The Effects of AIT-082 on ATP Levels in Cortical Neurons and Phosphorylation Levels in Cortical Neurons and Astrocytes in vitro

A Thesis Submitted to the College of Graduate Studies and Research
In Partial Fulfillment of the Requirements for the Degree of Master of Science In the Department of Anatomy and Cell Biology University of Saskatchewan Saskatoon

By

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Abstract

The research was designed to investigate the effects of AIT-082, a derivative of the purine hypoxanthine containing a para-amino benzoic acid moiety, on neural cells. AIT-082 has been shown to possess a number of neurotrophic and neuroprotective properties and to enhance memory. Furthermore, AIT-082 is undergoing clinical trials as a potential treatment for Alzheimer’s disease.

The first part of the study investigated the ability of AIT-082 to influence cellular ATP levels in cortical neurons. Decreased energy metabolism is a key point in Ying’s (Ying, 1996a) theory of the development of Alzheimer’s disease. Previous work with AIT-082 had shown that it could protect hippocampal neurons from cellular damage caused by sublethal doses of glutamate. Specifically, AIT-082 prevented neurite degeneration. Also, AIT-082 was shown to increase mitochondrial membrane potential, especially at the distal tips of the neurites, in hippocampal neurons. I hypothesized that AIT-082 was protecting the neurons by increasing the ability of the mitochondria to generate ATP and thereby increasing the amount of ATP available to the cell. ATP was collected and measured from cortical neuron cultures that were exposed to glutamate, AIT-082, glutamate and AIT-082. The ATP levels were compared to the ATP levels from cortical neuron cultures that were exposed to vehicle for glutamate and AIT-082. The results did not significantly increase ATP levels in cortical neurons following glutamate exposure.

The next set of experiments involved investigations into the ability of AIT-082 to influence phosphorylation events in neural cells. AIT-082 shares some neurotrophic and neuroprotective properties with a group of drugs called the immunophilin ligands. The neuroprotective properties of the immunophilin ligands are mainly due to their ability to
influence protein phosphorylation by inhibiting the activity of calcineurin a protein phosphatase. The first set of experiments used western blot techniques to measure serine peptide and threonine peptide phosphorylation levels in proteins from whole brain homogenates that were incubated with vehicle, AIT-082, and GMP. Both AIT-082 and GMP caused an increase in the level of serine peptide phosphorylation compared to vehicle but only the increase caused by GMP treatment proved to be significant. Further, threonine phosphorylation levels were significantly increased by GMP but not AIT-082.

Phosphorylation levels of short peptide sequences containing either a phosphorylated serine or threonine residue were also measured in neuronal and astrocytic cultures. The neuronal cultures were exposed to 4 h of hypoxia to mimic the conditions of reduced energy availability observed in Alzheimer’s disease brains. Astrocyte cultures were exposed to 4 h of hypoxia/ischemia for the same reason. Both cell types were allowed to recover for 0, 1, 4, 12 and 24 hours with or without AIT-082 following the insult. AIT-082 treatment did not significantly affect phosphorylation levels of proteins harvested from either neuron or astrocyte cultures at any time period. I conclude therefore, that AIT-082 is not able to influence phosphorylation of the short amino acid sequences containing phosphorylated serine or threonine residues that could be detected by the primary antibodies used in my experiments.
Acknowledgements

I am very grateful to my Supervisor, Dr. Bernhard Juurlink. He provided a place for me in his lab and an opportunity to pursue science in a creative and supportive environment.

I would also like to thank the members of my graduate advisory committee, Drs. N. Ovsenek and M. Desautels, whose guidance and suggestions were greatly appreciated. I am also grateful to Dr. P. Paterson who has kindly agreed to serve as my external examiner.

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permission to Use</td>
<td>ii</td>
</tr>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>ix</td>
</tr>
<tr>
<td><strong>1.0 Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Alzheimer’s disease</td>
<td>2</td>
</tr>
<tr>
<td>1.11 Neuritic Plaques and the Deleterious Network</td>
<td>3</td>
</tr>
<tr>
<td>1.111 APP Processing</td>
<td>3</td>
</tr>
<tr>
<td>1.112 Free Radicals and Oxidative Stress</td>
<td>4</td>
</tr>
<tr>
<td>1.113 Glutamate excitotoxicity and Ca(^{2+}) Homeostasis</td>
<td>6</td>
</tr>
<tr>
<td>1.114 Impaired Energy Metabolism</td>
<td>8</td>
</tr>
<tr>
<td>1.12 Phosphorylation</td>
<td>9</td>
</tr>
<tr>
<td>1.121 Kinases</td>
<td>10</td>
</tr>
<tr>
<td>1.122 Phosphatases</td>
<td>15</td>
</tr>
<tr>
<td>1.2 Purines and the Nervous System</td>
<td>18</td>
</tr>
<tr>
<td>1.3 AIT-082</td>
<td>22</td>
</tr>
<tr>
<td>1.4 Immunophilins and Immunophilin Ligands</td>
<td>27</td>
</tr>
<tr>
<td>1.5 Research Goals and Hypotheses</td>
<td>30</td>
</tr>
<tr>
<td><strong>2.0 Methods and Materials</strong></td>
<td>31</td>
</tr>
<tr>
<td>2.1 Animals</td>
<td>31</td>
</tr>
<tr>
<td>2.2 Reagents</td>
<td>31</td>
</tr>
<tr>
<td>2.3 Tissue Culture</td>
<td>31</td>
</tr>
<tr>
<td>2.31 Culture Preparation</td>
<td>31</td>
</tr>
<tr>
<td>2.311 Cortical Neurons</td>
<td>31</td>
</tr>
<tr>
<td>2.312 Astrocytes</td>
<td>32</td>
</tr>
<tr>
<td>2.32 Experimental Treatment</td>
<td>33</td>
</tr>
<tr>
<td>2.321 Immunofluorescence</td>
<td>33</td>
</tr>
<tr>
<td>2.322 Glutamate Excitotoxic Insult to Cortical Neurons</td>
<td>35</td>
</tr>
<tr>
<td>2.323 Hypoxic/Ischemic Insult to Astrocytes</td>
<td>35</td>
</tr>
<tr>
<td>2.324 Hypoxic Insult to Cortical Neurons</td>
<td>36</td>
</tr>
<tr>
<td>2.325 Whole Brain Homogenates</td>
<td>37</td>
</tr>
<tr>
<td>2.4 Biochemical Analysis</td>
<td>38</td>
</tr>
<tr>
<td>2.41 Neuronal ATP</td>
<td>38</td>
</tr>
<tr>
<td>2.42 Western Blots</td>
<td>39</td>
</tr>
<tr>
<td>2.5 Equations and Statistical Analysis</td>
<td>41</td>
</tr>
<tr>
<td><strong>3.0 Results</strong></td>
<td>42</td>
</tr>
<tr>
<td>3.1 Immunofluorescence</td>
<td>42</td>
</tr>
<tr>
<td>3.2 Glutamate Excitotoxicity</td>
<td>42</td>
</tr>
<tr>
<td>3.21 Cortical Neuron ATP Levels</td>
<td>43</td>
</tr>
<tr>
<td>3.3 Protein Phosphorylation</td>
<td>43</td>
</tr>
<tr>
<td>3.31 Brain Homogenates</td>
<td>43</td>
</tr>
<tr>
<td>3.32 Neural Cells</td>
<td>44</td>
</tr>
<tr>
<td>3.321 Hypoxic Neurons</td>
<td>45</td>
</tr>
<tr>
<td>3.322 Hypoxic/Ischemic Astrocytes</td>
<td>47</td>
</tr>
<tr>
<td><strong>4.0 Discussion</strong></td>
<td>49</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1: Cartoons of the molecular structures of AIT-082 and representative members of the purines and immunophilin ligands

Figure 2: Cartoon of the pathways that result in the generation of strong oxidants in the CNS

Figure 3: Photograph showing immunofluorescent confirmation of cellular identities of cortical neurons and astrocytes

Figure 4: Graphical representation of the effect of AIT-082 on cortical neuron ATP levels following exposure to glutamate

Figure 5: The effects of AIT-082 and GMP on whole brain homogenate serine protein phosphorylation

Figure 6: The effects of AIT-082 and GMP on whole brain homogenate threonine protein phosphorylation

Figure 7: The effects of AIT-082 on cortical neuron serine protein phosphorylation following 4h of hypoxia

Figure 8: The effects of AIT-082 on cortical neuron threonine protein phosphorylation following 4h of hypoxia

Figure 9: The effects of AIT-082 on cortical astrocyte serine protein phosphorylation following 4h of hypoxia/ischemia

Figure 10: The effects of AIT-082 on cortical astrocyte threonine protein phosphorylation following 4h of hypoxia
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>adenosine receptor 1</td>
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<td>A&lt;sub&gt;2A&lt;/sub&gt;</td>
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<td>Aβ</td>
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<td>acetylcholine esterase</td>
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<td>acetylcholine receptor</td>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>adenosine diphosphate</td>
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<td>adenosine monophosphate</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxasole-proprionic acid</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>APP</td>
<td>amyloid precursor protein</td>
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<td>BBB</td>
<td>blood brain barrier</td>
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<td>cyclic adenosine monophosphate</td>
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<td>cGMP</td>
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<td>CsA</td>
<td>cyclosporin A</td>
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<td>Dulbecco’s minimum essential medium</td>
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<td>DMSO</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>ethylene-diamine-tetra-acetic acid</td>
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<td>EGTA</td>
<td>ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
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<tr>
<td>FKBP</td>
<td>FK506 binding protein</td>
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<td>GABA</td>
<td>γ-amino butyric acid</td>
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<td>guanosine monophosphate</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GMP</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>H⁺</td>
<td>hydrogen or proton</td>
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<tr>
<td>H/I</td>
<td>hypoxic/ischemic</td>
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<td>IB-MECA</td>
<td>2-chloro-N⁶-(3-iodobenzyl)-5’-N-methylcarboxamidoadenosine</td>
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<td>IκB α, β, ε</td>
<td>inhibitory factor κ B α, β, ε</td>
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<td>IKK</td>
<td>Inhibitory factor κ B kinase</td>
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<tr>
<td>iNOS</td>
<td>inducible or immunologic nitric oxide synthase</td>
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<tr>
<td>IP</td>
<td>immunophilin</td>
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<tr>
<td>IPL</td>
<td>immunophilin ligand</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium</td>
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<tr>
<td>KA</td>
<td>kainic acid</td>
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<tr>
<td>LTP</td>
<td>long term potentiation</td>
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<td>Mg²⁺</td>
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<td>messenger ribonucleic acid</td>
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<td>MTP</td>
<td>mitochondrial transition pore</td>
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<tr>
<td>N₂</td>
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<td>neurofilament 200</td>
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<td>NFκB</td>
<td>nuclear factor κ B</td>
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<td>neurofibrillary tangle</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>Ni²⁺</td>
<td>nickel</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
</tbody>
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NT-3  neurotrophin 3
O₂  oxygen
P₁  purine receptor type 1 (adenosine)
P₂  purine receptor type 2 (ATP)
PBS  phosphate buffered saline
PBST  phosphate buffered saline with Tween 20
PC12  pheochromocytoma
PKA  cAMP dependent kinase
PKC  protein kinase C
PLA₂  phospholipase A₂
PLC  phospholipase C
PP₁  protein phosphatase 1
PP₂ₐ  protein phosphatase 2A
PP₂ₖ  protein phosphatase 2B
PP₂₉  protein phosphatase 2C
RNA  ribonucleic acid
ROS  reactive oxygen species
Ser  serine
SNAP25  synaptosomal associated protein 25 kD
TGFβ  transforming growth factor β
Thr  threonine
TPA  12-O-tetradecanoyl-phorbol-13-acetate
1.0 Introduction

AIT-082, 4-[[3-(1,6-dihydro-6-oxo-purin-9-yl)-1-oxopropyl]amino] benzoic acid, potassium salt (also known as letenprinim, NEOTROFIN™) is a derivative of hypoxanthine that contains a para-amino benzoic acid moiety. See figure 1A for the molecular structure of AIT-082 (Rathbone, 1998). Essentially, AIT-082 is a covalent linkage of inosine and p-acetamidobenzoic acid salt (inosine pranobex), developed because inosine pranobex augmented learning in a conditioned avoidance task in rats (Glasky et al., 1994; Rathbone et al., 1999). AIT-082 crosses the blood brain barrier (BBB) in useful quantities by a non-saturable mechanism (likely diffusion) and passes into the brain parenchyma (Taylor et al., 2000). There are also reports by two different groups that AIT-082 possesses memory enhancing properties (Gittis, 1999; Glasky et al., 1994).

In Swiss-Webster mice AIT-082 prolonged the duration of working memory in young mice as well as aged mice with mild to moderate memory deficits (Glasky et al., 1994). In addition to short term memory (tested by win shift paradigm) AIT-082 also improved long term memory (tested by passive avoidance paradigm) in mice (Taylor et al., 2000). Furthermore, AIT-082 improves memory deficits caused by age, ibotenic acid induced lesions and a number of amnestic agents (Taylor et al., 2000). This drug’s ability to cross the BBB combined with its memory enhancing properties made it an interesting candidate for investigation as a potential candidate for treatment in Alzheimer’s disease (AD). Indeed, in addition to the primary research presented here on the effects of AIT-082 in neural cells the drug has also been shown to enhance memory in patients with mild to moderate AD (Taylor et al., 2000).
1.1 Alzheimer’s disease

Alzheimer’s disease is the most common dementing disease in the western world. It has been estimated that one in twenty people 65 years and older are afflicted with AD; currently, approximately 238,000 Canadians suffer from AD. By 2031 that figure is expected to increase to an estimated 750,000 Canadians afflicted with AD (http://www.alzheimer.ca/eng-lish/disease/stats-people.htm). As well as its social significance, AD at an estimated cost of $5.5 billion (CAD) per year also has important economic consequences (http://www.alzheimer.ca/english/disease/stats-people.htm).

Clinically, AD is characterized by memory loss, personality changes, as well as signs of dysfunction in cortical function such as aphasia (partial or total inability to produce and understand speech), apraxia (inability to perform complex movements) and agnosia (total or partial loss of the ability to recognize familiar people or objects) (Morris, 1999). Pathologically, two types of lesions, neuritic plaques and neurofibrillary tangles (NFT), as well as loss of neurons from specific regions of the brain are characteristic features of AD. Both the senile plaques and the NFTs are found in the cerebral cortex and the CA1 region of the hippocampus (Hof, 1999). Significant loss of neurons also occurs in these regions but cell death has also been observed in subcortical neuronal populations in the nucleus basalis of Meynert and other areas of the basal forebrain, as well as more variable neuronal loss in the median raphe and locus ceruleus (Snyder and Sabatini, 1995). Although the cause of neurodegeneration in this disease is still unknown the two characteristic AD lesions suggest the involvement of certain pathological processes or at the very least the dysregulation of certain physiological processes. Neuritic plaques, for example, appear to be associated with a number of adverse processes that can contribute to cell death such as altered amyloid precursor
protein processing, increased free radical damage, glutamate excitotoxicity and abnormal Ca\(^{2+}\) homeostasis, and impaired energy metabolism that form the framework for Ying’s (Ying, 1996a; Ying, 1996b) “deleterious network” hypothesis in AD. The appearance of NFTs on the other hand, due to the hyperphosphorylated status of their main protein component, strongly indicate that there has been a break down in the regulation of neuronal phosphorylation in AD.

