Preventing Oxygen-Glucose Deprivation Induced Neuronal Death

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by

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Chapter 1. Introduction

1.1 Background

During a stroke, oxygen and glucose are deprived at the affected brain area. This deprivation results in cell death. When blood flow is restored and reperfusion occurs, an oxidative burst results from the abundance of oxygen and glucose now available. This results in the generation of reactive oxygen species (ROS) and alters the homeostasis of intracellular calcium levels. In addition to stroke, several other neurodegenerative diseases, such as Alzheimer Disease (1) or Parkinson Disease (2) share a similarity in that increased ROS production and elevated calcium levels can lead to premature cellular death through necrosis or apoptosis.

The purpose of these experiments will be to focus on preventing the reperfusion related cell death following oxygen/glucose deprivation (OGD). We will develop a model to replicate conditions that occur during the course of a stroke, as well as the oxidative burst that follows this insult. Additionally, we will examine compounds designed to target this process through both ROS production by targeting the mitochondrial protein, mitoNEET, as well as targeting calcium homeostasis through the blockade of voltage-gated calcium channels (VGCC).
1.2 The biology of a stroke

A stroke occurs when there is a sudden interruption of blood flow to the brain, either temporary or permanent. This disruption is caused by either a blockade (ischemic stroke) or a rupture of a cerebral artery (hemorrhage). Both forms can result in brain damage as the cutoff of blood supply causes a restriction in the delivery of oxygen and nutrients, most importantly glucose. Glucose is required for ATP production through cellular respiration and is essential for cell viability. When an ischemic stroke occurs, the effects are rapid due to the brain’s inability to store glucose or ability to respire without it (3).

The initial effects of a stroke are deleterious, but this damage is further exasperated due to reperfusion injury. Although oxygen is essential for tissue survival, it can have devastating effects following an ischemic event (4). Reperfusion injury occurs when oxygen and glucose become readily available following deprivation, and the result is not restoration of normal function, but rather a state of oxidative stress and excessive creation of reactive oxygen species (ROS). Although normally mammalian cells possess radical scavenging abilities as a defense against ROS, these antioxidant processes become overwhelmed after ischemia and reperfusion which results in an excess production of ROS (4).
Additionally, when these intracellular defense mechanisms become depleted, cells can undergo irreversible damage from oxidative stress (5). In addition to stroke, oxidative stress has been shown to be involved with several neurodegenerative diseases, including stroke and reperfusion injury (5). Mitochondria are thought to be the source of oxidative stress and ROS, as well as a potential therapeutic target for neurodegenerative and neurological disorders.

1.3 Cellular Respiration

Because of the huge demand for ATP in the brain, the CNS is filled with a large number of mitochondria, the energy-producing organelle of the cell. These mitochondria are responsible for the ATP-producing cellular respiration in the brain. Cellular respiration is divided into three main components: glycolysis, the citric acid cycle, and oxidative phosphorylation via the electron transport chain. Figure 1 illustrates these 3 processes showing the citric acid cycle and electron transport in the mitochondria. When oxygen is absent, these two processes cannot occur, and the cells can only utilize glycolysis for respiration, where it converts glucose into two molecules of pyruvate.
Figure 1. Cellular respiration can be divided into three metabolic processes: glycolysis, the citric acid cycle, and oxidative phosphorylation. Each of these occurs in a specific region of the cell; Glycolysis occurs in the cytosol, the citric acid cycle takes place in the matrix of the mitochondria and oxidative phosphorylation via the electron transport chain. This is carried out in the mitochondrial inner membrane. In the absence of oxygen, ATP is produced primarily through glycolysis if glucose is still available in the cytosol. Note, the end product of glycolysis is pyruvate. Pyruvate enters into the citric acid cycle to produce additional ATP. If respiration stops, pyruvate gets converted into lactic acid.

The first step in cellular respiration is glycolysis. In this process, glucose is converted into two molecules of pyruvate. The cell also nets two ATP and two nicotinamide adenine dinucleotides (NADH) in this process. This anaerobic process takes place in the cytosol outside of the mitochondria (Figure 2).
During glycolysis, the 6-carbon sugar, glucose, is converted into two molecules of pyruvate. This process results in a net gain of 2 ATP molecules and 2 NADH molecules. Glycolysis occurs in the cytosol.

During the next phase of respiration, pyruvate generated from glycolysis is transported into the mitochondria (Figure 3). Pyruvate is then oxidized into Acetyl CoA which enters the citric acid cycle. The citric acid cycle occurs in the mitochondrial matrix, and produces a great deal of energy in the form of ATP, NADH and FADH$_2$. 

**Figure 2.** During glycolysis, the 6-carbon sugar, glucose, is converted into two molecules of pyruvate. This process results in a net gain of 2 ATP molecules and 2 NADH molecules. Glycolysis occurs in the cytosol.
Figure 3. From glycolysis, pyruvate is transported into the mitochondria and loses a carbon dioxide to form acetyl-CoA. When acetyl-CoA is oxidized to carbon dioxide through the citric acid cycle, energy is released in the form of NADH, FADH$_2$, and ATP. The citric acid cycle occurs in the mitochondrial matrix.

Finally, in the last stage of cellular respiration, the electron transport chain moves electrons from electron donors to acceptors in a series of redox reactions. This process generates a significant amount of ATP for the cell. As seen in Figure 4, the electron transport chain consists of multiple complexes in the mitochondria. In this example, complex I accepts electrons from NADH which transfers them to Coenzyme Q (ubiquinone). From here the electrons are passed to complex III which in turn passes them to cytochrome C and finally to complex IV, which uses the electrons to reduce oxygen to water.
In the mitochondrial electron transport chain electrons move from an electron donor (NADH, ubiquinone, cytochrome C) to a terminal electron acceptor (O₂) via a series of redox reactions. These reactions are coupled to proton transfer in order to establishment of a proton gradient across the mitochondrial inner membrane. The resulting proton gradient is used to make ATP via ATP synthase through complex V.

