The impact of ROS scavenging on NMDA and AMPA receptor whole cell currents in pyramidal neurons of the anoxia tolerant western painted turtle

by

David Dukoff

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Cell & Systems Biology
University of Toronto

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ABSTRACT

Extended periods of oxygen deprivation cause brain death in mammals but the western painted turtle overwinters in anoxic mud for months without damage. Neural protection is achieved through decreases in the whole cell currents of N-methyl-D-aspartate and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (NMDAR and AMPAR) that are dependent on a mild increase in intracellular calcium from the mitochondria. The goal of this research was to determine if natural anoxic decreases in reactive oxidative species (ROS) serve as the signal to bring about these changes. Reductions in cellular ROS levels were demonstrated to have no effect on AMPAR currents or intracellular calcium and produced massive increases in NMDAR currents, indicating that ROS depression does not directly mediate anoxic alterations. Interestingly, mammalian neural tissue also experiences a similar increase in NMDAR whole cell current in response to reducing agents suggesting a possible conserved mechanism for normoxic receptor control.
ACKNOWLEDGMENTS

“Complete and finished.” That is what I have been told that the writing and submission of a master’s thesis feels like. Within a hundred or so pages students summarize two years of laboratory work, complete the requirements of their degree and move to the next phase of their careers. However, as I approach the end of this project, this feeling of closure has yet to sink in. During my first year living at the University of Toronto’s Graduate Residence, I was told that the reason so much of the building seemed raw and unfinished was due to the architect’s belief that “a graduate student’s work is never done”. Although I came to believe that this explanation was an excuse for ill-placed support struts and walls so thin you could hear your roommates shift their blankets, the phrase stuck a chord. Science is a potent addiction and no matter where my life goes after my degree I don’t think I will ever stop thinking like a researcher or cease studying the world around me. What is more, the people that have helped and supported me during my time here are similarly addicting. This section is for all of them.

I would like to begin by thanking the professors and administrative staff whose efforts have made my project and degree possible. I would like to thank Dr. Les Buck for everything he has done to support me during my time in the lab and make research fun. Aside from being a constant presence to help me with nearly every aspect of my project, he has also been there to trade jokes and offer advice on all manner of non-science related issues. He has kept me on track but never been afraid to let me make my own mistakes (of which there have been many) so that I can learn how to recover and be a better scientist. I would also like to thank my committee members for getting me this far. The advice provided by Dr. Martin Wojtowicz and Dr. David Lovejoy has helped shape the directions and aims of my experiments. More importantly, their questions and comments have kept me versed in information outside the scope of my project and reminded me of the value of the big picture. Finally, I would thank the administrative staff who make this department work and who put up with the strange creatures that are graduate students. A special thanks to Ian Buglass, Tamar Mamourian, Peggy Salmon, James Dix, and Trung Luu.

I would next like to thank my fellow students in our incredible department for the help and support they have shown me over the last two years. I would like to first thank David Hogg who, through unstinting assistance, opinionated discussions (often involving words I can’t include in a thesis) and stories about his family, has kept me going and reminded me that there is
more to life than what is under the microscope. Next I would like to thank Peter Hawrysh for being a seemingly infinite resource of technical advice, scientific explanations, dirty stories, nerdy references, awesome video games, and cold beer (not usually during work hours). I would like to thank Aaron Chowdhury for his electronic expertise, great sense of humor, inquisitive questions, and devious pranks that have kept me sharp, motivated, and protective of my laptop. I would like to thank Hilary Bond for her stories and comments which have helped shape my decisions about who I want to be. I would like to thank Lydia Farnell for giving me someone to trade country stories and country music with. I would also like to thank all of my fellow colleagues in the department whose friendships have kept me sane. A special thanks to Jessica Pressey, Louise de Lannoy, Zoltan Torontali, Dave Wasserman, Sabina Romanescu, Felix Gunawan and all the members of CSBGU. I would not have made it without all of you.

I would like to thank my friends and family outside the lab for always being there. My parents have provided unconditional love and support for every choice I have made. Through visits to take me out to lunch and time spent editing my writing, they have helped me in any way possible. I hope I have made them proud. I would like to thank my little brother Andrew for sending me music on lonely nights at the lab. I would like to thank David Darling and my godchild Kaylie for Sudbury vacations that recharged my batteries. Finally, I would like to thank my Toronto/Kitchener friends for creating occasions to get out of the lab and into the sunlight. Special thanks to Brittany, Serge, Robert and Vanessa for their efforts in this regard.

The last acknowledgement I would like to make is to science and nature (not to be confused with the professional journals). I went into biology for the same reason I spent my childhood taking things apart in my Dad’s workshop: I wanted to understand how things work. There are no greater puzzles or more complex mechanisms than those found in the natural world and this may be why I find myself, like so many others, completely obsessed with studying it. The famous biologist Lewis Thomas once wrote about scientific research, “I don't know of any other human occupation, even including what I have seen of art, in which the people engaged in it are so caught up, so totally pre-occupied, so driven beyond their strength and resources… There is nothing to touch the spectacle.” Thank you science for being so interesting.
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>Ψm</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>[Ca2+]i</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>8-PT</td>
<td>8-pentyltheophylline</td>
</tr>
<tr>
<td>5-HD</td>
<td>5-hydroxydecanoic acid</td>
</tr>
<tr>
<td>12h:12h</td>
<td>twelve hours of light and twelve hours of dark light cycle</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AFU</td>
<td>arbitrary fluorescence unit</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>APf</td>
<td>action potential frequency</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>BDM</td>
<td>butanedione-monoxime</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CaCl_{2}</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>Cl^{-}</td>
<td>chloride ion</td>
</tr>
<tr>
<td>CO_{2}</td>
<td>carbon dioxide</td>
</tr>
</tbody>
</table>
CM-H$_2$DCFDA  5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester

$C.p.$  $Chrysemys$ $picta$

Cys  cysteine

DTNB  5,5'-dithiobis-(2-nitrobenzoic acid)

DTT  dithiothreitol

EPSP  excitatory postsynaptic potential

EEG  electroencephalogram

ER  endoplasmic reticulum

Fig  Figure

Fmax  maximal fluorescence signal

Fmin  background fluorescence

GΩ  gigaohm

GABA  gamma-Aminobutyric acid

GPx  glutathione peroxidase

GSH  glutathione

GSSG  glutathione disulfide

H$^+$  proton

H$_2$O$_2$  hydrogen peroxide

HCO$_3^-$  bicarbonate

IMM  inner mitochondrial membrane

I/V  current/voltage

IV  intravenous

K$^+$  potassium ion

K$_{ATP}$ channel  ATP-sensitive potassium channel
KCl  potassium chloride
Kd   dissociation constant
kDA  kilo dalton
kHz  kilohertz
L    litre
MΩ   megaohm
MCU  mitochondrial calcium uniporter
Mg²⁺  magnesium ion
MgCl₂  magnesium chloride
min  minutes
mKₐtp  channel mitochondrial ATP-sensitive potassium channel
mM  millimolar
mmHg  millimeters of mercury
Mn²⁺  manganese ion
MnSOD  manganese superoxide dismutase
mOsM  milliosmole
MPG  N-2-mercaptopropionylglycine
mPTP  mitochondrial permeability transition pore
ms  millisecond
mV  millivolt
n  sample size
N₂  nitrogen gas
Na⁺  sodium ion
NaCl  sodium chloride
NAC    n-acetylcysteine
NaGTP  sodium guanosine triphosphate
NaATP  sodium adenosine triphosphate
NaH₂PO₄ sodium dihydrogen phosphate
NaHCO₃ sodium bicarbonate
Na-HEPES sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
nm     nanometre
nM     nanomolar
NMDA   N-Methyl-D-Aspartate
NMDAR  N-Methyl-D-Aspartate receptor
nNOS   nitric oxide synthase
NO     nitric oxide
O₂     molecular oxygen
O₂⁻    superoxide anion
ONOO⁻  peroxynitrite
pK₅₀    plasmalemmal ATP-sensitive potassium channel
PP     protein phosphatase
PP2B   calcineurin
PSD95  postsynaptic density protein-95
PSC    postsynaptic current
Q₁₀    temperature coefficient
ROS    reactive oxygen species
[ROS]ᵢ intracellular ROS concentration
s      seconds
$S.$ \textit{Spermophilus}

SA \quad \text{spike arrest}

S.E.M. \quad \text{standard error of the mean}

sGC \quad \text{soluble guanylate cyclase}

SOD \quad \text{superoxide dismutase}

$S.p.$ \textit{Spermophilus parryii}

t \quad \text{time}

TTX \quad \text{tetrodotoxin}

μM \quad \text{micromolar}

WTR \quad \text{water transport route}

X/XO \quad \text{xanthine/xanthine oxidase}
CHAPTER 1: INTRODUCTION

1.1 The importance of oxygen and low oxygen tolerance

Humans and most other vertebrates require a constant supply of oxygen to survive. Brief hypoxic exposure causes hyperventilation and tachycardia that temporarily compensate for decreased systemic oxygen but sustained anoxia for more than a few minutes results in neurological damage and eventually death [1, 2]. Aerobic life primarily depends on oxidative phosphorylation for the production of the cellular energy substrate - adenosine triphosphate (ATP), and if oxygen transport to tissues becomes compromised energy demands cannot be met and cellular death occurs [3, 4]. Despite these limitations, some vertebrates have developed the ability to survive under hypoxic or anoxic conditions for extended periods of time. Understanding the mechanisms through which tolerance is accomplished has applications within fields as broad as stroke prevention to space travel [5, 6].

Research has been conducted for decades on hypoxia and anoxia tolerant species to try and better understand their physiological and molecular adaptations. Running in parallel with this work has been research to try and understand the conditions that produced these adaptations. Related species to those with anoxia/hypoxia tolerance rarely possess the same capabilities, indicating a historical pressure that selected for the trait and brought about a speciation event. Understanding why some species have adapted to survive long term anoxia may help us better understand how they do it.

Within the northern latitudes a number of hypoxia tolerant mammals and anoxia tolerant reptiles and fish are found in environments that demonstrate seasonal temperature decreases and the development of ice cover. In order to survive these seasonal changes species have adapted various hibernation and overwintering strategies that involve tolerating extended periods of anoxia/hypoxia. It has been proposed that these survival strategies were selected for during periods of globally decreased temperature and increased ice cover. The most recent such event was the last glacial period.
1.2 Ice ages, glacial periods and their contributions to modern day biodiversity

When extensive ice sheets are present in the northern and southern hemispheres the period is designated as an ice age. Our planet has passed through four major ice ages since its formation and is currently experiencing the fifth that began 2.6 million years ago marking the beginning of the Quaternary Period [7]. Environmental conditions during ice ages alternate over tens of thousands of years between two opposing conditions. “Interglacial” periods are characterized by warmer global temperatures and glacial recession. “Glacial” periods are marked by colder global temperatures and ice sheet advances. Movement between these two periods is brought on by large scale changes in the amount of thermal energy present in the Earth’s atmosphere and oceans caused by alterations in the energy generated by the sun, changes in the distance of the Earth’s orbit, and adjustments in atmospheric composition. We are currently experiencing an interglacial period that began 11,400 years ago [7, 8].

The last glacial period began 120,000 years ago and at that time the regional temperatures and climates were nearly identical to those seen today. Glaciers expanded across the globe reaching a maximum between 26,500 and 20,000 years ago when glaciers and permafrost covered more than a quarter of the Earth’s surface [7, 8]. Approximately 28,000 years ago the surface temperatures of the oceans began to warm leading to slow rises in ocean levels and by 12,000 years ago most regions returned to climates close to what they had been seen previously. This marked the transition from glacial to interglacial [7, 8].

Worldwide biodiversity was not significantly affected by the initial interglacial-glacial transition or the sustained 100,000 yearlong glacial period itself. As ice sheets expanded most species were able to migrate to areas not covered by ice, commonly referred to as refugia. These areas were found sparsely throughout glaciated regions but primarily existed closer to the equator [9]. Changing climate and the addition of new plant and animal life caused these regions to begin to mimic the environments that had previously been found further north and south and as a result, migrating animals did not face extensive pressures to adapt or change [7]. Most species were forced into a third of the territory they occupied pre-glaciation, but mild population decreases and the establishment of new territorial limits prevented vast losses of biodiversity [7]. Increased glaciation decreased sea levels by 350 feet generating land bridges like from Alaska to
Siberia that allowed for migrations between refugia, further preserving diversity [7, 10]. However as the glacial cycle came to an end, a major global extinction took place.

This extinction event took place between 16,000 and 10,000 years ago, and unlike previous major global extinction events took place only on land. Nearly all large terrestrial mammals over 44 kg were wiped out [7, 10]. Similar extinctions did not occur during exit from previous glacial periods and the cause remains contested. The most supported hypotheses involve rapid environmental changes that caused the loss of much of the world’s mega flora, overhunting by early humans as they migrated into new regions following ice sheets recession, or a combination of the two [7, 10]. Whatever the cause, all of the continents experienced losses in large terrestrial animal life: South America (83%), North America (72%), Australia (88%), Eurasia (35%), and Africa (21%) [10]. The mass extinction left a number of environmental niches empty and provided surviving species with an opportunity to adapt and expand.

1.3 Life after glaciation and adaptations to low oxygen

About 10,000 years ago plant and animal life began to expand outwards from refugia as the ice sheets receded [7, 9]. Some of these species/subspecies were new, produced from the isolation of members of the same species in different refugia throughout the glacial cycle where genetic drift and varying selective pressures caused speciation events [11, 12]. Expansion of species from refugia following the last glacial cycle altered the biota from what existed pre-glaciation to a greater degree than seen during exit from previous glacial cycles [13, 14]. The new territory opened up by the mass extinction event was quickly colonized by smaller faster breeding species leading to changes in plant and animal life. Adaptations were developed to survive under these novel conditions increasing differences between new species/subspecies and establishing new territorial ranges. These adaptations were generally minor and most species/subspecies continued to live in similar habitats, experiencing only small changes in physical features, ranges or behaviours [15, 16]. However, following glacial retreat some species in North America and Europe expanded into regions that still experienced seasonal ice cover, reduced temperatures and decreasing available food fostering the development of distinct adaptations. Species with flight capabilities survived by developing adaptations to seasonally migrate south. Other small and less mobile species adapted to outlast the winters. Three
examples of such species are *Chrysemys picta belli*, *Spermophilus parryii parryii*, and *Carassius carassius*.

1.3.1 *Chrysemys picta belli*

The North American turtle *Chrysemys picta* (*C.p.*) is the most widely distributed turtle species in North America with a range extending from the Maritime Provinces west to British Columbia and south to Oregon, Louisiana, and Georgia [17]. Fossils have been found for the species indicating that they have been widely distributed for at least two, and possibly more than five million years [17-19]. Previous to the last glacial period *C.p.* existed as two forms divided along the Atlantic coastal and west of the Appalachian Mountains [20]. The expansion of glacial ice and the loss of vegetation forced both forms southward and isolated the turtles into three distinct populations that became distinct species with *C.p. picta* in the southeast Atlantic coast, *C.p. dorsalis* in the lower Mississippi and *C.p. belli* in the southwest [21-23]. During glacial recession all three of the *C.p.* species migrated out of refugia north and through interbreeding between *C.p. belli* and *C.p. dorsalis* it is believed a hybridized fourth intermediated taxon *C.p. marginata* came about [20]. Species eventually settled into their modern day locales with *C.p. picta* in the Atlantic Coastal region, *C.p. belli* on the Great Plains, *C.p. marginata* around the Great Lakes, and *C. p. dorsalis* within the southeastern United States [Figure 1.1] [20]. Recent genetic investigation has proposed that interbreeding between species has begun to break down the classic differentiations between groups and they may be more accurately regarded as a northern subspecies (C. p. picta, C. p. belli, C.p. marginata) and a southern subspecies (C. p. dorsalis) [24]. However within the scientific literature the classic separations still remain.