1.11 Neuritic Plaques and the Deleterious Network

1.111 APP Processing

Neuritic plaques are spherical, multi-cellular lesions. The plaque usually, but not always, contains a proteinaceous core. The core is composed of over 40 proteins (McGeer, 1999) but the main component is the β-amyloid peptide (Aβ). Degenerating axons and dendrites, i.e., dystrophic neurites, are intimately associated with the Aβ core and the plaque is surrounded by activated microglia and reactive astrocytes (Selkoe, 1999). There are also plaques that lack the dystrophic neurites and surrounding glial cells. These “diffuse plaques” are composed of non-fibrillar, but still highly amyloidogenic, Aβ (Selkoe, 1999).

Aβ is formed via proteolytic cleavage of the amyloid precursor protein (APP). APP is a 695-770 amino acid transmembrane protein (Mattson, 1994) that is processed by 3 proteases: α-, β- and γ-secretase (Lichtenthaler et al., 1999). α-secretase cleaves APP within the Aβ domain and therefore does not contribute to the accumulation of the protein. β-secretase cleavage, however, produces two fragments, one of which contains the entire amino acid sequence for Aβ (Busciglio et al., 1993; Higaki et al., 1995). γ-
secretase activity cleaves the Aβ containing fragment, producing the 40 and 42 amino acid Aβ peptides (Lichtenthaler et al., 1999). Inappropriate APP processing or metabolism as an important factor in AD pathogenesis is supported by the observations that AD is associated with: (i) inherited APP mutations and (ii) overexpression of APP resulting from chromosome 21 trisomy in Down’s syndrome (Yankner, 1996). Also, Aβ is neurotoxic and contributes to free radical formation and oxidative stress (Behl et al., 1994; Hensley et al., 1994), loss of Ca^{2+} homeostasis (Mattson, 1994; Mattson et al., 1992), glutamate excitotoxicity (Mattson, 1994; Yankner, 1996) and damage to energy metabolism (Copani et al., 1991; Kalaria, 1992).

1.112 Free Radicals and Oxidative Stress

The CNS consumes a disproportionately large quantity of oxygen when the body is at rest: approximately 2% of total body mass versus 20% oxygen consumption (Juurlink and Paterson, 1998). It derives almost all of its energy from oxidative metabolism of the mitochondrial respiratory chain that reduces O_{2} to H_{2}O by the addition of four electrons and four H^{+}. “Leakage” of electrons, particularly in such a high use system, produces the superoxide anion and hydrogen peroxide (Coyle and Puttfarcken, 1993). Approximately 3% of all oxygen consumed during mitochondrial respiration is partially reduced to the superoxide anion rather than completely reduced to water (Fridovich, 1986) and pathological processes that interfere with normal energy metabolism increase mitochondrial ROS output (Sheehan et al., 1997; Yankner, 1996). Generation of those two reactive oxygen species can lead to the production of a host of other free radicals that include both reactive nitrogen (nitric oxide radical and
peroxynitrite) and other oxygen species (hydroxyl radical, hypochlorite, and singlet oxygen). Unchecked these oxidative species can deplete cellular NADH, interfere with mitochondrial function, de-esterify and oxidize membrane lipids (disrupting the plasma membrane), oxidize RNA (Nunomura et al., 1999) and cause nuclear and mitochondrial DNA damage and mutagenesis (Juurlink and Paterson, 1998). For a more comprehensive review of ROS generation and its consequences in the CNS see Juurlink and Paterson, (1998) and Juurlink, (2001). Figure 2 depicts a cartoon diagram of the ROS species cascade (Juurlink, 2001).

In AD brains activated glial cells that are associated with the neuritic plaques provide another source of ROS (Akama and Van Eldik, 2000; McGeer, 1999). In the CNS microglia can be activated by Aβ (Araujo and Cotman, 1992; McGeer, 1999; Murphy et al., 1998) and act as resident macrophages. Activated microglia cells, like all macrophages, possess a respiratory burst system that can produce massive amounts of the superoxide anion. Normally, this powerful system is directed against invading organisms or tumour cells but in AD, where such targets are absent, the respiratory burst of microglia (unable to distinguish friend from foe) ends up causing oxidative damage to the surrounding neurons (McGeer, 1999). Reactive astrocytes contribute to Aβ induced oxidative stress in two ways. First, they prevent microglia from phagocytosing, and thus clearing, the Aβ cores of neuritic plaques (DeWitt et al., 1998). Second, they contribute directly as Aβ stimulates inducible nitric oxide synthase activity in astrocytes that ultimately results in peroxynitrite formation (Akama and Van Eldik, 2000). The combined action of microglia and astrocytes in the AD brain result in a significant
contribution to the oxidative stress experienced by neurons in the vicinity to the neuritic plaques.

1.113 Glutamate Excitotoxicity and \( \text{Ca}^{2+} \) Homeostasis

Glutamate is the principle excitatory neurotransmitter in the brain and under physiological conditions it regulates many important neuronal processes. Excessive stimulation of glutamate gated cation channels has been implicated as a very important factor for neuronal degeneration in epilepsy, stroke and a host of neurodegenerative disorders including AD (Coyle and Puttfarcken, 1993; Mattson, 1994). Glutamate binds to both ionotropic and metabotropic receptors. The G-protein linked metabotropic glutamate receptors do not mediate the neurotoxic effects of glutamate but they may modulate the effects of the ionotropic receptors (Coyle and Puttfarcken, 1993). The NMDA (N-methyl-D-aspartate), AMPA (\( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxasole-propionic acid) and KA (kainic acid) ionotropic receptors are named for their most potent pharmacological agonists and mediate neuronal cation influx. All of the receptors allow influx of \( \text{Na}^+ \) but the NMDA receptors, and to a lesser extent the KA and AMPA receptors, are able to allow \( \text{Ca}^{2+} \) into the cell (Juurlink and Paterson, 1998). Delayed neuronal degeneration following a brief exposure to a high concentration or prolonged exposure to a low concentration of glutamate is dependent on \( \text{Ca}^{2+} \) influx (Choi et al., 1987; Kato et al., 1991; Schwarcz et al., 1984) and is called excitotoxicity. Under pathological conditions the interdependence of \( \text{Ca}^{2+} \) influx and glutamate release results in a destructive spiral that greatly upsets \( \text{Ca}^{2+} \) homeostasis and leads to many cell
damaging processes. Furthermore, damage to plasma membranes as the result of oxidative stress can also allow the entry of additional Ca\(^{2+}\).

Calcium is the foremost second messenger mediating neuronal adaptive changes in response to external stimuli (Mattson, 1994). Therefore, it is not surprising that excessive perturbations of the homeostatic concentration of intracellular calcium can lead to a host of potentially pathological processes. The activity of many enzymes, including proteases, kinases, phosphatases and endonucleases, are regulated by calcium concentration and inappropriate enzyme activity can easily lead to cell damage. A group of proteases called the calpains provide a useful example of the deleterious consequences of inappropriate enzyme activation. The calpains are calcium dependent proteases that cleave xanthine dehydrogenase to form xanthine oxidase. Both xanthine dehydrogenase and xanthine oxidase catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid but xanthine oxidase uses O\(_2\) rather than NAD as the electron acceptor and the result is the formation of the superoxide anion thereby increasing oxidative stress (Sussman and Bulkley, 1990). Furthermore, the calpains cleave multiple target proteins and uncontrolled calpain activity can result in necrotic cell death (Juurlink and Paterson, 1998).

Mitochondrial function is closely linked to intracellular calcium concentrations and elevated intracellular Ca\(^{2+}\) causes futile mitochondrial Ca\(^{2+}\) cycling that consumes reduced pyrimidine nucleotides and damages mitochondria (Juurlink and Paterson, 1998). Damaged mitochondria produce greater amounts of ROS and exhibit a decreased ability to produce ATP (Richter and Kass, 1991). There is also evidence that increased Ca\(^{2+}\) can enhance A\(\beta\) production and its subsequent effects (Ying, 1996a).
1.114 Impaired Energy Metabolism

Compromised energy metabolism is an important facet of AD pathology. With respect to the AD brain Hoyer (Hoyer, 1991; Hoyer, 1993) reported that brain glucose and oxygen utilization, cerebral blood flow and ATP formation were all reduced significantly compared to control values. Additionally, Sorbi and colleagues (Sorbi et al., 1983) have shown that the activity of the pyruvate dehydrogenase complex was also reduced in brains from patients with AD. Furthermore, there are also defects in the mitochondrial electron transport chain (ETC) in AD brains. Parker and coworkers (Parker et al., 1994) have shown that mitochondria from AD brains showed an overall reduction in activity for all the electron transport chain complexes but the depression was most striking for the cytochrome c oxidase complex. As mentioned previously, reduced ETC efficiency also increases ROS production. The net result of decreased availability of substrate for the Krebs cycle and impaired efficiency of the electron transport chain is reduced availability of ATP.

Since energy metabolism affects most aspects of cellular function it is likely that the metabolic compromise observed in AD contributes to other pathological processes in the disease. In fact, decreased ATP impairs the action of cation pumps such as the Na\(^+\)/K\(^+\) ATPase that act to maintain membrane polarity (Silver and Erecinska, 1997) and the Ca\(^{2+}\)/H\(^+\) ATPase that act to clear excess intracellular Ca\(^{2+}\) (Siesjo, 1992). The resulting membrane depolarization encourages further Ca\(^{2+}\) influx that causes the release of neurotransmitters including glutamate that in its turn causes further membrane depolarization (Juurlink and Paterson, 1998). It has also been observed that declined energy metabolism and glucose deprivation lead to increased production of A\(\beta\).
(Gabuzda et al., 1994) and the formation of neurofibrillary tangle-like changes in hippocampal neurons *in vitro* (Cheng and Mattson, 1992; Planel et al., 2001). Like APP processing, Ca\(^{2+}\) homeostasis and oxidative stress, when it is malfunctioning energy metabolism has serious repercussions. These processes are interconnected and disturbance to any one of them can lead to breakdown of normal functioning of the others (Ying, 1996a; Ying, 1996b).

### 1.12 Phosphorylation

The presence of NFTs in Alzheimer’s disease, paired helical filaments made of hyperphosphorylated tau, indicates that abnormal phosphorylation is playing a role in the pathology of AD. The phosphorylation event is catalyzed by protein kinases that transfer the $\gamma$-phosphoryl group from ATP to the alcohol groups of serine and threonine or the phenol group of tyrosine on the side chains of the target protein. Kinases are commonly divided into two groups depending on the amino acid to which the transfer the phosphoryl group: *(i)* serine/threonine protein kinases and *(ii)* tyrosine kinases (Shtonda et al., 1999; Swope et al., 1999). Protein phosphatases are the enzymes that catalyze the removal of phosphoryl groups from their target proteins and can also be classified into two categories: *(i)* serine/threonine protein kinases and *(ii)* tyrosine kinases (Nairn et al., 1985). The serine/threonine phosphatases can be further divided into four general categories: protein phosphatases 1, 2A, 2B and 2C (PP\(_1\), PP\(_{2A}\), PP\(_{2B}\) and PP\(_{2C}\)) (Cohen, 1989; Morioka et al., 1999; Nairn et al., 1985). PP\(_{2A}\) and PP\(_{2B}\) are present at higher levels in the brain than in any other tissue.
In the brain, under normal physiological conditions the purpose of phosphorylation and dephosphorylation events is, generally, to control or modulate the function of a given protein target. This modulation can range from activating or deactivating an enzyme to merely increasing or decreasing its activity. Phosphorylation events can modulate ion passage through the cell membrane via receptors and ion channels. Protein phosphorylation can also affect neurotransmitter release, cytoskeleton assembly and stability, gene expression and protein-protein interactions. Because phosphorylation status can influence such a broad spectrum of protein functions it plays a vital role in propagating many second messenger cascades. A very useful quality of the phosphorylation event is in its reversibility, for example, an enzyme that is activated by a phosphorylation event can be deactivated by the removal of the phosphoryl group or an ion channels conductance can be modulated through the addition or removal of phosphoryl groups.

1.121 Kinases

Protein kinase C (PKC) and calcium/calmodulin dependent kinase II (CaMKII) are two important serine/threonine kinases in the CNS. PKC is present at higher levels in the brain than in any other tissue (Saitoh, 1989) and CaMKII represents as much as 0.4% of total brain protein (Micheau and Riedel, 1999; Nairn et al., 1985). In certain areas of the brain (e.g., the hippocampus) CaMKII can represent as much as 2% of total protein and is particularly concentrated at the post synaptic densities of excitatory synapses (Kennedy et al., 1983). Protein kinase C and CaMKII are both activated by Ca$^{2+}$ and an additional cofactor: phospholipids (especially phosphatidyl serine) for PKC and the calcium binding protein, calmodulin for CaMKII. The affinity of PKC for Ca$^{2+}$
can be greatly increased by diacylglycerol (DAG) to the point that it can be fully activated without a net increase in Ca\(^{2+}\) concentration if DAG is present (Kaibuchi et al., 1981). This effect can be mimicked by phorbol esters that resemble DAG such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Following activation, CaMKII can become auto-phosphorylated and achieve an active state independent of any requirements for calcium and calmodulin (Miller and Kennedy, 1986; Shtonda et al., 1999).

Both of the above kinases are involved in the process of long term potentiation (LTP) (Braun and Schulman, 1995; Micheau and Riedel, 1999; Paratcha et al., 2000). LTP involves the strengthening of synaptic connections and the formation of memories (i.e., learning). Furthermore, in the cortices of people with AD, a disease characterized by the loss of synapses followed by neuronal death and memory loss, levels of both these kinases are reduced in diseased brain compared to normal cortices (Cole et al., 1988; Nishizuka, 1986; Saitoh, 1989) indicating that not only are their functions important in the normal brain but that their dysfunction may be related to the pathological processes of AD. PKC and CaMKII also phosphorylate a number of proteins that could either directly or indirectly contribute to or influence pathological processes in AD.

Neurotransmitter receptors for the excitatory neurotransmitters acetylcholine and glutamate are both believed to be involved in AD. PKC phosphorylates serine moieties of the acetylcholine receptor (AChR) which results in an increase in the rapidity of receptor desensitization as well as a reduced sensitivity to acetylcholine thereby reducing the effectiveness of that neurotransmitter (Swope et al., 1999). In AD acetylcholine levels are already reduced and cholinergic neurons of the cortex appear to be particularly vulnerable to deleterious effects associated with reduced activity (Mufson et al., 2002).
The AMPA and kainate glutamate receptors are phosphorylated by both PKC and CaMKII. Phosphorylation of serine residues by these kinases potentiate AMPA receptor mediated currents and may also play a role in mediating AMPA receptor redistribution or insertion into the membrane (Swope et al., 1999). PKC also phosphorylates the NMDA glutamate receptor and appears to potentiate currents mediated by this receptor by increasing the incidence of the receptor channel opening and in some cases by decreasing the Mg$^{2+}$ block of the channels (Chen and Huang, 1991). The glutamate receptors, the NMDA receptor in particular, play important roles in a pathological process known as glutamate excitotoxicity (described previously in this document) that has been implicated in a number of neurodegenerative disorders including AD.