Occasionally, electrons may leak out of a complex to prematurely form oxygen free radicals. These are known as reactive oxygen species (ROS) and are primarily generated from complex I and III during electron transport (6).

1.4 Reactive Oxygen Species (ROS)

One of the mediators of several neurological disorders, such as stroke or ischemia, is oxidative stress (5). When oxidative stress injures cells, the damage can be irreversible because the intracellular defense mechanisms of the cell are destroyed (5). This insult causes the cells to be vulnerable against the damaging effects of ROS. Additionally, the accumulating oxidative damage can affect the efficiency of mitochondria and further increase the rate of ROS production (7).
Figure 4. The electron transport chain is a series of protein complexes in the inner mitochondrial membrane which help promote the movement of electrons. Oxygen serves as the final acceptor of electrons and is reduced to water. These reactions create a proton gradient which is used to make ATP through ATP synthase.

ROS are highly reactive molecules produced through a sequential reduction of O$_2$ (5). These include the hydroxyl radical (OH$^-$), hydrogen peroxide (H$_2$O$_2$) and superoxide (16). Normally, the mitochondria reduce greater than 98% of O$_2$ to water, but one to two percent of the O$_2$ will be reduced to ROS (8).
During ischemic stroke, there is an increase in oxidative stress and ROS production. This occurs during ischemia as well as reperfusion (9). A goal of stroke therapy is reperfusion and restoration of blood flow, as it is immediately needed to save brain tissue (9). However, this process has some harmful effects resulting from reoxygenation and increased ROS production (9). Many studies have shown that ROS plays a role in various neurological disorders, including ischemia and stroke (10). Increased generation of ROS is also a well-known initiator of apoptosis and necrosis in many cells (11).

1.5 Cell Death Mechanisms

Apoptosis, otherwise known as programmed cell death, is a fundamental component of the development and preservation of healthy cells in multicellular organisms (12). Although cells can be killed by many various factors, apoptosis is the death of cells in a controlled manner. For humans, excessive or insufficient apoptotic activities can lead to severe pathological issues (13).
Necrosis, on the other hand, is an uncontrolled form of cell death, arising from an injury or traumatic conditions such as that which occurs in tissue during an ischemic stroke (14). These cells lose plasma membrane integrity, causing them to swell and lyse and release their nuclear contents. However, although it is important for acute injuries, necrosis is not the normal mechanism by which cells die and can result in inflammatory responses which further destroys tissue (15).

Apoptotic cells are distinguished from this process by a distinct difference in morphology. Cells undergoing apoptosis display specific characteristics. In the beginning the cell begins the breakdown of chromatin in the nucleus, which leads to nuclear condensation and causes a “horse-shoe” like appearance. The cell then continues to shrink, and begins to package itself into a shape easily removable by macrophages. Finally, small membrane blebs can be seen, along with apoptotic bodies as the cell readies itself for programmed death (12).

Apoptosis can be triggered by various mechanisms. In the extrinsic apoptotic pathway, the cell itself has surface receptors that act as “death receptors.” These factors can be activated by certain ligands, which initiate a cascade of cell death events. One of these ligands is tumor necrosis factor (TNF). Other members of the TNF family can also induce
apoptosis. These include ligands such as: LT (lymphotoxin), FasL (fibroblast-associated ligand), TRAIL (TNF-related apoptosis inducing ligand), and DR3L (death receptor 3 ligand or TWEAK, also known as a weak homologue of TNF) (14).

Alternatively, commitment to death can be triggered by removing a death-inhibiting ligand (15). This form of cell death commences when a cell lacks the signals from its cell-surface survival-factor receptor (15). The absence of this signal initiates the endogenous death sequence within the cell, and the same final processes are activated. Mitochondria play an important role in amplifying the signaling from death receptors or lack of survival-promoting receptors (12).

Mitochondria also play an important role regulating intrinsic cell death. Death signals that originate from inside the cell such as DNA damage, oxidative stress, free radical damage or starvation, activate a group of proteins known as the Bcl-2 family of proteins. Some members of this class are anti-apoptotic, Bcl-2 and Bcl-xL, while others are pro-apoptotic such as Bad, Bax or Bid (12). Normally, the anti-apoptotic proteins reside in the outer mitochondrial membrane, and prevent cytochrome C release. When stress signals occur, the pro-apoptotic Bcl-2 proteins are activated
and relocate to the surface of the mitochondria where they inactivate the anti-apoptotic proteins (12).

When cytochrome C is released into the cytosol under pro-apoptotic conditions, it is able to interact with Apaf-1 (16). The binding of these two facilitates the recruitment of pro-caspase 9, and together forms the apoptosome (16). The apoptosome begins the caspase cascade and the induction of apoptosis.

At least fourteen caspases have been identified in mammals, and at least eight of these play a crucial role in apoptosis (13). Caspases are divided into two groups, initiators and effectors. An initiator caspase becomes active through the cells auto-apoptotic mechanisms. The initiator in turn will then activate the effector caspase. When the effector caspase becomes activated, it will begin cleavage of many cellular targets which are required for normal function, including structural proteins and nuclear proteins such as DNA repair enzymes (12).

Typically, initiator caspases 8 or 10 are activated which in turn leads to the activation of effector caspases 3 and 6 (12). These caspases then become responsible for the cleavage of many important intracellular
components, leading to the key morphological changes as discussed previously (16).