During migrations out of the refugia the northern turtle subspecies slowly adapted to survive conditions of seasonal ice cover, decreased temperatures and reduced food availability allowing for the expansion of their ranges [20]. They developed physiological and cellular adaptations to survive the winter submerged in insulating mud at the bottoms of frozen fresh water bodies in an unresponsive metabolically reduced state [25, 26]. Although the primary function of these adaptations was probably freezing avoidance and energy conservation it also produced capabilities for extreme anoxia tolerance as the overwintering environment required turtles to survive for months without oxygen. Under laboratory conditions *C.p. belli, picta*, and
marginata at 3°C have all been demonstrated to survive over 120 days of anoxia, compared to the southern subspecies (C.p. dorsalis) that does not overwinter and perishes after only 50 days under identical experimental conditions [27-29]. Anoxia tolerance is greatest within members of the subspecies found further north and the Western painted turtle (C.p. belli) that inhabits the most northern ranges has been demonstrated to withstand the longest periods of anoxia [27, 30, 31].

The overwintering capabilities of C.p. belli are thought to have arisen from enhancements of pre-existing diving abilities common to most turtle species [32, 33]. In comparative analysis between C.p. dorsalis and C.p. belli it was concluded that increased tolerance is the result of better capabilities to lower metabolic rate and limit lactate accumulation [27]. During overwintering energy is conserved via decreases in brain activity, heart contraction, protein synthesis, urea production and the maintenance of a comatose like state [34-38]. This leads to a 70-80% reduction in ATP turnover [34]. The remaining energy demands are met via glycolytic fermentation supported by enhanced glycogen stores within the liver (15% of the organs mass) [39]. The two units of lactate and free hydrogen ions produced for every unit of glucose anaerobically consumed are buffered by a high plasma bicarbonate concentrations and ion exchange systems between the shell and bones (comprising 37.5% of their total mass) [40]. Induction of the overwintering state occurs in response to low oxygen and does not appear to be controlled seasonally like within mammal hibernators [41].
Figure 1.1: The ranges of the four currently recognized *Chrysemys picta* subspecies. Figure adapted from online source [42].

1.3.2 *Spermophilus parryii parryii*

The arctic ground squirrel (*Spermophilus parryii parryii*) is the largest of the American ground squirrel genus and the most northern dwelling of the squirrel family. They are found throughout the Canadian Arctic, subsist socially in large burrows, and during the coldest 7 to 8 months of the year they hibernate in their dens at temperatures sometimes below 0°C [43, 44].

*S. parryii* originally arose in Asia from a common ancestor to the central Siberian *S. undulatus* approximately 100 000 years [45, 46]. The speciation event took place during the last
glaciation within a large refugium consisting of portions of eastern Siberia, Alaska, and the western Yukon Territories connected by the Bering Land Bridge [8, 11, 47-51]. The newly speciated S. parryii migrated to North America and further subdivided into a large arctic (S. p. parryii) and subarctic subspecies (S. p. plesius). Following glacial recession the two subspecies dispersed north into the regions they currently occupy today with S.p. parryii found north of the Brooks Range in Alaska and arctic Canada and S.p. plesius found south in interior Alaska and subarctic Canada [45, 46].

Like the western painted turtle the arctic ground squirrel survives the winter through physiological and cellular changes that conserve energy and allow them to tolerate low temperatures. During hibernation resting body temperature is reduced to between 2-10 °C, heart rate decreases (25 fold), neurological protein synthesis is arrested (0.04% of active levels) and blood flow to various brain regions is reduced (80-90%). These changes cumulatively result in glucose utilization rates that are 1-2% of resting levels [3, 52-55]. The resting respiration rate is also decreased (25 fold) and O₂ consumption is reduced (90-96%) allowing the squirrels to survive prolonged durations in burrows that can become hypoxic during the winter season [3, 56]. Metabolic needs are met through the breakdown of fat and lean muscle reserves. Increased levels of steroids/androgens and lipases three weeks prior to hibernation allows the squirrels to increase their mass by 42% (87% fat and 3% protein) ensuring an adequate fuel supply [57, 58].

Also like the western painted turtle, the adaptations utilized by the arctic ground squirrel to survive the winter season are believed to have been produced through modifications of existing capabilities common to ground squirrels as a whole. Several other grounds squirrel species hibernate although for shorter periods at not as low temperatures [59]. Most ground squirrel species also experience oxygen fluctuations within their burrows (6-20%) at different points of the year [60-62].

Ground squirrels do not remain quiescent throughout the hibernation period and at regular periods briefly warm and reverse depressions in brain activity and heart rate without awakening. These arousal periods are part of the torpor arousal cycle and although energetically costly, are thought to be necessary for the maintenance of neural tissue and to undergo sleep cycles that cannot be accomplished during hibernation [63]. They also allow squirrels to filter
out and excrete metabolic waste products that accumulate during the inactive state. The torpor arousal cycle and entrance/exit from the hibernation state are based on a circannual cycle and persists under constant photoperiods with food provided ad libitum [43, 64-66].

1.3.3 *Carassius carassius*

The crucian carp (*Carassius carassius*) is widely distributed throughout northern Europe with a range spanning from England to Russia [67]. The species lives within lakes and ponds that for several months every winter become anoxic as the surface freezes over preventing aquatic photosynthesis and atmospheric oxygen diffusion [68]. During this period only the crucian carp survives, remaining active in water at 2°C, without food, for up to 5.5 months [69].

Previous to the last glacial period, the crucian carp was widely dispersed across Eurasia. During glaciation, the species survived within bodies of water in southern and eastern refugia and re-dispersed to central and north European waters following ice sheet recession [70]. Because the carp are aquatic organisms it is unclear if their current range or adaptations to seasonal ice cover are the result of the post glacial extinction event or are products of previous glacial periods.

The physiological adaptations that allow the carp to survive under the ice involve decreases in metabolic rates and body temperature, similar to those seen in the species discussed above. Body heat production is reduced by 37%, whole organism protein synthesis is down down-regulated 50-95%, and heart rates is lowered from 88 to 10 beats per minute [71-73]. Although crucian carp remain active throughout the anoxic bout, they do experience a major reduction in activity within optical centres of the brain (90% decrease) that brings about blindness without deficits to survival [73, 74]. The cumulative down regulation of these processes decrease metabolic energy requirements to levels that can be met through anaerobic glycolysis. To accomplish this, the crucian carp also possess a large glycogen reserve accounting for 30% of the liver’s wet mass and 4.5% of the fish’s weight that allows them to survive anaerobically and without feeding through the winter [75]. In order to deal with lactate accumulation, the carp convert lactate to ethanol and release it through their gills into the surrounding environment [72]. Similar to the western painted turtle the crucian carp brings about
cellular and physiological changes in response to decreases in available oxygen and not as a result of circannual rhythms [76].

Similar to the other species discussed above the crucian carp’s adaptations for winter survival are believed to be the result of adjustments to pre-existing capabilities. The goldfish (Carassius auratus) is a cousin of the crucian carp that is found farther south in regions throughout Eastern Asia and sheltered within the same refugia during the last glacial period [70, 77]. In order to tolerate decreases in oxygen brought on by brief ice cover or algae blooms goldfish have adapted to tolerate several weeks of hypoxia or hours of anoxia [78].

1.4 Requirements for anoxia/hypoxia tolerance

It has been stated that in order for prolonged anoxia/hypoxia tolerance to be accomplished adaptations must exist that allow organisms to conserve energetic substrates and prevent self-pollution by end-product accumulation [79]. All of the species discussed above possess strategies to accomplish these goals, many of which are common between species.

Reduction of metabolic activities is the primary method through which both of these goals are met, termed “metabolic arrest”. Down regulations of ATP consuming processes decrease rates of substrate utilization and by-product production. During overwintering all of the species discussed above experience depressions in heart rate/blood flow, protein synthesis and brain activity. Decreasing environmental temperatures also allow the species to reduce resting body temperature and heat production. The temperature effects aid in the reduction of metabolic activities but are not solely responsible. Most organisms exhibit 2-3 fold depressions per 10 °C reduction (Q_{10}= 2-3) but the western painted turtle (Q_{10}= 8.4 at 3-10 °C), the arctic ground squirrel (Q_{10}=14.1 at 4-20 °C), and the crucian carp (Q_{10}=5.1 at 10–15°C) experience greater decreases indicting other mechanisms at work [37, 80, 81].

Reduced metabolisms are supported for prolonged periods by increased internal fuel stores. Both the crucian carp and the western painted turtles possess large livers with enhanced glycogen concentrations in order to support extended anaerobic metabolism [39, 75]. Squirrels increase fat stores and lean muscle prior to hibernation and aerobically metabolize all of it during
the winter season [57]. Waste products are still produced, although slowly and in small quantities that can accumulate over the anoxic period. The strategies to deal with this accumulation are diverse and less universal between the species discussed involving increases in buffering capabilities, exchange processes between shell/bone, arousal periods to filter blood/excrete wastes and the conversion of lactate into ethanol for expulsion into the environment [40, 72, 81, 82].

1.5 The importance of neuronal down regulation

The mammalian brain accounts for around 2% of total body mass but is responsible for 20% of the total body oxygen consumption making it the most energy demanding tissue [83, 84]. It is similarly responsible for a lower but significant percentage of the aerobic capacity within reptiles and fish [3, 37, 85]. All of the species discussed above undergo decreases in neuronal function during their overwintering periods believed to be vital for energy conservation and anoxia/hypoxia tolerance. In the absence of these neuroprotective changes, oxygen deprivation within the brain triggers a lethal cascade.

Approximately 50-60% of total brain ATP is used by cellular Na+/K+ ATPase in order to maintain and re-establish cellular ion gradients [83, 84]. The gradients are perturbed through the activities of post-synaptic currents, action potentials and neurotransmitter uptake [86]. The high proportion of energy devoted to simply maintaining homeostasis makes neurons extremely vulnerable to excitability during times of ATP depletion like hypoxia/anoxia. The loss of the membrane ion gradients results in a net outward movement of K+ ions preventing cells from repolarizing after firing. Na+ and Ca2+ channels become over activated and the whole cell is depolarized. The resulting Ca2+ influx into the cell triggers vesicular release of excitatory neurotransmitters such as glutamate into the synaptic cleft activating glutamatergic receptors on associated cells [87, 88]. Neurotransmitter excision is compromised by ATP depletion and glutamate lingers in the synapse causing receptor over activation and excessive Ca2+ influx.

Ca2+ plays important roles in second messenger pathways and metabolic regulation but in high quantities is toxic to the cell. Under normoxic conditions extracellular Ca2+ levels are maintained 10,000 times greater than intracellular levels through the activities of plasma
membrane transporters. Inside the cell calcium is stored in the mitochondria and endoplasmic reticulum or sequestered by calcium binding proteins [92-94]. These processes are ATP dependent and compromised by anoxia/hypoxia [95]. Excessive glutamatergic activation and compromised Ca\(^{2+}\) management capabilities result in excessive intracellular concentrations of free Ca\(^{2+}\) that cause cell swelling and trigger enzymatic cascades that eventually bring about cell death [85][96]. This is termed excitotoxic cell death (ECD).

1.6 Models for potential human application

Stroke represents the third leading cause of death in North America killing 14,000 Canadians each year and hospitalizing a further 46,000 that may suffer neurological impairments for the rest of their lives [89-91]. This represents a cost of $3.6 billion a year in physician services, hospital fees, lost wages, and decreased productivity [92]. About 80% of all strokes are caused by the interruption of blood flow to the brain due to a blood clot. The affected brain regions are deprived of glucose and oxygen (ischemia), triggering the neurotoxic set of events discussed above.

Tissue death caused by an obstruction of blood supply is termed infarction and cell death in the tissues localized to the blockage is an infarct. Until blood flow is returned the infarct grows in size affecting a greater proportion of the surrounding tissue. Within mammals, the size of this infarct can be reduced and cell death prevented through “ischemic preconditioning” that involves subjecting whole organisms to brief periods of ischemia prior to a prolonged lethal ischemic insults in order to reduce infarct sizes. Ischemic preconditioning was first demonstrated in canine myocardial tissue and has since been identified to offer protective roles in a multitude of mammalian models within organ systems including brain, heart, liver, small intestine, skeletal muscle, kidney and lung [93, 94]. In neurological evaluations in rats and gerbils the development of global ischemia through temporary surgical blockage of the carotid artery for 2-3 minutes reduced infarct sizes in response to lethal ischemia (5-10 minutes) by 53-98%. Neuroprotection from lethal ischemia required 24-72 hours to develop after the initial ischemic exposure and was maintained for 1-7 days [95-98].
The time span required for ischemic preconditioned neuroprotection to be achieved and the short amount of time it is maintained for limit practical applications within the field of medicine. As well, repeatedly exposing patients to brief ischemic periods is dangerous and impractical. Attempts have been made to pharmacologically mimic the protective response but have met with limited success in human application [94, 99]. Finally, ischemic preconditioning has been demonstrated to reduce the size of infarcts but not prevent them all together resulting in minor to moderate brain damage.

The hypoxia/anoxia tolerant organisms discussed above do not suffer neurological impairments during their hibernation periods. Studying the mechanisms through which their tolerances are achieved may offer an alternative route to dealing with conditions brought on by blood flow reduction or may aide in our understanding and successful application of the mechanisms controlling ischemic preconditioning. Preconditioning reveals that even within mammals there is capacity to increase the hypoxia/anoxia tolerance of a tissue and the underlying mechanisms may be similar to those used by anoxia-tolerant species. Of the three species discussed above, the western painted turtle is the ideal model for study. As a terrestrial vertebrate they are a better comparative model for potential human application than the crucian carp. The neurological mechanisms permitting anoxia tolerance are triggered rapidly in response to oxygen deprivation and not controlled circanually like the arctic ground squirrel. Lastly the western painted turtle brain is tolerant to ischemia as well as anoxia [100].

1.7 Turtle synaptic organization

The anatomy and synaptic organization of the turtle brain has been investigated within various turtle species and some of the structures have been compared against those found within other organisms [101-104]. The turtle dorsal cortex shares several important anatomical similarities with the mammalian neocortex including afferent and efferent connections to various regions of the brain and electrically innervated organs [104-106]. However, although the extrinsic connectivity of the turtle dorsal cortex resembles neocortex, the intracortical structure of the turtle is greatly simplified compared to that of the mammal. Instead of a six-layered isocortex, the turtle has only three cortical layers (molecular, cell and subcellular) that are composed of two general classes of neurons: pyramidal cells that form the main output of the
cortex, and stellate interneurons [104, 107, 108]. The turtle brain has similarities to the mammalian brain allowing for a degree of comparability in the context of anoxic/ischemic investigation. However, it should be stressed that the resemblance between organs is limited. Electrophysiological analyses have been performed within the dorsal cortex of the turtle *Pseudemys scripta elegans* in order to better characterize the pyramidal and stellate cell types. The pyramidal cells have their somata in the cell layer, extend several densely spined apical dendrites through the molecular layer to the pia, and have spiny basilar dendrites directed through the subcellular layer toward the ependymal border. Physiologically, pyramidal cells have relatively prolonged action potentials that show marked frequency adaptation during a sustained suprathreshold current pulse. In contrast, stellate cells have their somata in the molecular and subcellular zones, and their dendrites are horizontally oriented and spine-free. Stellate cells have relatively brief action potentials and show little or no spike frequency adaptation [101].

1.8 Anoxia mediated neuroprotective mechanisms

As discussed above, entrance into the overwintering state by the western painted turtle is associated with major decreases in metabolic processes. Although the effects of decreasing temperature aid in reducing metabolism (*Q*₁₀ effects) oxygen deprivation is the primary trigger for metabolic down regulation. Turtles submerged in tanks at 20 °C experience a four-fold depression in the frequency of heart contractions, and after 8 hours exhibit an 80% reduction in metabolic rate [35]. Similarly, submergence at 25 °C decreases protein synthesis and urea production by 92% and 72% respectively after 12 hours [36]. Anoxic down regulation of metabolic activity leads to reductions in neuronal activity and the induction of a comatose-like state as a result of spike and channel arrest.