Plasma membrane Ca$^{2+}$ pump activity is a very important mechanism for maintaining cellular Ca$^{2+}$ homeostasis (Borle, 1981; Smallwood et al., 1988). This pump catalyzes the ATP dependent exchange of the divalent calcium cation for the monovalent hydrogen cation (i.e., a proton). The results of removing Ca$^{2+}$ from the cytoplasm are two-fold in neurons. First, it controls the active state of numerous enzymes that are activated by increases in Ca$^{2+}$. Second, it helps to repolarize the cell membrane following the rapid increase in Ca$^{2+}$ following neurotransmitter binding to synaptic receptors. Phosphorylation of the Ca$^{2+}$/H$^{+}$ ATPase by PKC causes a significant increase in Ca$^{2+}$ flow through the pump (i.e., a 5-7 times increase in V$_{max}$ but essentially no change in K$_{m}$ for Ca$^{2+}$) (Smallwood et al., 1988). Na$^{+}$/K$^{+}$ ATPases are responsible for maintaining resting membrane potential in neurons. Na$^{+}$/K$^{+}$ ATPases act to return the membrane potential to its resting state following extended periods of depolarization. Phosphorylation by PKC, as well as the cAMP dependent kinase (PKA), stimulates the Na$^{+}$/K$^{+}$ ATPase thereby hastening membrane repolarization (Therien and Blostein,
Together, phosphorylation of these two ion pumps helps to restore membrane polarization as well as Ca\textsuperscript{2+} homeostasis. When those two states are excessively or inappropriately altered they can contribute to a potentially cytotoxic cascade that involves ATP depletion, mitochondrial dysfunction, glutamate excitotoxicity and free radical generation (Juurink and Paterson, 1998) that, as discussed previously, contributes to many pathological conditions in the brain including trauma, stroke, AD and other neurodegenerative disorders.

Phosphorylation by PKC and CaMKII can also influence gene expression. NF\kappa B is a transcription factor that has been shown to be involved in neuronal apoptosis and survival. Activation of this transcription factor prior to experimental insults such as glutamate, A\beta, reactive oxygen species exposure or glucose deprivation, protects cells against apoptosis (Barger and Mattson, 1996; Cheng et al., 1994; Kaltschmidt et al., 1997). Also, post-mortem examination of brains from patients with neurodegenerative disorders such as Parkinson’s disease and AD show increased NF\kappa B activity closely linked with the degenerative processes (Hunot et al., 1997; Kaltschmidt et al., 1997). NF\kappa B is activated by glutamate receptor binding, membrane depolarization, and excitotoxic and apoptotic insults (Grilli et al., 1996; Guerrini et al., 1995; Kaltschmidt et al., 1995). Peptides called I\kappa B\alpha, -\beta, and -\epsilon bind to inactive NF\kappa B and conceal its nuclear localization signal (Whiteside and Israel, 1997). When a class of kinases, I\kappa B kinases (IKK) -\alpha and -\beta, phosphorylate the inhibitory molecules NF\kappa B moves to the nucleus to influence gene expression (Lilienbaum and Israel, 2003). It is at this level that the kinases PKC and CaMKII act, in response to increases in Ca\textsuperscript{2+} concentration, by phosphorylating the IKKs. Inhibition of PKC inhibits NF\kappa B mediated transcription and
activation of PKC results in NFκB activity (Bonizzi et al., 1999; Bren et al., 2000; Han and Logsdon, 2000; Lin et al., 2000a; Lin et al., 2000b; Pieper and Riaz ul, 1997; Tando et al., 1999; Wooten et al., 1999). PKC seems to exert these effects via IKKβ but not –α (Lallena et al., 1999). Also, when CaMKII is inhibited IKK cannot become activated and in the presence of a constitutively active CaMKII NFκB also becomes active (Hughes et al., 2001). Thus kinases play a vital role in controlling the activity of a transcription factor that is important not only in cell death associated with AD but many pathological processes.

PKC and CaMKII phosphorylate two proteins that are directly involved in AD pathology: tau and APP (Alonso et al., 1996; Gandy et al., 1988; Lichtenthaler et al., 1999; Micheau and Riedel, 1999; Sironi et al., 1998). Both tau and APP are precursors of the major protein components of the characteristic AD lesions. NFT’s are accumulations of paired helical filaments in the perikarya of neurons. These paired helical filaments are composed of hyperphosphorylated tau. The wild type tau plays a role in promoting and stabilizing microtubule assembly and its ability to perform these tasks is inhibited by phosphorylation (Alonso et al., 1996; Baudier and Cole, 1987; Grundke-Iqbal et al., 1986; Iqbal et al., 1994; Micheau and Riedel, 1999; Sironi et al., 1998; Sternberger et al., 1985; Trojanowski and Lee, 1994; Wang et al., 1996). Both PKC and CaMKII in addition to a number of other kinases have been shown to phosphorylate tau (Braun and Schulman, 1995; Brewton et al., 2001; Ekinci and Shea, 1999; Micheau and Riedel, 1999). Phosphorylation of the serine-262 (Ser262) residue of tau is believed to be primarily responsible for decreasing tau’s ability to bind to microtubules (Biernat et al., 1993; Drewes et al., 1995). Sironi and colleagues (Sironi et
al., 1998) showed that almost half of all phosphorylation events at that site were the result of CaMKII activity. As mentioned previously, the main protein component of senile plaques is Aβ that originates from the larger APP. γ-secretase cleavage under normal conditions produces more Aβ40 than Aβ42 (Lichtenthaler et al., 1997; Maruyama et al., 1996; Suzuki et al., 1994; Tienari et al., 1997). Aβ length is important to AD pathology because increased length of the hydrophobic C-terminus (i.e., Aβ42 compared to Aβ40) promotes early deposition of fibrillar Aβ in familial AD (Mills and Reiner, 1999). Lichtenthaler and colleagues (Lichtenthaler et al., 1999) showed that mutating various amino acids within transmembrane domain of the Aβ contain β-APP fragment (i.e., the putative γ-secretase recognition or cleavage sequence) could alter the ratio of Aβ42/Aβ40. Mutation of threonine-43 to phenylalanine (cannot be phosphorylated) resulted in an increase in the Aβ42/Aβ40 ratio (Lichtenthaler et al., 1999) allowing for the possibility that phosphorylation of the fragment at this amino acid may discourage γ-secretase cleavage that favours Aβ42 formation. PKC and CaMKII both phosphorylate APP on serine and threonine residues (Gandy et al., 1988). The researchers were not able to conclude what effect the phosphorylation events they observed would have on APP processing. However, they did point out that similar phosphorylation events on the interleukin-2 receptor and epidermal growth factor receptor by PKC resulted in internalization and suggested that in APP the phosphorylation events may regulate its internalization and metabolism (Gandy et al., 1988).

1.122 Phosphatases
Although protein phosphatase activity represents the enzymatic counter point to kinase activity, the protein kinases have generally been viewed as the undisputed champions of cellular signalling (Sontag, 2001). Not surprising given that common wisdom regarding the protein phosphatases used to be that they were “boring, lazy, constitutive ‘housekeeping’ enzyme(s)” (Sontag, 2001). The two most common serine/threonine kinases, PP$_{2A}$ and PP$_{2B}$ (commonly referred to as calcineurin) in the brain provide ample evidence that phosphatase activity serves not only to counter act kinase activity but to provide novel control opportunities for receptors, enzymes, cytoskeletal elements and transcription factors as will be demonstrated by the following examples. It is becoming increasingly clear that phosphatase activity plays a major role in regulating both physiological and pathological processes.

The catalytic activity, subcellular localization and broad substrate specificity (Nairn et al., 1985) of PP$_{2A}$ appear to be regulated by subunit composition, post translational modifications of the subunits and a diverse range of enzyme inhibitors (Goldberg, 1999; Sontag, 2001). Calcineurin, on the other hand, exhibits more restricted substrate specificity (Nairn et al., 1985) and is activated by the presence of calmodulin, Ca$^{2+}$ and another divalent cation (Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$ or Ni$^{2+}$) to act as a cofactor (Li and Chan, 1984). Once activated the traditionally recognized responsibility of the phosphatases to keep the signalling output of activated kinases in check obviously results in the reversal of the effects (such as the ones described above) the kinases initiated. The following examples demonstrate the unique roles PP$_{2A}$ and calcineurin play in the brain and how they can contribute to AD.

The importance of kinase action in tau achieving the hyperphosphorylated state seen in AD is indisputable. However, experiments by Planel and his colleagues (Planel
et al., 2001) show that phosphatase action may be of even greater consequence in the development of hyperphosphorylated tau. It is known that PP$_{2A}$ is a major tau phosphatase (Sontag et al., 1999). In experiments involving starved mice, which induces tau hyperphosphorylation similar to that in AD (Planel et al., 2001), Planel and colleagues, observed decreases in tau directed tau protein kinase I/glycogen synthase kinase 3β, cyclin dependent kinase 5, and PP$_{2A}$ activities. This indicates that activation of those two kinases at least are not necessary for tau hyperphosphorylation and that PP$_{2A}$ activity is of major importance for maintaining normal tau (Planel et al., 2001). Gong et al (Gong et al., 1995) have shown that in AD the activity of PP$_{2A}$ is also reduced. Furthermore, proper tau activity is restored by phosphatase activity of PP$_{2A}$ (as well as calcineurin and PP$_{1}$ activity though with decreasing effectiveness) (Wang et al., 1996).

Nitric oxide (NO) is a free radical gas, produced by nitric oxide synthase (NOS), that functions as a diffusible neurotransmitter and inter- and intracellular signalling molecule in the brain (de la Monte et al., 2003; Schmidt and Walter, 1994). In some instances NO can react with the super oxide anion to produce the oxidant peroxynitrite. Peroxynitrite inhibits mitochondrial respiration and causes oxidative damage to cellular macromolecules (Juurlink and Paterson, 1998) and in this way is believed to contribute to AD (de la Monte et al., 2003; Law, 2001; Luth et al., 2002). There are three NOS enzymes: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible or immunologic NOS (iNOS) (Morioka et al., 1999). Both nNOS and eNOS are activated by Ca$^{2+}$/calmodulin while iNOS is Ca$^{2+}$-independent (Bredt and Snyder, 1990; Marletta, 1994; Yun et al., 1996). Phosphorylation of NOS by PKC inhibits its activity while
calcineurin activity reverses that effect (Bredt et al., 1992; Dawson et al., 1994). Reduction in NOS activity is believed to be the basis of the neuroprotective effects of several inhibitors of calcineurin, such as the immunophilin ligand FK506 discussed below.

1.2 Purines and the Nervous System

 The purines are ubiquitous molecules in biological systems that include: bases (guanine and adenine), nucleosides (guanosine and adenosine) and nucleotides (ATP, ADP, AMP, GTP, GDP and GMP) as well as their metabolic products (inosine, xanthine and hypoxanthine) (Rathbone et al., 1999). See figure 1B, 1C and 1D for the molecular structures of some representative members of the purine family (Lewin, 2000; Linden, 1994). The purine bases and their pyrimidine complements form the building blocks of DNA and RNA. The nucleotide ATP is unquestionably the most common form of cellular energy and it and other nucleotides and nucleosides (particularly GTP) are involved in many biochemical and energy transfer pathways within the cell (Rathbone et al., 1999). Nucleotides such as GDP (in association with G-proteins) and the cyclic nucleotides (cAMP and cGMP) act as important second messengers during signal transduction (Rathbone et al., 1999). In the CNS it has been recognized for sometime that adenosine (Rathbone et al., 1999), ATP (Burnstock, 1972; Geffen and Livett, 1971; Richardson and Brown, 1987; White, 1977) and GTP (Rathbone et al., 1999) have important roles in neurotransmission and neuromodulation. More recently however, it is becoming clear that the purines are involved in a number of processes that extend over longer time frames than the milliseconds to seconds that characterize neurotransmission.
(Rathbone, 1999). These trophic processes involve growth of neurites, neuroprotection and even managing cell number and growth.

Several in vitro studies have shown that purines, particularly guanosine and GTP, increase neurite outgrowth. In PC12 cells, guanosine and GTP (Gysbers et al., 2000; Gysbers and Rathbone, 1992; Gysbers and Rathbone, 1996a; Gysbers and Rathbone, 1996b) are capable of independently inducing neurite outgrowth. Furthermore, both guanosine and GTP act synergistically with NGF to greatly increase the proportion (beyond that achieved by either purine or NGF treatment alone) of neurite bearing PC12 cells (Gysbers et al., 2000; Gysbers and Rathbone, 1992; Gysbers and Rathbone, 1996a; Gysbers and Rathbone, 1996b) in PC12 cultures. Guanosine, GTP and GMP (but not adenosine or ATP) were also very effective in increasing neurite growth (increasing length and number of branches) in hippocampal neuron cultures (Juurlink, 1998b). Juurlink and Rathbone (Juurlink, 1998b) observed that effects of GTP were evident on both axonal and dendritic neuritic processes. The effects of adenosine and its analogues on neurite outgrowth from PC12 cells are unclear. Gysbers and Rathbone (Gysbers and Rathbone, 1992; Gysbers and Rathbone, 1996b) report that neurite outgrowth is elicited in response to those purines while Braumann et al (Braumann et al., 1986) report contradictory results.

Adenosine appears to be protective against hypoxia/ischemia, trauma, excitotoxicity and neurotoxic substances and these protective properties appear, for the most part, to be mediated via the purine receptors. The P₁ receptors preferentially bind to adenosine while the P₂ receptors preferentially bind ATP (Burnstock, 1976). The P₂ receptors can be divided into both metabotropic (P₂Y) and ionotropic (P₂X) type receptors with at least seven distinct members for each subtype (Abbracchio and Burnstock,
The $P_1$ receptors are metabotropic and can be divided into four subtypes: $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$. The four subtypes are distinguished on the basis of agonist and antagonist binding preferences as well as G-protein coupling and signalling pathways. The $A_1$ receptor typically inhibits adenylyl cyclase via the $G_{i\alpha 1-5}$-proteins (Londos et al., 1980; van Calker et al., 1979) though it can also affect guanylyl cyclase, $K^+$ channels, voltage gated $Ca^{2+}$ channels (Collis and Hourani, 1993), and phospholipase $A_2$ and $C$ (PLA$_2$ and PLC) activities (Collis and Hourani, 1993; Gerwins and Fredholm, 1992). $A_2$ receptor activation is usually associated with the activation of adenylyl cyclase via $G_S$-proteins (Olah and Stiles, 1995) and can also increase PKC activity that in turn modulates N-type $Ca^{2+}$ channel gating. The $A_3$ receptors are the least well characterized of the $P_1$ receptors and have been reported to inhibit adenylyl cyclase via the $G_i$-protein and stimulate $Ca^{2+}$ release from internal stores via the $G_q$-protein (Rathbone, 1999). For a complete review of the purine receptors see Poulson and Quinn, 1998 and Olah and Stiles, 1995.

There are many examples of various adenosine receptor agonists protecting neural tissue from hypoxic/ischemic (H/I) and excitotoxic insults. Following induction of global forebrain ischemia in rats, the adenosine analogues and $A_1$ receptor agonists 2-chloroadenosine, chloro-$N^6$-cyclopentyladenosine and $N^6$-cyclopentyladenosine strikingly reduced neuron loss in the CA1 region of the hippocampus of rats (Evans et al., 1987) and lowered mortality and neuron loss in gerbils (Rudolphi and Schubert, 1997; Von Lubitz et al., 1994). Furthermore, a number of reports show that 2-chloroadenosine was neuroprotective when co-injected into the striatum with various excitotoxic agents including NMDA, kainate, quisqualate and ibotenate, (Arvin et al., 1994).
21

1989; Arvin et al., 1988; Finn et al., 1991). Two other A\textsubscript{1} receptor agonists, R-N\textsuperscript{6}-phenylisopropyladenosine and N\textsuperscript{6}-cyclohexyladenosine, reduced kainate neurotoxicity in the hippocampus and other areas of the brain while A\textsubscript{1} receptor antagonists, 8-cyclopentyl-1,3-dipropylxanthine and 8-cyclopentyl-1,3-dimethylxanthine, potentiated kainate toxicity (MacGregor et al., 1998; MacGregor et al., 1997; MacGregor et al., 1996; MacGregor et al., 1993; MacGregor and Stone, 1992; MacGregor and Stone, 1993; Matsuoka et al., 1999).