1.6 Calcium Homeostasis in the Cell

Calcium regulation is vital for proper cell function and essential for cell survival. Modifying neuronal intracellular concentration of Ca^{2+} can alter many responses, including neurotransmitter release, synaptic transmission and apoptotic mechanisms (17). In many neurodegenerative disorders, the function of neurons is reduced due to improper calcium signaling and inappropriate changes in intracellular Ca^{2+} levels. In fact, an increase of intracellular Ca^{2+} concentration [Ca^{2+}] is considered the single most important contributor to neurodegeneration and neuronal cell death following an ischemic event (17).

Calcium homeostasis is a tightly regulated process involving many molecules, structures, and pathways. Inside the cell, calcium levels are regulated through intracellular stores in the endoplasmic reticulum, various pumps, and ion channels. Under normal conditions, there is a large driving force of Ca^{2+} into the cell. This force has both a concentration and electrical component. The concentration gradient across the plasma
membrane is large and approximately 1 mM outside of the cell and 100 nM within the cell (18). The electrical potential across the membrane also creates a large gradient, with the inside being 60-90 mV more negative than the outside potential (19). This vast electrochemical gradient is managed by three basic mechanisms. The cell membrane itself helps to regulate this process as the lipid bilayer is Ca\textsuperscript{2+} impermeable. In addition, specific Ca\textsuperscript{2+} pumps, powered by ATP as well as the Na+/Ca\textsuperscript{2+} exchanger are vital for regulating [Ca\textsuperscript{2+}]\textsubscript{i} (20). Finally, the mitochondria and endoplasmic reticulum play a major role in the uptake of Ca\textsuperscript{2+} (20).

Calcium influx can occur through voltage-gated and ligand-gated channels. The activity of these channels is regulated by various intracellular signaling pathways, including calmodulin binding and activation, phosphorylation of protein kinases, and G-coupled protein binding (21). Voltage-gated calcium channels (VGCC) are permeable to Ca\textsuperscript{2+} and found only in excitable cells, those which can be activated by depolarization of the membrane or activation of a ligand-gated or receptor-operated Ca\textsuperscript{2+} channel (ROCC) (22). Voltage-gated channels are classified as L, N, T, or P/Q type.
The L-type Ca\(^{2+}\) channels (LTCC) have been shown to be a major entryway for Ca\(^{2+}\) into the cell from the extracellular space (22), thus LTCC blockers have been investigated for their protective effects in ischemia and stroke. Nimodipine is an antagonist to LTCC and reduces the increase in intracellular Ca\(^{2+}\), which normally follows a cerebral insult (17). However, nimodipine is not very effective in crossing the blood-brain barrier.

In postsynaptic neurons, the release of neurotransmitters can activate excitatory receptors such as \(N\)-methyl-D-aspartate (NMDA) receptors or \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. NMDA receptors require the dual binding action of glutamate and glycine for activation (23). Upon binding, the channel is non-specific for cations and allows Ca\(^{2+}\) and Na\(^{+}\) to flow into the cell, while Mg\(^{2+}\) flows out.

When a stroke occurs, the delivery of oxygen and glucose is restricted. This results in an impairment of the bioenergetics required for preservation of the neuronal ionic gradients (24). During cellular stress resulting from ischemia, the regulatory mechanisms which normally withhold Ca\(^{2+}\) from entering the cell are compromised, resulting in a rapid or prolonged elevation of intracellular Ca\(^{2+}\) levels (25). This increase will lead to a number of devastating consequences for the cell. As progressively larger
amounts of extracellular Ca\textsuperscript{2+} continue to influx, excessive release of glutamate will occur (26). This is known as excitotoxicity, and will lead to increased activation of NMDA receptors, again resulting in increased Ca\textsuperscript{2+} influx. In addition, oxidative stress will cause a rapid decline in ATP levels, further insulting cellular mechanisms. Because Ca\textsuperscript{2+} pumps utilize ATP as their fuel source, this will compromise the mechanisms that normally keep Ca\textsuperscript{2+} from entering the cell, again resulting in an increase in intracellular Ca\textsuperscript{2+} levels.

1.7 Calcium and Mitochondria

Mitochondria also play an important role in calcium homeostasis. Ca\textsuperscript{2+} enters the through the mitochondrial Ca\textsuperscript{2+} Uniporter (MCU). Entry is driven by the membrane potential during Ca\textsuperscript{2+} uptake as membrane potential decreases. Therefore a large accumulation of Ca\textsuperscript{2+} in the cell will cause a collapse in membrane potential (27). Ca\textsuperscript{2+} influx through LTCC also plays a critical role in mitochondrial Ca\textsuperscript{2+} overload and downstream mitochondrial and cellular dysfunctions. Additionally, elevation of the level of intracellular Ca\textsuperscript{2+} is responsible for activation of ROS-generating enzymes and formation of free radicals by the mitochondria (28).
In addition to ROS production, an increase in mitochondrial Ca\textsuperscript{2+} levels will trigger the formation of the mitochondrial permeability transition (MPT) pore (29). The MPT pore is an assembly of proteins, from both the inner and outer mitochondrial membranes, into a large channel which allows the release of both Ca\textsuperscript{2+} and cytochrome C into the cell (29). Cytochrome C binding with Apaf-1 will initiate the activation of caspase-9, which will activate caspase-3 and begin the apoptosis pathway.
Chapter 2. Neuroprotection Strategies

2.1 Oxygen/Glucose Deprivation of Primary Cortical Neurons

Cell lines are a mainstay of research in most areas of cell biology. They can be stored in liquid nitrogen and grown up when needed and even shared with other labs throughout the world (30). However, continuous cell lines are not commonly used for central nervous system studies, as these cells do not form defined dendrites and axons while dividing and since they are cancer cells, they do not respond to insults as normal neurons would (31). Most scientists work with primary cultures instead, with the most used being cortical and hippocampal cultures. There are several reasons for this popularity in culture selection. The stages of neuron development in these cultures have been well-documented and characterized and are consistent from lab to lab (31). They also form clearly-defined dendrites and make large synaptic networks (31). Additionally, these cells have been utilized for over twenty years in laboratories, so an extensive database can be provided when beginning new experiments (31).
Cortical cultures are obtained from late-stage embryos, most specifically, embryonic day 18 (E-18), as this provides optimal time for neuronal growth. The generation of pyramidal neurons at this point is essentially complete. Cells cultured on this day will also contain few glial cells, a major source of contamination in neuronal cultures (31).