1.8.1 Spike arrest

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter within the mature central nervous system of vertebrates and mediates wide spread electrical depression in most neurons [109-111]. Activation of postsynaptic GABAₐ receptors increases chloride (Cl⁻) conductance through the channel’s pore region. Activation of postsynaptic GABAₐ receptors increases K⁺ conductance via G-protein coupling to K⁺ channels. The reversal potentials for Cl⁻
and K+ are near neuronal resting potential and opening of GABA receptors generates an inhibitory shunting current near the GABA reversal potential (E_{GABA}) that impedes action potential (AP) generation [112]. Within the brain of the western painted turtle, extracellular GABA concentrations are elevated to 90 times normoxic levels after 4 hours of anoxia. As well GABA_A-receptor density increases by 22% and 29% after 12 and 24 hours of anoxia, respectively [113, 114]. This produces decrease in whole brain activity demonstrated by reductions in action potential frequency (AP_f), field potentials and electroencephalogram (EEG) activity in the anoxic turtle [115-117]. Depressed electrical activity is termed “spike arrest” and serves to conserve ATP throughout the anoxic period [118].

1.8.2 Channel arrest

Activation of post-synaptic N-methyl-D-aspartate receptors (NMDAR) is the primary route for extracellular Ca^{2+} entry. NMDARs are hetero-tetramers formed by two GluN1 and two GluN2 subunits (previously denoted as NR1 and NR2) of which there are four subtypes in vertebrates (GluN2A-D) [Figure 1.2]. In mammals GluN2A and GluN2B are expressed throughout the forebrain including the hippocampus, GluN2C is restricted to the cerebellum and GluN2D is found predominantly in the midbrain [119]. Binding of glutamate to the GluN1 and the co-agonist glycine to the GluN2 is necessary to trigger Na^+/Ca^{2+} influx and K+ outflow [120]. NMDAR activation is inhibited at resting membrane potentials by a magnesium ion (Mg^{2+}) block within the pore region. The blockade is maintained in a voltage-dependent manner and removed by cellular depolarization following postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) activation. AMPARs are hetero-tetramers composed of different combinations of the four GluA (previously denoted as GluR) subtypes (GlurA1-4) each possessing a binding site for glutamate [Figure 1.2]. AMPARs are permeable to Na^+, K^+ and also Ca^{2+} in configurations without GluR2. AMPARs are opened when two of the glutamate binding sites become occupied producing a depolarizing current, the removal of the Mg^{2+} block from NMDARs, and Ca^{2+} influx [121]. Activation of these receptors and the resulting Ca^{2+} influx plays important roles in learning, memory, synaptic plasticity and cognitive function [122].
As previously discussed, glutamate concentrations are rapidly elevated following oxygen deprivation within anoxia intolerant nervous systems. The resulting excessive Ca\(^{2+}\) influx is produced primarily by NMDAR and AMPAR over activation. In the anoxic brain of the western painted turtle, excessive glutamate release is partially prevented through GABA mediated decreases in cell firing that keep glutamate levels relatively constant throughout (∼1 μM) [113, 123]. Adaptations are also present that decrease the degree to which NMDAR and AMPAR are activated by glutamate during anoxia. Following 40 minutes of anoxia NMDAR and AMPAR currents are decreased by 45-65% and 50-60%, respectively [124-128]. Rapid changes are believed to be the result of reversible modifications made to the receptor subunits that affect open time [129]. Over long term anoxia (3-21 days) NMDARs are removed from the cellular membrane demonstrated through a 40% reduction in GluN1 subunit abundance and a depression of Ca\(^{2+}\) permeability after 6 weeks [5]. It is unclear if a similar mechanism exists for AMPARs. Rapid and long term changes in NMDARs and AMPARs are collectively termed “channel arrest” and are utilized to conserve energy and prevent ECD [79].
Figure 1.2: *NMDAR and AMPAR structures.*

A, schematic diagram of the NMDA and AMPAR receptor complexes. B, schematic diagram of the NMDAR and AMPAR subunit arrangements. Figures adapted from online sources [130, 131]

1.9 Mechanisms controlling “channel arrest”

Initial decreases in NMDAR and AMPAR whole cell currents are triggered rapidly in response to oxygen deprivation. The mechanisms controlling these changes are believed to be signalled by changes associated with anoxia. The effects of anoxia mediated changes in adenosine, ATP-sensitive K⁺ (K\textsubscript{ATP}) channels and intracellular calcium have all been investigated.

1.9.1 Increases in adenosine

A temporary but significant decrease (20%) in ATP concentrations occurs in the brain of the western painted during transition into anoxia. This decrease lasts for approximately an hour before metabolic processes are reduced sufficiently for energetic costs to be met by anaerobic
glycolysis [34]. When ATP breakdown exceeds ATP production, AMP begins to accumulate in the cell and 5’-nucleotidase activity increases. 5’-nucleotidases are a family of enzymes found in the vertebrate CNS and inhibited by ATP. When activated they break down AMP into adenosine [3, 132].

Adenosine is a “retaliatory metabolite” produced in response to oxidative stress in a number of vertebrates [133]. Extracellular adenosine increases trigger protective changes through interactions with adenosine receptors in different tissues (A1, A2A, A2B, and A3) [134]. Changes involved in neuroprotection include: suppression of pre-synaptic excitatory neurotransmitter release (ex: glutamate), stimulation of anaerobic glycolysis, increased cerebral blood flow and reduction of neuronal excitability via inhibition of Ca2+ channels/activation of G protein-coupled inwardly-rectifying K+ channels [3, 134-138]. Neuronal extracellular adenosine levels after 100 minutes of anoxia are 12 fold of normoxic concentrations [139]. This increase is temporary and as metabolic processes are depressed in the turtle as discussed above, energy needs are met by anaerobic glycolysis and normal ATP concentrations are re-established. After 300 minutes adenosine concentrations return to baseline levels [139].

Increased extracellular adenosine levels in the western painted turtle brain increase resistance to K+ efflux in order to preserve cellular ion gradients. This is mediated by binding of the A1 receptor [140]. Adenosine and A1 receptor activation has also has also been implicated as a mediator of NMDAR channel arrest. Within neural tissue from the western painted turtle normoxic adenosine addition reduces NMDA-mediated Ca2+ entry by 62 %. This decrease is abolished by perfusion of the A1 antagonist 8-pentyltheophylline (8-PT) [141]. However during anoxia, A1 receptor antagonism does not block decreases in NMDAR open time or NMDA-mediated Ca2+ entry [142]. This suggests that adenosine is capable of reducing NMDAR activity but is not a necessary component of anoxia-mediated NMDAR channel arrest.

1.9.2 Activation of ATP-sensitive K+ (K_{ATP}) channels

Like adenosine production, K_{ATP} channel activity is controlled by ATP concentrations. These channels, found in the plasma membrane and internal organelles like the mitochondria, are gated by high concentrations of ATP and activated when levels are low like during oxidative
stress. These channels are found within the brain of vertebrates, particularly in the hippocampus [97]. \( K_{\text{ATP}} \) channels are comprised of pore-forming Kir subunits and ATP-binding sulfonyl urea subunits (SUR) [143]. The configuration and combination of the subunits varies based on the type of membrane they are found within. The plasmalemmal \( K_{\text{ATP}} \) (p\( K_{\text{ATP}} \)) channel is composed of two Kir6.1 and Kir6.2 bound to two SUR1 and SUR2 [144]. The mitochondrial \( K_{\text{ATP}} \) (m\( K_{\text{ATP}} \)) is also thought to be a heteromultimer consisting of mitoKir and mitoSUR and containing neither Kir6.1 or Kir6.2 [143, 145].

The freshwater turtle (\textit{Trachemys scripta}) is another anoxia tolerant turtle species able to survive at least 48 h of anoxia at 25°C [146]. Within this species increased p\( K_{\text{ATP}} \) channel activity is necessary during anoxia for the maintenance of cellular ion gradients. \( K^+ \) efflux from neurons is reduced by 50% within one hour of anoxia, but abolished with administration of the \( K_{\text{ATP}} \) channel blocker glibenclamide [147]. Increases in m\( K_{\text{ATP}} \) channel activity work in concert with adenosine to block glutamate release within the first 3 hours of anoxia. Addition of the \( K_{\text{ATP}} \) channel blocker BDM or the adenosine receptor antagonist theophylline produced increase in glutamate release of approximately 70% above anoxic levels. At 5 hours, adenosine inhibition produces an approximate 100% increase in glutamate release but \( K_{\text{ATP}} \) channel blocking has no effect [136].

In the western painted turtle, mitochondrial specific \( K_{\text{ATP}} \) (m\( K_{\text{ATP}} \)) channels are involved in anoxia-mediated down regulation of NMDAR and AMPAR currents. Addition of the m\( K_{\text{ATP}} \) channel activator diazoxide under normoxic conditions reduces both whole-cell AMPAR and NMDAR currents to levels comparable to anoxia. Inversely m\( K_{\text{ATP}} \) blockade with 5-hydroxydecanoate (5-HD) abolishes anoxia-mediated reduction of NMDAR and AMPAR currents [125, 126]. Activation of m\( K_{\text{ATP}} \) channels is also associated with an uncoupling of turtle mitochondria by 10–20% and reduced \( Ca^{2+} \) uptake into the mitochondria [126] [148]. The resulting rise in intracellular \( Ca^{2+} \) levels can bring about downstream effects in NMDAR and AMPAR activity.
1.9.3 Increases in intracellular calcium

Following the onset of anoxia in turtle neural tissue, intracellular calcium ([Ca$^{2+}$]$_i$) concentrations increase [126, 128, 149]. The [Ca$^{2+}$]$_i$ increase is necessary for the development of anoxia mediated AMPAR/NMDAR channel arrest. The inclusion of the Ca$^{2+}$ chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) in the pipette solutions of whole-cell patch clamp recording electrodes abolishes anoxia-mediated reductions in AMPAR/NMDAR currents [125, 129]. Increases in [Ca$^{2+}$]$_i$ occur in the absence of extracellular calcium, indicating that Ca$^{2+}$ is released from an intracellular source [126, 128, 149]. Within neurons Ca$^{2+}$ is stored in the mitochondria and the endoplasmic reticulum (ER). Inhibition of ER specific calcium release via ryanodine does not abolish anoxia mediated NMDAR current reductions, indicating mitochondrial specific Ca$^{2+}$ release is responsible for anoxia mediated changes [126]. As discussed above activation of mK$_{ATP}$ channels is associated with mitochondrial uncoupling and reduced Ca$^{2+}$ uptake. Activation of mK$_{ATP}$ channels with diazoxide or levcromakalim produces increases in [Ca$^{2+}$]$_i$ and decreases in NMDAR/AMPAR currents of similar magnitude to those seen during anoxia. The addition of the mK$_{ATP}$ antagonists 5-HD or glibenclamide prevented both of these changes [125, 126]. Increases in [Ca$^{2+}$]$_i$ trigger the activation of a variety of intracellular regulating proteins that can serve as the intermediate step in signalling NMDAR/AMPAR current down regulation in response to mitochondrial Ca$^{2+}$ release.

1.9.4 Enhancements of calmodulin and phosphatase activity

Calmodulin is a calcium-binding messenger protein expressed in all eukaryotic cells. Calmodulin can modulate NMDAR directly by binding to the COOH terminus of the NMDAR GluN1 subunit and inactivating the channel in a Ca$^{2+}$-dependent manner [150]. Within the western painted turtle inhibition of calmodulin with calmidazolium attenuates anoxia mediated decreases of NMDAR currents within neural tissue [129]. Inhibition has yet to be evaluated on anoxia mediated AMPAR current reductions.

NMDAR activity can also be reversibly modulated by intracellular kinases and protein phosphatases (PPs), some of which are controlled by intracellular calcium concentrations. Dephosphorylation via PP1, PP2A and the Ca$^{2+}$/calmodulin-dependent phosphatase PP2B


(calcineurin) all attenuate NMDAR activity [151-154]. Within the anoxic turtle brain, addition of the PP1 inhibitor okadaic acid or the PP1/PP2A inhibitor calyculin A prevent anoxia-mediated attenuation of NMDAR currents [129]. The inhibition of calcineurin via cypermethrin similarly attenuates NMDAR currents over the short term (20 minutes) but not the long term suggesting that calcineurin might only play a role in early attenuation [129]. The effect of phosphatases on anoxia mediated AMPAR current reductions have yet to be tested.

![Figure 1.3: Schematic illustration of anoxia-mediated changes contributing to channel arrest.](image)

1.10 Reactive oxidative species (ROS)

Other cellular changes aside from decreases in intracellular ATP concentrations occur during anoxia that may contribute to “channel arrest” and/or “spike arrest”. One such change is reductions in reactive oxidative species (ROS). All reactive molecules and free radicals produced as unwanted by-products from the incomplete reduction of diatomic oxygen (O$_2$) during oxidative metabolism are termed as ROS [155]. All aerobic organisms produce ROS and have developed cellular anti-oxidant defence systems that prevent concentrations from reaching harmful levels. Under normoxic conditions, intracellular ROS levels are maintained at relatively
low concentrations but never completely removed. During oxygen deprivation in anoxia tolerant organisms, oxidative phosphorylation halts and ROS levels decline to immeasurable levels [155]. Within the brain of the western painted turtle CM-H$_2$DCFDA fluorescence evaluations have demonstrated that ROS concentrations decrease by 25% within 10 minutes of anoxia [156].

ROS are primarily produced within the mitochondria between complexes and I and III where 2-3% of all O$_2$ consumed is left partially reduced as the highly reactive superoxide anion (O$_2$$^-$) [Figure 1.3] [157, 158]. This molecule is short lived and in most cases binds with water either spontaneously or through induction by mitochondrial superoxide dismutase (SOD) to produce hydrogen peroxide (H$_2$O$_2$) [159-161]. H$_2$O$_2$ is one of the longest lived cellular ROS but is eventually converted to water and O$_2$ by catalase or glutathione peroxidase (GPx) [93, 162]. Levels of H$_2$O$_2$ are directly linked to metabolic activities and increases or decreases can serve to signal cellular functions such as apoptosis, gene expression, and the activation of cell signaling cascades via reversible oxidation of cysteine (Cys) thiol residues on signalling proteins and membrane ion channels [163-167]. H$_2$O$_2$ is cell permeable and capable of moving through the lipid bilayer by free diffusion or through aquaporins (AQP)/water channels and oxidizing extracellular as well as intracellular Cys residues. In this manner, H$_2$O$_2$ acts as an inter and intra cellular messenger [168-171]. The oxidized Cys residues can be reduced by elements of the antioxidant defence system or can react with other sulfide compounds to produce disulfide bridges [Figure 1.3] [165, 168-171].

Although there remains controversy over whether CNS neurons contain aquaporins neurons do exhibit low water permeability under resting conditions and are capable of moving water through alternative means such as membrane cotransporters (ex: KCC1) [172]. H$_2$O$_2$ is very water soluble and should be capable of following the movement of water through available channels similar to movement through APQs [168, 173, 174]. For the purposes of this thesis, the routes for facilitated water transport (aquaporins, cotransporters, or other) will be grouped and designated as water transport routes (WTRs) for the sake of simplicity.

Another source of cellular ROS is nitric oxide (NO) produced by neuronal NO synthase (nNOS). nNOS is tethered to the NMDAR by the postsynaptic density protein-95 (PSD95) [175, 176]. nNOS produces NO from arginine and O$_2$ and is triggered by the activated Ca$^{2+}$-binding
protein calmodulin (CaM) [Figure 1.3] [177-179]. Tethering of nNOS to PSD-95 and Ca\(^{2+}\) dependent activation couple the production of NO to NMDAR activation. Like \(\text{H}_2\text{O}_2\) NO is short lived, capable of moving through the lipid bilayer by free diffusion or through WTRs, and can reversibly oxidize cysteine residues through a processes termed S-nitrosylation. NO can also react with \(\text{O}_2^-\) and form peroxynitrite (ONOO\(^-\)) that is another effective oxidizing agent. It can also activate soluble guanylate cyclase (sGC) and produce cyclic guanosine monophosphate (cGMP) capable of triggering downstream changes that include further ROS generation [168, 179-182]. [Figure 1.3].