Though A\textsubscript{1} receptor agonists seem to be clearly neuroprotective the effects of A\textsubscript{2} receptor binding are a lot less straightforward. As increased adenylyl cyclase activity resulting from A\textsubscript{2} receptor activation is the reverse of A\textsubscript{1} receptor activation, i.e., decreased adenylyl cyclase activity (Poulsen and Quinn, 1998), it is receptor antagonists rather than agonists that mediate neuroprotection by the A\textsubscript{2} receptors. A\textsubscript{2A} receptor antagonist 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazol{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol reduced kainate mediated excitotoxicity of the CA3 region of the hippocampus (Jones et al., 1998) and caffeine (an A\textsubscript{1} and A\textsubscript{2A} receptor antagonist) administered chronically for 3 weeks before bilateral carotid occlusion and hypotension induced ischemia improved brain magnetic resonance and histopathological changes (Sutherland et al., 1991). However, when administered acutely caffeine potentiated ischemic damage (Sutherland et al., 1991).

When A\textsubscript{3} receptor agonists (inhibit adenylyl cyclase activity) are administered chronically or at low concentrations they appear to possess neuroprotective properties. The selective A\textsubscript{3} receptor agonist 2-chloro-N\textsuperscript{6}-(3-iodobenzyl)-5’-N-methylcarboxamidoadenosine (Cl-IB-MECA) at low concentrations exerted trophic effects and inhibited astrocyte cell death in vitro (Abbracchio and Burnstock, 1998;
Abbracchio et al., 1997b) and N⁶-(3-iodobenzyl)-5’-N-methylcarboxamidoadenosine (IB-MECA) administered chronically protected hippocampal neurons and reduced mortality in gerbils following global forebrain ischemia (Von Lubitz et al., 1999; Von Lubitz et al., 1994). However, those same compounds administered acutely or at high concentrations had precisely opposite effects. High concentrations of Cl-IB-MECA increased cerebellar granule cell death (Sei, 1997) and reduced astrocyte viability (Abbracchio and Burnstock, 1998; Abbracchio et al., 1997a) while acute treatment of ischemic gerbils with IB-MECA increased the mortality rate and damage to hippocampal neurons (Von Lubitz et al., 1999; Von Lubitz et al., 1994) in vivo.

The apparently contradictory results above are called the effect inversion (Jacobson et al., 1996) and may occur for different reasons depending on the drug and the target receptor. Acute administration or administration of high doses may rob an agonist of its receptor specificity or in other cases perhaps chronic administration results in receptor desensitization. While the neuroprotective and neurotrophic properties of the purines are well known and documented there has not been much success in finding a practical pharmacological use for them (Rathbone, 1999). However, there has been some success with synthetically derived purines such as AIT-082.

1.3 AIT-082

AIT-082 has been observed to have trophic effects in the nervous system. Substances with trophic properties affect the development, structure or maintenance of neural cells and tissue over a longer time frame than that associated with neural transmission (Rathbone et al., 1999). Neurotrophic effects include plastic changes involved with LTP, memory and learning, collateral sprouting of nerve processes, cell
number regulation via apoptosis and even neuroprotection against toxic stimuli. Though it is a somewhat artificial division of cellular roles I intend to deal with the protective aspects of AIT-082’s trophic properties separately from its other trophic properties.

Neurotrophic factors are polypeptides that support the growth and differentiation of neurons in developing nervous systems and promote neuron survival in adults. AIT-082 treatment served to increase mRNA levels of three neurotrophic factors: nerve growth factor (NGF), neurotrophin 3 (NT-3) and basic fibrillary growth factor (bFGF) in astrocyte cultures (Di Iorio et al., 2001). It also caused NGF and another neurotrophic factor, transforming growth factor β (TGFβ), to be released into the culture medium (Ciccarelli, 1998; Rathbone, 1998). Furthermore, medium from astrocyte cultures thus ‘conditioned’ was able to protect hippocampal neuron cultures from NMDA-mediated toxicity (Caciagli, 1998). In vivo, AIT-082 was able to induce production of mRNA of brain derived neurotrophic factor (BDNF) following spinal cord lesions (Crocker, 1997) and NGF mRNA in basal forebrain following stereotaxic injection of ibotenic acid (Rathbone et al., 1999). Also, AIT-082 treatment of astrocyte cultures resulted in an increase in the production of the purines adenosine and inosine. As mentioned previously, adenosine also has some well documented neuroprotective properties.

AIT-082 has been shown to induce or encourage processes that represent the most recognizable neurotrophic activities: neurite growth (Bintner, 1999; Juurlink, 1998b; Middlemiss et al., 1995) and axon sprouting (Ramirez et al., 2002; Rathbone et al., 1999). AIT-082 promotes axon growth in primary cultures enriched in hippocampal neurons (Bintner, 1999; Juurlink, 1998b). The axon complexity of neurons grown under control conditions were compared to those grown in the presence of 1.0 µM AIT-082.
Axon complexity was measured as the number of times a given axon intersected the concentric circles of a grid applied to the neuron as described by Ang et al (1993). After five days in culture the axon complexity of control neurons reached a plateau but axon complexity of AIT-082 supplemented neurons continued to steadily increase for up to 7 days (Bintner, 1999; Juurlink, 1998b).

Experiments with PC12 cells also demonstrate the ability of AIT-082 to induce neurite growth. Without NGF PC12 cells proliferate but do not differentiate but when NGF is added to the culture medium the cells stop dividing and differentiate into sympathetic neuron-like cells and extend neurites (Greene, 1982). PC12 cells are cultured without astrocytes and therefore need exogenously supplied NGF to differentiate. Middlemiss et al (1995) have shown that not only does AIT-082 treatment increase the proportion of PC12 cells bearing neurites beyond that achieved by NGF alone it also: (i) increases the proportion of cells with more than one primary neurite, (ii) shifts the concentration response curve of NGF to the left, and (iii) increases the proportion of cells with neurites in cultures treated with optimal amounts of NGF (Middlemiss et al., 1995). Therefore, not only does AIT-082 promote neurite growth, it can act directly on neuron-like cells and seems to do so in a manner that is different from but synergistic with NGF mediated neuritogenesis (Middlemiss et al., 1995).

There are also a couple lines of evidence that AIT-082 can exert neurite growth in vivo. Rathbone et al (1999) reported that AIT-082 administration caused a small but significant enhancement in nerve sprouting start time into denervatated skin in rats. The sprouting was NGF-dependent; however, unlike exogenous NGF treatment, AIT-082 did not cause hyperalgesia (i.e., increased sensitivity to pain).
In response to reports that AIT-082 increased NGF mRNA (above) Ramirez et al (2002) decided to investigate the effects of AIT-082 on septodentate sprouting, a process believed to be regulated by NGF, after entorhinal cortex lesions. They observed that AIT-082 exerted stimulatory effects on lesion-induced sprouting, as measured by an increase in acetylcholinesterase (AChE) label in the outer molecular layer of the ventral dentate gyrus, compared to saline (Ramirez et al., 2002). Thus, AIT-082 is effective \textit{in vivo} as well as \textit{in vitro} and has the ability to stimulate anatomically appropriate sprouting.

Many of the above examples of the effects of AIT-082 on neural cells appear to rely on the presence of neurotrophic factors particularly NGF. In contrast to those studies, Lahiri and colleagues (Lahiri et al., 2000) have demonstrated AIT-082 activity that is opposite to that of NGF in the same system. Lahiri and coworkers (Lahiri et al., 2000) measured intracellular synaptophysin levels from PC12 cells as well as secreted synaptophysin levels in the culture medium. Synaptophysin is synaptic vesicle-associated integral membrane protein that is involved with neuronal transmission and is used as a protein marker for presynaptic terminals (Scheller, 1995). Synaptophysin is reduced in AD brains and can be used to measure synaptic number, density and (obliquely) neuronal transmission (Lahiri et al., 2000). They found that while NGF treatment resulted in a decrease in both intracellular and secreted synaptophysin AIT-082 caused synaptophysin levels, intracellular and secreted, to increase (Lahiri et al., 2000). They also found that AIT-082 increased the levels of synaptosomal associated protein of 25 kD (SNAP25) another presynaptic terminal protein (Lahiri et al., 2000). These results suggest that AIT-082 may be able to improve neurotransmission at the
presynaptic terminal and therefore perhaps improve some of the cognitive deficits seen in AD.

AIT-082 seems to be effective in protecting neurons against a number of compounds that induce excitotoxicity via the glutamate receptors. AIT-082 conditioned medium from astrocyte cultures protected hippocampal (as mentioned already) and cortical neuron cultures from NMDA-mediated toxicity (Caciagli, 1998; Ciccarelli, 1998). AIT-082 also protected hippocampal neurons, in vitro, from a sub-lethal dose of L-glutamate (Bintner, 1999; Juurlink, 1998b) even when AIT-082 was added to the culture medium after the neurons were exposed to glutamate. AIT-082 also increased hippocampal neuron mitochondria membrane potentials (visualized using a rosamine derivative) (Bintner, 1999). The more distal the mitochondria were in the neurites (from the soma) the lower their membrane potentials were and it was those mitochondria that were most affected by AIT-082 treatment (Bintner, 1999). This suggests that not all of AIT-082’s neuroprotective properties are mediated by changes in gene expression.

Experiments by two labs have demonstrated AIT-082’s neuroprotective efficacy in vivo. Local NMDA administration caused an almost 50% reduction in choline acetyltransferase activity in hippocampal neurons that was reversed by AIT-082 treatment (Caciagli, 1998; Di Iorio, 1999). The heterocyclic glutamate analogue kainate induces excitotoxic damage to cells that possess a large number of ionotropic glutamate receptors such as the CA3 pyramidal neurons in the hippocampus (Di Iorio et al., 2001). Kainate injection also causes limbic motor seizures in rodents (a model for human temporal lobe epilepsy) (Di Iorio et al., 2001). AIT-082 therapy did not change the kainate-induced seizures in Sprague-Dawley rats; however, it did decrease kainate-induced mortality and weight loss (in surviving animals) (Taylor et al., 2000).
Furthermore, AIT-082 treatment of kainate-stressed rats greatly reduced delayed hippocampal neuron death as assessed by glutamic acid decarboxylase activity and histological examination of hippocampi (Taylor et al., 2000). In a model of spinal cord injury, AIT-082 treatment resulted in a host of positive outcomes: fewer reactive glial cells, less necrotic tissue and cavitation, increased in nuclear staining and cell number and less swelling caudal to the lesion (Middlemiss, 1999). These histological improvements were accompanied by functional recovery in foot orientating and open field walking tests (segmental reflex and gross locomotor recovery respectively) (Middlemiss, 1999).

1.4 Immunophilins and Immunophilin Ligands

The search for safe, reliable drugs to suppress the immune system following tissue and/or organ transplant led to the discovery of a small group of drugs collectively termed the immunophilin ligands (IPLs). As their name suggests these drugs bind to members of a group of molecules called the immunophilins (IPs). The IPLs include the drugs cyclosporin A (CsA), which binds to the cyclophilins and FK506 that binds to the FK506 Binding Proteins (FKBPs). The protein complexes formed from the interaction of the IPs and their ligands interact with a broad range of signal transduction systems particularly those that relate to Ca$^{2+}$ and phosphorylation (Snyder and Sabatini, 1995). The immunosuppressive properties of the IPLs are believed to result from the drug-immunophilin complex inhibiting the activity of the Ca$^{2+}$/calmodulin dependent phosphatase, calcineurin (for review see Snyder and Sabatini, 1995; Sabatini et al., 1997). The immunophilin FKBP12 is present at high levels in nervous tissue. In situ hybridization shows FKBP12 mRNA at levels that are 10 to 50 times (Sabatini et al.,
higher in the brain than anywhere else in the body (Steiner et al., 1992). The molecular structure of some of the immunophilins shares some similarities with AIT-082 and there is also an accumulating body of evidence that the IPLs possess neuroprotective and neurotrophic properties that are in many cases similar to those observed with AIT-082. See figure 1E, 1F and 1G for the molecular structures of some representative immunophilin ligands (Harding et al., 1989).

Both FK506 and CsA possess neuroprotective properties. Dawson and coworkers (Dawson et al., 1993a) have shown that the immunophilin ligands mitigate excitotoxic damage to neuronal cells. Treatment with either 1 μM of FK506 or CsA significantly reduced excitotoxic cell death mediated by NMDA glutamate receptors. The drugs have also been shown to be neuroprotective in vivo by reducing the volume of ischemic damage to rat cortex following middle cerebral artery occlusion (Sabatini et al., 1997; Sharkey and Butcher, 1994; Sharkey et al., 1996). It has been proposed that the neuroprotective action of the IPLs lies in their ability to reduce nitric oxide formation by inhibiting calcineurin activity. Inhibition of calcineurin activity results in increased phosphorylation of a number of proteins including nNOS (Snyder et al., 1998) and iNOS (Trajkovic et al., 1999). The phosphorylated forms of nNOS and iNOS have lower catalytic activities and therefore produce less NO under excitotoxic conditions.

Cyclosporin A also appears to play a protective role by preventing apoptosis resulting from the formation of the mitochondrial transition pore (MTP). The open MTP (approximately 3 nm aperture) is permeable to solutes of up to 1500 Da (Massari and Azzone, 1972). The consequences of MTP opening are diminished ATP production due to destruction of the H⁺ electrochemical gradient, increased ATP consumption by
enzymes that are activated via the release of Ca\textsuperscript{2+} sequestered by the mitochondria, and release of proapoptotic peptides (e.g. cytochrome c and apoptosis inducing factor) from the intermembrane space to the cytosol (Bernardi, 1996; Fall and Bennett, 1999; Parone et al., 2002). These events form a destructive spiral that unless interrupted ultimately leads to cell death.

FK506 has been shown to augment neurite growth in SH-SY5Y and PC12 cells and in rat and chick sensory ganglia explants (Gold et al., 1995; Lyons et al., 1994; Steiner et al., 1997). The drug increased neurite growth in the cultures only when applied in conjunction with NGF. Although NGF was not added to the sensory ganglia explants it was presumed to be present as a result of the mixed cellular population. It has been shown that IPLs increase both the number and length of neurites in dopaminergic neuronal cultures (Costantini et al., 1998) and stimulate axonal regeneration and functional recovery following injury to peripheral nerves (Gold et al., 1995) and spinal cords (Bavetta et al., 1999). More recent experiments have demonstrated neurotrophic properties in human cells. Avramut and colleagues (Avramut et al., 2001) have shown that FK506 treatment of second trimester fetal brain cultures result in an increase in cell number (MAP-2 staining indicates a large portion of the increase was in neurons) and dendrite extension.

AIT-082 and the immunophilins share neuroprotective properties as well as the ability to stimulate neurite outgrowth. I hypothesize that in addition to sharing with the immunophilin ligands a similar molecular structure and the abilities to protect against glutamate mediated neurotoxicity and stimulate neurite outgrowth, that AIT-082 is also able to affect neural protein phosphorylation.
1.5 Research Goals and Hypotheses

AIT-082 has been shown to enhance memory and neurotrophic (both neuritogenic and neuroprotective) properties. It has also become the subject of clinical trials to determine its efficacy as a treatment for AD. In view of those results the goals of this research project were:

1. To determine if AIT-082 is able to cellular ATP levels.
2. To determine if non-adenosine purines (GMP and AIT-082) are able to affect phosphorylation of proteins from brain tissue.
3. To determine if AIT-082 can affect protein phosphorylation following conditions that (as in AD) reduce energy metabolism and increase Ca\(^{2+}\) influx in specific neural cell types (i.e., neurons and astrocytes).

