of OGD, remarkably by 2 hrs of reperfusion cells are in the recovery phase morphologically.
4. Substrate supplementation (L-Glutamine) decreases toxicity but substrate inhibition increases toxicity only in the BSS group.
5. Iron is not a major cause of oxidative stress in this model of OGD.
6. There is a massive rapid and persistent increase in oxidative stress during OGD in cells in DMEM.
7. Free radical generation has been demonstrated using the same indicator 5’6’CMDCF-DA on an individual cell basis (confocal microscopy) and in a population as well (flow cytometry) of neuronal PC12 cells.
8. Free radical generation (ROS) is responsible for the increased cell death during OGD in the DMEM group, this was confirmed using parallel PI staining.
9. Free radical mediated lipid peroxidation is increased during OGD in cells in DMEM (TBARS assay).
10. Anti-oxidants SOD and Catalase are protective, their mechanism of action is probably extracellular in my model of OGD.
11. NGF is protective in this model of in vitro ischemia in a dose dependent manner, one of its main mechanism of neuroprotection is enhancing endogenous anti-oxidant defences.

12. Hypothermia is protective in this model, a possible mechanism could be either a decrease in free radical generation or intracellular calcium release.

13. Preconditioning is not neuroprotective in neuronal PC12 cells against OGD, using the paradigms mentioned.

Preliminary experiments with the calcium indicator indicated a parallel increase in calcium during oxidative stress, considerable controversy exists in literature whether a rise in calcium follows or precedes free radical generation. Detailed experiments will be done later.

A. Alternative substrate theory. (Figure 39)

My model of OGD was conceived with a view to compare cytotoxicity in differentiated PC12 cells exposed to OGD in 2 different experimental solutions. One insult representing complete absence of substrates, was given in the more typically used BSS (see Figure 5). The other model represented 'incomplete substrate deprivation' as can occur in certain conditions of ischemia in vivo and used culture medium (DMEM) (formulations in Appendix 1). Culture medium has a number of amino acids, vitamins, cofactors and iron in addition to the basic composition of BSS, which is primarily inorganic salts. Hence culture medium should be able to enhance the survival of differentiated PC12 cells because PC12 cells are known to consume essential amino acids, glutamine and arginine (Figure 17). If that is the case, they should survive better in DMEM than in BSS, under conditions of oxygen and glucose deprivation. But completely contrary to my expectations, cell death was increased both acutely as well as at 24 hrs after the insult. My first explanation was that alternative substrates present in DMEM were being consumed by PC12 cells, but as energy requirements could not be met with completely by these alternative substrates, free radicals were being generated. The result (Figure 21) shows the increase in oxidative stress during OGD and reperfusion in DMEM, which supports this theory.
**Figure - 39**

Probable Models To Explain Cytotoxicity During OGD/GD/OD In Neuronal PC12 Cells

**OXIDATIVE STRESS\= CYTOTOXICITY**

**A. Alternative substrates utilization**

- Indirect evidence

**Factors Against This Theory**

- * Substrate utilization should decrease cell death; L-Glutamine & Glucose decrease cell death
- * 2DDG increases cell death in BSS, not in DMEM, could ‘X’ block glycolysis & gluconeogenesis in DMEM?
- * SOD & Catalase are neuroprotective

**B. Extracellular Iron in DMEM**

- Direct evidence

**Factors against iron alone**

- * No increase in cell death in BSS with addition of iron

**Factors Supporting This Theory**

- * Undiff cells show a similar response & release DA under stress
- * Intra/extra cellular oxid’n of DA-toxic
- * SOD & Catalase protective (? EC)
- * DA toxicity increases in 0 glucose
- * Increase ROS during OGD

**C. DA release & Auto-oxidation**

- Indirect evidence
But the following facts do not conform to this theory.

i). If the alternative substrates present in DMEM can be consumed by the cells to fulfill their energy demands, then cell death should decrease in cells in culture medium, under conditions of OGD/GD, not increase (see Result Figure 10). As ATP synthesis would continue till the available substrates are depleted.