1.11 The role of ROS in anoxia-intolerant vertebrates

Under normoxic conditions, the role of ROS in cellular signalling has been well documented in a number of anoxia-intolerant organisms. Changes in ROS increase and decrease the activities of a number of ion channels and intracellular proteins [183, 184]. Changes in the NMDAR redox state and intracellular ROS levels affect NMDAR activity. Like anoxia-mediated regulation, these changes appear to be affected by the phosphorylation state of the NMDAR.

1.11.1 Redox changes affect NMDAR but not AMPAR activity

The structure and function of several membrane bound receptors containing Cys residues are sensitive to redox conditions [185]. Specific Cys residues in the NMDAR form redox modulatory sites whose oxidation/reduction state affects the activity of the receptor. Redox modulatory sites have been demonstrated to exist within GluN1, GluN2A and GluN2B subunits [186-188]. Experiments using site-directed mutagenesis in rat and frog oocytes have identified residues within the GluN1 (Cys744, Cys798, Cys 79 and Cys 308) and GluN2A (Cys 87, Cys 320 and Cys 399) subunits whose alteration affects redox modulation but the site in the GluN2B has yet to be localized. All of the sites identified have been localized to extracellular regions of the receptor subunits within their respective species. However, there is some controversy as to whether intracellular or transmembrane redox sites also exist but have yet to be identified [185, 187-189].
The effect of redox modulation on NMDA receptors has been observed in a wide variety of tissue preparations including intact chick retina, frog oocytes, rat retinal ganglion, hippocampal, cortical and striatal slices and post-mortem human cortex [188, 190-198]. Evaluations of NMDAR activity within these preparations using methods including intracellular, extracellular, and patch-clamp recording have demonstrated that addition of oxidizing agents (DTNB and GSSG) decrease receptor activity while reducing agents (DTT) increase receptor activity. The increases produced through reduction are greater than decreases through oxidation (6-11 times), suggesting that redox sites are maintained in a more oxidized state [190, 196, 199].

Unlike the NMDAR, no modulatory redox sites have been found in the structures of the AMPAR GluR subunits. Within many of the same tissue preparations used to demonstrate the effects of NMDAR redox modulation, AMPA addition has been shown to be unaffected by oxidizing or reducing agents [190, 196-198].

1.11.2 Alterations in ROS affect NMDAR activity

Like reducing/oxidizing agents, changes in ROS have been shown to alter NMDAR activity. Within rat cortical neurons oxygen free radicals generated by xanthine and xanthine oxidase (X/XO) are capable of reversing increases in NMDAR activity brought on by reducing agents and decreasing activity similar to oxidizing agents [191, 196]. The inhibition of mitochondrial ROS production has also been demonstrated to effect NMDAR activity. Rotenone is a complex I inhibitor and decreases mitochondrial ROS production in mitochondria from rat brain, heart, liver, dog brain and human cell cultures [157, 200-202]. This is accomplished through inhibition of electron transfer from iron-sulfur centers in complex I to ubiquinone [Figure 1.3]. In whole cell patches of dopamine neurons from slices of rat midbrain the addition of rotenone increases the average amplitude of evoked NMDAR currents by 162% without affecting AMPAR activity [203].

The effect of NO manipulation has also been evaluated in cultured rat cortical neurons and frog oocytes through Ca^{2+} imaging and whole cell patch clamp recordings. Like oxidizing
agents and ROS generators, the application of NO generators produced marked decreases in NMDAR activity [189, 204, 205].

1.11.3 NMDAR redox regulation is blocked by kinase inhibition

As discussed above, increased phosphatase activity is associated with decreases in NMDAR activity during anoxia in the turtle brain [151-154]. There is some evidence to suggest that phosphorylation of the NMDAR is necessary for oxidation/reduction of the redox modulatory site. Electrophysiological recordings in hippocampal rat slices demonstrated increases in NMDAR-mediated synaptic responses with DTT application. Increases were blocked by inhibition of Ca²⁺/calmodulin-dependent protein kinase II but not by inhibition of the protein phosphatases PP1 or calcineurin [196]. Similarly in horizontal slices from rat ventral midbrain rotenone augmentation of N-methyl-D-aspartate (NMDA)-evoked currents were inhibited by the inclusion of a tyrosine-kinase in intracellular pipettes [206]. These findings may indicate that the NMDAR redox site is only accessible when the NMDAR is phosphorylated.
**Figure 1.4:** The mechanisms of ROS production and cysteine oxidation.

A, depiction of the mitochondrial electron transport chain showing the sites of reactive oxygen species production and rotenone inhibition. B, depiction of NMDAR coupled nNOS activation and nitric oxide synthesis. C, depiction of the effect of H$_2$O$_2$ and NO oxidation on cysteine residues. Figures adapted from (A) Pamenter 2007 and (B) Nakamura 2007 [156, 179].
1.11.4 mK\textsubscript{ATP} mediated ROS increases affect ischemic preconditioning

Changes in ROS have recently been linked to the development of protection produced through ischemic preconditioning. Like anoxia mediated NMDAR/AMPAR downregulation in the brain of the western painted turtle, activation of the mK\textsubscript{ATP} channel has been identified to be involved in ischemic preconditioning. Within cardiac tissue from rats and rabbits the addition of the selective mK\textsubscript{ATP} antagonist 5-HD prevents the development of protective effects from ischemic preconditioning [207-209]. In neural tissue from rats and pigs the addition of the mK\textsubscript{ATP} agonist diazoxide protects the brain against ischemic and anoxic stresses similar to ischemic preconditioning [210-213].

Activation of mK\textsubscript{ATP} channels in the western painted turtle is believed to trigger a mild increase in intracellular calcium necessary for the development of downstream changes. In ischemic preconditioning however the activation of mK\textsubscript{ATP} channels and downstream changes are associated with increased production of mitochondrial ROS. In rat hippocampal slices, isolated rabbit hearts, and human atrial-derived cell lines the addition of the ROS scavenger N-2-mercaptopropionylglycine (MPG) reversed diazoxide mediated protection similar to 5-HD addition [214-216]. Fluorescent investigation in human atrial derived cell line, rabbit myocardial tissue, and rat retinal tissue has demonstrated that mK\textsubscript{ATP} channel activation increases oxidation of the detective probes indicative of increases in cellular ROS levels [216-218]. This seems to indicate that mK\textsubscript{ATP} channel opening increases ROS generation that then triggers the development of the preconditioned state.

There is also some evidence to suggest that mK\textsubscript{ATP} channels are more favourably activated in the presence of mitochondrial ROS or NO. Within rat myocytes, reconstituted bovine ventricular myocardium mitochondria, embryonic chick ventricular myocytes, rabbit ventricular myocytes, rat dorsal root ganglion neurons, and rat cardiac submitochondrial particles the addition of NO donators, O\textsubscript{2}\textsuperscript{-} generators, ROS scavengers and exogenous H\textsubscript{2}O\textsubscript{2} have all been demonstrated to affect mK\textsubscript{ATP} activity [219-224]. Increased ROS caused mK\textsubscript{ATP} channels to become less inhibited by ATP and more readily activated. This increase has been attributed to
increased oxidation of cysteine residues in the SUR subunit, similar to what occurs in the NMDAR redox site [223]. In this way, mK_{ATP} activation during ischemic precondition may cause channel oxidation and promote further activity in a positive feedback loop [221].

1.12 The antioxidant defence system

Antioxidants are substances that when present at low concentrations, compared to that of an oxidizable substrate, significantly delays or inhibits oxidation [225]. The antioxidant defence system protects the cell from unwanted reactions by maintaining levels of ROS generated through processes like nNOS activation and mitochondrial oxidative phosphorylation at low concentrations [226].

The three intracellular antioxidants most relevant to nNOS and mitochondrial ROS generation are superoxide dismutase (SOD), catalase and glutathione (GSH)/glutathione peroxidase (GPx). SOD is found within the mitochondria of all eukaryotes and differentiated from SOD found in chloroplasts and prokaryotic organisms by the type of metal cofactor (MnSOD) [227]. SOD catalyses the dismutation of O_2^•−, produced from oxidative phosphorylation, into H_2O_2 at a reaction rate that is 10,000-fold faster rate than spontaneous dismutation (O_2^•− + O_2^•− + 2H^+ => 2H_2O_2 + O_2) [228]. SODs are nuclear encoded and there is some evidence to suggest that synthesis is sensitive to environmental stressors and can be altered in response to changes in ROS production [227]. Intracellular concentrations of H_2O_2 are managed through a wide range of enzymes, including catalase and GPx. Catalase forms an intermediate complex with H_2O_2 and generates water and O_2 (2H_2O_2 => O_2 + 2H_2O). GPx catalyzes the reaction of GSH with H_2O_2 to produce oxidized glutathione (GSSG) the helps to maintain a redox balance in the cellular compartments (2GSH + H_2O_2 => GSSG + 2H_2O). A central nucleophilic cysteine residue is responsible for the high reductive potential of GSH, allowing it to scavenge cytotoxic H_2O_2 and reacts non-enzymatically with other ROS like singlet oxygen, superoxide radicals, hydroxyl radicals, and NO [226, 227]. The cumulative actions of these and other antioxidants maintain the intracellular environment within a reduced state [168].
As discussed above both H$_2$O$_2$ and NO are capable of leaving the cell through free diffusion or WTRs and interacting in the extracellular environment. The resulting extracellular ROS concentrations are managed by antioxidants produced within the cell and transported to the extracellular environment. GSH, catalase and GPx act in the extracellular environment but generally exist at lower concentrations than found within the cell [229]. The major extracellular thiol is free L-Cys that exists in both a reduced (Cys) and oxidized (Cyss) form, although in the plasma the oxidized form is predominant. Influx and efflux of reduced Cys between the intracellular environment may contribute to extracellular redox status and help to maintain a Cys/Cyss balance [168]. Due to lower antioxidant concentrations and the movement of ROS out of the cell, the extracellular environment exists within a predominantly oxidized state [168].

Antioxidants in both the extracellular and intracellular environments can act to reverse ROS reactions, such as by reducing thiol residues in proteins oxidized through ROS interactions. Aside from scavenging roles, GSH and free L-Cys have been implicated to act as the primary intracellular and extracellular protein reductants, respectively [230]. Intracellular and extracellular levels are maintained relatively constant in most cells and act to establish a baseline balance with ROS products between oxidation and reduction states of cellular proteins [168, 230]. During anoxia in the western painted turtle when ROS concentrations fall the balance can be disturbed leading to a shift towards protein reduction in both the intracellular and extracellular environment.

1.13 ROS regulation of GABA in anoxic turtle cortex

As previously discussed one of the mechanisms through which turtles reduce ATP consumption within the brain during the over wintering period is through increases in GABA receptor postsynaptic currents (PSCs) that serve to decrease whole brain activity via spike arrest [113, 114]. Recent investigation within our lab has demonstrated that in cortical slices from the western painted turtle under normoxic conditions, ROS scavenging increased GABAergic PSC amplitudes similar to anoxic exposure. Application of the mitochondrial complex IV inhibitor cyanide decreased intracellular ROS to the same extent as anoxia and also resulted in a similar increase in GABAergic PSCs. Finally GABAergic PSC increases from ROS scavenging and
anoxia were both reversed by exogenous application of H$_2$O$_2$ [231]. These findings demonstrate that anoxic decreases in mitochondrial ROS production signal increases in GABA release.

1.14 Rationale for study

Decreases in reactive oxidative species following entrance into anoxia within the western painted turtle have been documented [156] [231]. Pharmacologically scavenging ROS or inhibiting mitochondrial ROS synthesis under normoxic conditions increases GABAergic PSCs similar to anoxia [231]. These findings demonstrate that ROS decreases play a role in signalling anoxia-mediated changes. Aside from increases in GABA release, during anoxia neuroprotective decreases in NMDARs and AMPARs currents also occur and are associated with mild increases in intracellular calcium. The primary aim of this study was to determine whether pharmacological reduction of cellular ROS under normoxic conditions produced decreases in whole-cell evoked NMDAR/AMPAR currents similar to those seen during transition from normoxia to anoxia. The secondary aim was to evaluate whether the mild increase in Ca$^{2+}$ associated with receptor down regulation is also affected by changes in ROS.

Within anoxia-intolerant vertebrates decreases in ROS and reduction of the NMDAR redox site are associated with increased NMDAR activity. Because the western painted turtle experiences decreases in ROS during entrance into anoxia, you would expect to see a similar increase in activity if a similar mechanism were present. Instead NMDAR activity is downregulated suggesting that either the structure of the NMDARs in the turtle brain are different from those seen in other documented vertebrates and respond to ROS changes in an opposing manner or an anoxia mediated mechanism is present that overrides and reverses normal redox modulation. The mechanism will also need to down regulate AMPAR activity, that is documented to be unaffected by ROS modulation.

Based on the material discussed above on ion channel control during normoxia and anoxia, there are a couple of viable routes through which anoxia-mediated channel arrest could be accomplished. Redox control could be overridden through increases in the activity of cellular phosphatases. As discussed above, there is evidence that NMDARs may need to be phosphorylated in order for redox modulation to take place. Increases in phosphatase activity
suggested to occur during anoxia in the western painted turtle may be sufficient to override and reverse redox modulation. The increases in phosphatase activity may be also triggered by ROS changes. As discussed above the activity of the mK$_{ATP}$ channel can be affected by its oxidation/reduction state and in anoxia-intolerant organisms is preferentially activated in the presence of ROS. The mK$_{ATP}$ subunit that exists in the turtle brain may be modified such that it reacts to decreases in ROS/ increased reduction by becoming more easily activated and triggering anoxia-mediated increases in intracellular calcium and phosphatase activity. Based upon this reasoning I propose the following:

**Hypothesis:** Decrease in reactive oxidative species during anoxia triggers a down regulation of NMDAR and AMPAR whole cell currents via an increase in the activity of mK$_{ATP}$ and a subsequent increase in intracellular calcium.

**Aims:**

1. *Pharmacological reduction of cellular ROS will reduce normoxic NMDAR/AMPAR currents and addition of H$_2$O$_2$ under anoxia conditions will increase NMDAR/AMPAR currents.*

Using whole-cell patch-clamp techniques NMDAR/AMPAR whole cell currents will be measured following pharmacological manipulation of ROS under normoxic and anoxic conditions.

2. *Decreases in ROS will trigger increases in cytosolic calcium levels comparable to those measured during anoxia.*

The fluorescent Ca$^{2+}$ indicator dye, Oregon Green, will be used to measure changes in cytosolic calcium levels following anoxic exposure and/or pharmacological manipulation of ROS.
CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

This study was approved by the University of Toronto Animal Care committee and conforms to the relevant guidelines for the care and handling of experimental animals as outlined in the Guide to the care and Use of Experimental animals as determined by the Canadian Council on Animal Care.

Adult male and female western painted turtles were purchased from Niles Biological Inc. (Sacramento, CA, USA). Animals were housed in an indoor aquarium equipped with a flow-through de-chlorinated freshwater system, a heating lamp, and a basking platform. The water temperature was maintained at 20°C. The turtles were given continuous access to food, and were kept on a 12h:12h light-dark photoperiod.