Obtaining neuronal hippocampal tissue from a rat pup at E-18 is a complex and intricate task. Pregnant dams are anesthetized and a cesarean section performed to gather the pups. The brains are dissected from each pup following the protocol published by Kaech and Banker, 2006 (31). However, recently this tissue has become available to purchase through a vender. To obtain primary neurons in a more resourceful and practical way, we utilized the services of Brainbits, LLC ®. Brainbits dissect regions of embryonic rat and mouse brain for the isolation of live neurons in twenty minutes (32). The company was established after developing a media that could keep brain tissue preserved for weeks under refrigeration. This media allows viable cells to be shipped all over the world to be utilized for culturing purposes. We used Brainbits ® to obtain cells quickly, and in a more cost-effective manner then dissecting them ourselves.
For studies of neuronal development, pharmacology, and neurotoxicity, a commonly used media for cell culture is Neurobasal. This medium is derived from Dulbecco’s Modified Eagle Medium (DMEM) but used specifically for neuronal cell lines. Brewer et al. optimized this media from DMEM by lowering the concentration of several amino acids (alanine, cysteine, proline and Vitamin B12), in addition to eliminating ferrous sulfate, glutamate and aspartate (33). B27, a serum-free supplement is also added to the neurobasal for growth and long-term viability of primary neurons. Cortical neurons have been shown to be more viable in this media, then DMEM with any supplements (33).

In addition, rat tail collagen type I has been used for years as a reliable substrate for neuronal tissue. This substance is easy to use and readily obtainable, and allows cells to adhere properly to culture dishes (34). The method for collagen coating, as well as the procedure for other substrate coating techniques is described in detail in Banker and Goslin's book, Culturing Nerve Cells (30).

As a first step to this study, a reliable model must be established to properly mimic hypoxic conditions, as well as reperfusion related effects following an ischemic event. Oxygen-glucose deprivation models are
commonly utilized in replicating stroke conditions. These models utilize hypoxia chambers, which are an easy way to create a controlled oxygen-free environment. The chamber has an air tight seal with fast gas exchange properties which create a hypoxic environment that does not fluctuate (35). Cells are placed in the chamber which is attached to a flow meter measuring gas flow in liters per minute. The chamber is flushed for several minutes with the desired gas mixture, and then can be sealed off and placed in an incubator for the time specified by the researcher. It is important to have a gas mixture of 5% CO$_2$ with the 95% N$_2$ for the hypoxic conditions, so cell death results from oxygen deprivation and not an acidic environment. The solution, 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES) is also widely used in cell culture as a buffer for maintaining physiological pH (36).

To determine cell death, we used the lactate dehydrogenase assay. Lactate dehydrogenase (LDH) is a glycolytic enzyme that is a key component in the conversion of pyruvate to lactate under anaerobic conditions (37). LDH release signifies both cell death and loss of membrane integrity. The LDH assay can assess both of these situations by measuring cytoplasmic LDH levels to quantify cell death, or by
measuring membrane integrity through the amount of cytoplasmic LDH released into the medium (38).

This assay is based on the reduction of NAD$^+$ by LDH. The reduction of NAD$^+$ to NADH is utilized in the stoichiometric conversion of a tetrazolium dye (38). If the cell is dead or has a disrupted membrane, there will be an increase in the amount of substrate converted. This increase can be measured spectrophotometrically using a microplate reader.

2.2 Targeting mitoNEET

A group of compounds called the glitazones have been shown to bind to mitochondria, and prevent neuronal damage after cerebral reperfusion injury (39). These compounds are members of the thiazolidinedione (TZD) family, and are a class of insulin sensitizers (see structures below) (40). Studies have shown that glitazones have agonist activity at the peroxisome proliferator-activated receptor γ (PPARγ) which accounts for their beneficial effects in type 2 diabetes (40). However, the action at PPARγ is not thought to be completely responsible for the prevention of oxidative damage following a stroke.
Recent studies have identified and isolated the protein where these compounds bind. This mitochondrial associated protein was found to contain the amino acid sequence, Asn-Glu-Glu-Thr (NEET), and was therefore named mitoNEET (40). It was determined that the glitazones are ligands to this protein and mitoNEET is an iron-containing outer membrane protein as depicted in Figure 6 (41). Wiley suggested that mitoNEET may play a role in the control of mitochondrial respiratory rates which has been confirmed in our laboratory (41) (40).

**Figure 5.** Structure of 3 glitazones from the TZD family
Recent data from our lab have indicated that when ligands bind to mitoNEET, the oxidative capacity of the mitochondria and complex I substrates are altered (41). Complex I is a known source of ROS production, therefore utilizing this protein pharmacologically could serve as a potential target for neurodegenerative disease by reducing its ability to generate ROS.

Figure 6. Location of mitoNEET protein on the outer mitochondrial membrane.

After the discovery of the glitazone's binding affinity for mitoNEET, a group of compounds were designed containing a thiazolidinedione (TZD) ring with a similar structure to target the mitoNEET. Binding studies have shown these compounds, known as the NL compounds, are successful
ligands to mitoNEET. The possible binding sites for the NL compounds are shown in Figure 7 (41).