I hypothesized that AIT-082, by increasing neuronal mitochondrial membrane potential would increase cellular ATP levels. The increase in ATP would allow the neurons to cope with the excess membrane depolarization and Ca\(^{2+}\) influx that accompanies glutamate excitotoxicity. Alternatively, the increase in mitochondrial membrane potential could represent closure of the mitochondrial transition pore and thus be protective by preventing the release of pro-apoptotic proteins (e.g., cytochrome c) from the intermembrane space.

I also hypothesized that because of their similarities to the IPLs the non-adenosine purines (GMP and AIT-082) would be able to alter protein phosphorylation in neural tissue following insults that caused impaired metabolism and increased intracellular Ca\(^{2+}\) concentration. Furthermore, I hypothesized that because of the
closeness of AIT-082 in form and function to the immunophilin ligands that any alteration in phosphorylation would likely result from changes in phosphatase rather than kinase activity.

2.0 Methods and Materials

2.1 Animals

All animals used in the performance of the experiments described here were obtained from the University of Saskatchewan’s Animal Resources Centre. Pregnant CD1 mice were obtained on day 14 of pregnancy and maintained overnight in the Department of Anatomy and Cell Biology’s animal facility. Adult male CD1 mice, between 20-25 grams, were also obtained from the Animal Resources Centre.

2.2 Reagents

Unless otherwise noted all reagents used were obtained from Sigma Chemical Company, St. Louis, MO. Mouse anti-phosphoserine (16B4), and anti-phosphothreonine primary antibodies were obtained from Calbiochem, San Diego, CA. Goat anti-mouse IgG secondary antibody conjugated with horse radish peroxidase for use in western blot analysis was purchased from BioRad, Inc., Hercules, CA.

2.3 Tissue Culture

2.31 Culture Preparation

2.311 Cortical neurons.

Cortical neurons from CD1 mice were cultured as previously described by Juurlink and Walz (Juurlink, 1998a). Briefly, neopallia were isolated from E15 mouse
embryos, washed with Puck’s solution, trypsined with 0.2% trypsin (1:250 trypsin obtained from Gibco-BRL) for 2 minutes at room temperature and trituated with a glass Pasteur pipette. Cells were counted and plated on Falcon 60 mm x 15 mm tissue culture dishes coated with poly-D-lysine at a density of 3x10^6 cells per dish. Dishes with cultures intended for immunofluorescence analysis had 6 coverslips placed at the bottom of the dish before the addition of poly-D-lysine. The cells were plated in a medium consisting of Dulbecco’s minimal essential medium (DMEM, from Gibco-BRL) supplemented with 15 mM HEPES, 14 mM NaHCO₃, 30 mM glucose, and 2 mM glutamine and incubated at 37°C for one hour. The plating medium was then aspirated to remove non-neuronal cells and replaced with the primary growth medium, DMEM supplemented with 15 mM HEPES, 14 mM NaHCO₃, 30 mM glucose, 2 mM glutamine, 0.1% insulin (v/v), and 5% (v/v) horse serum (Summit Biotechnology, Ft. Collins, CO). On the third day in culture the antimitotic agent fluorodeoxyuridine (10 µM) plus uridine (40 µM) was added to the cultures to minimize contamination of the cultures by cells capable of proliferation. The cells were fed with secondary medium, DMEM supplemented with 15 mM HEPES, 14 mM NaHCO₃, 30 mM glucose, 0.3 mM glutamine, 2.0 mM pyruvate, 0.1% insulin (v/v), and 5% (v/v) horse serum on day 4 in culture. The cultures were used on days 5-6.

2.3.12 Astrocytes.

As for the cortical neurons, the mouse astrocytes were cultured as described previously by Juurlink and Walz (Juurlink, 1998a). Newborn CD1 mice were killed by an overdose of an anaesthetic, Halothane, in accordance with Canadian Council on
Animal Care regulations. The skin of the mice was disinfected by first briefly submerging the bodies in 2% iodine/70% ethanol followed by submersion in 70% ethanol for 60 s. Neopallia were isolated and the tissue was disassociated into a single-cell suspension. The cells were planted in Falcon 100 mm x 20 mm tissue culture dishes at a density of $5 \times 10^3$ cells per dish. For the first 24 h the growth medium consisted of 7.5 mM glucose, 15 mM NaHCO$_3$, and 10% horse serum (v/v) in DMEM. For the next two weeks the cultures were fed 3 times per week with a medium of 25 mM sorbitol, 15 mM NaHCO$_3$, and 10% horse serum in DMEM. Astrocytes are able to use sorbitol as a source for carbon; therefore, other neural cells that require glucose will not survive to contaminate the astrocyte cultures. After two weeks the medium was switched back to the original growth medium to allow the astrocytes to reach confluency. The day before the confluent astrocyte cultures were used, usually between days 17-21, they were fed with serum free medium.

2.32 Experimental Treatment

2.32.1 Immunofluorescence

Immunofluorescence was used to ascertain the identity of the cells produced by the above tissue culture protocols. Three groups of coverslips were probed with primary antibodies against GABA, the neuronal protein neurofilament 200 (NF200) and, and an astrocyte marker protein, GFAP. Fluorescent labelled secondary antibodies: goat anti-rabbit IgG labelled with FITC (DakoCytomation, Carpintaria, CA) and goat anti-mouse IgG conjugated with Cy3 (Jackson ImmunoResearch Laboratories, Ltd., West Grove, PA), were used to visualize the results of the primary antibody probe. Two groups of coverslips were examined for GABA immunopositivity. The first group was pre-
incubated with 1.0 µM GABA for 30 min in serum free medium (DMEM, 10 mM HEPES) and quickly washed twice in serum free medium and once in 0.01 M PBS. The cells were fixed with Zamboni fixative (0.2% picric acid, 4% formaldehyde in 0.2M phosphate buffer; pH 7.2-7.4) and 0.125% glutaraldehyde for 15 min. The second group of coverslips was not pre-incubated with GABA but was otherwise treated the same. The final set of coverslips was washed as above and fixed in Zamboni fixative without glutaraldehyde for 15 minutes. After fixation all three groups were washed 3x5 minutes in cold 0.01M PBS.

The fixed cells were permeabilized with 5% horse serum, 1% BSA and 1% Triton X-100 in 0.01M PBS for 20 min at room temperature. The coverslips of permeabilized cells were divided into four groups. The first group consisted entirely of coverslips that had been pre-incubated with 1.0 µM GABA were incubated in 1:1000 (v/v) rabbit anti-GABA and 1:200 (v/v) mouse anti-NF200. The second group consisted of coverslips were fixed with Zamboni plus glutaraldehyde but were not pre-incubated with GABA. This group was incubated with 1:1000 (v/v) rabbit anti-GABA and 1:400 mouse anti-GFAP. Coverslips that were not pre-incubated with GABA and had been fixed in Zamboni fixative without glutaraldehyde made up groups three and four. These coverslips were probed with either 1:200 (v/v) mouse anti-NF200 or 1:400 (v/v) mouse anti-GFAP.

All of the coverslips were incubated in the primary antibody for 30 min at room temperature and washed 4x5 min in 0.01 M PBS. All coverslips were incubated in a secondary antibody solution containing 1:100 (v/v) anti-rabbit IgG-FITC and 1:200 (v/v) anti-mouse IgG-Cy3 for 30 min at room temperature and washed 4x5 min in 0.01 M
PBS. Coverslips were mounted on glass slides in Citifluor (Marivac, Ltd., Montreal) containing 10 µg/µL Hoechst (Sigma).

2.322 Glutamate Excitotoxic Insult to Cortical Neurons

L-Glutamate dissolved in distilled H₂O and AIT-082 (Neotherapeutics, Irvine, CA) was prepared in serum free medium. Glutamate (100 µM) was added to the secondary growth medium and the cultures were incubated at 37°C for 10 minutes to stress the cells. Then the cultures were given 1.0 µM AIT-082 and incubated at 37°C for an additional 20 min. For comparison, cultures were also prepared that received vehicle treatment in place of glutamate, AIT-082, or both glutamate and AIT-082. The neurons were harvested in strong acid solution for ATP analysis or harvesting buffer (as described below) for western blots. A bicinchoninic acid (BCA) protein assay (Smith et al., 1985) was used to determine the total protein content of the harvested cultures with bovine serum albumin as the protein reference standard. Total ATP content of the samples was expressed as a percentage of control ATP levels.

2.323 Hypoxic/Ischemic (H/I) Insult to Astrocytes

Astrocyte cultures were exposed to hypoxic and ischemic conditions and were allowed to recover in the presence or absence of 1.0 µM AIT-082. Under these experimental conditions ischemia shall be defined as the removal of energy metabolism substrates. H/I medium was prepared by bubbling 25 mM NaHCO₃ supplemented DMEM with a 5% CO₂/1% O₂/balance N₂ gas mixture (Praxair, Mississauga, ON). Astrocyte cultures were fed H/I medium and placed in a modular incubator chamber.
(Billups-Rothenburg, Inc., Del Mar, CA) and the chamber was flushed with the 5% CO2/1% O2/balance N2 gas mixture for 15 minutes. The cultures were incubated at 37°C for 4 hours and then the cultures were fed standard secondary medium, described above, with or without 1.0 µM AIT-082. The astrocyte cultures were allowed to recover under normoxic conditions for 1, 4, 12 and 24 hours. Two additional groups of astrocyte cultures were also set up. The first group was maintained under standard conditions (i.e., the cultures were fed standard medium and maintained in a normoxic atmosphere) with and without 1.0 µM AIT-082 for 1 hour. The second group was exposed to the H/I conditions described above but was supplemented with 1.0 µM AIT-082. This second group, along with an equal number of AIT-082 unsupplemented dishes, was harvested immediately following the 4-hour incubation period. Astrocyte cultures were harvested in harvesting buffer (described below). A (BCA) protein assay (Smith et al., 1985) was used to determine the total protein content of the harvested cultures with bovine serum albumin as the protein reference standard.

2.3.24 Hypoxic Insult to Cortical Neurons

Cortical neurons were subjected to hypoxic insult in much the same manner as astrocytes were exposed to H/I. Secondary medium was bubbled with a 5% CO2/1% O2/balance N2 gas mixture to make it hypoxic; however, the medium was fortified with all the supplements described above in section 2.3.11. The neuronal cultures were fed with the hypoxic medium and placed in the modular incubator chambers. As with the astrocytes the chambers were flushed with the hypoxic gas mixture for 15 minutes and the sealed chambers were incubated at 37°C for 4 hours. Following incubation the
cultures were fed normoxic medium, with or without 1.0 µM AIT-082, and allowed to recover for 1, 4, 12 and 24 hours before they were harvested. Also, two additional groups of cortical neuron cultures were set up. The first group was maintained under standard conditions (i.e., the cultures were fed standard medium and incubated in a normoxic atmosphere) with and without 1.0 µM AIT-082 for 1 hour. The second group was exposed to the hypoxic conditions described above but was supplemented with 1.0 µM AIT-082. This second group, along with an equal number of AIT-082 unsupplemented dishes, was harvested immediately following the 4-hour incubation period. Cortical neuron cultures were harvested in harvesting buffer (described below). A BCA protein assay (Smith et al., 1985) was used to determine the total protein content of the harvested cultures with bovine serum albumin as the protein reference standard.

2.325 Whole Brain Homogenates

The whole brain homogenization procedure and buffer solution were modified from the procedure outlined by Steiner et al (1992). Adult, male CD1 mice were killed with 70% CO2/balance air (Praxair, Mississauga, Ont.) in accordance with Canadian Council on Animal Care regulations. The carcasses were placed on ice and their brains were removed. The whole brains were placed in Puck’s solution on ice and the cerebellum and brain stem were dissected from the rest of the brain and discarded. The remaining brain tissue was finely chopped before being homogenized.

The tissue was homogenized in a buffer (1.0 mM EGTA, 2.0 mM dithiothreitol in 50 mM Hepes; pH 7.4) at a wet-weight of 0.1g/mL. The homogenization buffer was supplemented with either 1.0 µM AIT-082, 1.0 µM GMP, or vehicle (water). The whole
brain homogenates were centrifuged at 10,000*g for twenty minutes and the supernatants were transferred to fresh tubes. Supernatants were centrifuged again at 100,000*g for 1 h to remove the mitochondria and other small insoluble material. The supernatant was incubated for 20 minutes at 37°C with 17 µg/mL phosphatidylserine, 10 µM free Ca²⁺, 100 µM ATP and either 1.0 µM AIT-082, 1.0 µM GMP, or vehicle.

The soluble proteins were precipitated by incubation at room temperature for 20 minutes with 5% trichloroacetic acid. The samples were centrifuged at 10,000*g for 10 minutes and the supernatants were discarded. The pellet was washed with ice-cold acetone. The pellets were vortexed in the acetone and then centrifuged briefly at 10,000*g. The acetone was aspirated the wash was repeated an additional 2 times. Finally, the pellet was allowed to dry before it was resuspended in harvesting buffer (described below). A BCA protein assay (Smith et al., 1985) was used to determine the total protein content of the harvested cultures with bovine serum albumin as the protein reference standard.

2.4 Biochemical Analysis

2.4.1 Neuronal ATP.

Cortical neuron cultures were used to measure AIT-082’s effect on neuronal ATP. Before the cultures were harvested they were placed on ice and washed once with cold 0.01 M PBS. The cells were harvested in 2.0 M perchloric acid. Macromolecules such as lipids, DNA and proteins are less soluble in the low pH of a strong acid solution and therefore make the isolation of small molecules more straightforward. Additionally, perchlorate itself becomes insoluble when it is neutralized in a potassium solution.
cells were centrifuged at 10,000*g for 10 min and the supernatants were transferred to fresh tubes and neutralized to pH 7.8 with 2.0 M K$_2$CO$_3$. The samples were stored at approximately –80°C until needed. The protein pellet was saved for determination of total protein content.

The ATP content of the samples was quantified using an adenosine 5’-triphosphate (ATP) bioluminescent assay kit (Sigma, St. Louis, MO) according to the manufacturer’s instructions (Technical Bulletin No. BAAB-1). Briefly, ATP is quantified by measuring the amount of light produced as a result of the oxidation of D-luciferen by firefly luciferase. ATP is consumed in the first step of the reactions and when ATP is the limiting reagent the amount of light produced is proportional to the amount of ATP present in the sample. The bioluminescence of the samples was measured with the TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

2.42 Western Blots

Astrocyte and cortical neuron cultures were placed on ice and harvested by scraping in harvesting buffer. The harvesting buffer contained: 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1.0 mM sodium vanadate, 1% (v/v) Triton X100, and 10% protease inhibitor cocktail. The protease inhibitor cocktail contained 50 µg/mL leupeptin, 50 µg/mL pepstatin A, 100 µg/mL aprotinin, 2.0 mM phenylmethylsulfonyl fluoride (PMSF) in 50% DMSO (v/v) and was added to the harvesting buffer just before use.

Western blot analysis was performed on proteins harvested from neurons subjected to hypoxic insult, astrocytes subjected to H/I insult, and whole brain
Western blots performed on proteins from hypoxic neurons, H/I astrocytes and whole brain homogenates were probed with antibodies raised against amino acid sequences containing phosphorylated serine or threonine (anti-phospho-Ser, or -Thr) residues. Standard western blotting techniques were performed from the manual Molecular Cloning: A Laboratory Manual 2nd edition (Sambrook et al., 1989). All western blot apparatus used in the following experiments was also purchased from BioRad Inc. The following describes the protocol used to probe with the anti-phosphoserine and anti-phosphothreonine antibodies.