2.2 Dissection and whole-cell patch-clamp recording protocol

Turtles were selected at random and decapitated. Whole brains were rapidly excised from the cranium of western painted turtles and cortical sheets were isolated and placed in artificial cerebral spinal fluid (aCSF; mm: 107 NaCl, 2.6 KCl, 1.2 CaCl₂, 1 MgCl₂, 2 NaH₂PO₄, 26.5 NaHCO₃, 10 glucose, 5 imidazole, pH 7.4; osmolality 280–290 mosmol l⁻¹). Individual cortical sheets (3-4) were cut medially from the visual cortex of each cerebral hemisphere [Figure 2.1].
Cortical sheets were placed in an RC-26 chamber with a P1 platform (Warner Instruments, CT, USA). During recordings of NMDAR activity, cortical brain tissues were bathed in standard aCSF. During recordings of AMPAR activity, cortical tissues were bathed in a modified high Mg$^{2+}$ aCSF solution [4 MgCl$_2$] in order to block NMDAR activity, and isolate AMPAR currents specifically.

aCSF solutions were gravity perfused into the chamber via 1-L glass bottles attached to intravenous (IV) drippers. A three way valve allowed for transitions to be made between initial and experimental bottles during recordings. Conditions of normoxia were developed by bubbling with 95% O$_2$/5% CO$_2$. Investigations performed under anoxic conditions were accomplished by switching to a secondary bottle of aCSF bubbled with 95% N$_2$/5% CO$_2$. The perfusion tubing was double jacketed and gassed with 95% N$_2$/5% CO$_2$ to maintain anoxic conditions. As well, a plastic rim with anoxia tubing connected was placed around the saline bath to gently gas 95% N$_2$/5% CO$_2$ during anoxic conditions. All experiments were conducted at room temperature (22 °C).

Whole-cell recordings were performed using 5-8 MΩ micropipettes produced from borosilicate glass capillary tubes with a P-97 micropipette puller model (Sutter Instruments). Micropipettes were fire-polished using a MF-830 Microforge (Narashige Group). Pipette solution contained the following (in mM): 8 NaCl, 0.0001 CaCl$_2$, 10 Na-HEPES, 130 potassium gluconate, 1 MgCl$_2$, 0.3 NaGTP, and 2 NaATP (adjusted to pH 7.4 and osmolarity 290-300 mOsM). Cell-attached 1-20 GΩ seals were obtained using the blind-patch technique of Blanton et al. (1989). To break into the cell, the recording electrode potential was voltage-clamped to -80 mV and a sharp pulse of negative pressure was applied. Once the whole-cell configuration was
established, cells were given at least 2 minutes to acclimate before series resistance was measured, which normally ranged from 20-100MΩ. Patches were discarded if series resistance varied by >25% over the course of an experiment. Data was collected at 5-10 kHz using an Axopatch-1D amplifier, a CV-4 head stage, and a Digidata 1200 interface (Axon Instruments) and stored on computer using Clampex 10 software (Axon Instruments). A fast-step perfusion system (VC-6 perfusion valve controller and SF-77B fast-step perfusion system; Warner Instruments) was used to deliver pharmacological agents to the tissue.

Figure 2.1: Diagrammatic representation of cortical sheet excision and experimental setup.

Figure adapted from an online source [232].
2.3 Neuron identification

All investigations were carried out within pyramidal cells. Neurons were identified as pyramidal or stellate based on the expressed electrophysiological properties. Previous to all whole cell patch clamp experiments cells were current clamped and a current was injected for 450 ms to cause a 30 mV depolarizing change in membrane potential and action potential firing. Characterization was accomplished by examining the adaptation pattern and amplitudes of action-potential firing and comparing these to the standards previously developed for differentiating between stellate and pyramidal neurons [101].

2.4 Evoked NMDAR and AMPAR current amplitude recordings

After patches stabilized neurons were voltage-clamped to a holding potential of -80 mV for the entirety of the recording period. All recordings were carried out within the same time frame in order to ensure comparability. Recordings were made at 0 min, 10 min, 30 min, 50 min, 70 min, and 90 min [Figure 2.2]. Access resistance was measured at the beginning and end of each recording and if resistance fluctuated by >20% from the initial value patches were discarded. Initial recordings at 0 and 10 were made under normoxic conditions to establish a baseline for comparison and confirm currents were stable. The initial amplitude recording at t= 0 min was set to 100% and all subsequent recordings were normalized to that control value for comparison purposes. Patches were discarded if t=10 min varied by more than 20% from t= 0 min. Experimental conditions were induced immediately after the establishment of a stable baseline and currents were evaluated after 20 and 40 minutes. Normoxic conditions were then reintroduced in order to reverse experimental changes and evaluated after 20 and 40 minutes.
The peak current values were taken from the recordings and used for the purposes of comparison and statistical analysis.

Tetrodotoxin (TTX) was administered via drip perfusion 5 minutes previous to all recordings in order to prevent the opening of voltage gated sodium channels and ensure currents elicited were only from NMDAR/AMPAR activation. The only exception was during H$_2$O$_2$ experiments where 5 minute TTX addition was carried out 10 minutes prior to all recordings. This allowed for H$_2$O$_2$ to be drip perfused 5 minutes prior to recordings to optimize its effects. The effects of TTX have been shown to extend beyond 15 minutes ensuring that NMDAR/AMPAR currents were isolated throughout the extended recording period. After TTX administration cortical sheets were drip perfused with NMDA or AMPA for 3-10 seconds to induce a current response. The same application time was then used for every subsequent recording performed on the same neuron within a single experiment.

![Patch Aquired Timeline](image)

**Figure 2.2:** *NMDAR and AMPAR whole-cell patch clamp recording timeline.*

NMDAR and AMPAR current amplitude measurements were all taken at specified time points (top line) during varying treatments (bottom).
2.5 Fluorescence investigation of cytosolic Ca\(^{2+}\) changes

To assess changes in cytosolic Ca\(^{2+}\) cortical slices were loaded with the Ca\(^{2+}\)-sensitive dye Oregon-Green 488 BAPTA-1 AM (Invitrogen). Oregon-Green 488 BAPTA-1 was chosen due to its high Ca\(^{2+}\) affinity (Kd \(\approx\)170 nM). Tissue sheets were incubated individually in an opaque vial containing 2 mL of aCSF with 40 uM Oregon-Green (from a 1 mM Oregon-Green stock solution in dimethylsulfoxide and 20% pluronic acid). Cortical slices were loaded within the vials for 3 hours at 3-5 °C and subsequently washed in unmodified aCSF for 15 minutes. Following dye loading slices were placed in a flow-through recording chamber as previously described for whole-cell patch clamp experiments. During anoxic experiments a plastic cuff was placed around the objective in order to gently gas 95% N\(_2\)/5% CO\(_2\) across the bath.

Oregon-Green was excited at 488 nm using a DeltaRam X high-speed random access monochromator and a LPS220B light source (Photon Technology International) at a bandwidth of 4.5 nm. Fluorescence measurements were acquired at 1 second intervals using an Olympus BX51W1 microscope and U-CMAD3 camera. Fluorescence emission measurements at 488 nm were acquired at 10 second intervals under 400x magnification using an Olympus BX51W1 microscope (Olympus Canada Inc.) and Rolera-MGi Digital EMCCD camera (QImaging). Camera gain and exposure time values were 4.0 and 3 s, respectively. Previous to all recordings excitation scans were performed from 450 to 550 nm in order to confirm peak excitation at 488 nm and successful cellular loading of Oregon Green.

Fluorescent recordings were all carried out within the same time frame to ensure comparability. The time frame was reduced compared to that utilized within whole cell patch
clamping experiments to compensate for the rapid rate of photo bleaching and ensure that the fluorescence signal was not exhausted. Cells were recorded under normoxic conditions for 10 minutes to obtain a stable baseline, followed by a treatment period for a further 10 minutes, and concluded with normoxic reperfusion for a final 10 minutes [Figure 2.3]. All fluorescent experiments were conducted with bulk perfusion as drip perfusion proved unreliable and affected image stability. Solutions were gas bubbled in the same manner as solutions used for whole cell patching. After the reperfusion period, aCSF flow was halted and tissues were incubated with the Ca$^{2+}$ ionophore ionomycin (2 µmol) for 5 minutes to increase extracellular Ca$^{2+}$ uptake and obtain the maximal fluorescence signal ($F_{\text{max}}$). This was followed by addition of MnCl$_2$ (2 mM) to preferentially bind the Oregon green dye and quench fluorescence so that the minimal fluorescence signal could be obtained ($F_{\text{min}}$). The protocol and concentrations used were based on previous investigations [149, 233].

A minimum of 10 neurons were selected for analysis in each experimental recording period. In order to ensure good dye uptake and cellular health neurons exhibiting low to moderate basal calcium fluorescence were chosen. The change in fluorescence was determined from a line of slope drawn over the treatment area and compared against a parallel line of slope across the pre and post treatment area (resting fluorescence signal) [see Figure 3.7 for illustration]. The difference between these lines at the midpoint of the experimental period ($F$) was taken and divided by the established range of fluorescence ($F_{\text{max}}$-$F_{\text{min}}$) to establish the change in Ca$^{2+}$ fluorescence. Expressed as the function: Ca$^{2+}$ Change= ($F$/($F_{\text{max}}$-$F_{\text{min}}$)) x 100. The percent change value for all neurons selected within a trace was averaged and used as a single $n$-value for final statistical analysis.
**Figure 2.3:** *Oregon Green fluorescent recording timeline.*

Fluorescent recordings were all carried out within the timeframe with the treatment applications (bottom) all at the same time points (top).

2.6 Whole cell spontaneous neuronal activity recordings

Spontaneously firing pyramidal neurons were utilized to assess changes in neuronal activity. Whole cell patches were obtained using the same procedure as described above. Neurons displaying spontaneous activity were confirmed to be pyramidal cells in the same manner as used in evoked NMDAR/AMPAR recordings. Following successful identification neurons were recorded over a 40 minute time frame. Cells were recorded under normoxic conditions for 5 minutes to establish a measurement of baseline activity, followed by a treatment period for 20 minutes, and concluded with normoxic reperfusion for a final 15 minutes [Figure 2.4]. All experimental solutions were administered via bulk perfusion. AP frequency (APf) was evaluated for 2 minutes after the cell was allowed to stabilized for 1 minute (t=1-3 min). This value was set to 1 and all subsequent values were normalized to the value for comparison purposes. APf values for pre-treatment/normoxia (t=3-5 min), treatment (t=23-25 min), and washout/recovery periods (t=38-40 min) were evaluated within the last 2 minutes of each treatment period.
Figure 2.4: *Whole cell spontaneous activity recording timeline.*

Spontaneous activity recordings were all carried out within the timeframe with the treatment applications (red) all at the same time points (black). AP was evaluated for 2 min within the various treatment periods (blue).

2.7 Pharmacology and drug application

During NMDAR/AMPAR current measurements drugs were applied either by drip perfusion alone or in conjunction with bulk perfusion. Drip perfusion was utilized for agents whose mode of action required little exposure time or whose extended presence could prove toxic to the cell. 1 μM TTX, 300 μM NMDA and 50 μM AMPA were concentrations based on previous publications within our laboratory and were all exclusively drip applied as all agents could be toxic during longer exposures [125-127, 234]. 50 μM H$_2$O$_2$ represents the primary mitochondrial ROS product and was used to test the effects of increased ROS presence on receptor activity. Concentrations were identical to those used in previous experiments with turtle cortical neurons, where 50 μM was the lowest concentration in which a significant change was detected in CM-H$_2$DCF ROS fluorescence [231]. H$_2$O$_2$ was drip perfused for 5 minutes only in order to try and
limit excessive reactions. Drip solutions were appropriately bubbled with 95% O₂/5% CO₂ or 95% N₂/5% CO₂ to ensure they were normoxic/anoxic.

Drugs were bulk perfused in the same manner as the normoxic and anoxic solutions for both electrophysiological and fluorescence experiments. Separate 1 litre IV bottles were used and filled with the respective drug solutions and transition between conditions was made using a three way valve. All drug agents were bubbled with 95% O₂/5% CO₂ in order to ensure they were normoxic. Cell permeable ROS scavengers N-2-mercaptobacopropionylglycine (MPG, 0.5 mM) and n-acetylcysteine (NAC, 0.5 mM) were used to artificially decrease normoxic ROS and test the effects of whole cell ROS decreases on NMDAR/AMPAR currents. Concentrations were identical to those used in previous experiments with turtle cortical neurons where 30 minutes of 0.5 mM MPG/NAC decreased CM-H₂DCF ROS fluorescence comparable to what was documented for anoxia within the same time frame [231]. Rotenone (25 μM) was utilized to inhibit mitochondrial complex I, prevent mitochondrial specific ROS production and determine the role of mitochondrial ROS in NMDAR/AMPAR control. A rotenone concentration of 25 μM was previously validated as safe in turtle cortical slices [156].

2.8 Measurement of oxygen tension

Oxygen concentrations within the recording chamber during induced anoxic periods were tested using an E101 oxygen electrode (Cameron Instruments Co.) containing 1M KCl and 20 μM sodium azide connected through a Model 1900 polarographic amplifier (A-M Systems) to a LabPro interface (Vernier Software and Technology) for computer data collection. Logger Pro 3.8.4.2 was used for data collection at a sampling rate of 2 seconds. Oxygen tension was
calibrated to approximately 160 mmHg by placing the electrode in a glass bottle bubbled with 21% O\textsubscript{2}/79% N\textsubscript{2} and to 0 mmHg in a glass bottle bubbled with 95% N\textsubscript{2}/5% CO\textsubscript{2}. The electrode was submerged in aCSF within a chamber identical to that used during fluorescent and whole-cell patch-clamp recordings.

2.9 Chemicals

Tetrodotoxin was purchased from Tocris Bioscience (Ellisville, MO, USA) and Oregon Green 488 was purchased from Invitrogen Life Technologies (Burlington, ON, Canada). All other chemicals were obtained from Sigma-Aldrich Chemical Co. (Oakville, ON, Canada).

2.10 Statistical analysis

Electrophysiological and fluorescent data was analyzed using SigmaPlot software package version 11.0 (Systat Software, Inc., San Jose, CA). NMDAR/AMPAR whole-cell peak current data and Oregon-Green fluorescence data was analyzed following division by a factor of 1000 and root arcsine transformation with a one-way RM ANOVA (Tukey test) to compare the means of normoxic controls and treatments within treatment groups. Results are expressed as means and standard error of the means (S.E.M.). Significance was determined at p < 0.05.
CHAPTER 3: RESULTS

3.1 Pre-current recording evaluations

3.1.1 Anoxic conditions are produced within the bathing chamber

To confirm the recording chamber was anoxic during perfusion with \( \text{N}_2/\text{CO}_2 \) - bubbled saline, an \( \text{O}_2 \) electrode was used to measure the change in the partial pressure of oxygen during the transition from normoxic to anoxic conditions. The solution was bubbled with room air at an approximate partial pressure of 160 (~21% \( \text{O}_2 \)) mmHg, and after transition to aCSF bubbled with 95% \( \text{N}_2/5\% \text{ CO}_2 \) the pressure fell to 0.6 mmHg (~0% \( \text{O}_2 \)) after 10 minutes [Figure 3.1]. This value was at the limits of detection of the \( \text{O}_2 \) electrode and was not different from the level measured in the \( \text{N}_2/\text{CO}_2 \) - bubbled bottle.
Figure 3.1: *Change in oxygen tension during normoxic to anoxic transition.*

Sample trace representing oxygen tension over the course of 16 minutes in the recording chamber. The switch to anoxic conditions was made at $t = 5$ min and the return to normoxia was made at $t = 11$ min. Complete anoxic conditions were met after approximately 10 minutes.

3.1.2 All recordings were conducted within pyramidal cells

Pyramidal neurons were ideal for this investigation due to their abundance within the turtle cortex and their use of glutamate as their primary excitatory neurotransmitter. Previous publications within the turtle cortex have primarily conducted experiments within these cell types. To confirm that all neurons tested were pyramidal cells the adaptation pattern and amplitudes of action-potential firing of all cells were tested previous to NMDAR/AMPAR current recordings. In order to generate the necessary electrophysiological traces for comparison cells were current clamped and then current injected for 450 ms to generate a 30 mV depolarizing change in membrane potential and action potential firing. Cells that displayed action potentials exhibiting marked frequency adaptation during a sustained injection of current
about firing threshold, as well as firing two discrete sizes of action potential were classified as pyramidal cells. Cells that exhibited no spike frequency adaptation, uniform spike amplitudes, and a short but large undershoot of membrane potential following action potentials were classified as stellate cells. The difference between cell types was distinct when traces were compared [Figure 3.2].