ii). Glutamine is an alternative substrate used by PC12 cells, as expected cell death during OGD decreased in PC12 cells when L-Glutamine was present in the medium during the insult (see Result Figure 17). During hypoxia, when glucose is available as a substrate, cell death is decreased (see Result Figure 16).

iii). The next experiment was the addition of 2 DDG, a competitive glycolysis inhibitor. If the cells have been using alternative substrates, then cell death should increase after the addition of 2DDG. On the contrary, cell death in the DMEM group is not significantly affected, this means some other entity is already blocking glycolysis, so essentially the addition of 2DDG does not make any difference at all. (see Results Figure 18). Cell death in the BSS group increased after the addition of 2DDG, perhaps cells in this group when exposed to OGD continue to utilize the glycogen stores present and thereby are able to survive better.

iv). SOD & Catalase which act primarily extracellular are able to protect cells in this model of OGD/GD. This cannot be explained using the 'alternative substrate theory'.

B. Iron as a cause of oxidative stress and cell death in culture medium with iron. (Results Figure 19)

Another proposed mechanism can be iron, as detailed earlier (see Result Figure 19), iron is a pro-oxidant both in vitro and in vivo. It acts as a catalyst for many free radical mediated reactions ultimately leading to cell death. Culture medium (DMEM) has iron in the form of ferric nitrate nonahydrate at a concentration of 0.0001g/L. I added iron in exactly the same formulation and concentration to BSS. If iron is a major cause of oxidative stress and ultimately cell death in culture medium, cell death should now be the same in both cell groups. Completely contrary to my expectations, cell death was not increased in the BSS group. The only possible explanation is that iron alone is not an initiator of oxidative stress, probably other components are needed wherein iron can then act as a catalyst. On the basis of an extensive literature review, I now propose another
mechanism for the increase in cytotoxicity during OGD in cells in DMEM as compared to BSS (see Figure 40).

PC12 cells are known to release neurotransmitters NE and DA (See Introduction section 5) in response to hypoxia, ischemia, metabolic stress and pharmacological agents. These cells are derived from rat Pheochromocytoma (adrenal medullary tumour), therefore they represent a model of sympathetic neurons and are a catecholaminergic cell line. They are used extensively as a model in vitro system to study Parkinson's disease, because of their ability to release dopamine and the presence of a dopamine transporter. Detailed mechanisms of toxicity caused by auto-oxidation or enzymatic oxidation of DA is in Discussion.
Figure 40: Proposed Cellular Mechanism Of Cytotoxicity; A New Model

DMEM-G-O (OGD)  BSS-G-O  DMEM/BSS +G-O (OD)  DMEM+G+O CONTROLS
Oxidative/ Metabolic stress

DA released

Intracellular Toxic DA metabolites

Inhibits Glycolytic Enzymes depletes GSH +ROS

H₂O₂

Interacts with extracellular methionine (pro-oxidant)

Cysteine forms cysteinylation of H₂O₂ production

Fe catalysed OH⁻ formation (Fenton reaction)

Vicious cycle of H₂O₂ production

No DA released

No metabolic stress as glycolysis continues

DA released No mediators of EC toxicity

No H₂O₂

No inhibition of glycolysis

Glycogen stores enhance survival

Protein damage

Intra/extracellular toxicity Protein/lipid DNA damage
C. Dopamine As A Primary Mediator of Cytotoxicity In My Model Of OGD And Reperfusion

C.1 Proposed mechanism of toxicity during OGD/GD in DMEM

In my model (see Figure 39) during OGD and GD, the primary event of importance is the absence of glycolysis, this causes metabolic stress, which is known to increase intracellular calcium levels as well as ROS generation. A rise in intracellular calcium then causes phosphorylation of synaptic vesicle proteins triggering the release of DA and NE.