Briefly, 40 µg of protein was concentrated from each sample by precipitating the protein from a predetermined volume of the sample (calculated using the total protein concentrations obtained from the BCA protein assay) using ice-cold acetone. One part sample plus four parts acetone was incubated at –20°C for 10 minutes. The acetone/sample mixture was centrifuged at 15,000 g for 10 minutes and the supernatant was aspirated and discarded. The protein pellet was allowed to dry before being resuspended in 1x loading buffer (50 mM Tris-Cl, pH 6.8; 100 mM dithiothreitol; 2% electrophoresis grade SDS; 0.1% bromophenol blue; 10% glycerol). The samples were then loaded onto a 10% polyacrylamide gel submerged in 1x Tris-glycine electrophoresis buffer (25 mM Tris; 250 mM electrophoresis grade glycine, pH 8.3; 0.1% SDS) and electrophoresed (200 mA; constant 110 V) for 30-40 minutes. A BioRad PowerPac 300 power supply was used.

Proteins were transferred to a PVDF membrane using the BioRad wet transfer apparatus. The gel-membrane holders were submerged in cold transfer buffer (39 mM glycine; 48 mM Tris base; 0.037% SDS; 20% methanol) in a specific order (i.e., holder
A in position 1 and holder B in position 2) and exposed to a 500 mA (maximum), 110 V (constant) electrical current for 1 h at 4°C. The position of the gel-membrane holders was switched, holder 1 in position B and holder 2 in position A, after 30 min to ensure even transfer of proteins from both gels.

The membranes were incubated in an agitated blocking solution, 5% bovine serum albumin (w/v) in 1x PBST, for 1 h at room temperature. The blocked membranes were then incubated in the primary antibody solution: 2.5% bovine serum albumin (w/v) in 1x PBST plus either 1:1000 mouse anti-phosphoserine or 1:1000 mouse anti-phosphothreonine on a FinePCR tube rotator (Finemould Precision Ind. Co.) for 1 h at room temperature. The probed membranes were washed 3x10 min with 1x PBST containing 2.5% bovine serum albumin at room temperature.

Finally, the membranes were probed with a horse radish peroxidase conjugated goat anti-mouse IgG secondary antibody (Biorad). The secondary antibody was diluted to 1:10,000 (v/v) in 1x PBST plus 2.5% bovine serum albumin (w/v). The membranes were incubated for 30 min at room temperature on a FinePCR tube rotator. After the membranes were probed with the secondary antibody they were subjected to three washes: 3x10 min in 1x PBST plus 2.5% (w/v) bovine serum albumin, 3x10 min in 1x PBST, and 3x10 min in 0.01 M PBS all at room temperature.

Proteins were detected using DuPont NEN Renaissance chemiluminescence reagents (Mandel Scientific, Ltd., Guelph, ON) according to the manufacturer’s instructions. Densitometric analyses of protein levels were done using NIH image analyzing software.

2.5 Equations and Statistical Analysis
The following equations were used to determine percent ATP and phosphorylation respectively.

\[
\text{Percent ATP} = \left( \frac{[\text{Sample ATP}]}{[\text{Mean Control ATP}]} \right) \times 100 \quad (1.0)
\]

\[
\text{Percent Phosphorylation} = \left( \frac{\text{Sample SI}}{\text{Mean Vehicle SI}} \right) \times 100 \quad (2.0)
\]

The data compiled for the ATP experiments and the phosphorylation experiments involving the specific neural cell types experiment were analyzed for statistical significance using the Two-way analysis of variance (ANOVA) and Holm-Sidak method for multiple comparisons test. The tests were performed using the SigmaStat 3.0 software package. The whole brain homogenate data was tested for statistical analysis using a one way ANOVA and the Tukey-Kramer method for multiple comparisons test.

3.0 Results

3.1 Immunofluorescence

Cells from CD1 mouse cultures of cortical neurons and astrocytes were probed with antibodies against specific marker proteins for those two cell types. The antibody binding was visualized using secondary antibodies tagged with fluorescent markers. GABA staining was equally effective in staining neurons that were pre-incubated with GABA and those that were not pre-incubated. Cells from cortical neuron cultures displayed both GABA and NF200 staining (see figure 3A and 3B) indicating that the cells were indeed neurons. Cells from neuronal cultures that were probed with primary antibodies against GABA and GFAP were positive for the former marker but not the latter (see figure 3C and 3D). This indicates that not only are the cells neuronal but that
the cultures are free of astrocyte contamination. Finally, cells from astrocyte cultures were positive for GFAP staining confirming their cellular identities (see figure 1E and 1F). Cells from astrocyte cultures were not positive for NF200 (not shown).

3.2 Glutamate Excitotoxicity

3.21 Cortical Neuron ATP Levels

ATP concentrations from the glutamate excitotoxicity experimental groups were normalized with sample protein content and entered into the equation 1.0. ATP measurements from cultures that were treated with AIT-082 alone (90.0 ± 13.1; n=9) were not significantly different (p>0.05) from control cultures (100.0 ± 23.2; n=9). ATP measurements from cultures that were treated with glutamate (66.1 ± 6.5; n=9) showed a significant reduction (p<0.001) in ATP levels compared to control ATP levels. However, ATP levels from cultures that were exposed to glutamate excitotoxicity followed by AIT-082 treatment (70.8 ± 13.0; n=9) were not significantly different (p>0.05) from those cultures that were exposed to glutamate excitotoxicity alone. I repeated the experiments three times. See figure 4 for a graph summarizing the data and the statistically significant differences between treatment groups.

3.3 Protein Phosphorylation

3.31 Brain Homogenates

I examined the phosphorylation status of soluble proteins from CD1 mice whole brain homogenates. The homogenates were divided into three groups: vehicle-treated, GMP-treated and AIT-082-treated. I analyzed the samples using western blot analysis and measured the effects of AIT-082 treatment on generalized serine and threonine containing sequence phosphorylation levels. I measured the effects of AIT-082 on
phosphorylation signal of the whole lane, as well as, of a specific 43.4 kD band for each treatment group. I repeated the brain homogenate experiments three times. The values for phosphorylation signal intensity (SI) for each sample were entered into equation 2.0.

I found that for measurement of whole lane signal intensity GMP-treated brain homogenates ($142.5 \pm 36.2; n=9$) have a significantly higher percent serine containing sequence phosphorylation ($p<0.05$) than vehicle-treated brain homogenates ($100 \pm 6.5; n=8$). I found that AIT-082 ($124.5 \pm 28.1; n=8$) produced a smaller, but statistically non-significant ($p>0.05$) increase in percent serine containing sequence phosphorylation compared to the vehicle-treated group. See figure 5A and 5B.

The results for the 43.4 kD band mirrored those for the whole lane. Serine containing sequence phosphorylation signal intensity for the 43.4 kD band from GMP-treated homogenates ($179.7 \pm 57.3; n=9$) is significantly higher ($p<0.01$) than that from vehicle-treated brain homogenate ($100 \pm 29.8; n=8$). Again, as for the whole lane, the AIT-082 brain homogenates ($142.7 \pm 21.9; n=8$) showed a smaller non-significant ($p>0.05$) increase in phosphorylation signal intensity. See figure 5A and 5C.

When I examined the results for threonine containing sequence phosphorylation signal intensity I found that for the whole lane signal the results were close to those I observed for serine containing sequence phosphorylation. The whole lane signal from the GMP-treated homogenates ($118.8 \pm 6.5; n=9$) are significantly higher ($p<0.001$) than those from the vehicle-treated brain homogenates ($100 \pm 10.3; n=9$). The difference seen is smaller than that observed for serine containing sequence phosphorylation. AIT-082 treatment did not produce a significant change ($96.8 \pm 9.2; n=9$) in threonine containing sequence phosphorylation compared to vehicle treatment. See figures 6A and 6B. There is no difference in threonine containing sequence phosphorylation signal intensity
for the 43.4 kD band. The small differences observed between the vehicle-treated homogenates (105 ± 17.6; n=9) and the GMP and AIT-082-treated brain homogenates (109.9 ± 81.1, 80.6 ± 41.4; n=9) was rendered insignificant (p>0.05) due to the variation of signal intensity within the groups. See figures 6A and 6C.

3.32 Neural Cells

I also examined proteins harvested from cortical neuron and astrocyte cultures using western blot techniques, for the effect of AIT-082 treatment on both serine and threonine containing sequence phosphorylation signal of the whole lane, as well as, of a specific 46 kD band for each treatment group. The neuronal cultures were exposed to hypoxic conditions and the astrocytic cultures were exposed to hypoxic and ischemic conditions before the cultures were allowed to recover with or without 1.0 µM AIT-082. I compared both hypoxic cortical neuron cultures and H/I astrocyte cultures to unstressed cultures incubated with or without 1.0 µM AIT-082 as well as a treatment group that was stressed with or without 1.0 µM AIT-082 and harvested without being allowed to recover. I replicated all phosphorylation experiments with neural cells a minimum of three times. As with the brain homogenates all values are expressed as a percentage of the mean signal intensity for the unstressed, non-AIT-082-treated group according to equation 2.0. For the following sets of experimental results I have indicated treatment groups with a number followed by either a positive (+ve) or negative (-ve) sign. The number indicates the recovery time (in hours) and the positive or negative sign indicates the presence or absence, respectively, of AIT-082 in the recovery medium.
3.3.21 Hypoxic Neurons

There is no significant difference (p>0.05) in whole lane serine containing sequence phosphorylation signal intensity between AIT-082-treated and untreated groups over the course of the 24 h recovery period following the hypoxic insult. There is also no significant difference (p>0.05) between the control groups (control -ve 100 ± 0; control +ve 100 ± 31.8; n=13) and the stressed groups during recovery (1-ve 89.0 ± 24.4, 1+ve 84.8 ± 25.3; 4-ve 87.3 ± 28.0, 4+ve 86.7 ± 22.2; 12-ve 86.4 ± 27.1, 12+ve 82.4 ± 29.3; 24-ve 67.5 ± 20.8, 24+ve 87.1 ± 27.5; n=9-16). Furthermore, I found no significant difference (p>0.05) between the two groups that were harvested immediately following the hypoxic insult (0-ve 60.7 ± 36.9, 0+ve 65.7 ± 31.3; n=10); however, the no recovery treatment groups with and without AIT-082 have significantly lower (p<0.05) total serine containing sequence phosphorylation signal intensity than either the control group without AIT-082 or control group with AIT-082. Analysis with the two way ANOVA indicates that this difference is the result of the hypoxic treatment and is unaffected by AIT-082. See figures 7A and 7B.

There is no significant difference in serine containing sequence phosphorylation signal intensity of a 46 kD band between AIT-082-treated and untreated groups over the course of the 24 h recovery period following the hypoxic insult. There is also no significant difference (p>0.05) between the control groups (control -ve 100 ± 0; control +ve 93.7 ± 32.0; n=13) and the stressed groups during recovery (1-ve 98.5 ± 43.0, 1+ve 93.3 ± 33.9; 4-ve 96.9 ± 40.0; 4+ve 89.1 ± 32.6; 12-ve 89.8 ± 25.6, 12+ve 81.8 ± 24.5; 24-ve 95.3 ± 40.6, 24+ve 108.2 ± 41.4; n=9-16) or the groups that were harvested immediately after the period of hypoxia (0-ve 92.3 ± 30.8, 0+ve 79.5 ± 37.3; n=10). See figure 7A and 7C.
When I examined the neurons for changes in threonine containing sequence phosphorylation I found there are no significant differences in signal intensity between any of the treatment groups for either whole lane signal intensity (control -ve 100 ± 0; control +ve 93.7 ± 32.0; 0-ve 92.3 ± 30.8, 0+ve 79.5 ± 37.3; 1-ve 98.5 ± 43.0, 1+ve 93.3 ± 33.9; 4-ve 96.9 ± 40.0; 4+ve 89.1 ± 32.6; 12-ve 89.8 ± 25.6, 12+ve 81.8 ± 24.5; 24-ve 95.3 ± 40.6, 24+ve 108.2 ± 41.4; n=9-14) or signal intensity of the 46 kD band (control -ve 100 ± 0; control +ve 99.8 ± 25.7; 0-ve 115.4 ± 33.6, 0+ve 106.9 ± 35.0; 1-ve 132.1 ± 37.1, 1+ve 111.9 ± 40.8; 4-ve 103.5 ± 32.8; 4+ve 96.9 ± 35.8; 12-ve 91.2 ± 35.7, 12+ve 108.1 ± 30.7; 24-ve 111.5 ± 15.5, 24+ve 91.2 ± 35.7; n=9-14). See figure 8A, 8B and 8C.

3.322 Hypoxic/Ischemic Astrocytes

There is no significant difference (p>0.05) in whole lane serine containing sequence phosphorylation signal intensity between AIT-082-treated and untreated groups over the course of the 24 h recovery period following the hypoxic/ischemic insult. There is also no significant difference (p>0.05) between the control groups (control -ve 100 ± 3.5; control +ve 99.9 ± 23.5; n=13) and any of the other groups before or during the recovery period (1-ve 99.8 ± 38.5, 1+ve 109.5 ± 34.1; 4-ve 110.9 ± 66.4, 4+ve 104.7 ± 53.8; 12-ve 109.7 ± 37.5, 12+ve 125.0 ± 40.6; 24-ve 90.8 ± 33.4, 24+ve 122.8 ± 48.5; n=8-12). The 4 h hypoxic ischemic treatment did cause a significant reduction in serine containing sequence phosphorylation of the non recovered groups (0-ve 80.7 ± 22.2, 0+ve 74.4 ± 17.59; n=8) that was unaffected by AIT-082. See figures 9A and 9B.
There is no significant difference \((p>0.05)\) in serine containing sequence phosphorylation signal intensity of a 46 kD band between AIT-082-treated and untreated groups over the course of the 24 h recovery period following the hypoxic/ischemic insult. There is also no significant difference \((p>0.05)\) between the control groups (control -ve 100 ± 13.4, control +ve 97.7 ± 68.4; \(n=13\)) and the stressed groups during recovery (1-ve 98.2 ± 33.2, 1+ve 121.5 ± 43.8; 4-ve 125.1 ± 60.6; 4+ve 95.8 ± 33.2; 12-ve 121.1 ± 67.9, 12+ve 120.3 ± 46.4; 24-ve 86.5 ± 53.6, 24+ve 106.1 ± 23.8; \(n=8-12\)); however, once again 4 h of H/I caused a significant reduction in the phosphorylation of the serine containing sequence in the unrecovered groups (0-ve 71.8 ± 28.2, 0+ve 51.9 ± 26.6; \(n=8\)) that was unaffected by AIT-082. See figure 9A and 9C.

There is no significant difference \((p>0.05)\) in whole lane threonine containing sequence phosphorylation signal intensity between AIT-082-treated and untreated groups over the course of the 24 h recovery period following the hypoxic/ischemic insult. There is also no significant difference \((p>0.05)\) between the control groups (control -ve 100 ± 2.5; control +ve 89.8 ± 21.5; \(n=11\)) and the stressed groups during the recovery period (1-ve 84.8 ± 43.1, 1+ve 83.0 ± 31.3; 4-ve 69.6 ± 40.5, 4+ve 66.7 ± 30.6; 12-ve 78.3 ± 37.2, 12+ve 91.83 ± 37.5; 24-ve 75.4 ± 45.9, 24+ve 71.9 ± 33.2; \(n=8-10\)). There are significant differences in whole lane signal intensity between the control groups and the two groups that were harvested immediately after the hypoxic/ischemic insult. Signal intensity for the unrecovered lanes is significantly reduced compared to the control lane (0 –ve 47.9 ± 25.6, 0 +ve 53.0 ± 26.7; \(n=8\) \(p<0.001\)) and the reduction in threonine containing sequence phosphorylation is unaffected by AIT-082-082. See figures 10A and 10B.
There is no significant difference (p>0.05) in threonine containing sequence phosphorylation signal intensity of the 46 kD band between AIT-082-treated and untreated groups over the course of the 24 h recovery period following the hypoxic/ischemic insult. There is also no significant difference (p>0.05) between the control groups (control -ve 100 ± 18.1, control +ve 79.3 ± 27.6; n=11) and the stressed groups during recovery (1-ve 94.2 ± 51.1, 1+ve 104 ± 83.0; 4-ve 78.2 ± 72.3; 4+ve 70.6 ± 63.2; 12-ve 105 ± 91.3, 12+ve 121 ± 88.2; 24-ve 55.1 ± 51, 24+ve 74.7 ± 74.3; n=8-10). The groups that were harvested immediately after the period of hypoxia (0-ve 34.2 ± 35.8, 0+ve 31.9 ± 42.2; n=8) show significantly lower threonine (p=0.03) containing sequence phosphorylation than the control lane. The reduction in signal intensity was unaffected by AIT-082 treatment. See figure 10A and 10C.

4.0 Discussion

The ability of AIT-082 to cross the blood brain barrier, its memory enhancing properties and its apparent lack of toxicity make it an attractive candidate for treating AD. Neurotrophic and neuroprotective properties have been attributed to AIT-082 but the mechanism or mechanisms behind these effects are largely unknown. For example, in astrocytes AIT-082 has been shown to increase the mRNA for a number of neurotrophic factors including NGF (Di Iorio et al., 2001), increase the secretion of NGF (Ciccarelli, 1998; Rathbone, 1998) and potentiate certain neurotrophic effects of NGF on PC12 cells (Lahiri et al., 2000; Middlemiss et al., 1995). It also increases secretion of adenosine (Caciagli, 1998; Rathbone, 1998). Though a lack of NGF has been proposed as a possible cause of AD and adenosine has been shown to possess many neuroprotective properties, the actions of AIT-082 cannot be explained in terms of its
effects on NGF and adenosine because AIT-082 has been shown to act directly on neurons without requiring glial cells to mediate those effects (Rathbone, 1999). Furthermore, Juurlink’s lab (unpublished data) has shown that receptor antagonists against A₁, A₂A, A₂B, A₃ and P₂ receptors do not block the drug’s ability to reduce neuronal damage caused by glutamate.

4.1 AIT-082 and ATP

In spite of the fact that the primary cause of AD is not known, the classical lesions associated with the disease provide a good starting point for investigations into the mechanisms of drugs, such as AIT-082, that are believed to be possible effective treatments. As I mentioned previously, the classical AD lesions suggest the involvement of certain pathological processes or the dysregulation of physiological processes. Neuritic plaques are clearly involved in a system of pathological processes referred to as the “deleterious network” by Ying (Ying, 1996a; Ying, 1996b).

Inappropriate APP processing, excessive free radical production, decreased energy metabolism and loss of Ca^{2+} homeostasis all cause neuronal damage. However, Ying (Ying, 1996a; Ying, 1996b) proposes that those processes are interconnected and that induction of any one can induce the others. This creates a self-perpetuating cytotoxic cascade that ultimately leads to the development of AD. It has also been proposed that a similar deleterious cascade leads to cell death in stroke (Sweeney et al., 1995). The corollary of that hypothesis is that restoration of any one of those processes to its normal state may be able to disrupt the cascade and allow the affected cells to recover. Several observations regarding the effects of AIT-082 indicated that the drug may be able to disrupt components of the deleterious network. AIT-082 protects
neurons in vivo and in vitro against excitotoxic damage induced by glutamate (Bintner, 1999; Juurlink, 1998b), kainate (Di Iorio et al., 2001), and NMDA (Caciagli, 1998; Ciccarelli, 1998). Given that excitotoxic damage is mediated by excessive Ca\(^{2+}\) influx it is possible that neuroprotective properties of AIT-082 are mediated by an ability to control intracellular Ca\(^{2+}\) concentrations. This possibility is strengthened by the observation that AIT-082 increases mitochondrial membrane potentials in neurons, thus potentially increasing the ability of these organelles to sequester Ca\(^{2+}\). However, Ca\(^{2+}\) concentrations play critical second messenger roles in cells throughout the body and if AIT-082 can alter intracellular Ca\(^{2+}\) concentrations in neurons it would also be able to do the same in other cell types. As there is no evidence that Ca\(^{2+}\) homeostasis has been disrupted in other cellular populations in the body of AD patients such alterations in Ca\(^{2+}\) homeostasis by AIT-082 would likely be deleterious. Indeed, Ca\(^{2+}\) channel blocking drugs designed for stroke therapy have not proven successful due in part to cardiovascular side effects (Juurlink and Paterson, 1998); however, no problems with tolerability or safety were observed in a preliminary clinical study of AIT-082 (Grundman et al., 2002). A likely alternate candidate to Ca\(^{2+}\) homeostasis regulation, for the neuroprotective activity of AIT-082, from the deleterious network, is energy metabolism.

Restoring energy metabolism to normal levels, or at least, increasing ATP production beyond that typical of AD neurons could protect cells against excitotoxic damage by providing energy for both the Na\(^+\)/K\(^+\) ATPases and Ca\(^{2+}/H^+\) ATPases. Activity of those pumps is critical for maintaining membrane polarity, restoring intracellular Ca\(^{2+}\) concentrations and ultimately preventing cell damage (Novelli et al., 1988; Siesjo, 1992; Silver and Erecinska, 1997). The possibility that AIT-082 was
promoting increased neuronal ATP levels was supported by a few different observations. First Juurlink and Rathbone (Juurlink, 1998b) observed that treatment with 1.0 μM AIT-082 increased axon length and complexity in hippocampal neuron cultures compared to controls after day 5 in culture. They proposed that the plateau in axon growth seen in control cultures may be due to limitations in energy metabolism that were overcome when AIT-082 was added to the culture medium. Second, AIT-082 treatment increased mitochondrial membrane potential (Bintner, 1999), measured by uptake and retention of rhodamine-123 (a rosamine derivative). The more distal mitochondria were from the soma the greater the increase in mitochondrial membrane potential. By increasing the mitochondrial membrane potential AIT-082 may have the ability to reinforce the electrochemical proton gradient that drives ATP synthesis by the F₀F₁ complex. Third, exposure of hippocampal neurons to a sublethal dose of L-glutamate caused neuronal damage beginning at the distal tips of the axons. Treatment of the cultures with AIT-082 supplemented medium prevented much of the axonal degeneration (Rathbone, 1998).

Taken together, the above three observations led me to hypothesize that by increasing mitochondrial membrane potential AIT-082 promotes neurite growth and neuronal survival following excitotoxic insult by increasing ATP production. This was a particularly attractive hypothesis because there are no apparent deleterious effects associated with increased ATP availability. Also, neurite degeneration appears to be an early indicator of neuronal degeneration in AD (Cotman, 1999) that correlates well with cognitive decline in the disease (Masliah et al., 1991; Sze et al., 1997). Preliminary results in cerebellar neuron cultures appeared to indicate that AIT-082 did increase ATP
content (Bintner, 1999); however, the current experiments show AIT-082 had a small but not significant effect on the ATP content of cortical neuron cultures that were exposed to a sublethal dose of L-glutamate. Although my results do not support the hypothesis it may be possible that AIT-082 indeed increases ATP production by mitochondria in those regions of the cell that show the greatest increase in mitochondrial membrane potential (i.e., the distal portions of the axons). If that is the case it may be that in the affected regions the increased availability of ATP would be sufficient to resist or reverse the effects of exposure to sublethal quantities of glutamate, while the overall increase in ATP content of the neuronal cultures was too small to detect.

This possibility could be explored in a couple of different ways. The first way would involve the use or development of an ATP measurement system more sensitive than the one used in my experiments to try and detect the small change in ATP levels that would correspond to increased ATP production in the distal tips of the neurons. The second way would be to develop a culture system that would allow the isolation of the axons, thus allowing measurement of ATP in the cellular compartment that is of the greatest interest (i.e., most likely to be affected by AIT-082 treatment). The advantages of the first method would be that the experiments would be able to continue with established tissue culture techniques. The drawbacks of trying to find or develop a more sensitive measurement system, however, would ultimately favour the second strategy. The measurement system used in my experiments is reliable and very sensitive and development of an even more sensitive measurement technique would not address the possibility that it is the mitochondria located in the distal portions of the axons (i.e., the mitochondria that showed the greatest response to AIT-082) that are producing more ATP.
4.2 AIT-082 and Phosphorylation

In AD the neurofibrillary tangles bear evidence that there has been a perturbation in the regulation of enzymes that control phosphorylation. Hyperphosphorylated tau accumulates in affected neurons and causes the breakdown of microtubules. Furthermore, loss of regulatory control of cellular phosphorylation can potentially affect many critical cellular functions such as gene expression, intracellular ion concentrations, neurotransmitter release, and protein-protein interactions. The presence of hyperphosphorylated tau in AD indicates that either the activity of one or more kinases has been upregulated, or the activity of one or more phosphatases has been suppressed.

In AD protein levels of two major protein kinases, PKC and CaMKII, are reduced (Cole et al., 1988; Nishizuka, 1986; Saitoh, 1989) and the activities of other kinases, tau protein kinase I/glycogen synthase kinase 3β, and cyclin dependent kinase 5, that are important in normal tau phosphorylation, are reduced during experimental conditions that induce tangle formation (Planel et al., 2001). Planel et al (2001) also demonstrated that one of the protein phosphatases, PP2A, also has reduced activity in starved mice. A breed of knockout mice for calcineurin develops hyperphosphorylated tau very similar to that in AD indicating the importance of that phosphatase to the development of the disease. In light of those observations it would seem likely that in AD, at least in regards to tau, although kinase activity is compromised it is abnormal phosphatase activity that is the major contributor to tau hyperphosphorylation. However, decreased phosphatase activity, particularly calcineurin activity, is not always harmful.
There is some evidence to support the concept that non-adenosine purines, particularly AIT-082, can affect protein phosphorylation in a manner that may be able to influence the pathological processes of AD. Furthermore, the immunophilin ligands share some neurotrophic and neuroprotective properties with AIT-082, such as stimulating neurite outgrowth from PC12 cells (Gold et al., 1995; Lyons et al., 1994; Steiner et al., 1997), axonal regeneration and functional recovery (Bavetta et al., 1999; Gold et al., 1995) and protection against excitotoxic damage (Dawson et al., 1993b). Many of the neuroprotective properties of the immunophilin ligands lie in their ability to inhibit calcineurin activity (see section 1.4). Inducible nitric oxide synthase activity is inhibited by PKC phosphorylation and promoted by dephosphorylation by calcineurin. The immunophilin FK506 protects cortical neurons from glutamate excitotoxicity by inhibiting calcineurin activity that in turn results in lowered iNOS activity and lower production NO (a precursor of peroxynitrite and an ROS itself) (Bredt et al., 1992; Dawson et al., 1993b). In cerebellar granule cells, FK506 also protected cells against glutamate excitotoxicity, but in this case the authors suggested that the immunophilin ligands protected the cells by preventing the collapse of mitochondrial membrane potentials (Ankarcrona et al., 1996), another trait that AIT-082 appears to share with the immunophilin ligands. It is of interest, therefore, to evaluate the ability of AIT-082 to influence phosphorylation status.

4.21 Non-adenosine Purines and Phosphorylation in Whole Brain Homogenates

Protein phosphorylation is a very common post translational modification and most proteins are phosphorylated to some degree. Therefore, if AIT-082 is able to influence protein phosphorylation there would be so many potential targets that
identifying them individually could be very time consuming and difficult. Instead, I chose to investigate the effects of AIT-082 on protein phosphorylation by examining its effects on protein harvested from whole brain homogenates using western blots and antibodies raised against phosphorylated serine and threonine residues. For comparison I also investigated the effects of GMP on protein phosphorylation in whole brain homogenates. Guanosine based purines, like AIT-082, were able to increase neurite outgrowth from PC12 cells (Gysbers and Rathbone, 1992; Gysbers and Rathbone, 1996b) while other purines, e.g., inosine, adenosine and 5’ AMP, were not (Braumann et al., 1986).

When I analyzed the western blots I saw that both of the non-adenosine purines increased signal density of the total protein sample when probed with an antibody against phosphorylated serine residues but only the increase in the GMP-treated samples was significant. I observed the same trend in serine containing sequence phosphorylation signal for a 43.4 kD protein band. When I probed the western blots with an antibody that recognized phosphorylated threonine the results were somewhat different. GMP-treated homogenates still showed a significant increase in signal intensity for the entire sample but AIT-082 treatment did not result in any increase in signal intensity over control levels. I observed no significant increase in signal intensity of the 43.4 kD marker band in homogenates treated with either of the two non-adenosine purines when they were probed with the anti-phosphothreonine antibody.

These results show that GMP but not AIT-082 has the ability to significantly influence phosphorylation on both serine and threonine residues. However, there are some technical considerations that had to be taken into account when I interpreted these results. The first consideration was the relative frequency of phosphorylation events that
occur on serine and threonine residues. The vast majority of phosphorylation events (>90%) involve serine residues while a much smaller percentage (<5%) take place on threonine residues and the remainder involve tyrosine. It may be that small changes in threonine containing sequence phosphorylation (compared to serine containing sequence phosphorylation) will still exert significant effects in cells. Second, the full extent of the ability of non-adenosine purines to influence phosphorylation may be further obscured by the fact that the antibodies I used only recognize one consensus sequence containing the phosphorylated residues. This technical limitation would likely have the greatest impact on detecting significant changes in the less frequent threonine containing sequence phosphorylation events. Finally, by examining whole brain homogenates I am not able to draw any conclusions about which cell type is affected by the observed changes in protein phosphorylation levels and changes in phosphorylation density of protein(s) from one cell type may be diminished by the presence of proteins from all the cell types. To address the uncertainty of which cell type the non-adenosine purines affect phosphorylation in I examined protein phosphorylation in cortical neuron and astrocyte cultures. By examining those two specific cell types I also hoped to determine whether AIT-082 treatment was able to result in a significant alteration in phosphorylation levels.

4.22 Hypoxic Neurons, AIT-082 and Phosphorylation

Examining neurons for possible influence by AIT-082 on protein phosphorylation status allowed me to investigate the possibility that the small increase in serine containing sequence phosphorylation status I observed in the brain homogenate experiments is more significant in this cell type. Also, by subjecting the neuronal
cultures to hypoxic insult I was able to create a situation that mimics at least part of the pathological system that exists in AD, specifically reduced O$_2$ availability (see section 1.114). Finally, hypoxic insults have been shown to induce the activity of cellular machinery that includes an enzyme of particular interest, the calcium/calmodulin dependent protein phosphatase calcineurin (Morioka et al., 1999; Taylor et al., 1999).

I did not observe any significant difference in protein phosphorylation signal for the total sample or for the 46 kD marker band as revealed by anti-phosphoserine antibody binding. This indicates that the small increase in serine containing sequence phosphorylation status that I observed in AIT-082-treated brain homogenates cannot be attributed to a significant increase in neuronal protein phosphorylation. There was also no appreciable difference between AIT-082-treated and AIT-082-untreated control cultures probed with an anti-phosphothreonine antibody. Furthermore, I was not able to observe any significant difference in protein phosphorylation status (serine containing sequence or threonine containing sequence based) in any of the recovery groups. Samples of proteins harvested from neuron cultures that were exposed to hypoxia and allowed to recover for 0, 1, 4, 12 and 24 hours in the presence of AIT-082 showed no significant difference in phosphorylation status compared to the cultures that recovered without AIT-082. Conditions of hypoxia did cause a decrease in serine containing sequence phosphorylation levels. Neither AIT-082 supplemented nor AIT-082 unsupplemented cultures revealed any significant difference from control cultures with respect to serine containing sequence or threonine containing sequence phosphorylation status.

4.23 Hypoxic/Ischemic Astrocytes, AIT-082 and Phosphorylation
I examined astrocytes for possible influence by AIT-082 on protein phosphorylation status for the same reason I conducted the hypoxia experiments with astrocytes. I conducted the experiments with astrocytes to allow me to investigate the possibility that the small increase in serine containing sequence phosphorylation status I observed in the brain homogenate experiments is more significant in this cell type. Astrocyte cultures are more resistant to hypoxic insult than neuronal cultures (Sochocka et al., 1994) so instead I subjected the astrocytic cultures to H/I insult. As I mentioned previously, the ischemic component in these experiments means that substrates for energy metabolism were removed from the culture medium. Again, I was able to create a situation that mimics at least part of the pathological system that exists in AD, specifically reduced O₂ availability (see section 1.114). Like hypoxic insults, H/I insults are known to involve the activity of calcineurin (Morioka et al., 1999; Taylor et al., 1999).

Treatment with 4 h of hypoxia/ischemia consistently reduced phosphorylation of both serine and threonine containing sequences in astrocytes. However, AIT-082 does not appear to cause any significant change in serine containing sequence or threonine containing sequence phosphorylation status in any of the trial groups or the control samples (i.e., not subjected to H/I) for either the total sample or the 46 kD marker band. From these results I can only conclude, as with the results from the neuronal hypoxia experiments, that the small increase in phosphorylation status observed in AIT-082-treated brain homogenate samples is not the result of a greater increase in astrocyte protein phosphorylation status. Therefore, the increase in serine containing sequence phosphorylation status I observed in AIT-082-treated whole brain homogenate samples could be result of a significant increase in some other brain cell type, the result of some
unknown process or interaction present in the brain homogenate but not neuron or astrocyte experiments, or (most likely) the result of chance and therefore insignificant.

Examining protein phosphorylation did not result in many interesting results, except for the ability of GMP to increase serine containing sequence and threonine containing sequence phosphorylation status in whole brain homogenates. However, the high degree of variability in many of the sample groups is a problem that needs to be addressed. As mentioned previously I measured changes in phosphorylation status using antibodies raised against short, specific sequences that contained the phosphorylated serine or threonine residues. This creates a situation in which many potential phosphorylation events are not being identified and as a result the system is not very precise. Also, the use of anti-phospho-serine and –threonine antibodies in western blot analysis creates many technical difficulties that often result in overexposed films that are difficult to analyze and interpret. These problems could, perhaps, be addressed in two different manners.

The first way would be to examine some specific phosphatase targets. Given that calcineurin is known to play a role in neuronal response to hypoxia targets like iNOS (or other common calcineurin targets) may be appropriate candidates for western blot analysis. A second way would rely less on the targeting of specific proteins and more on providing a more accurate view of the effects of AIT-082 on general phosphorylation status. There will be technical hurdles to overcome, of course, but if a method were devised to supply a radioactively labelled source of phosphate to the cells for ATP synthesis the resulting changes in protein phosphorylation status would be more amenable to clean, precise documentation that western blots probed with anti-phospho antibodies.
5.0 Conclusions

Based on my stated research goals and hypotheses I conclude that the present experiments have shown that:

1. AIT-082 is not able to alter neuronal total ATP levels. However, further experiments as I described may be able to refine and/or clarify the results presented in this thesis.

2. GMP but not AIT-082 is able to influence phosphorylation of specific serine and threonine containing sequences from brain tissue.

3. AIT-082 did not affect protein phosphorylation status during conditions that (as in AD) reduced energy metabolism and increased Ca$^{2+}$ influx.

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Figure 1: Cartoons of AIT-082 and representative members of the purines (B and C) and the immunophilin ligands (D and E). Molecular structures of AIT-082 (A), adenine (B), guanine (C), FK506 X=O, R=H (D), and cyclosporin A (E). The cartoon of AIT-082 was taken from Rathbone et al, 1998; the cartoons of adenine and guanine were adapted from Lewin, 2000; the cartoons of the immunophilin ligands were adapted from Harding et al, 1989.
Figure 2: Some pathways that generate free radicals, reactive oxygen species and other strong oxidants. The cartoon was adapted from Juurlink, 2001. The reactions are not all balanced nor do they all include intermediary steps. Some oxidant scavenging pathways present in the original diagram have been removed. Superoxide molecules can interact (1) to form singlet oxygen. The superoxide anion can also react with nitric oxide to form peroxynitrite (2) that in turn can give rise to hydroxyl radicals and nitrogen dioxide (3). Superoxide dismutase (SOD) can scavenge superoxide to form molecular oxygen and hydrogen peroxide (4). Hydrogen peroxide, in the presence of transition metal ions, can form the hydroxyl radical (5). Carbon centered lipid radicals are formed when the hydroxyl radical abstracts an electron from a polyunsaturated fatty acid (6). The lipid radical can react with molecular oxygen to form a peroxyl radical that in turn abstract an electron from another polyunsaturated fatty acid that initiates a lipid peroxidation chain reaction (7) that can cause extensive plasma membrane damage. The resulting oxidative stress can activate NFκB. The importance of transition metal ion scavenging (8) becomes clear from (6) and (7). Lipid peroxides can also degrade into strong oxidants such as 4-hydroxynonenal (9). The superoxide anion, in the presence of transition metal ions, can convert sugars into strong oxidants such as dicarbonyl glyoxal (10) that in turn interact with proteins to form advanced glycation endproducts (AGEs).
Figure 3: Neural cell identity demonstrated by immunofluorescent staining of cells harvested from CD1 mice and grown in culture as described by Juurlink and Walz, 1998. The top two panels show cortical neurons double labelled with primary antibodies against neuronal proteins: neurofilament 200 and GABA. Anti-GABA binding was visualized with an FITC labelled secondary antibody (A) and anti-NF200 binding was visualized with a Cyb III labelled secondary antibody (B). Panels C and D show that the cortical neurons (anti-GABA staining in C) do not display GFAP immunopositivity (D). Panels E and F both show cortical astrocytes stained with a primary antibody against astrocyte marker GFAP and a Cy III labelled secondary antibody. The astrocyte cultures were negative for anti-NF200 binding (not shown).
Figure 4: The effect of AIT-082 treatment on relative ATP levels from CD1 mouse cortical neurons subjected to glutamate excitotoxicity. CD1 mouse cortical neuron cultures were organized into four groups and treated with either vehicle/vehicle (Blank above), vehicle/1.0 µM AIT-082 (AIT-082), 100 µM L-glutamate (Glutamate), or 1.0 µM AIT-082/100 µM L-glutamate (AIT-082 and Glutamate). Treatment with AIT-082 only had no significant effect on ATP levels. Neurons exposed to L-glutamate showed a significant reduction in ATP levels compared to control (indicated by an asterisk). Glutamate also caused a significant decrease in ATP levels compared to control (indicated by an asterisk) that was not affected by the subsequent addition of AIT-082. Error bars represent standard deviations. All experimental and control groups (n=9) were replicated three times. Statistical significance was determined using a two way ANOVA test and the Holm-Sidak method for multiple comparison versus control test.
Relative ATP Levels

Control

AIT-082 only

Glutamate only

Glutamate and AIT-082

*
Figure 5: Effect of non-adenosine purines on the phosphorylation of proteins from CD1 mouse brain homogenates. A- Western blots stained with an antibody that recognizes a short amino acid sequence with a phosphorylated serine residue. Lane 1: control lane, brain homogenate was incubated with blank purine vehicle; Lane 2: GMP lane, brain homogenate was incubated with 1.0 µM guanosine monophosphate for 20 min; Lane 3: AIT-082 lane, brain homogenate was incubated with 1.0 µM AIT-082 for 20 min. B - Graph of total phosphorylation signal density expressed as a percentage of control treatment phosphorylation. GMP treatment caused a significant increase in phosphorylation. AIT-082 also caused a small, though not significant, increase in protein phosphorylation. C - Graph of phosphorylation signal density of a 43.4 kD band expressed as a percentage of control treatment phosphorylation. The phosphorylation of the 43.4 kD band marked above (A) follows the same trend seen in 3B. Only the increase seen in GMP treated homogenates is considered significant. All control and experimental groups (n=9) were replicated three times. Error bars in graphs represent standard deviations. Statistical significance was determined using a one way ANOVA test and the Tukey-Kramer Multiple Comparisons Test.
A

Relative Phosphorylation levels

Vehicle  GMP  AIT-082

B

Relative Phosphorylation levels

Vehicle  GMP  AIT-082

C

Relative Phosphorylation levels

Vehicle  GMP  AIT-082
**Figure 6:** Effect of non-adenosine purines on the phosphorylation of proteins from CD1 mouse brain homogenates.  

A - Western blots stained with an antibody that recognizes a short amino acid sequence with a phosphorylated threonine residue.  Lane 1: control lane, brain homogenate was incubated with blank purine vehicle; Lane 2: GMP lane, brain homogenate was incubated with 1.0 µM guanosine monophosphate for 20 min; Lane 3: AIT-082 lane, brain homogenate was incubated with 1.0 µM AIT-082 for 20 min.  

B - Graph of total phosphorylation signal density expressed as a percentage of control treatment phosphorylation.  GMP treated homogenates displayed a significantly higher signal density than either control or AIT-082 treated homogenates.  AIT-082 treated homogenates were not significantly different from control.  

C - Graph of phosphorylation signal density of a 43.4 kD band expressed as a percentage of control treatment phosphorylation.  Though the phosphorylation of the 43.4 kD band marked above (A) follows the same trend seen in 3B the differences are not significant.  All experimental and control groups (n=9) were replicated three times.  Error bars in graphs represent standard deviations.  Statistical significance was determined using a one way ANOVA and the Tukey-Kramer Multiple Comparisons Test.
A

B

Relative Phosphorylation levels

Vehicle GMP AIT-082

C

Relative Phosphorylation levels

Vehicle GMP AIT-082
Figure 7: The effect of AIT-082 on neuronal protein serine phosphorylation status following 4 h hypoxia.  
A - Western blot of proteins harvested from CD1 mouse cortical neuron cultures stressed with 4 h of hypoxia. Control cultures were not stressed, the numbered lanes indicate the time in hours the cultures were allowed to recover under normoxic conditions before they were harvested. ‘-’ or ‘+’ signs indicate the absence or presence of 1.0 μM AIT-082 in the culture medium during recovery. Lane 0 + was supplemented with 1.0 μM AIT-082 during the 4 h of hypoxia.  
B - Data presented as a ratio of signal density for the entire lane/total signal density for the control - lane. AIT-082 treatment did not significantly affect serine containing sequence phosphorylation. The 4 h hypoxic insult did cause a significant reduction in serine containing sequence phosphorylation signal compared to control conditions (shown in the graph as *)  
C - Data presented as a ratio of signal density for a 46 kD band/46 kD band density for the control - lane. Neither AIT-082 treatment nor hypoxia caused a significant alteration in serine containing sequence phosphorylation of the 46 kD band at any time period examined. All control and experimental groups (n=9-16) were replicated at least three times. Error bars in graphs represent standard deviations. Statistical significance was determined using a two way ANOVA test and the Holm-Sidak method for multiple comparison versus control test.
**Figure 8:** The effect of AIT-082 on neuronal protein threonine phosphorylation status following 4 h hypoxia.  

**A** - Western blot of proteins harvested from CD1 mouse cortical neuron cultures stressed with 4 h of hypoxia. Control cultures were not stressed, the numbered lanes indicate the time in hours the cultures were allowed to recover under normoxic conditions before they were harvested. ‘-’ or ‘+’ signs indicate the absence or presence of 1.0 µM AIT-082 in the culture medium during recovery. Lane 0 + was supplemented with 1.0 µM AIT-082 during the 4 h of hypoxia.  

**B** - Data presented as a ratio of signal density for the entire lane/total signal density for the control - lane. Neither AIT-082 treatment nor hypoxia significantly altered threonine containing sequence phosphorylation at any time period examined.  

**C** - Data presented as a ratio of signal density for a 46 kD band/46 kD band density for the control - lane. Neither AIT-082 treatment nor hypoxia significantly altered threonine containing sequence phosphorylation at any time period examined. All control and experimental groups (n=9-16) were replicated at least three times. Error bars in graphs represent standard deviations. Statistical significance was determined using a two way ANOVA test and the Holm-Sidak method for multiple comparison versus control test.
**Figure 9:** The effect of AIT-082 on astrocyte protein serine phosphorylation status following 4 h hypoxia and ischemia.  

**A** - Western blot of proteins harvested from CD1 mouse cortical neuron cultures stressed with 4 h of hypoxia/ischemia. Control cultures were not stressed, the numbered lanes indicate the time in hours the cultures were allowed to recover under normoxic conditions before they were harvested. ‘-’ or ‘+’ signs indicate the absence or presence of 1.0 µM AIT-082 in the culture medium during recovery. Lane 0 + was supplemented with 1.0 µM AIT-082 during the 4 h of hypoxia.  

**B** - Data presented as a ratio of signal density for the entire lane/total signal density for the control - lane. The 4 h period of hypoxia and ischemia (H/I) significantly decreased serine containing sequence phosphorylation (indicated by an asterisk) compared to control. AIT-082 treatment during recovery period did not significantly alter serine containing sequence phosphorylation at any time period examined.  

**C** - Data presented as a ratio of signal density for a 46 kD band/46 kD band density for the control - lane. The 4 h period of H/I significantly decreased serine containing sequence phosphorylation (indicated by an asterisk) compared to control. AIT-082 treatment during recovery period did not significantly alter serine containing sequence phosphorylation at any time period examined. All control and experimental groups (n=8-13) were replicated at least three times. Error bars in graphs represent standard deviations. Statistical significance was determined using a two way ANOVA test and the Holm-Sidak method for multiple comparison versus control test.
Figure 10: The effect of AIT-082 on astrocyte protein threonine phosphorylation status following 4 h hypoxia/ischemia. A - Western blot of proteins harvested from CD1 mouse cortical astrocyte cultures stressed with 4 h of hypoxia/ischemia. Control cultures were not stressed, the numbered lanes indicate the time in hours the cultures were allowed to recover under normoxic conditions before they were harvested. ‘−’ or ‘+’ signs indicate the absence or presence of 1.0 μM AIT-082 in the culture medium during recovery. Lane 0+ was supplemented with 1.0 μM AIT-082 during the 4 h of hypoxia. B - Data presented as a ratio of signal density for the entire lane/total signal density for the control - lane. The 4 h period of hypoxia and ischemia (H/I) significantly decreased threonine containing sequence phosphorylation (indicated by an asterisk) compared to control. AIT-082 treatment during recovery period did not significantly alter threonine containing sequence phosphorylation at any time period examined. C - Data presented as a ratio of signal density for a 46 kD band/46 kD band density for the control - lane. The 4 h period of H/I significantly decreased serine containing sequence phosphorylation (indicated by an asterisk) compared to control. AIT-082 treatment during recovery period did not significantly alter serine containing sequence phosphorylation at any time period examined. All control and experimental groups (n=8-11) were replicated at least three times. Error bars in graphs represent standard deviations. Statistical significance was determined using a two way ANOVA test and the Holm-Sidak method for multiple comparison versus control test.