Figure 3.2: Spike frequency adaptation in pyramidal and stellate neurons.
A) Response of a representative pyramidal neuron to a suprathreshold pulse of current. B) Response of a representative stellate neuron to a suprathreshold pulse of current.

3.2 Whole-cell NMDAR current experiments

3.2.1 Normoxic NMDAR currents are stable throughout the time course

The 90 minute experimental time frame utilized for all NMDAR pharmacological experiments was based on previous experiments by our laboratory within the turtle cortex. To confirm that currents remained constant and changes brought on by pharmacological
manipulation were not artifacts, normoxic NMDAR current controls were conducted for the entirety of the experimental time course. To ensure recordings were stable throughout the time course, cells that experienced a greater than 20mV change in membrane potential from t=0 min were discarded. Normalized NMDAR peak currents did not change after 10, 30, 50, 70 and 90 minutes of normoxic saline perfusion (93.83 ± 8.00% [n=8], 97.55 ± 9.40% [n=8], 98.77 ±8.56 [n=8], 95.52 ± 9.67% [n=8] and 98.16 ± 10.23% [n=4], respectively) [Figure 3.3].

3.2.2 Normoxic NMDAR current amplitudes are reduced during anoxia

NMDAR whole cell currents in turtle cortical pyramidal neurons undergo a large approximately 50% reduction in amplitude during anoxia [127, 129, 149]. In order to confirm that these decreases could be replicated and generate comparisons for pharmacological manipulations anoxic conditions were induced and evaluated. Following 20 and 40 minutes of anoxia NMDAR peak current amplitudes were reduced comparable to what has been previously documented (61.93 ± 8.57% [n=7, p= 0.004] and 58.58 ± 8.97% [n=7, p= 0.008], respectively). The effects were mostly reversed after 20 minutes of normoxic reperfusion (82.72 ± 12.16% [n=6]) and completely reversed after 40 minutes of normoxic reperfusion (98.17 ± 4.21% [n=5]) [Figure 3.3].
Figure 3.3: Effect of anoxia on whole cell NMDAR currents.

A. Percentage normalized NMDAR peak current amplitudes after 20 min (t=30, blue bars) and 40 min (t=50, red bars) of treatment followed by 20 min (t=70, green bars) and 40 min (t= 90, purple bars) of washout. Dashed lines represent normoxic baseline controls taken at t=10 min (red) and 40 min anoxic treatment value (blue). Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05).

B-C, Paired sample NMDAR currents of the initial baseline recording (t= 10 min) and following 20 min of the indicated experimental treatment (t= 30 min).
3.2.3 NMDAR current amplitudes are increased by ROS scavenging

In order to test the hypothesis that decreases in intracellular ROS concentrations ([ROS]$_i$) during anoxia serve to trigger decreases in whole cell NMDAR currents, the ROS scavengers MPG and NAC were administered separately under normoxic conditions. Following 20 and 40 minutes of scavenging, large increases in NMDAR peak current amplitudes were observed for MPG (201.42 ± 8.25% [n=6, p< 0.001] and 195.06 ± 16.31% [n=4, p< 0.001], respectively) and NAC (192.46 ± 19.86 % [n=6, p= 0.046] and 208.93 ± 37.32% [n=4 p= 0.035], respectively). The effects of these increases often resulted in hyperactivity, depolarization and the loss of the patch. Within the recordings that were maintained, normoxic reperfusion for 20 and 40 minutes failed to reverse drug effects from MPG (182.84 ± 16.32% [n=3, p< 0.001] and 186.67 ± 5.33% [n=3, p< 0.001], respectively) and only mildly reversed the effects of NAC (168.35 ± 26.05% [n=3] and 139.55 ± 24.74% [n=3], respectively) [Figure 3.4]. In only one recording for both experiments were the effects completely reversed after a period of 110 minutes for MPG and 120 minutes for NAC [Data Not Shown].
Figure 3.4: Effect of ROS scavenging on whole cell NMDAR currents.

A. Percentage normalized NMDAR peak current amplitudes after 20 min (t=30, blue bars) and 40 min (t=50, red bars) of treatment followed by 20 min (t=70, green bars) and 40 min (t= 90, purple bars) of washout. Dashed lines represent normoxic baseline controls taken at t=10 min (red) and 40 min anoxic treatment value (blue). Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). B-C. Paired sample NMDAR currents of the initial baseline recording (t= 10 min) and following 20 min of the indicated experimental treatment (t= 30 min). Abbreviations: N-2-mercaptopyrrolpropionyl glycine (MPG), n-acetylcysteine (NAC).
3.2.4 NMDAR current amplitudes are decreased by $H_2O_2$ addition

In order to evaluate the effect of increases in $[\text{ROS}]_i$ on receptor activity the primary mitochondrial ROS product $H_2O_2$ was administered under normoxic conditions. $H_2O_2$ administration was limited to 5 minutes prior to recordings in order to prevent/limit $H_2O_2$ toxicity. At 20 and 40 minutes of the treatment regimen significant decreases in NMDAR peak current amplitudes were observed ($80.24 \pm 3.11\%$ [n=9, $p=0.023$] and $78.42 \pm 4.43\%$ [n=5, $p=0.036$], respectively). The effects were completely reversed after reperfusion for 20 ($93.23 \pm 7.11\%$ [n=4]) and 40 minutes ($99.26 \pm 7.78\%$ [n=4]) [Figure 3.5].

3.2.5 Blocking mitochondrial ROS production increases NMDAR currents

In order to determine if the increases in NMDAR activity due to decreases in $[\text{ROS}]_i$ are from a mitochondrial or non-mitochondrial source the mitochondrial complex I inhibitor rotenone was used to block mitochondrial ROS production under normoxic conditions. After 20 and 40 minutes of rotenone treatment increases in NMDAR peak current amplitudes were observed ($216.57 \pm 30.14\%$ [n=5, $p=0.017$] and $232.26 \pm 28.47\%$ [n=4, $p=0.01$], respectively). The effects were reversed after 20 and 40 minutes of reperfusion ($100.08 \pm 2.89\%$ [n=3] and $104.56 \pm 3.44\%$ [n=2], respectively) [Figure 3.5].
Figure 3.5: Effect of H$_2$O$_2$ manipulation on whole cell NMDAR currents.

A, Percentage normalized NMDAR peak current amplitudes after 20 min (t=30, blue bars) and 40 min (t=50, red bars) of treatment followed by 20 min (t=70, green bars) and 40 min (t=90, purple bars) of washout. Dashed lines represent normoxic baseline controls taken at t=10 min (red) and 40 min anoxic treatment value (blue). Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). B-C, Paired sample NMDAR currents of the initial baseline recording (t=10 min) and following 20 min of the indicated experimental treatment (t=30 min). Abbreviations: hydrogen peroxide (H$_2$O$_2$).
3.2.6 Anoxia attenuated NMDAR currents are not reversed by ROS addition

In order to test if the effects of anoxia are reversed by the re-introduction of ROS, H$_2$O$_2$ was added after anoxic reductions of NMDAR were measured. As a result the time frame was modified. Decreases in NMDAR peak current amplitudes were confirmed after 20 and 40 minutes of anoxia (69.62± 3.45% [n=5, p= 0.031] and 66.56 ± 5.96% [n=5, p= 0.013], respectively). Anoxic conditions were maintained and H$_2$O$_2$ was administered over a further 40 minute period. H$_2$O$_2$ administration did not reverse anoxia induced changes after 20 or 40 minutes (58.60 ± 6.73% [n=5, p= 0.001] and 54.36 ± 5.57% [n=5, p< 0.001], respectively). The decreases produced from H$_2$O$_2$ administration were not statistically significant from those produced by anoxia alone [p >0.05]. Normoxic reperfusion only reversed the effects of anoxia and H$_2$O$_2$ mildly after 20 minutes (59.00 ± 11.48% [n=3, p= 0.006]) and moderately after 40 minutes (75.36 ± 10.10% [n=3]) [Figure 3.6].
Figure 3.6: Effect of \( \text{H}_2\text{O}_2 \) addition on anoxia-attenuated whole cell NMDAR currents.

A, Normalized NMDAR peak current amplitudes after 20 and 40 min of anoxia followed by 20 and 40 min of \( \text{H}_2\text{O}_2 \) treatment and concluded with 20 and 40 min of washout (n=6). Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). B, paired sample NMDAR currents of a 10 min control and 20 min of anoxia (t= 30 min), and 20 min of \( \text{H}_2\text{O}_2 + \) anoxia (t= 70 min). Abbreviations: hydrogen peroxide (H2O2).
3.3 Whole-cell AMPAR current experiments

3.3.1 Normoxic AMPAR currents are stable throughout the time course

The time course utilized for AMPAR experiments was the same as that used for NMDAR experiments. AMPAR currents under normoxic conditions were examined throughout the experimental time course to confirm that current amplitudes remained stable. Normalized AMPAR peak current amplitudes did not change after 10, 30, 50, 70 and 90 minutes of normoxic saline perfusion (99.85 ± 4.40% [n=6], 96.92 ± 7.10% [n=6], 97.15 ± 7.01 [n=6], 104.94 ± 2.72% [n=4], 105.95 ± 0.78% [n=4] respectively) [Figure 3.7].

3.2.2 Normoxic AMPAR current amplitudes are reduced during anoxia

Like NMDARs, AMPARs also undergo large decreases in current amplitudes under anoxic conditions. In order to generate anoxic decreases for comparisons to pharmacological conditions anoxia was induced and currents were induced and evaluated within the established time frame. Following 20 and 40 minutes of anoxia AMPAR peak current amplitudes were reduced to a comparable degree of to those previously published (69.95 ± 5.69% [n=5, p= 0.006] and 55.16 ± 6.04% [n=5, p< 0.001], respectively) [125, 234]. The effects were reversed after 20 and 40 minutes of normoxic reperfusion (98.76 ± 4.86% [n=4] and 101.32 ± 5.19% [n=4] respectively) [Figure 3.7].
Figure 3.7: Effect of anoxia on whole cell AMPAR currents.

A. Percentage normalized AMPAR peak current amplitudes after 20 min (t=30, blue bars) and 40 min (t=50, red bars) of treatment followed by 20 min (t=70, green bars) and 40 min (t=90, purple bars) of washout. Dashed lines represent normoxic baseline controls taken at t=10 min (red) and 40 min anoxic treatment value (blue). Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). B-C, Paired sample AMPAR currents of the initial baseline recording (t=10 min) and following 20 min of the indicated experimental treatment (t=30 min).
3.3.3 AMPAR current amplitudes are unaffected by ROS scavenging

In order to test the hypothesis that decreased [ROS], has an inhibitory effect on AMPAR current amplitudes the ROS scavengers MPG and NAC were administered separately under normoxic conditions. No changes in AMPAR peak current amplitude were found after 20 or 40 minutes for MPG (105.40 ± 4.61% [n=6] and 94.71 ± 7.30% [n=4], respectively) or NAC (100.48 ± 3.21 % [n=7] and 103.48 ± 2.80 % [n=6], respectively). Currents remained unchanged through 20 and 40 minutes of normoxic reperfusion for MPG (95.26 ± 3.29 % [n=4] and 97.81 ± 12.60 % [n=3]) and NAC (98.62 ± 2.81% [n= 4] and 97.04 ± 3.86 % [n=3]) [Figure 3.8].

3.3.4 AMPAR current amplitudes are unaffected by H₂O₂ addition

In order to evaluate if increases in [ROS] bring about changes in AMPAR currents the primary mitochondrial ROS product H₂O₂ was administered under normoxic conditions. Normoxic AMPAR peak current amplitudes were not altered by 20 or 40 minutes of H₂O₂ addition (100.00 ± 3.35% [n=11] and 99.19 ± 2.44% [n=7], respectively). AMPAR current amplitudes remained unchanged through 20 and 40 minutes of normoxic reperfusion (106.33 ± 3.91% [n=5] and 100.00 ± 2.95% [n=3], respectively) [Figure 3.8].
**Figure 3.8:** Effect of ROS manipulation on whole cell AMPAR currents.

A. Percentage normalized AMPAR peak current amplitudes after 20 min (t=30, blue bars) and 40 min (t=50, red bars) of treatment followed by 20 min (t=70, green bars) and 40 min (t= 90, purple bars) of washout. Dashed lines represent normoxic baseline controls taken at t=10 min (red) and 40 min anoxic treatment value (blue). Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). B-C, Paired sample AMPAR currents of the initial baseline recording (t= 10 min) and following 20 min of the indicated experimental treatment (t= 30 min). Abbreviations: n-acetylcysteine (NAC), N-2-mercaptopropionyl glycine (MPG), hydrogen peroxide (H2O2).
3.4 Oregon green intracellular calcium assessments

3.4.1 Excitation scans confirmed dye loading in turtle brain sheets

Dye-loading within the cells was confirmed by running excitation scans from 450 nm to 550 nm prior to all recordings. Peak excitation was observed around 488 nm in all dye loaded cell but was absent in non-dye loaded cells [Figure 3.8].

![Figure 3.9: Oregon Green excitation scans.](image)

A-B, sample excitation scans from 450 nm to 550 nm in Oregon Green dye-loaded (A) and non-dye-loaded (B) neurons.

3.4.2 Normoxic calcium concentrations are stable throughout the time course

The time course for fluorescent experiments was different from that utilized for whole cell recordings. Photo bleaching occurs after extended periods of exposure that can cause a loss of the fluorescent signal. The experimental time frame consisted of a 10 minute normoxic time
period that allowed for the fluorescent signal to stabilize, followed by 10 minutes of pharmacological treatment, and 10 minutes of normoxic recovery. To confirm that the Oregon Green fluorescence was stable throughout the experimental time course traces were collected under normoxic conditions through a 30 minute time period. Under normoxic conditions Oregon Green fluorescence did not change significantly with respect to baseline (0.61 ± 0.77% [n= 6]) and decreased at a steady rate due to photo-bleaching and/or dye exocytosis [Figure 3.10].

3.4.3 Intracellular calcium concentrations are increased by anoxia

The entrance into anoxia is associated with a mild increase in intracellular calcium [126, 128, 149]. These experiments were replicated to confirm previous findings and generate rates of change for comparison to pharmacological treatments. Anoxic administration mildly increased Oregon Green fluorescence (16.94 ± 3.29% [n= 6, p< 0.001]) [Figure 3.10].

3.4.4 Intracellular calcium concentrations are unaffected by ROS scavenging

As previously discussed intracellular calcium is increased as a result of anoxia. In order to determine if decreases in ROS served to bring about the mild increases in calcium associated with anoxia the ROS scavengers MPG and NAC were separately applied during an experimental time course. Oregon green fluorescence was not significantly altered with respect to the baseline by MPG or NAC addition (0.56 ± 0.64% [n= 6] and 0.96 ± 1.28% [n= 6]) [Figure 3.10].
3.4.5 Intracellular calcium concentrations are unaffected by H₂O₂ addition

H₂O₂ was bulk perfused onto the slice as drip perfusion proved unreliable and affected image stability. H₂O₂ bulk concentrations were altered to be comparable to those brought on by drip perfusion into the recording bath during electrophysiological recordings (drip= 50 uM, bulk= 5 uM). H₂O₂ addition did not significantly alter fluorescence in respect to baseline (0.84 ± 1.39% [n= 6]) [Figure 3.10].
**Figure 3.10:** *Effect of oxygen and ROS manipulation on Oregon Green fluorescence.*

Percentage normalized changes in Oregon Green fluorescence post treatment. Data are expressed as means ± S.E.M. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). Abbreviations: Arbitrary Fluorescence Units (AFU), N-2-mercaptopropionyl glycine (MPG), n-acetylcysteine (NAC), Hydrogen peroxide (H2O2).
Figure 3.11: Sample Oregon Green fluorescence traces.

Sample traces of Oregon Green fluorescence from neurons treated as indicated. Changes in fluorescence were calculated between two parallel tangents, as illustrated in B. C, Illustration of the method by which Fmin and Fmax were determined. Abbreviations: Arbitrary Fluorescence Units (AFU), N-2-mercaptopropionyl glycine (MPG), n-acetylcysteine (NAC), Hydrogen peroxide (H2O2).
3.5 Action potential frequency assessments in spontaneously active pyramidal cells

3.5.1 Action potential frequency is reduced by ROS scavenging

As previously discussed entrance into anoxia is associated with an increase in GABAergic PSCs that serve to bring about SA and inhibit/decrease neuronal firing [115-117]. This is most clearly seen within neurons that exhibit spontaneous activity and fire continuously at regular intervals. Recordings from these cells within turtle cortical sheets demonstrate a 70% reduction in action potential frequency (APf) with the transition to anoxia [112]. ROS scavenging with MPG and NAC has also been shown to bring about increases in GABAergic PSCs similar to anoxia [231]. Therefore, in order to determine if decreases in ROS result in SA similar to that seen during anoxia, spontaneously active pyramidal cells were treated with MPG during APf recordings. APf decreased by approximately 97% after 18 minutes of MPG treatment (0.04 ± 0.04 [n=4, p< 0.001]) that was reversed and increased after 13 minutes of normoxic reperfusion (2.33 ± 0.44 [n=4, p= 0.016]) [Figure 3.11].
Figure 3.12: Changes in spontaneous activity in response to ROS scavenging.

A, Summary of APf expressed as fold change relative to pretreatment baseline. Data are expressed as means ± S.E.M. Dashed line represents normoxic baseline APf. Asterisks (*) indicate data significantly different from the paired normoxic control (p < 0.05). B, Raw spontaneous activity sample trace. Abbreviations: N-2-mercaptopropionyl glycine (MPG).
CHAPTER 4: DISCUSSION

4.1 Control normoxic and anoxic NMDA and AMPA currents

In agreement with previous findings normoxic NMDA and AMPA administration produced consistent repeatable currents throughout the 90 minute time course of the experiment. The introduction of anoxia produced a decrease of approximately 50% in both NMDA and AMPA currents within 20 minutes consistent with previous reports [126, 149, 234]. This served to further confirm neurological stability and pharmacological validity.

4.2 Pharmacological manipulation of ROS

4.2.1 The NMDAR modulatory redox site in turtle cortical slices

Cellular ROS scavenging with both MPG and NAC produced massive increases in NMDAR whole cell currents contrary to the anoxia mediated decrease typically observed in the western painted turtle cortex. Inversely, the addition of H$_2$O$_2$ under normoxic conditions decreased NMDAR whole cell currents. Although this indicates that decreases in ROS are not responsible for NMDAR attenuation during anoxia, the findings are consistent with what has been documented within neural tissue from other vertebrates and may reveal a conserved mechanism of NMDAR control during normoxia [190-198].

As discussed above, pharmacological increases in ROS or the addition of oxidizing agents has been documented to decrease NMDAR activity within tissue preparations from
mammals, amphibians, and birds. Pharmacological decreases in ROS or the addition of reducing agents has also been shown to produce increases in NMDAR activity within these same groups. These changes have been previously attributed to alterations in the oxidation/reduction state of extracellular cysteine residues found within the NMDAR GluN1 (Cys744, Cys798, Cys 79 and Cys 308), GluN2A (Cys 87, Cys 320 and Cys 399), and/or GluN2B subunits (Cys not yet localized) that collectively make up NMDAR redox sites [187-189]. The changes documented within cortical slices from the turtle during ROS manipulation may also be attributed to alterations in the NMDAR redox site. Specific regions within NMDAR subunits have been documented to be highly conserved between vertebrate species. Comparisons of the Brown ghost knifefish GluN1 amino acid structure found an 88% sequence similarity to the amino acid structures in frog, duck, rat and human [235]. Similarly, the amino acid structures of the four NR2 subtypes possess high levels of conservation between species within sequences coding the lining of the ion pore, glutamate binding pockets, and C-terminal region [119, 236]. The redox modulatory sites on the GluN1 and GluN2 subunits may be similarly conserved between species.

Increases and decreases in NMDAR current amplitude between ROS scavenging and addition were not uniform. The decreases in receptor currents with H$_2$O$_2$ addition were only ~20% compared to the ~100% increase with ROS scavenging. This is consistent with what has been seen in other vertebrates where the addition of reducing agents produced increases that were 6-11 times greater than decreases produced through oxidation [190, 196, 199]. Our findings suggest that the turtle NMDAR redox modulatory site is maintained closer to a fully oxidized than fully reduced form under normoxic conditions, in agreement with what has been documented in other vertebrate species. This provides support for the Cys residues being found on the extracellular portion of the receptors, where the environment is predominantly oxidized.
It may also indicate a maintained ROS presence in close proximity to the receptors and redox sites.

The level of recovery from treatment varied drastically between ROS scavenging and addition. Although recovery from H₂O₂ was achieved within the 40 minute reperfusion period, MPG/NAC recovery was not and cells often depolarized and did not recover preventing the exploration of a longer recovery period. This is consistent with what has been documented in other vertebrates where decreases in NMDAR activity induced through the application of oxidizing agents were consistently reversed with washout, but increases in NMDAR activity induced through reductive agents were maintained. The effects of reducing agents have only been documented to be reliably recovered to baseline through the addition of oxidizing agents [196, 237]. The reasoning behind the lack of recovery from scavenging/reducing agents may be explained by slow cellular ROS production and slow degradation of scavenging/reducing agents. As discussed above, ROS are produced slowly in the cell (only 2-3% of all mitochondrial O₂ consumed) maintained at low levels and may move slowly across the extracellular membrane [168]. It may take a long time for baseline levels to be recovered following ROS scavenging and/or re-oxidize cysteine residues affected by scavenging agents. Furthermore, scavenging and reducing agents may take a long time to be removed from the cell or to be degraded through oxidation, further delaying the re-establishment of baseline ROS levels and NMDAR activity. The addition of oxidizing agents would help in re-oxidization of redox sites and the degradation of scavenging/reducing agents, potentially explaining why this tactic was successful in reversing the effects of reducing agents on NMDAR activity. Cells are likely to be better equipped to deal with ROS increases then their absence as the complete removal of ROS is unlikely for most aerobic life.
The risks of NMDAR over activation and resulting ECD are common to all vertebrates and NMDAR activity must be tightly regulated to prevent it. Ca\(^{2+}\) entry must be maintained within a range where intake concentrations are great enough to signal downstream changes but small enough to be taken up by the mitochondria and ER so that they don’t bring on toxic effects. Most cellular activities are maintained within a desired range via negative feedback systems allowing products generated by specific processes to regulate the actions of the processes themselves. NMDAR Ca\(^{2+}\) dependent ROS production via nNOS or mitochondrial activation may be a mechanism through which NMDAR activity is regulated among vertebrates.

4.2.2 Mitochondrial produced ROS in NMDAR redox modulation

The mitochondrial complex I inhibitor rotenone decreases ROS production in various vertebrate tissues including neuronal tissue [157, 200-202]. Rotenone addition in turtle cortical slices produced increases in NMDAR currents comparable to what was seen with MPG/NAC ROS scavenging. This suggests that changes in mitochondrial ROS production affect NMDAR activity. Full recovery from rotenone treatment was achieved within the reperfusion period unlike what was seen with scavenging agents. This supports the concept that the recovery delay seen during scavenging is the result of agents taking a long period to be degraded and removed from the cell.

The finding that mitochondrial ROS production affects NMDAR activity does not rule out a role for NO generated from nNOS. During whole cell patch experiments, TTX application and voltage clamping would have prevented natural glutamate release and blocked NO
production except during NMDA addition. Because nNOS activity directly linked to NMDAR activation and NO can oxidize cysteine residues in a similar manner to H$_2$O$_2$, it is very likely that both NO and mitochondrial ROS play a role in NMDAR control. In studies within other vertebrates the addition of nNOS inhibitors increases baseline NMDAR currents while artificial NO generators bring about receptor attenuation [241, 242]. However, nNOS inhibitors do not prevent mitochondrial ROS increases triggered by NMDAR activation, suggesting that the mechanisms function independently of each other [238, 239]. Further investigation is necessary to determine the role and contribution of NO in NMDAR redox control.

Mitochondrial ROS generation is increased by NMDAR activation within mammalian neural cultures. NMDA mediated ROS increases were not prevented by nNOS inhibitors but were blocked by the removal of extracellular calcium, rotenone/ other mitochondrial uncouplers, and Ca$^{2+}$ uniporter inhibitors [238, 239]. This suggests that increased mitochondrial ROS is generated as a result of NMDAR specific Ca$^{2+}$ influx and increased mitochondrial Ca$^{2+}$ uptake. If Ca$^{2+}$ influx were to exceed the capabilities of the storage systems like mitochondrial uptake, then Ca$^{2+}$ would begin to spread through the cell causing toxicity. It is therefore not unreasonable that mitochondrial uptake might influence NMDAR activity.

Mitochondrial calcium uptake is primarily accomplished through the actions of the mitochondrial calcium uniporter (MCU) located on the inner mitochondrial membrane (IMM). The outer mitochondrial membrane is permeable to most large ions (<6000 DA) allowing for unaided Ca$^{2+}$ entry. The IMM is not as permeable and transporters are required to allow for Ca$^{2+}$ movement [240]. The MCU becomes active in response to large concentrations of localized Ca$^{2+}$ known as microdomains that in excitable cells need to be 500-600 nM in order to generate a
response [241]. Activity is driven by the large negative potential of the mitochondrial matrix established by the actions of the respiratory chain. Electrophoretic uptake of Ca\(^{2+}\) into the mitochondrial matrix through the uniporter partially dissipates the electrochemical gradient [242, 243]. Compensation is accomplished via Ca\(^{2+}\) induced increases in electron transport chain activity that pump H\(^+\) out of the matrix helping to re-establish the electrochemical gradient and also bringing about increases in ATP production [242]. Enhancements in ROS production can be accomplished through the increases in ETC activity, generating ROS as a by-product. There is also some evidence to suggest that matrix Ca\(^{2+}\) can partially dislocate cytochrome c from the IMM thereby slowing down the electron transfer from complex III to complex IV and enhancing ROS generation at the Q cycle [239, 244]. Ca\(^{2+}\) within the matrix is transient and the actions of multiple Ca\(^{2+}\) efflux mechanisms, the primary one in excitable tissues being the Na\(^+\)/Ca\(^{2+}\) exchanger, work in concert to move Ca\(^{2+}\) back into the cytosol [242, 245]. Storage within the mitochondrial matrix can be quite high and has been documented in excess of 100 uM [246]. However, the capacity is not unlimited and if Ca\(^{2+}\) overload occurs and intake exceeds efflux mitochondrial membrane potentials can collapse leading to excessive Ca\(^{2+}\) efflux and the triggering of apoptosis [247, 248].

4.2.3 A potential mechanism for ROS mediated NMDAR control

For NMDAR activity to be regulated via oxidation/reduction ROS synthesis must not only be tied directly to receptor activity, ROS production must be accomplished within close proximity to the channel. The redox site will be maintained in a preferentially oxidized state by likely existing within the extracellular environment, but changes in intracellular ROS changes will only be effective signals if ROS production occurs within the vicinity of the NMDAR. As
discussed above, this may involve NO produced by nNOS that is tethered to NMDAR via PSD-95. However, for mitochondrial ROS generation to be effective in modulating activity as shown the rotenone experiments, mitochondrial localization must also take place.

ROS are produced from the mitochondria in relatively small quantities (2-3% of $O_2$ consumed) that are maintained at low levels through the actions of cellular antioxidant defence systems. This coupled with high reactivity leads to small and inconsistent mitochondrial ROS levels that exist throughout the cytoplasm limiting their signalling ability to short distances [163, 249]. Furthermore, in order for ROS to affect extracellular redox sites ROS must pass into the extracellular environment through diffusion or WTRs where it can then be further degraded by extracellular antioxidants [168-171]. Therefore in order for ROS control to be effective and consistent, target cysteine residues must be in close proximity to the mitochondria. NMDARs are primarily located within post synaptic densities of the excitatory synapse and mitochondria are similarly packed into these synapses in order to provide the ATP necessary for synaptic activities [250-252]. These synaptic regions are high in membrane ion channels that transport water (WTRs) potentially allowing for an efficient flux of generated ROS across the membrane [172]. Recent investigation in mammalian neurons found NMDAR specific $Ca^{2+}$ entry was far more rapidly taken up by the mitochondria than $Ca^{2+}$ entry through other channels [253]. This tight coupling of mitochondrial $Ca^{2+}$ uptake to NMDAR activation is suggested to be the result of the organelles and receptors being maintained within close proximity to each other in order to better control cytosolic $Ca^{2+}$ levels [253, 254]. There is a considerable body of work in non-excitatable cells that suggests that rapid mitochondrial $Ca^{2+}$ uptake requires high levels of $Ca^{2+}$ to be present in close proximity (~2-5 uM) to the mitochondria and that the endoplasmic reticulum and mitochondria are positioned so that excess calcium leaving the ER is high enough to signal this
action and be quickly sequestered [255-259]. Within excitable cells like neurons this high Ca$^{2+}$ concentration has been shown not to be required to activated rapid uptake, but localized cytosolic Ca$^{2+}$ levels of above 500–600nM are still required to stimulate influx supporting the tight coupling concept [241].

Given my experimental findings and those of similar experiments conducted in other organisms oxidation/reduction of the NMDAR redox site appears to have a similar effect across multiple vertebrate species suggesting a common mechanism of control. Using information collected from other studies I submit the following potential mechanism for mitochondrial ROS mediated NMDAR control under normoxic conditions. I do this in order to try and better explain how ROS control might be accomplished so that that I can better explain later how anoxia mediated mechanisms may override these processes. The proposed mechanism for is as follows:

NMDAR activation causes an influx of extracellular Ca$^{2+}$ into the cell that serves to signal changes downstream changes via activation of receptor localized Ca$^{2+}$-dependent enzymes. Excessive calcium that does not become bound is rapidly taken up by localized mitochondria to prevent it from spreading out through the cell and undergoing unwanted reactions. Ca$^{2+}$ freely flows across the permeable mitochondrial outer membrane and is then taken up into the matrix via the action of the MCU. Ca$^{2+}$ entrance stimulates increases in ETC activity to compensate for the inward ion flow, generating increased levels of ROS as result. The ROS produced will rapidly move out of the mitochondria and into the cytosol. ROS may then move across the extracellular membrane and into the extracellular environment by simple diffusion or through WTRs where it can interact with NMDAR redox sites in close proximity, reversibly oxidizing the modulatory redox site and decreasing the receptors open time in
response to stimulus. The cumulative action of multiple mitochondria undergoing these processes will serve to generate a cloud or microdomain of ROS that will help maintain NMDARs primarily in an oxidized state and resist the actions of antioxidant enzymes. Increases in NMDAR activation will bring on increases in $\text{Ca}^{2+}$ influx and subsequent ROS generation that will increase receptor oxidation and decrease sensitivity. Similarly decreases in NMDAR activation will lead to decreases in ROS allowing for enzymes like GSH/L-Cys to reduce a greater portion of receptors and increase sensitivity. Through this negative feedback mechanism $\text{Ca}^{2+}$ influx can be maintained within a range that brings about necessary changes without becoming high enough to overtake mitochondrial uptake mechanisms and trigger overload [Figure 4.1].

Although this mechanism will maintain NMDAR activity within a normal range during normoxia it will not function under anoxic conditions as mitochondrial ROS generation requires $\text{O}_2$. The loss of cellular oxygen will halt oxidative phosphorylation and allow for cellular ROS levels to be quickly depleted. The method of maintained NMDAR inhibition will quickly be lost as the receptors are rapidly reduced leading to hyper excitability. Activation of the receptors will trigger a massive influx of $\text{Ca}^{2+}$ bringing about excitotoxic cell death. In order for the western painted turtle to survive prolonged anoxia another mechanisms must serve to override ROS control and trigger the decreases in NMDAR activity documented to occur.
Figure 4.1: Diagrammatic representation of the proposed mechanism for ROS mediated NMDAR control under normoxic conditions.