1. DA released then interacts with pro-oxidants methionine in the culture medium forming methionine sulfoxide a toxic oxidant (Sakagami H. et al., 1998) (see Appendix 1 for formulation).
2. DA interacts with cysteine, another constituent of culture medium to generate cysteinyl DA, a highly toxic metabolite which causes protein damage.
3. In the presence of iron acting as a catalyst, the highly toxic hydroxyl radical is formed (Fenton Reaction).
4. Auto-oxidation of DA leads to the formation of toxic quinones which are the mediators of extensive lipid/protein damage (Figure 37).
5. A vicious cycle of massive generation of $\text{H}_2\text{O}_2$ occurs primarily extracellularly, this now acts on the cell producing the effects of oxidative stress (analogous to adding exogenous $\text{H}_2\text{O}_2$).
6. Meanwhile within the cell, under metabolic stress, ROS is generated and intracellular oxidation of DA occurs by enzymatic mechanisms(Figure 38) generating toxic byproducts of DA metabolism DOPAL and DOPET. Intracellular nonenzymatic auto-oxidation of DA also occurs generating massive amounts of free radicals
7. Free radicals inhibit glycolytic enzymes (see Discussion section16), therefore the cell is unable to utilize any substrates, ATP levels fall rapidly, pumps fail, and ionic homeostasis is lost – ultimately causing cell death.

Indirect evidence in favour of this proposed mechanism

1. Undifferentiated and differentiated cells show a similar pattern of cell death on exposure to OGD in culture medium.
2. Rapid early demonstration of oxidative stress in this model.
3. Inhibition of glycolysis is known to increase the sensitivity of PC12 cells to DA toxicity. This has been demonstrated in my experiments.
4. SOD and Catalase are anti-oxidants whose primary site of action is extracellular, this protection has been demonstrated in my model.
5. Differentiation in NGF increases the anti-oxidant defences (detailed in Discussion section 14), this is a probable mechanism in my model of in vitro ischemia.
6. Hypothermia is decreases cell death probably by decreasing free radical generation, intracellular calcium or the release of DA extracellularly.

C2. **What happens to cells in BSS exposed to OGD?**

1. During OGD, DA is released. BSS contains only inorganic salts, there are no amino acids or other factors that are pro-oxidants. So, DA does not interact with any other pro-oxidants and there is no major extracellular generation of generation of H₂O₂.
2. It is probable that cells in BSS continue to utilize the glycogen stores, within cells, thus helping their survival during OGD and GD (detailed in Discussion section 1).
3. In case of delayed cell death, another possibility is that the DA released during the insult is washed out at reperfusion and therefore these cells are protected during the 24 hrs following an insult. In contrast cells in DMEM are severely affected during the insult, and the remaining cells that have reached an irreversible stage in the process of cell death, die. Therefore there is increased cell death in the DMEM group, inspite of DA released during the OGD being washed out at reperfusion.

C3. **What happens during hypoxia in both DMEM and BSS?**

As has been detailed earlier, oxygen deprivation alone is not toxic, because cells have glucose, there is less metabolic stress and therefore less intracellular generation of ROS or increase in calcium. Consequently in both DMEM and BSS cells are able to survive oxygen deprivation in the presence of glucose.
C4. Control cells in DMEM show minimal toxicity; why?

The control cells in DMEM+Glucose+Oxygen, are not stressed because the preferred substrate glucose is available under aerobic conditions facilitating metabolism. Differentiation and the presence of NGF throughout the experiment boosts antioxidant defenses, therefore the cells are able to survive well even in the absence of serum for 24 hrs.

C5. Undifferentiated cells show an increase in cytotoxicity in controls.

When undifferentiated cells were exposed to OGD, a similar pattern in cell death is observed in the immediate cell death group, but delayed cell death was considerably increased in the control group (see Results section 5).

I propose the following mechanism for the increase in cell death in the controls of undifferentiated cells.

1. Cells in DMEM and BSS follow a similar pattern of cell death as neuronal PC12 cells, the mechanism is explained as above.
2. Control cells are in DMEM+G under aerobic conditions, immediate cell death is not increased because cells are in a healthy environment. The cells are not stressed and therefore there is no release of DA.
3. In case of delayed cell death, cells are kept in 0 serum for 24 hrs, undifferentiated cells have not been treated with NGF and therefore their anti-oxidant defences in the absence of serum are poor.