**Figure 7.** Possible binding sites found in mitoNEET using SiteFinder (MOE, Chemical Computing Group) (A) Identification of the five possible sites (B) Pioglitazone docked in sites 1 and 2

The NL-compounds were designed to be structurally similar to the glitazones, but they did not display the PPARγ agonistic effects of the glitazones. Preliminary data suggest that these compounds uncouple respiration by targeting mitoNEET which causes a biochemical cascade resulting in altered mitochondrial function.
Considering the receptor independent actions of PPARγ, it is apparent that TZDs play a role in mitochondria and respiration. Therefore, this study will focus on four specific glitazones designed to target mitoNEET. The structures and IC₅₀ values for the NL-compounds, 1-4, are shown in Table 1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
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<tbody>
<tr>
<td><strong>NL-1</strong></td>
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</tr>
<tr>
<td><strong>NL-2</strong></td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td><strong>NL-3</strong></td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td><strong>NL-4</strong></td>
<td><img src="image4" alt="Structure" /></td>
</tr>
</tbody>
</table>

**Table 1.** Structures and IC₅₀ values of NL compounds
Based on preliminary data, each of these compounds acts as a mitoNEET ligand, but elicits slightly different effects. As shown in Figure 8, each compound appears to have an inverse relationship in its ability to inhibit respiration vs. uncouple phosphorylation (unpublished data). In addition, NL-3 appears to produce no activity, and negates the activity of other NL compounds if added first.

Figure 8. Preliminary data of novel ligands developed to target mitoNEET. They cause the inhibition of state III respiration (A) which is inversely proportional to their ability to uncouple phosphorylation (B). n=1 for each data point which was extracted from a dose response curve for each compound using tissue from the same animal. These data are consistent with other experiments evaluating the NL compounds.
These compounds will be used to evaluate the potential role of mitoNEET in protecting neurons after ischemic conditions and reperfusion. These data will also be compared to their ability to uncouple versus inhibit pyruvate-driven respiration for each compound. This information will be essential in developing future drug candidates as new NL-compounds are synthesized.

2.3 Targeting L-type Calcium Channels

Previous studies have examined LTCC blockers as well as NMDA receptor antagonists as neuroprotective agents. These studies have shown beneficial effects, but are limited due to their low blood brain barrier penetration (42). However, a new group of compounds have been developed which target both receptors and allows for a novel way of approaching neuroprotection (1986 and 2000). These compounds are polycyclic cage derivatives and a member of the pentacycloundecylamine (PCU) family. The compound 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01) has earlier been identified as both an LTCC blocker as well as an NMDA antagonist. In
addition, a group of polycyclic triquinalamines (Figure 9) were also tested (LB compounds).

Figure 9. Structures of polycyclic cage compounds including NGP1-01.
Chapter 3. Materials and Methods

3.1. Cell Culture

Primary neurons were obtained from BrainBits® (Springfield, IL USA). Cells were obtained from rats on embryonic day 18 and stored in 2 mL B27/Hibernate (Invitrogen) at 4 °C. Cells were prepared as described elsewhere with slight modifications (33) (43). Cells were plated within one week of delivery and stored at 4 °C until preparation.

Prior to the plating of cells, 96-well plates were coated with collagen type I, (Invitrogen #A10483—1). To prepare cells for plating, 1 mL of medium was removed from the tube containing the brain tissue, and discarded. Using a silanized 9 inch Pasteur pipet, the tissue and remaining medium were then carefully triturated by sucking the tissue into the pipet and immediately dispensing the contents back into the same container. Care was taken to not create bubbles during this process. The trituration was repeated 10-15 times, or until the tissue was evenly dispersed. Then a 1 mL pipette was used to aspirate the solution 5-10 additional times to ensure thorough dispersion.

For two 96-well plates, the mixture of cells and media was added to approximately 6 mL of media specifically prepared for this experiment.
The media consisted of Neurobasal (Invitrogen, 500 mL) with 5% fetal bovine serum (FBS) and antibiotics/antifungal (penicillin-streptomycin-fungizone, 1:100) along with 2 mL of B27 and 1.25 mL glutamine. This mixture was used specifically for oxygen-glucose deprivation studies.

Cells were added to each well of the plate in 50 µL quantities. An additional 50 µL of media was added on top of the cells in each well, and plates were slowly shaken for proper mixing. Cells were then incubated at 37 °C with 5% CO₂ for exactly 1 week to allow for differentiation. Media was changed every other day starting on day 2 after plating. One half of the media was removed, and the other half was replaced with fresh Neurobasal prepared for this study.

For oxygen-glucose deprivation studies, cells were not plated in any row on the edge of the plate in order to minimize any “edge-effect” erroneous results (figure 10). The red line is drawn through all wells which were not used during this study. This limited the available wells from 96 to 60.
3.2. Oxygen-Glucose Deprivation and Compound Testing

Oxygen-glucose deprivation was achieved by utilizing a modular incubator chamber obtained from Billups-Rothenberg, Inc. The chamber was connected through tubing to a gas tank, which provided a controlled atmosphere inside the chamber of 95% N\textsubscript{2} and 5% CO\textsubscript{2}.

To achieve complete hypoxia, the chamber was flushed at a rate of 20 liters/min for 4 minutes. Prior to treatment, glucose-free RPMI 1640 (Cellgro®) media with galactose and antibiotics was placed in a fluted flask and placed in the chamber for the 4 minute flushing duration. The

\textbf{Figure 10. Wells not used during oxygen-glucose deprivation experiments}
entire chamber (with the media) was then placed in a 37° incubator overnight.