1. NMDAR influx triggers Ca²⁺ influx.
2. Ca²⁺ is taken up into the mitochondrial matrix by the MCU.
3. Ca²⁺ influx triggers increases in ETC activity to re-establish the membrane potential.
4. Increases in ETC activity increase H₂O₂ generation.
5. Increased H₂O₂ moves into the extracellular environment through WTRs or through simple membrane diffusion.
6. H₂O₂ oxidizes NMDAR redox sites and decreasing receptor activity.
7. Cellular antioxidants reduce NMDAR redox sites if H₂O₂ levels are low.
4.2.4 AMPAR redox insensitivity

AMPAR currents were unaffected by MPG, NAC or H$_2$O$_2$ consistent with what is seen in mammalian tissue with oxidizing/reducing agents suggesting that the mechanisms for AMPAR control in respect to ROS changes are conserved between species [196-198, 203].

The reasoning behind why there exists a mechanism for ROS mediated NMDAR control but not AMPAR may be a direct result of how oxidation of the NMDAR leads to inhibition of its activity. AMPARs activation produces excitatory postsynaptic potentials (EPSPs) that cause neuronal depolarization and the removal of Mg$^{2+}$ from the pore region of the NMDAR allowing activation and Ca$^{2+}$ influx [260-263]. It is through this indirect mechanism of action that AMPARs exhibit control over intracellular Ca$^{2+}$ influx. Changes to the modulatory redox site may override AMPAR control by affecting the efficiency to which NMDARs can maintain Mg$^{2+}$ within the pore region. Treatment of mammalian neural tissue with rotenone resulted in increases in NMDAR activity as previously discussed, but also documented that NMDAR currents lost the characteristic region of negative-slope conductance that is normally produced by voltage-dependent block by Mg$^{2+}$. NMDAR increases were mimicked by low extracellular Mg$^{2+}$ and rotenone induced increases were antagonized by a high levels of Mg$^{2+}$. Rotenone addition had no effect on AMPAR currents [203, 206]. This suggests that oxidation/reduction alters the ability of the NMDAR to maintain Mg$^{2+}$ within the pore region and overrides/circumvents AMPAR control.
The lack of AMPAR down regulation in response to decreased [ROS]; further supports the concept that ROS changes are not responsible for NMDAR/AMPAR current decreases in the anoxic Western painted turtle brain and that a secondary mechanism must be at work.

4.2.5 Anoxia-mediated block of NMDAR redox control

Anoxic decreases in NMDAR currents were not reversed by the addition of H$_2$O$_2$ confirming that ROS changes are not responsible for anoxia mediated changes. H$_2$O$_2$ addition to anoxia-attenuated NMDAR currents appeared to further reduce whole cell currents (~10%), but it is not clear if this decrease was simply the result of extended anoxia. Average currents after 90 minutes of anoxia with H$_2$O$_2$ reached ~54% of normoxic levels that was not significantly different from that seen after 30 minutes of anoxia in control experiments (~58%) or previous publications (~52%) [149]. H$_2$O$_2$ addition alone produced decreases after 40 min of approximately 22% from normoxic baseline compared to addition after anoxia that produced after 40 min only an approximate 12% change. This would seem to support the concept that the secondary mechanism for triggering anoxic NMDAR activity decreases exists that overrides the mechanisms of ROS control. However without anoxic controls for a comparable period of time the assumption cannot be verified. Regardless, the results confirm that another mechanism aside from ROS decreases is responsible for the induction of NMDAR down regulation. The most likely candidate for these changes is changes in cellular calcium from an intracellular source.
4.3 A role for anoxia-mediated calcium increases in blocking redox control

A mild increase in cytosolic calcium from the mitochondria is seen during anoxia in the western painted turtle and is necessary to bring about decreases in NMDAR and AMPAR whole cell currents [125, 264]. Investigations using Oregon Green Ca$^{2+}$ fluorescence confirmed an anoxic increase in intracellular calcium similar to what has been previously documented (~15%) [149]. However, the addition of MPG, NAC and H$_2$O$_2$ failed to elicit any change in the fluorescent Ca$^{2+}$ signal demonstrating that ROS changes do not play a role in the anoxia-mediated increase in cytosolic Ca$^{2+}$. This serves to confirm that a secondary mechanism for anoxia-mediated changes is at work and suggests that mitochondrial release of Ca$^{2+}$ is central to its activity.

Increases in mitochondrial Ca$^{2+}$ release are associated with activation of the calcium-binding messenger protein calmodulin (Ca$^{2+}$/calmodulin). Ca$^{2+}$/calmodulin is a small Ca$^{2+}$ binding protein that is highly conserved within eukaryotes, present in high concentrations in the brain and capable of modulating a number of different enzymes and cellular processes [265, 266]. Within the western painted turtle inhibition of Ca$^{2+}$/calmodulin has been demonstrated to prevent decreases in NMDAR activity. Ca$^{2+}$/calmodulin may also activate the Ca$^{2+}$/calmodulin-dependent phosphatase PP2B (calcineurin), whose inhibition has also been shown to inhibit early anoxia mediated NMDAR downregulation. Interestingly inhibition of PP1 and PP2A was also shown to inhibit NMDAR decreases suggesting a dependency between both dephosphorylation and calmodulin interaction [129]. The sites of modification and binding offer means for NMDAR/AMPAR control that may also serve to override the mechanism of ROS mediated NMDAR regulation.
Calmodulin has been shown to exert effects via binding to two identified sites (CBS1 and CBS2) the C0 terminus of the GluN1 via destabilization of the NMDARs from the cytoskeleton [267-269]. It has also been demonstrated that phosphorylation of the GluN1 serine residues decreases affinity for calmodulin, an effect that can be reversed by the actions of PP1 and PP2A [150]. This has led to the hypothesis that during anoxia, PP1 and 2A dephosphorylate the serine residue on the C-terminus of the GluN1 subunit allowing for the binding of Ca\(^{2+}\)/calmodulin and an attenuation of NMDAR function [264]. As discussed above NMDAR redox modulatory sites have been identified on the GluN1, (Cys744, Cys798, Cys 79 and Cys 308) GluN2A (Cys 87, Cys 320 and Cys 399) and GluN2B (not yet localized) [187-189]. GluN1 dephosphorylation or calmodulin binding both offer a potential means to block modulatory redox sites, preventing oxidation/reduction and overriding ROS control. Furthermore, calcineurin dephosphorylates the GluN2A subunit (not GluN1) that besides having been shown to contribute to glycine desensitisation and NMDAR current reduction, offers a means through which modulatory redox sites on the GluN2A subunit may be blocked [267, 270]. It is also possible that calmodulin induced NMDAR cytoskeletal dissociation may bring about destabilization the modulatory redox site leading to a loss of ROS control. AMPAR activity has not been demonstrated to be regulated through calmodulin binding but has been identified to be controlled by dephosphorylation. Increases in calcineurin activity dephosphorylate Ser845 on the GluA1 subunit triggering a decrease in AMPAR function [271-273].

In support of this mechanism, inhibition of intracellular calcium release and subsequent phosphatase activity has been associated with increases in NMDAR activity under anoxic conditions. Addition of the calcium chelator BAPTA increased NMDAR whole cell currents by
35% after 20 minutes of anoxia. Similarly addition of the PP1/PP2A inhibitor calyculin A increased NMDAR whole cell currents by 45% after 40 minutes of anoxia. Finally, addition of the calmodulin inhibitor calmidazolium increased NMDAR whole cell currents by 20% after 40 minutes of anoxia [129].

The mechanism by which calcium is released from the mitochondria has yet to be established. However recent investigation has suggested that it is the result of mK<sub>ATP</sub> activation and subsequent mitochondrial permeability transition pore (mPTP) formation. The decreases in AMPAR and NMDAR activity seen during anoxia are mimicked by mK<sub>ATP</sub> activators and blocked by mK<sub>ATP</sub> inhibitors [125, 126]. Furthermore, inhibition of mK<sub>ATP</sub> prevents the mild increase in intracellular Ca<sup>2+</sup> characteristic of anoxia, while activation produces Ca<sup>2+</sup> increases of a similar magnitude [126]. mK<sub>ATP</sub> channels are traditionally gated by high levels of ATP under normoxia. During anoxia ATP production at the level of the mitochondria decreases causing local ATP levels to be reduced potentially leading to the opening of mK<sub>ATP</sub> channels. Opening then leads to an influx of K<sup>+</sup> that can trigger mPTP formation as a result of mitochondrial membrane depolarization. The mPTP is a temporary channel that can be formed in the inner mitochondrial membrane and permit Ca<sup>2+</sup> release. The formation of a singular large mPTP often bring on excessive Ca<sup>2+</sup> release and toxic results, but the formation of several small mPTPs has been shown to bring about safe levels of Ca<sup>2+</sup> efflux [274]. Within the western painted turtle, the addition of an mPTP activator produced decreases in NMDAR currents similar to those seen during anoxia that were abolished by the addition of a Ca<sup>2+</sup> chelator. The addition of the mPTP activator also produced an increase in Ca<sup>2+</sup> fluorescence identical to that seen during anoxia [149]. The mPTP is naturally formed under conditions of mitochondrial stress such as K<sup>+</sup> mediated uncoupling that can trigger mild to moderate membrane depolarization (10-20%) [275].
Furthermore, the pore is traditionally unstable and short lived causing it to quickly collapsing and potentially only produce a mild increase in intracellular calcium [276].

4.4 Roles for redox sensitive GABA release

Entrance into anoxia is associated with a decrease in spontaneous AP frequency of approximately 70%, which is attributed to increases in GABAR activity [112]. Recently decreases in ROS were shown to produce increases in GABAergic PSC activity suggesting that they play a role in GABAergic anoxic signalling [231]. In support of these findings MPG addition reduced spontaneous AP frequency similar to anoxia although to a greater degree (~98%). These results serve to confirm that although decreases in ROS during anoxia are not responsible for NMDAR and AMPAR down regulation they do appear to be involved in the processes controlling GABAergic activity increases.

Recovery from MPG treatment resulted in a major increase in firing above previous baseline levels (~74%). This may be the result of NMDAR over excitation and Ca\(^{2+}\) influx, triggered by ROS decreases as previously discussed. As discussed previously, MPG may take a long time to be removed from the cell leading to NMDAR currents increases that were not reversed after 40 minutes of normoxic reperfusion. Therefore, even though GABA mediated ROS effects were quickly reversed the NMDAR augmentation may have remained and resulted in excessive Ca\(^{2+}\) influx, increased neuronal firing, and generated the AP frequency increases seen.
Interestingly, increases in GABAergic PSCs will serve to decrease/inhibit postsynaptic glutamate release preventing NMDAR and AMPAR activation. Through this mode of action GABA changes in response to low oxygen may serve as a preliminary level of protection, preventing NMDAR from activating in a reduced/overexcited and triggering ECD long enough for the mechanisms controlled via mitochondrial Ca\(^{2+}\) release to take effect.

4.5 Potential mechanism of NMDAR/AMPAR control under anoxic conditions

The mechanisms proposed above for control of NMDAR activity through redox changes will maintain intracellular Ca\(^{2+}\) influx within a normal range under normoxic conditions. However, during anoxia O\(_2\) and ROS depletion will produce a state of NMDAR hyperexcitability that has the potential to produce massive increases in Ca\(^{2+}\) intake. Within the brain of the western painted turtle ECD is not seen to occur and NMDAR/AMPAR receptors experience major down regulations in activity. This suggests that a secondary mechanism of control is activated by the anoxic state that can override the mechanism of ROS/redox control.

Given my and others findings in the brain of the western painted turtle during oxygen deprivation I propose the following mechanism of anoxia-mediated NMDAR/AMPAR control: as oxygen concentrations are depleted within neuronal tissue oxidative phosphorylation will begin to cease. This will result in mitochondrial localized decreases in ATP triggering the opening of m\(K_{ATP}\) channels. Influx of K\(^+\) through these channels will trigger mild/moderate loss of the mitochondrial membrane potential and bring about the formation of multiple small mPTPs. Ca\(^{2+}\) will move rapidly through these channels that do to their instability quickly collapse. The resulting mild cytosolic Ca\(^{2+}\) increase will bind to calmodulin activating it.
Ca\(^{2+}\)/calmodulin can then bind directly to the NMDAR GluN1 subunit either with or without the aid of dephosphorylation via PP1 and PP2A. This binding will serve to inhibit receptor activity as well as block the GluN1 redox modulatory sites overriding the mechanisms of ROS control.

The activation of calcineurin via calmodulin binding will serve to dephosphorylate the GluN2A subunit contributing to receptor down regulation and GluN2A modulatory redox site destabilization. Calcineurin will also dephosphorylate the Ser845 on the GluA1 subunit of the AMPAR down regulating activity. While these changes are occurring increases in GABAergic activity triggered by decreasing ROS levels will serve to prevent glutamate release and subsequent NMDAR/AMPAR activation [Figure 4.2].
Figure 4.2: Diagrammatic representation of the proposed mechanism for anoxia mediated NMDAR/AMPAR downregulation.

1. Low oxygen halts ETC activity.
2. Local ATP levels decrease.
3. Decreasing ATP triggers mKATP activation.
5. Mitochondrial membrane potential decrease triggers MPTP formation and Ca2+ efflux.
6. Intracellular Ca2+ activates calmodulin and protein phosphatases. Calmodulin binding and dephosphorylation of regions on the GluN1 block NMDAR redox sites and contribute to decreases in receptor activity.
7. Calmodulin binds and activates calcineurin. Calcineurin dephosphorylation of regions on the GluN2A also block NMDAR redox sites and contribute to decreases in receptor activity. Dephosphorylation of regions on the AMPAR GluA1 contribute to downregulation.
4.6 Concluding remarks and directions for future research

These results suggest that changes in oxidation/reduction state of the NMDAR modulatory redox site lead to increases and decreases in activity respectively, without effect on AMPAR. This is in agreement with what has been demonstrated within investigations in other vertebrates. To our knowledge this is the first case where evidence for this mechanism has been demonstrated in reptiles. Our findings also support the hypothesis that these redox changes are regulated by natural ROS levels, and in this way receptor function can be conservatively regulated between vertebrates under normoxic conditions. Our research demonstrated that this mechanism is not responsible for NMDAR/AMPAR downregulations seen during anoxia within the western painted turtle, but found evidence to support an alternative mechanism involving mitochondrial Ca$^{2+}$ release.

In order for a better determination of the validity of the mechanisms proposed, a number of questions still need to be answered and different experiments should be conducted. (1) It has yet to be conclusively established how anoxia-mediated changes override ROS control, though there is evidence to suggest it occurs through mitochondrial Ca$^{2+}$ release. The mK$_{ATP}$ activator diazoxide has been previously documented to produce anoxic decreases in NMDAR currents similar to those seen during anoxia. Diazoxide mediated decreases under normoxia should be evaluated in conjunction with MPG/NAC in order to determine if scavenging effects are mitigated. (2) The effects of phosphatases or calmodulin on AMPAR activity have yet to be evaluated in the western painted turtle brain. Anoxia mediated AMPAR currents should be evaluated in the presence of inhibitors for PP1, PP2A, calmodulin, and calcineurin in order to determine if which if any of these groups is responsible for anoxia mediated AMPAR
downregulation. (3) The role of NO in NMDAR control has yet to be firmly established. nNOS inhibitors should be used in the same manner as roteone to determine if their blockade produces comparable increases to ROS scavenging.

Understanding neurological anoxia tolerance is critical to many of the medical problems faced within our society today. Stroke represents the third leading cause of death in North America and with both childhood and adult obesity rates increasing across North America the number of incidents is predicted to increase [277]. The development of preventative and fast acting measures, like seen in the overwintering capabilities of the western painted turtle, may offer a means to save thousands of lives.
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