24 hrs of serum deprivation increases ROS generation, leading to rise in intracellular calcium, release of DA and extracellular toxicity. Intracellular ROS also oxidizes DA releasing further free radicals. As antioxidant defences are poor in absence of NGF, cell death is increased.

18. Future Directions And Possible Implications

At the end, the unanswered question remains – HOW PHYSIOLOGICAL IS PHYSIOLOGICAL/BALANCED SALT SOLUTION? (see Figure 41) This project actually began with my idea that cells exposed to OGD in culture media would be in a more physiological solution as compared to BSS, which has no actual counterpart in vivo. Typically neuroscientists are using BSS as
1. Is Balanced Salt Solution ‘Physiological’?

2. The mechanisms underlying cytotoxicity are different in cells exposed to ischemia in culture medium and BSS. ROS generation is increased in cells exposed to OGD in DMEM, not in BSS.

3. Changes in mitochondrial morphology are early and persistent in cells exposed to OGD in DMEM.

4. OGD in DMEM is a model representing ‘incomplete ischemia’.

5. DA release is a potentially critical mechanism in OGD and may play a role in striatal ischemia.

6. Is this model representative of ‘Ischemia + Parkinson’s disease’???
the medium in which all in vitro experiments are carried out. But such a situation is never present in vivo, besides cells are grown in culture medium and moved into BSS for experiments. It is possible, changes like these need to be considered if lab-based research is to benefit mankind. This may also explain why very often new pharmacological strategies often never reach the stage of clinical trials. My experiments will contribute to our understanding of ischemia and highlight the problems inherent in studying such a complex phenomena even in a relatively ‘simple’ in vitro model.

The majority of studies in PC12 cells using OGD/GD/OD are done in BSS, few that have been done using DMEM have studied other aspects. This is the first study that documents the wide variation in results between using BSS and culture medium for inducing OGD/GD/OD. This is also the first study to describe the mitochondrial morphology in neuronal PC12 cells during OGD. PC12 cells are a model for Parkinson’s disease, whether a component of ischemia is initially the triggering event leading to Parkinson’s disease is not known. It is known that striatal ischemia releases DA in massive amounts and cell death is also increased, could be explained possibly using this in vitro model (detailed in Discussion section 16).

At the same time caution must be exercised in interpreting these results; it is necessary to consider that PC12 cells are an immortalized cell line and may respond atypically. All my experiments were done in zero serum as the LDH assay was being used to determine cell death, and bubbling the experimental solution was initially used to induce physical hypoxia. Zero serum is not possible in vivo.

The primary focus of my future work will be to demonstrate the release of DA in my cells following OGD. HPLC analysis will be done to determine intra/extracellular DA toxic metabolites (semi-quinones and cysteinyld DA) and hydrogen peroxide comparing between both experimental groups. I also would like to study specifically the effects of simultaneous addition of methionine, cysteine and ferric nitrate addition to BSS during OGD. There is a lot of controversy in literature regarding the initiator of cytotoxicity, the question is what comes first- is it free radical generation or is it increased intracellular calcium? I would like to use parallel imaging with dual channels and confocal microscopy to study both free radical generation and intracellular calcium at early time points. I intend to study oxidative stress during the early phase of OGD and determine the earliest time points when changes first occur.
Appendix – 1

Constituents of DMEM g/L
(Sigma Aldrich chemicals Cat. No. 5030)

L-Arginine·HCl 0.084
L-Cystine·2HCl 0.0626
Glycine 0.030
L-Histidine·HCl·H2O 0.042
L-Isoleucine 0.105
L-Leucine 0.105
L-Lysine·HCl 0.146
L-Methionine 0.03
L-Phenylalanine 0.066
L-Serine 0.042
L-Threonine 0.095
L-Tryptophan 0.016
L-Tyrosine·2Na·2H2O 0.10379
L-Valine 0.094
Choline Chloride 0.004
Folic Acid 0.004
Myo-Inositol 0.0072
Niacinamide 0.004
D-Pantothenic Acid Hemicalcium 0.004
Pyridoxal·HCl 0.004
Riboflavin 0.0004
Thiamine·HCl 0.004
Calcium Chloride·2H2O 0.265
Ferric Nitrate·9H2O 0.0001
Magnesium Sulfate (Anhydrous) 0.09767
Potassium Chloride 0.4
Sodium Chloride 6.4
Sodium Phosphate Monobasic (Anhydrous) 0.109
NGF2.5S (25ng/ml)
Antibiotic-Antimycotic (Penicillin-Streptomycin-Amphotericin B)
mixture 1ml/100ml
Appendix – 2

Balanced Salt Solution (BSS)

Components

1. \( \text{NaCl} \) (116 mM) – 6.78 g/L;
2. \( \text{KCl} \) (5.4 mM) – 0.40 g/L;
3. \( \text{MgSO}_4 \times 7\text{H}_2\text{O} \) (MgSO\(_4\)) (0.8 mM) – 0.963 g/L;
4. \( \text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O} \) (1mM) – 0.1379 g/L;
5. \( \text{CaCl}_2 \) (CaCl\(_2\) x \(2\text{H}_2\text{O}\)) (1.8mM) – 0.2646 g/L;
6. \( \text{NaHCO}_3 \) (26mM) – 2.184 g/L;
7. NGF2.5S (25ng/ml)
8. Antibiotic-Antimycotic (Penicillin-Streptomycin-Amphotericin B) mixture 1ml/100ml
Appendix - 3

Krebbs-Ringer- HEPES (KRH)

Components

1. NaCl (125 mM) – 7.3 g/L
2. KCl (5 mM) – 0.3724 g/L
3. HEPES (25 mM) – 5.958 g/L
4. Glucose (6 mM) – 1.08 g/L
5. NaHCO₃ (5 mM) – 0.42 g/L
6. MgSO₄ (1.2 mM) – 0.1442 g/L
7. KH₂PO₄ (1.2 mM) – 0.632 g/L
8. CaCl₂ (1.2 mM) – 0.1764 g/L
9. NGF 2.5S (25 ng/ml)
10. Antibiotic-Antimycotic mixture 1 ml/100 ml (Penicillin-Streptomycin-Amphotericin B)
Appendix - 4

Collagen Preparation

- method of Bornstein (1958)
- type 1 collagen only
- collect tails and store at -20°C
- sterilize in 70% ethanol for 20 minutes and then rinse in sterile DDW (double distilled water)
- using alcohol sterilized pliers and a hemostat, pull the pieces of collagen apart, and cut the tendons with sterile scissors
- transfer the tendons into a pre-weighed dish of DDW (corning glass petri-dish)
- after 3-4 tails, weigh tendons and calculate weight
- for each gram of tendon, need 150 ml of sterile 0.1% acetic acid solution, but make only 2 grams - 0.1 ml/100 ml
- extract for 24-48 hours at 4°C with periodic agitation
- transfer mixture to sterile 50 ml poly carbonate centrifuge tubes (oak ridge reusable tubes), and centrifuge at 30000 x g for 30 minutes or 10 K (10000 rpm) for 30 minutes at 2°C; pour using magnet to hold stir bar in place
- pipette supernatant down to fibres, use supernatant as 100% stock collagen
- may store at 4°C for up to several months
- for coating culture dishes, dilute with DDW (final concentration used 25%)
REFERENCES


11. Bazan N. G. Free arachidonic acid and other lipids in the nervous system during early ischemia and after electroshock. In: Function and metabolism of


197. Teng K. K., Angelastro J. M., Cunningham M. E., Farinelli S. E., Greene L. A. Cultured PC12 Cells: A model for neuronal function, differentiation and survival. Cell and Tissue culture and associated techniques, 244-250.


212. **Vanden Hoek T. L., Shao Z., Li C., Zak R., Schumacker P. T Becker L. B.**


217. **Yamamoto K. T., Hayakawa H., Mogami H., Akai F., Yanagahara T.**