Before placing the cells in the hypoxia chamber, the neurobasal media was replaced with 50µL of the oxygen-glucose free media from the chamber. The cells were then placed in the chamber with no lid to allow for maximum exposure to the gas flushing. Again, the chamber was flushed with gas for 4 minutes and then placed back in the incubator for 2 hours at 37 °C.

The compounds to be tested were prepared in RPMI media with glucose and 5% FBS. Following the 2 hour incubation period, the cells were removed from the chamber, and 50µL of drug or control media was added to each well. The cells were then placed back in a 37 °C incubator for a 6 hour period.

Pictures were taken prior to hypoxia, post-hypoxia, and 6 hours after replacement of oxygen/glucose from vehicle and drug-treated cells using a VWR Vista Vision inverted microscope at either 100 or 250 times magnifications as described in the figure legends. The images were captured using a Moticam 2300 camera at 3 M pixels.
3.3 Cell Viability

In order to quantify cell death, lactate dehydrogenase (LDH) activity was measured in an assay kit obtained from Cayman Chemical Company, Ann Arbor, MI. LDH is rapidly released from cells when apoptosis or necrosis has occurred, and the membrane has been disrupted. The Cayman LDH Cytotoxicity Assay Kit uses a coupled two-step reaction to measure the amount of LDH which has been released into the media.

The first step of the reaction is shown below. LDH catalyzes the reduction of NAD$^+$ to NADH and H$^+$ by the oxidation of lactate to pyruvate as shown in Figure 11.

![Chemical reaction diagram](image)

**Figure 11.** The oxidation of lactate to pyruvate.
In the next step of this reaction, diaphorase catalyzes the reduction of a tetrazolium salt (INT) to highly-colored formazan, using the newly formed NADH and H⁺ (Figure 12). Diaphorase is an enzyme that catalyzes the reduction of various dyes which act as hydrogen acceptors from the reduced form of phosphopyridine nucleotides, such as NADH (44).

\[ \text{LDH} \quad \text{Pyruvic acid} \]
\[ \text{NAD}^+ \quad \text{NADH} + \text{H}^+ \]
\[ \text{Formazan} \quad \text{Diaphorase} \]
\[ \text{Tetrazolium} \]

**Figure 12.** The reduction of Tetrazolium to Formazan.

When INT has been converted to formazan, the absorbance can be assessed, as formazan has a strong absorbance between 490 and 520nm.
Prior to use, the LDH standard was reconstituted with 1.8 mL of assay buffer and kept on ice during the assay. The glucose free media and regular RPMI with glucose media was mixed in equal portions, in order to replicate the test sample. Six test tubes were then obtained and labeled 1-6. Then 475 µL were placed in tube 1, while 250 µL were placed in tube 2-6. 25 µL of the standard was then added to tube 1. After thorough mixing, 250 µL of the contents of tube 1 were placed into tube 2. Again, the media was mixed thoroughly, and the serial dilution continued for tubes 3-5. Tube 6 was left as a blank, with no standard added.

Prior to performing the assay, the cell-based assay buffer tablet was dissolved in 100 mL of distilled water. Then 600 µL of this assay buffer was then used to reconstitute the diaphorase. The diaphorase was then aliquotted into 100 mL portions, and those not used were frozen, while the aliquot needed for this experiment was kept on ice.

To make 10 mL of reaction solution, which is sufficient for 1 96-well plate, 100 µL of the following were each added to 9.6 mL of assay buffer:

- NAD+ (100X)
- Lactic Acid (100X)
- INT (100X)
- Reconstituted Diaphorase from above
Each well received 100 µL of this reaction solution. The plate was then shaken gently and let sit at room temperature for 30 minutes. After the 30 minutes, the absorbance was read at 490 nM on a Molecular Devices Spectra Max 340 PC microplate reader. There were six to twelve wells replicated for each substance tested, as described in the figures.

The LDH is released into the medium when cells membrane integrity is lost; therefore culture plates were centrifuged at 500 x g for 5 minutes to collect the media from away from the cells. For each plate, the standards occupied the first 6 wells of both rows A and B (see figure 13). Each set of test articles were in their own plate separate from other compounds. Once all plates were prepared, they were incubated at room temperature for 30 minutes and then placed in the microplate reader. The absorbance was read at 490nm.

![Figure 13. Six standards occupy the first 6 wells of row A and B.](image)
Statistics were done using InStat version 3. A Student-Newman Keuls multiple comparison test was used to test for statistical differences. GraphPad and Prism5 were used to create all graphs of data.
Chapter 4. Results

4.1 Oxygen/Glucose deprivation model

The first part of this study was to develop an appropriate model for simulating stroke conditions in neuronal cells. An efficient and convenient way of obtaining neurons was to purchase them from Brainbits®. The cells obtained were embryonic day 18 Sprague/Dawley cortical cells. The cells came packaged in a proprietary media, Hibernate®, with an additional aliquot reserved for cell plating purposes. At the beginning of this study, this media was utilized. Because this media was designed to preserve neurons, especially for shipping and transportation purposes, we did not see cell death when using the hypoxia chamber and glucose-free media, as described in the material and methods section. Figure 14 shows cell survival following 24 hours of oxygen-glucose deprivation.

Because cells incubated in Hibernate® was observed to be resistant to oxygen-glucose deprivation, it was determined that a modification to the media would be necessary to achieve cell death. After switching the media to Neurobasal without Hibernate®, cell death was visually noticeable, as well as apparent in LDH assay results. Figure 15 shows visible cell death following a 2 hour oxygen-glucose deprivation.
Figure 14. Bright field microscopy shows neurons grown in Hibernate shown prior to hypoxia treatment (A) and following a 24 hour oxygen-glucose deprivation with 6 hour exposure to glucose and oxygen (B).

Figure 15. Bright field microscopy shows neurons grown in Neurobasal shown prior to hypoxia treatment (A) and following a 2 hour oxygen-glucose deprivation with 6 hour exposure to glucose and oxygen (B).
4.2 Neuroprotection by the NL-compounds

The first set of compounds tested were the NL-compounds which target mitoNEET. The compounds were dissolved in DMSO in 10 mM stock solutions and then each diluted to 10 µM in media prior to testing. The compounds were applied following a two-hour incubation period in a hypoxia chamber with glucose-free media. Dilutions of the test articles were made in RPMI media with glucose to observe the effects of reperfusion following hypoxic conditions.

There are four NL-compounds and each was tested at 10 µM to observe which substance showed protection from reperfusion-associated cell death. The results from these assays (Figure 16) show a significant increase in cell viability for the compounds NL-1, NL-2, and NL-3 when compared to vehicle treatment alone (p<0.5 and n=10-12). NL-4 showed no significant protection of cell death.

Due to the use of NL-1 in additional studies, further characterization was performed on this compound. A dose response was conducted to identify efficacious concentrations of the drug. In this experiment, we found that 5 and 10 µM showed significant protection in our OGD model. Shown in Figure 17 are the results from the dose response for NL-1.
Figure 16. NL compounds target the mitochondria during reperfusion associated cell death following hypoxia conditions. NL1, NL2, and NL3 show significant protection compared to control media. Error bars are average +/- SD, where N=10-12. *p<0.05 denotes statistical significance.
Figure 17. Dose response of NL-1 in µM. 10uM shows protection similar to that seen in Figure 1. Error bars are average +/- SD, where N=10-12. *p<0.05 denotes statistical significance.
These data are supported visually by microscopic examination of the cells. Figure 18 shows the severe impact of 2 hours oxygen/glucose deprivation on our neuronal cells when compared to controls (17.A and 17.B). After the reintroduction of glucose and oxygen, 6 hours later majority of the cells had lost their cell membrane integrity (17.C). However, when treated with 10 μM NL-1, 6 hours after the reintroduction of glucose and oxygen the cells looked at least as good as when they came out of the oxygen/glucose deprivation chamber. Combined, these data suggest that NL-1 was capable of significantly reducing damage that occurs during the oxidative burst associated with the reintroduction of oxygen/glucose.
Figure 18. Bright field microscopy shows neurons before hypoxia (A), following a 2 hour incubation period (B), following a 6 hour reperfusion period (C) and following a 6 hour reperfusion period and treated with NL-1 (10 µM). These cells were plated at high density. Cells were magnified to 250 X.
4.2 Targeting the L-type Calcium Channels

The triquinylamines (LB compounds) were also tested in an oxygen-glucose deprivation model. Unfortunately, as shown in figure 19, although 3 compounds showed significant protection, the protection observed did not warrant further investigation. Surprisingly, NGP1-01 showed approximately 75% protection as determined using the LDH assay (Figure 20). A dose response was performed at 0.01 µM, 0.1 µM and 1 µM of NGP1-01 and significance was found at all concentrations tested (p<0.5, n=5-11, Figure 20).

Figure 19. LB compounds tested at 10 µM. Compounds do not show any significant protection. Error bars are average +/- SD, where N=10-12. *p<0.05 denotes statistical significance.
Figure 20. NGP dose response tested at 0.01, 0.1 and 1 µM. Compounds do not show any significant protection. Error bars are average +/- SD, where N=10-12. *p<0.05 denotes statistical significance.

Figures 21 and 22 show 2 separate experiments with compound NGP1-01. The neuroprotection of NGP1-01 is apparent in both experiment 1 (Figure 19, 250x magnification) and experiment 2 (Figure 20, 100x magnification).
Figure 21. Bright field microscopy shows neurons before hypoxia (A), following a 2 hour incubation period (B), following a 6 hour reperfusion period (C) and following a 6 hour reperfusion period and treated with NGP1-01 (1µM) (D). Cell magnification was at 250x.

These photographs support the remarkable protection predicted by the LDH assays. In fact, the cells looked significantly better after the reintroduction of oxygen / glucose when compared to just being removed from the oxygen / glucose deprivation chamber. These data suggest that not only did NGP1-01 protect the neurons, but allowed the neurons to successfully start cellular repairs.
**Figure 22.** Bright field microscopy shows neurons before hypoxia (A), following a 2 hour incubation period (B), following a 6 hour reperfusion period (C) and following a 6 hour reperfusion period and treated with NGP1-01 (1µM). Cell magnification was at 100 x.
Chapter 5. Discussion and Conclusions

5.1 Development of the Oxygen-Glucose Deprivation Model

The purpose of this study was to investigate the effectiveness of two different sets of compounds in a stroke model. To mimic hypoxic conditions, an oxygen-glucose deprivation model was developed using primary cortical neurons. As previous studies have demonstrated this method as an effective way to simulate stroke-like conditions in cells (29) (45) (46).

The neurons obtained from Brainbits® were a convenient source of acquiring embryonic day 18 Sprague/Dawley hippocampus, and were therefore utilized exclusively for this study. The cells were delivered packed on dry ice, and kept at 4 °C until plated for use. Initially at the beginning of these experiments, we utilized the media that was packaged with the cells. This is a proprietary media, known as Hibernate® and is supplied solely by Brainbits®. Hibernate® is a nutrient media used for the maintenance of neural tissue and cells. Cells arrived in a tube of Hibernate® and although Brainbits® advises to use cells right away for highest yield, the neurons are still viable after a week of storage at the proper temperature. This demonstrates the durability of cells when
cultured in Hibernate®. When the goal of an experiment is to deprive cells of oxygen and glucose and examine cell death, using a media that helps prevent cell death is not an optimal choice for this project. This was discovered after several attempts of utilizing the hypoxia chamber and glucose-free media. The neurons survived both the hypoxia insult as well as reintroduction of oxygen and glucose. Figure 14 shows merely a slight decline in cell structure, as compared with that observed in Figure 15 after the discovery of the protective effect of Hibernate®.

In order to quantify cell death properly, medium needed to be used which wasn’t designed to protect cells for shipping and storage purposes. When plating cells, we utilized neurobasal media from Invitrogen and added B27, glutamate, fungizone and FBS as specified in the Brainbits® protocol, but removed cells from Hibernate® media. LDH assays showed experiments following this modification were successful in inducing cell death following oxygen-glucose deprivation. This adjustment to the study is important for any future studies utilizing Brainbits® cells and media, and should be taken into consideration before using.
5.2 Glitazones and oxygen-glucose deprivation

Previous research has indicated a possible role in glitazones and neuroprotection. Based on this literature, we evaluated several TZDs (NL-compounds) in an oxygen-glucose deprivation model. From our studies, we found three NL-compounds showed statistically significant protection of oxygen-glucose deprivation (OGD). In particular, NL-1, the lead compound was able to reduce cell death due to OGD by approximately 50%. NL-2 and NL-3 also showed varying degrees of protection, while NL-4 displayed no discernible protection. The protection observed by compound 3 was surprising considering that it did not alter pyruvate driven mitochondrial respiration. This would indicate that the binding of NL-3 to mitoNEET may provide protection through an as yet unknown mechanism.

Based on the findings of NL-1, and its possible role in protection, we conducted a dose-response assessment looking at 1, 2.5, 5, and 10 µM. We saw a dose dependent decrease in cell death from OGD, with 5 and 10 µM being the most significant. Being able to determine the concentrations in which protection is afforded without toxicity is important for determining dosing in animal studies. In fact, when NL-1 was given at 10 mg/Kg in a rodent ischemia model, brain concentrations were predicted
to reach approximately 4 μM after 1 hour of dosing (unpublished data from our lab). At this dose, approximately 50% protection was observed (unpublished data from the Mdzinarishvili lab, NEOMED), which is consistent with the protection found in the OGD model using primary neurons.

These finding are consistent with the previous literature on glitazones which shows specific functionality related to PPAR-Ƴ receptor-independent effects. A review by Feinstein et al. suggested that TZDs inhibit complex I activity, and this disruption in cellular energy could produce the insulin-sensitizing effects of the glitazones as discussed previously (47). Additionally, the glitazones have been demonstrated as ligands to mitoNEET. Wiley et. al. demonstrated that mitoNEET is an iron-containing protein in the mitochondria, that is involved in mitochondrial respiration. Feinstein also suggests that by binding to mitoNEET, the TZDs can possibly block pyruvate driven respiration (47).

As shown from previous data, the NL-compounds are known mitoNEET ligands. NL-1 is an uncoupler of oxidation-phosphorylation in the mitochondrial electron transport chain which suggests these actions may be attributable to its binding affinity of mitoNEET. As shown in Figure 55, when NL-1 binds to mitochondrial mitoNEET, there is an alteration in the
oxidative capacity of the mitochondria and complex I substrates. Because complex I has been shown to be a source of ROS production, utilizing this protein pharmacologically could serve as a potential target for neurodegenerative disease.

Future studies could utilize NL-1 as a lead compound for clinical development in reperfusion-related effects of a stroke. Based on these data and the protection found, it is suggested that additional possible signaling pathways exist. These will be evaluated in the future (Figure 23).

Figure 23. Future studies of the TZD compounds will focus on several ROS generating and apoptosis pathways which are associated with the TZDs and mitochondria.
5.3 PCU Compounds and Oxygen-Glucose Deprivation

Current literature suggests that calcium plays a pivotal role in neurodegeneration. During cellular stress, two major entry pathways of calcium into the cell are the VGCC and NMDAR. In this study, we evaluated NPG1-01 and several of its derivatives in an OGD primary neuronal toxicity model.

Previous studies with NGP1-01 in animal models of stroke showed that it was neuroprotective in both permanent and transient ischemic stroke models resulting in greater than 50% protection (48) (49). The work done by Kiewert et al. showed that NGP1-01 was a dual blocker of both the VGCC and the NMDA receptor/ion channel (50). This suggested that NGP1-01 may be neuroprotective via blocking of calcium influx associated with apoptosis. Given that NGP1-01 was neuroprotective in stroke and able to modulate excessive calcium influx into neurons, we investigated NGP1-01 and derivatives thereof for their protective effects in our reperfusion injury model. The goal was to find lead compounds which would have clinical relevance when a patient arrives at the hospital after a stroke episode. The data presented here show that NGP1-01 was significantly more neuroprotective than the LB compounds. In particular, NGP1-01 was shown to be neuroprotective at concentrations as low as 10
nM. This value is significantly lower than the IC$_{50}$ for the VGCC and suggests that NGP1-01 may have additional activities not already discovered. These activities could include antioxidant activity, inhibition of apoptosis, or regulating mitochondrial and/or endoplasmic reticulum Ca$^{2+}$ stores. Interestingly the LB compounds did not show any neuroprotection. Given that some of the polycyclic cage compounds have a higher affinity for the VGCC, these data were quite surprising. These findings would suggest that altering the cage portion of NGP1-01 abrogates an as yet identified neuroprotective property of this compound.

NGP1-01 and several of its derivates have characteristics of being brain permeable, and easily passes through the blood-brain barrier. This suggests that they may serve as lead compounds in the development of clinical candidates which can be used for the treatment of stroke. Future studies will be focused on elucidating the exact mechanism of neuroprotection within this novel class of compounds, in particular NGP1-01.
References:


