Extreme Resistance to Oxygen Deprivation in Brain Tissue from the Naked Mole-Rat

BY

BETHANY L PETERSON
B.S., University of Arkansas, 2005

THESIS

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Defense Committee:

A Don Murphy, Chair
Thomas Park, Advisor
John Leonard
Chris Fall, Bioengineering
John Larson, Psychiatry

This thesis is dedicated to my son, without whom it might not ever have been accomplished.

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BLP

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LIST OF ABBREVIATIONS

ACSF	Artificial cerebral spinal fluid
ATP	Adenosine triphosphate
CA1	*Cornu Ammonis* area 1
cAMP	Cyclic adenosine monophosphate
DG	dentate gyrus
DNA	Deoxyribonucleic acid
EEG	Electroencephalography
EPO	Erythropoietin
EPSP	Excitatory postsynaptic potential
GABA	Gamma-aminobutyric acid
hAGS	hibernating Arctic ground squirrel
HIF	Hypoxia-inducible factor
ibeAGS	interbout euthermic Arctic ground squirrel
LTP	Long-term potentiation
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Ngb	Neuroglobin
NMDA	N- methyl-D-aspartate
NMDAR	N- methyl-D-aspartate receptor
NMR	Naked mole-rat
OGD	oxygen and glucose deprivation
P	Postnatal day

LIST OF ABBREVIATIONS (continued)

PKA	Protein kinase A
ROS	Reactive oxygen species

SUMMARY

The naked mole-rat lives in large colonies living completely underground, a very unique way of life. This leads to an environment where the air is low in oxygen and high in carbon dioxide. Adaptations in blood and metabolism have been previously reported in the naked mole-rat that help it survive these conditions. My study is aimed at specifically examining the tolerance to low oxygen (hypoxia) in the brain of this unusual species. My working hypothesis was that naked mole-rats retain neonatal protective characteristics against hypoxia into adulthood.

First we exposed whole animals to no oxygen (anoxia) to see how long they survived. A group of naked mole-rats was exposed for around 6 minutes and still recovered once put back in room air. This is remarkable when compared to mice, which only survived around 45 seconds and did not recover. We then measured the levels of ATP in the brains of adult naked mole-rats compared to adult mice and neonatal mice. This is important because ATP is the energy of the brain and oxygen is needed for its synthesis. We found that ATP stores in adult naked mole-rat brain depleted slower than in adult mouse brain, however, it was not as slow as in neonatal mouse brain.

Next, since many hypoxia-tolerant model systems are able to prevent an increase in internal calcium that leads to cell death during hypoxia, we wanted to establish if naked mole-rats could prevent this as well. We determined that in hippocampal brain slices from naked mole-rats the increase in internal calcium was significantly reduced compared to both neonatal and weanling mice.

Lastly, we wanted to find out if the adult naked mole-rat brain retained more of the NMDA receptor subunit NR2D into adulthood compared to adult mouse brain. This subtype

closes during hypoxia and is usually expressed more in neonatal mammals than in adults. We revealed that adult naked mole-rats do have a higher proportion of the NR2D subunit in adulthood compared to mice.

These finding lead us to conclude that naked mole-rats are retaining neonatal protective characteristics against hypoxia.

1. INTRODUCTION

1.1 Adaptations to extreme environments

The ability of living organisms to adapt to a wide variety of extreme environmental conditions has resulted in life all over the globe, including places of extreme temperature, salinity, and pH. The microbe *Thermus aquaticus* is able to live in the 80°C pools at Yellowstone Park (Brock and Freeze, 1969). *Mesenchytraeus solifugus*, is a species of ice worm, and as the name suggests, they can live in glaciers and snowfields because their cells and enzymes can function in low temperatures (Napolitano, et al., 2004). The brown tree frog of New Zealand is able to freeze its body solid during the winter and defrost itself once spring comes (Bazin, et al., 2007). Ice fish, which includes several different species, live in the seas of the Antarctic that would freeze other fish (Burton and Burton, 1970). Many bat species have a tolerance for inhaling ammonia levels toxic to other animals (Studier and Fresquez, 1969).

In order to survive in conditions that we would consider hazardous, these animals have evolved important adaptations to overcome environmental challenges. For example, *Thermus aquaticus* has a thermostable enzyme, fructose-1,6-diphosphate aldolase, that can maintain function at temperatures that would degrade enzymes in most other animals (Freeze and Brock, 1970). *Mesenchytraeus solifugus* has high levels of ATP and it increases energy levels in response to drops in temperature (Napolitano, et al., 2004). Several frogs that freeze protect their organs by dehydration and increasing levels of glucose to prevent crystallization (Costanzo, et al., 1993). Ice fish have blood that lacks hemoglobin but contains a kind of protein antifreeze (Eastman and Lannoo, 2004). Animals that are ammonia-tolerant have special adaptations in the respiratory tract that change the interaction between ammonia and the mucous layer, or they have ways of neutralizing it (Studier and Fresquez, 1969).

Here I examine the naked mole-rat, which lives in a chronically low oxygen environment. I explore the tolerance of brain tissue and hope to uncover some of the mechanisms behind it.

1.2 **General background of naked mole-rats**

Naked mole-rats, *Heterocephalus glaber*, are indigenous to northern East Africa, where they live in sealed subterranean burrows. They have lived in this environment since the early Miocene Era (Brett, 1991; Bennett and Faulkes, 2000). They are rodents, roughly the size of mice, with short limbs that allow them to go forward and backwards at equal speeds. Like many subterranean animals, naked mole-rats are blind. As the name suggests, the animals have evolved to be almost entirely hairless. However, they have retained ten rows of body hairs to detect objects around them (Crish et al., 2003). They use their constantly growing strong teeth, which protrude through the skin above and below their lips, to burrow their tunnel systems (Park et al., 2010).

Naked mole-rats are the only known poikilothermic mammals (Buffenstein and Yahav, 1991). They have a unique eusocial lifestyle similar to that of bees and termites, with only one or two breeding female queens (Jarvis, 1981). It is not uncommon for a naked mole-rat to live an extraordinarily long life up to 30 years (Buffenstein, 2005; Bennett and Faulkes, 2000), and they also show a profound resistance to cancer (Seluanov et al., 2009; Liang, et al., 2010).

1.2.1 **Adaptations in the naked mole-rat to a harsh environment**

The African naked mole-rat displays a number of adaptations for living in a habitat where food sources are scarce and where water is very limited for extended periods of time.

Likewise, they live in areas in which oxygen levels are extremely low and the carbon dioxide levels are markedly higher compared with typical living conditions (Bennett and Faulkes, 2000).

Naked mole-rat foraging can best be described by referring to the "food-aridity" hypothesis: Animals that burrow in hardened soil, where food sources are patchy, have difficulty surviving in small groups. Therefore, the animals must cooperate for foraging purposes – a system that promotes reproductive altruism and communal care of offspring (Bennett and Faulkes, 2000).

Specific to surviving in this harsh environment, the kidneys of this species are adapted to concentrate and retain the little water it extracts from the rare roots and tubers it finds (Urison and Buffenstein, 1994). They have an unusually low body temperature and metabolic rate (Gesser, et al., 1977). Their hemoglobin has a higher affinity for oxygen that helps it survive in an environment with limited food and oxygen. This hemoglobin is able to unload oxygen in the tissue because NMR have an altered concentration of 2,3 BPG. (Johansen, et al., 1976).

In addition to the physiological and behavioral adaptations mentioned above, profound adaptations in central and peripheral nervous systems recently have been reported. These include peripheral insensitivity to acidosis (Park, et al., 2008). In fact, naked mole-rats lack a sense of pain from such chemical irritants as capsaicin and ammonia, and they are absent a sense of inflammatory pain (Park, et al., 2008; LaVinca, et al., 2009). In the central nervous system naked mole-rats show a remarkable resistance to low levels of oxygen (Larson and Park, 2009). These putative adaptations make the naked mole-rat well-suited to a high-carbon-dioxide (acidic) and low-oxygen environment.

Naked mole-rats experience extremes in carbon dioxide and oxygen because of the unique combination of living a completely subterranean life and in groups comprising hundreds

of individuals. These two factors – many animals living together and breathing the same poorly ventilated air – lead to an environment that is high in carbon dioxide and low in oxygen (Bennett and Faulkes, 2000). The focus of my research is to explore the nature of the adaptations that make brain tissue from naked mole-rats resistant to low oxygen.

1.3 **Oxygen use and hypoxic injury**

Oxygen is needed by all cells in the body to produce adenosine triphosphate (ATP), the energy molecule of the body. The electron transport chain, which takes place by a series of protein complexes in the mitochondria, transfers electrons from an electron donor, NADH, to an electron acceptor, oxygen, which is then reduced to water. This is done to transfer protons across the matrix membrane. This creates an electrochemical proton gradient across the membrane. The protons can then flow back across the membrane (down the concentration gradient) through the ATP synthase enzyme. ATP synthase uses this proton flow to force part of the enzyme to rotate, which phosphorylates ADP to ATP (Alberts et al., 2010).

In brain cells from rats and mice, a drop in the oxygen level can lead to cell death over several steps and multiple routes (Bickler and Donohoe, 2002; Bickler, 2004). Without oxygen, ATP levels rapidly decline, which halts all metabolic activity (Erecinska and Silver, 2001). Since the maintenance of the membrane potential relies on ATP-dependent ion pumps, the cell membrane will depolarize. This causes an excess influx of sodium and water into the cell, causing the cell to swell (Hansen, 1985). There is also a calcium influx through voltage-gated calcium channels. As the sodium gradient is lost, sodium-glutamate cotransporters release glutamate into the extracellular space (Rossi and Brown, 2000). This glutamate then activates postsynaptic glutamate receptors, particularly N-methyl-D-aspartate (NMDA) channels, which

leads to a glutamate cascade. The activated NMDA channels are responsible for a significant part of the calcium influx. Since calcium in large amounts is toxic to the cell, the cell often experiences calcium-dependent cell injury or cell death (Deshpande et al, 1987; Lee et al, 1991; Lipton, 1999).

1.4 **Oxygen homeostasis and adaptation mechanisms**

There are many regulatory factors to control the oxygen distributed all over the body. If oxygen becomes too low (hypoxia) there won't be enough ATP generated to maintain metabolism. However, if there is too much oxygen (hyperoxia) then reactive oxygen species (ROS) are formed which can cause damage to DNA. The lung epithelium is one place where oxygen levels can be sensed and it is able to produce mucus, cytokines and chemokines, hormones, growth factors, and enzymes in response to damage (Haddad, 2002). Also, the heme protein can act as an oxygen sensor to activate genes (Kietzmann et al., 1992; Hochachka et al., 1996). There are many oxygen sensitive ion channels, receptors, transcription factors and neuromodulators (Bickler and Donohoe, 2002), a few of the well studied examples are discussed below.

1.4.1 **Hypoxia-inducible factor 1 (HIF-1)**

HIF-1 is a basic helix-loop-helix transcription factor that is expressed in all mammalian cell types. Its expression is increased as oxygen is decreased, because it is hydroxylated by oxygen-dependent propyl hydroxylases and targeted for proteolytic destruction under normoxia. Therefore, it becomes stabilized under hypoxic conditions (Wang et al., 1995; Wenger 2002). HIF-1 regulates many genes, such as the vascular endothelial growth factor (VEGF) and

erythropoietin (EPO). The EPO gene mediates increased oxygen delivery to cells and mediates adaptations in the cell to the lower oxygen level, for example increasing glucose transporters and glycolytic enzymes (Beck et al., 1993; Iyer et al., 1998; Wenger, 2002). HIF-1 is also required for embryonic development and used by tumor cells to grow.

1.4.2 **Nuclear Factor-κB**

NF-κB is a dimeric transcription factor that regulates a large number of genes associated with the immune and inflammatory response. It can be activated by cellular stresses, such as cytokines, radiation, and oxidative stress (ROS). It was first discovered for its role in regulating the expression of the immunoglobulin κ light chains in B lymphocytes. NF-κB are sequestered inside the cell next to the IκB protein, which gets degraded upon activation. This exposes the nuclear localization signal on NF-κB allowing it to enter the nucleus to activate its target genes. Therefore, NF-κB plays a critical role in the early events of the molecular response to ROS (Haddad, 2002).

1.4.3 **Adenosine**

The vertebrate brain has several mechanisms to protect itself from toxic events from hypoxia (and other stressors) on a very short-term basis. One of these mechanisms is the release of adenosine.

Adenosine is a neuromodulator that can work presynaptically and postsynaptically. It is released during times of physiological stress, such as hypoxia. There are four known adenosine receptors, A1, A2A, A2B, and A3. They are G-coupled protein receptors. The A1 subtype is the primary receptor responsible in inhibiting nerve cells in the central nervous system and is most

likely linked to a variety of G proteins. One G protein linked to the A1 receptor primarily inhibits the activity of adenylyl cyclase causing the levels of cAMP in the cell to decrease. The A1 receptor is also shown to be coupled to ion channels, such as potassium (Haas and Greene, 1984) and to phospholipase C. Another action of the A1 receptor is to inhibit the release of transmitter at the presynaptic terminal and to reduce neuronal activity (Dunwiddie and Haas, 1985; Latini and Pedata, 2001). The exact mechanisms are unknown and probably vary greatly depending on where the receptor is located. Mechanisms have been shown to differ between presynaptic or postsynaptic location, brain regions, and species. However, many of the effects of adenosine have been identified.

When adenosine receptors are blocked in the turtle brain, the concentration of external potassium increases rapidly to a plateau, which depolarizes the cell. This is because adenosine acts on the potassium channel responsible for potassium efflux and causes it to arrest, which leads to decrease potassium leakage. During anoxia, the turtle brain slice shows evidence that the potassium flux is significantly reduced as well as a decrease in the internal concentration of calcium mediated through NMDA receptors (Pek and Lutz, 1997). Adenosine plays a role in the decreased activity of NMDA receptors by decreasing the open probability time by acting though adenosine receptors (Bickler, 1998).

Adenosine also plays a role in the mammalian brain. By activating A1 receptors presynaptically, adenosine works to inhibit neurotransmitter release in excitatory synapses but not inhibitory ones (Katchman and Hershkowitz, 1993). The mechanism is unknown for how the release is modulated, but the action can be reduced by activation of protein kinase C. By activating A1 receptors postsynaptically, adenosine hyperpolarizes the cell. It also works to

enhance the slow conductance potassium current after hyperpolarization and the accommodation of firing (Fredholm and Dunwiddie, 1988).

1.5 **Hypoxia tolerant model systems**

The mechanisms described above can protect brain tissue from the harmful effects of transient hypoxia, but only for a short period of time in most cases. However, there are several animal species that have evolved to withstand much longer periods of hypoxic conditions and presumably have mechanisms to increase protection even more. For example, certain types of fish experience hypoxia when shallow ponds freeze over. Several species of frogs experience some hypoxia when they are in estivation. During this time the frogs reduce their metabolism, rely on lipid oxidation for energy, and retain water through use of cocoons and urea accumulation (Storey KB, 2002). Many of the hypoxia tolerant model system that have been studied in terms of mechanisms of protection in the brain are outlined below.

1.5.1 **Turtle**

Several species of turtles have been found to be hypoxia tolerant and have been studied in great detail. For example, the Western painted turtle can survive anoxia for 5 months at 1-3°C during winter dormancy (Bickler and Donohoe, 2002), and the fresh water turtle, *Trachemys scripta,* can withstand anoxia for days (Lutz and Milton, 2004). This is accomplished by creating a state of deep hypometabolism.

Turtles have complex and involved mechanisms to be able to withstand low levels of oxygen that start with their normoxic state. During normoxia, they maintain a high glycogen energy store that is fivefold greater than the rat's store (Lutz et al., 2003). They also have lower

enzyme activities and ion channel densities compared to mammals. For example, the voltage-gated sodium channel has around a third of the density (Edwards et al., 1989). However, the density of inhibitory receptors, like GABAa, are similar to the rat (Lutz and Leone-Kabler, 1995) and the δ-opioid receptors are four times the density of mammals, which is thought to help protect against glutamate excitotoxicity (Xia and Haddad, 2001). They also have high normoxic levels of NF-κB, which helps recover from trauma, and heat shock protein 73 (Hsp73), which protects against denaturing of proteins from hypoxia damage (Lutz and Prentice, 2002; Snoeckx et al., 2001).

In addition to this, turtles have even more methods for survival during anoxic periods. First, they decrease the demand for ATP and then are able to maintain survival at basal levels of ATP for the long-term. They are able to suppress ATP use by 70-80% so that all energy needs can be met by anaerobic glycolysis (Hochachka and Lutz, 2001). Ion channels are down regulated. Potassium flux is reduced by 50% in the first hour of anoxia and continues to fall to 35% of the normoxic level by the second hour, which is mediated by adenosine and the activation of Katp channels by the depletion of ATP, which increases the conductance of potassium to hyperpolarize the cell (Chih et al., 1989; Pek-Scott M, Lutz PL, 1998), and voltage-gated sodium channels are decreased by 42% (Perez-Pinzon et al., 1992). NMDA receptor activity is reduced by 50-60% in the first 8 minutes of anoxia by dephosphorylating the receptor, which prevents excess calcium influx that can lead to cell injury. This is mediated by phosphatase 1 or 2A and adenosine (Bickler et al., 2000; Bickler and Donohoe, 2002; Buck and Bickler, 1998). In order to prevent the increase in extracellular glutamate, turtles prevent vesicular release with adenosine and continue the use of the glutamate uptake transporters by

opening Katp channels which hyperpolarize the neurons (Nilsson and Lutz, 1991; Milton and Lutz, 1998; Milton, 2002).

These things combine to depress the neural activity. However, the activity does not stay depressed constantly during an extended time of hypoxia/anoxia. There are periodic short burst of brain activity that vary in frequency. These burst are probably regulated by the neurotransmitters that are continued to be release and uptaken to maintain the integrity of the circuitry and to check whether oxygen has been made available (Milton, 2002; Milton and Lutz 1998).

Once oxygen becomes available, the neuronal activity is rapidly restored. ROS are a potential problem during reoxygenation. ROS are very reactive and can cause damage to DNA, oxidize lipids and proteins, and can lead to apoptosis. Turtles protect against this damage, they are able to use the high level of NF-κB they maintain during normoxia (Lipton, 1999; Lutz et al., 2003; Lutz and Milton, 2004).

1.5.2 **Hibernating ground squirrel**

Arctic ground squirrel, when hibernating (hAGS) and awake, termed interbout euthermic (ibeAGS), can survive short durations (30 minutes) of oxygen and glucose deprivation (OGD), however, hAGS's tolerate it better (Christian et al., 2008). In their natural habitat, hAGS survive through major decreases in cerebral blood flow during times of hibernation and can return to a normal flow without neurological damage. AGS tolerance to low oxygen has been studied in great detail and it has been found that it involves a modification of NMDA receptors during times of hibernation to decrease activity (Ross et al., 2006).

The distribution of the NMDA receptor subunit 1 (NR1), which is common to all NMDA receptors, in the AGS is similar to other species throughout the central nervous system. There is also not a difference between ibeAGS and hAGS NR1 expression and distribution, suggesting NMDA receptors are modified by another means (Zhao et al., 2006b). The NR1 protein abundance is similar in hAGS and ibeAGS in membrane fractions; therefore, internalization of NMDA receptors does not contribute to the down regulation of NMDA receptor activity in hAGS (Zhao et al., 2006a). Phosphorylation of the NMDA subunit NR1 is known to enhance NMDA receptor activity (Liu and Zhang, 2000). The hAGS has significantly less phosphorylation of the NR1 subunit than ibeAGS or rat. A decrease in NMDA receptor phosphorylation is likely to contribute significantly to the down regulating effect (Zhao et al., 2006a). This strategy is also used by turtles, neonatal rats, and newborn piglets (Bickler, 1998; Mishra et al, 2001; Fritz et al., 2002). The amino acid Ser897 of the NR1 subunit is phosphorylated by PKA. Thus, PKA activity might be lowered or serine phosphatases activity might be higher in hAGS. Further evidence showed AP5, a competitive NMDA receptor antagonist, did not reduce the glutamate-induced rise in the concentration of internal calcium in hippocampal slices in hAGS. This suggests that the glutamate-induced increase in internal calcium was not through NMDA receptors (Zhao et al., 2006b).

AGS neurons survive oxygen-glucose deprivation even though there was a rapid ATP depletion. They also survived independent of hibernation state or season. Therefore, protective mechanisms are downstream from ATP loss. It was also shown that they do not rely on glycogen or oxidative phosphorylation to meet energy demands, however they may have high levels of high-energy phosphates. The survival of these neurons is still unknown but appears to involve improving the maintenance of the cellular homeostasis by keeping the levels of certain

mitogen-activated protein kinases (MAPK) important for survival at baseline (Christian et al., 2008).

1.5.3 **Diving Seals**

Seals are capable of going on long dives up to 2 hours long. Seals use a high concentration of hemoglobin in blood and myoglobin in muscle to increase the capacity to store oxygen as a way to cope with hypoxia (Lenfant et al., 1970). In one of the first studies on hypoxia tolerance in seals, harbor seals and dogs were put through forced dives while critical arterial and cerebral venous oxygen tensions were measured and while being monitored with an EEG. The dive lasted until the EEG rhythm consisted mainly of the appearance of low frequencies, an endpoint that has been established for acute asphyxial hypoxia in laboratory mammals. It was shown in the harbor seals that when in forced dives they have the same residual blood oxygen content as the dog at the endpoint of the EEG. However, the seals reached critical levels of hypoxia five minutes before the endpoint meaning there is some cerebral tolerance to hypoxia (Kerem and Elsner, 1973). In other words, brain activity continued even after blood oxygen levels were exhausted, suggesting seals had neural mechanisms of protection in addition to their high concentration of hemoglobin.

This cerebral tolerance is reflective of intrinsic properties of the neurons. This was further shown in adult hooded seals. Changes in membrane potential during hypoxia were measured using intracellular recording from the pyramidal layer of isolated visual cortex slices. During normoxia, mice and seals have similar membrane potentials around -60 to -70 mV. However, during ten minutes of hypoxia, mice cells depolarized by around 65 mV whereas the seal cells only depolarized by around 13 mV (Folkow et al., 2008).

There are some cellular differences in neurons from seals that contribute to their hypoxia tolerance. Levels of neuroglobin (Ngb), a globin similar to hemoglobin, that is expressed in neurons, did not differ in brains from seals, rats, and mice. However, in the seals, Ngb, along with cytochrome c, was located primarily in astrocytes unlike in mice and rats were they almost exclusively reside in neurons. This may help to further maintain the supply of oxygen to the cell as it has been shown to do in some hypoxia-tolerant mollusks. This set up could also protect the neurons from ROS generated during oxygen metabolism (Mitz el al., 2009).

1.5.4 **Fruit Flies**

The fruit fly, *Drosophila melanogaster,* can survive in anoxia for up to 5 hours without suffering from morphological abnormalities and can then go on to behave normally in their life cycle (Haddad, 2006). Because of this ability, experimenters wanted to see if they could raise flies to live chronically in lower levels of oxygen, then determine how the genes of these flies were altered. The experiment started at a level of 8% oxygen, since most flies can survive at this level. After living at this level for 3-5 generations, the oxygen level was dropped by 1%. It was continued in this way until an oxygen level of 4% was reached. The phenotype for these flies included a decreased recovery time from anoxic stupor, an increased rate of oxygen consumption in hypoxia, and decreased body size and mass due to decreased cell number and size (Zhou et al., 2007). To determine if this hypoxia tolerance was a stable heritable trait the flies were put back into normoxia for 8 generations then re-exposed to the 4% oxygen environment. 80% of the flies still survived the low oxygen environment indicating that the genes are altered in a heritable way. By analyzing the genes of these flies compared to wild type flies it was found that mostly genes

encoding for proteins related to immunity were up-regulated and mostly genes encoding proteins related to metabolism were down-regulated (Zhou et al., 2008).

1.5.5 **Neonatal mammals**

It has been known for decades that infant mammals are tolerant to hypoxia during the embryonic and neonatal periods. In mice this tolerance persist for about one week after birth (Adolph, 1948). Neonatal animals have to be able to withstand hypoxia, since brain tissue oxygen tension is around 10 mm Hg in utero. Once the animal is born the brain tissue oxygen tension increases to 30 mm Hg (Bickler et al., 2004). Consequently, the neonatal animal must have many mechanisms to avoid the negative effects from hypoxia.

Neonatal mammals have a slowed rate of ATP depletion during hypoxia. One reason for this could be that because they have smaller, less branched neurons that make fewer synapses, which leads to a lower energy demand (Thurston and McDougal, 1969). More recently, it was found that neonates produce more ATP anaerobicly, reduce ATP utilization, and reduce their sensitivity to glutamate to slow intercellular calcium increases during hypoxia compared to adult (Hochachka et al., 1996; Bickler et al., 1993). Neonatal neurons also better retain sodium gradients during anoxia (Jiang and Haddad, 1992), which helps prevent the release of glutamate through the sodium-dependent re-uptake transporters that happens during hypoxia in adults (Szatkowski and Attwell, 1994; Rossi and Brown, 2000). Less glutamate is released in neonates than mature animals in general (Bickler and Hansen, 1998).

In addition to these protective mechanisms, neonatal mammals also have more NMDA receptors with the subunit NR2D, which has a smaller percentage of open time when in a hypoxic environment; thus, it also leads to a decrease in internal calcium accumulation (Bickler

et al., 2003). A key feature in reducing calcium entry in neonates during hypoxia involves the structure of the NMDA receptor, which changes with age. NMDA receptors are ion channels permeable to monovalent cations and calcium. Activation requires the binding of glutamate and glycine, and there is an additional magnesium block at resting membrane potential. NMDA receptors are made up of one NR1 subunit combined with one or more NR2 or NR3 subunits. There are 8 different splice variants of the NR1 subunit, 4 different NR2 subunits, and 2 different NR3 subunits. The subunit profile changes as the animal develops and ages (Collingridge and Watkins, 1994). The NR2 subunit profoundly controls the biophysical and pharmacological activity of the receptor (Takahashi et al., 1996; Waters and Machaalani, 2004). The subunits NR2B and NR2D are highly expressed in neonatal brains. As the animal matures, these subunits are replaced with high expression of NR2A and NR2C (Monyer et al., 1994). These subunits also vary in expressions between brain regions in adult rat brain. NR2A is expressed throughout the adult rat brain. NR2B is primarily expressed in the forebrain. NR2C is dominant in the cerebellum. And lastly, NR2D is predominantly expressed in the thalamus, midbrain, and brainstem, as well as some expression in the cortex and hippocampus (Dunah et al., 1996). However, NR2D declines significantly in expression with age (Wenzel et al., 1996). This pattern of expression is similar among brains from mouse, rabbit, frog and humans (Laurie et al., 1997).

During the developmental change in the NR2 subunit profile, there is a decrease in tolerance to hypoxia (Vannucci and Hagberg, 2004). As mentioned, adult brains are vulnerable during times of hypoxia because NMDA channels are over-activated due to glutamate release causing a large calcium influx into the cell, which can lead to cell death. However, it has been found that neonates actually had less intracellular calcium present during hypoxia compared to adult brains (Friedman and Haddad, 1993; Bickler and Hansen 1998). This is interesting because

neonates have more intracellular calcium present when the NMDA receptors are activated by only NMDA application in a non-hypoxic environment. Also, normal NMDA activity in the developing brain is high. NMDA receptors with the NR2D subunit had a reduced current during hypoxia due to a decrease in the time the channel was open. The reduced time the channel is open leads to a smaller increase in internal calcium and, ultimately, less cell death. This explains at least one reason why neonatal animals are more resistant to hypoxia (Bickler et al., 2003).

Neonatal and adult NMDA receptors are different in many other ways as well. The neonatal NMDA receptor profile, in comparison to the adult NMDA receptor profile, has a lower sensitivity to the magnesium channel block, a higher sensitivity to glycine, longer EPSPs, increased ability to induce markers of synaptic plasticity like LTP, as well as an increase of the calcium influx during excitotoxicity mentioned before (McBain et al., 1994; Scheetz and Constantine-Paton, 1994; Aamodt and Constantine-Paton, 1999). Hypoxic injury leads to cell death in both ages, but apoptotic death predominates in neonatal neurons, while necrotic death predominates amongst mature neurons exposed to hypoxia (Johnston et al., 2001). The neonatal brain also downregulates the total number of NMDA receptors during hypoxia and creates changes in receptor binding, making the receptor more difficult to activate, after the onset of hypoxia (Tingley et al., 1993; Waters and Machaalani, 2004).

1.5.6 **Naked mole-rats**

In a previous experiment from our lab, tolerance to hypoxia in brain slices was quantified in naked mole-rats by using a hippocampal slice preparation (Larson and Park, 2009). This is a well-established preparation for assessing responses to low oxygen because the hippocampus is highly metabolic but low in energy stores and, therefore, vulnerable to low

oxygen (Mitani et al., 2005). These experiments showed that naked mole-rat brain tissue retains functionality under low oxygen conditions for much longer than brain tissue from mice. First, hippocampal slices from a mouse and a naked mole-rat were recorded under anoxia, where oxygen is replaced with nitrogen in the bath and recording chamber. In order to determine if the tissue remained responsive, EPSPs were recorded in the CA1 region of the hippocampus while the Schaffer-commissural fibers were stimulated at 20-second intervals. The mouse responses declined rapidly and synaptic field potentials ceased a short time after the onset of anoxia. In contrast, the naked mole-rat response slowly declined after the onset of anoxia and maintained the ability to generate synaptic field potentials throughout the entire anoxic period, however the field potentials were reduced. The tissue also was able to recover after the anoxic period. It was further shown that it took an average of 40 minutes to abolish synaptic field potentials in the naked mole-rat, but it took only 12 minutes in the mouse.

Temperature effects were also examined because body temperatures are different for naked mole-rats and mice and because temperature is known to greatly affect responses to anoxia (Morris et al., 1991). In its burrows in Africa, naked-mole rat body temperature is around 30°C, whereas a mouse's body temperature is around 35°C. The data so far was taken at 30°C, but similar data were also collected at 35°C. Both the naked mole-rat and the mouse showed a more rapid decline in function at 35°C, but the difference between species remained proportional. The data is even more remarkable when the animals are compared under their respective body temperature.

It is hypothesized that the neonatal model system might be the most relevant to the naked mole-rat. There are many similarities between naked mole-rats and neonatal mammals. Since naked mole-rats live a eusocial lifestyle, most animals never become sexually mature.

Characteristics related to hypoxia tolerance that both neonatal mammals and naked mole-rats have include insensitivity to adenosine and lack of paired-pulse facilitation. Larson and Park (2009) applied an adenosine receptor antagonist to the bath to test if naked mole-rats also used this mechanism. They found that the antagonist did not reduce naked mole-rat's tolerance to anoxia. Thus, excessive adenosine release is not a mechanism used by naked mole-rats to avoid hypoxic injury. In fact, the naked mole-rat was less sensitive to adenosine effects than mice.

Another similarity between naked mole-rats and neonatal mice is the lack of the basic synaptic phenomenon of paired-pulse facilitation. In most species tested, stimulating the brain with two depolarizing electrical pulses 200 ms apart will cause the response to the second pulse to increase relative to the response to the first pulse. This is because the first electrical pulse depolarizes the cell, which activates voltage-gated calcium channels creating a calcium influx that will allow for the release of a neurotransmitter. Shortly after the calcium enters the cell it is sequestered into internal stores as well as pumped out of the cell. If the second pulse occurs before the majority of the calcium can be removed from the cytoplasm, the calcium from the second pulse will add to the calcium already present leading to a facilitation of neurotransmitter release.

1.6 **Current study**

The purpose of the current study is to further explore the mechanisms that allow for hypoxia tolerance in the naked mole-rat. The current hypothesis is that naked mole-rats retain many of the protective characteristics of neonates into adulthood.

The current study will determine (1) if adult naked mole-rat brain has a higher ATP store, and if it is depleted slower during hypoxia compared to adult mouse brain, (2) if naked mole-rat

brain has a reduced internal calcium concentration during hypoxia compared to mouse brain, and (3) if naked mole-rat brain retains a neonatal-like NMDA receptor profile into adulthood.

The following three chapters are manuscripts to be published addressing each of these topics.

2. Adult Naked Mole-rat Brain Shows Slowed ATP Loss During Complete Ischemia

To be submitted to Brain Research

2.1 Abstract

Brain slices from adult African naked mole-rats show an extreme tolerance to hypoxia, although the underlying mechanisms of protection are not fully known. The goal of the present study was to determine if ATP loss during hypoxia was slowed in brain tissue from naked mole-rats as is the case for two other models systems of hypoxia tolerance, freshwater turtles and neonatal mammals. We found that brain tissue from adult naked mole-rats retained appreciable levels of ATP longer than brain tissue from adult mice (≥ 10 min for naked mole-rats versus ≤ 5 minutes for mice). The present results support our working hypothesis that slowed or arrested brain development (neotany) accounts in part for hypoxia tolerance in adult naked mole-rat brain.

2.2 Introduction

Brain tissue from naked mole-rats has been shown to be extremely tolerant to hypoxia (Larson and Park, 2009; Peterson et al., 2011a), a feature well suited to their challenging environment. Naked mole-rats live in eusocial colonies of hundreds of animals in a completely subterranean lifestyle (Jarvis, 1981). The combination of many individuals sharing the same limited air supply leads to a chronically low oxygen environment (Brett, 1991; Bennet and Faulkes, 2000).

Naked mole-rats are known to have two major homeostatic adaptations that are advantageous for living in chronic hypoxia. Their hemoglobin has an unusually high affinity for oxygen (Johansen et al., 1976), and their weight-specific metabolic rate is about one-third less than that of other rodents (Buffenstein and Yahav, 1991). Hypoxia tolerance in *in vitro* brain

slices indicates additional adaptations for living under chronic hypoxia. Hippocampal brain slices from naked mole-rats maintain synaptic transmission under low oxygen concentrations that cause function to cease in slices from mice. And, under anoxia (nominally zero oxygen), slices from naked mole-rat maintain function 3 to 4 times longer than slices from mice (Larson and Park, 2009). In additional experiments, also using the hippocampal brain slice technique, we found that the increase in internal calcium concentration from hypoxia was reduced in naked mole-rat compared to mice (Peterson et al., 2011a). This is important because calcium toxicity is a major factor in hypoxia-associated cell damage and cell death. Naked mole-rats also retain more of the NMDA receptor subtype NR2D into adulthood compared to mice (Peterson et al., 2011b). NMDA receptors with NR2D subunits have a higher percent close time during hypoxia compared to receptors without NR2D (Bickler et al., 2003). Again this relates to calcium because NMDA receptors pass toxic amounts of calcium during hypoxia. In mammals in general, the higher proportion of NR2D found in neonates is thought to be a major contributor to the hypoxia tolerance of neonatal brain tissue.

Hypoxia tolerance, a reduced calcium effect during hypoxia, and the presence of a high proportion of NR2D are characteristics that adult naked mole-rats share with neonates of other mammalian species. These shared characteristics, and others (Larson and Park, 2009) have led us to propose that the hypoxia tolerance of naked mole-rat brain may reflect a slowed or arrested development in this species. In addition to the characteristics listed above, neonatal mammalian brains have a reduced utilization and depletion rate of ATP during hypoxia compare to adult brains (Thurston and McDougal, 1969; Duffy et al., 1989; Kass and Lipton, 1989; Bickler et al., 1993). Therefore, the goal of the present study was to determine if ATP depletion during hypoxia in naked mole-rat brain tissue was similar to that of neonatal mice versus adult mice.

During severe hypoxia or ischimia, ATP is one of the first things that is affected. Once levels of ATP have dropped, a cascade of events begins that ultimately can lead to cell death. Brain tissue uses ATP to maintain ion gradients, therefore once ATP levels decline the neuron depolarizes, causing an excess influx of sodium and water into the cell. As the sodium gradient is lost, sodium-glutamate co-transporters reverse and glutamate is pumped into the extracellular space (Rossi and Brown, 2000). The excess glutamate activates glutamatergic receptors, such as NMDAR and AMPAR. There is an influx of calcium through the NMDA receptors, which is toxic to the cell (Lipton, 1999; Bickler, 2004). Some hypoxic tolerant model systems slow the decline of ATP during hypoxia: turtles and neonatal mammals (Hochachka and Lutz, 2001; Bickler et al., 1993). However, not all hypoxia model systems are able to prevent ATP decline, such as the hibernating ground squirrel (Christian et al., 2008).

In the present study we measured ATP in adult naked mole-rat brain exposed to different durations of complete ischemia, and compared the data to that from adult and neonatal mice. In addition, we performed a simple *in vivo* experiment to document anoxia tolerance in intact adult naked mole-rats. This is because even though naked mole-rats show tolerance *in vitro*, and there are anecdotal accounts about tolerance in whole animals, to our knowledge there have thus far been no quantitative reports.

2.3 **Materials and Methods**

2.3.1 **Animals**

Experiments were performed on male and female C57BL/6 mice (bred from stock obtained from Charles River Laboratories, Wilmington, MA) and naked mole-rats of both sexes (born in colonies maintained in our laboratories) housed under normoxic laboratory conditions.

Experiments were conducted on adult mice (>2 months), neonatal mice (postnatal day 6), and adult naked mole-rats (>1 year). Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

2.3.2 ***In vivo*** **tolerance to anoxia**

Animals were placed into a standard mouse cage and lid with a combined volume of 10.5 liters. The cage was prefilled with 100% nitrogen via a tube connected to a nitrogen tank. Nitrogen was continuously infused at 10 liters/min throughout the experiment. The filter top of the cage was sealed with aluminum foil except for a small area to allow the nitrogen to exhaust. An animal was observed until its last gasp (last respiration). We continued to apply nitrogen for an additional 20 seconds (mice) or 120 seconds (naked mole-rats), then we removed the animal from the test cage and into normal room air. Animals were video recorded throughout these procedures with a digital cam quarter for later analysis. Recordings were used to determine the precise amount of time to the animal's last gasp.

2.3.3 **Brain tissue preparation and ATP analysis**

For ATP measurement, we followed a modified version of the complete ischemia by decapitation protocol used by Lowry, et al (1964) and for tissue preparation we followed the protocol described by Bickler et al. (1993). Animals were decapitated and the brains were quickly removed and cut in half along the midline. One half (control) was immediately denatured and stored on ice. The other half was incubated at room temperature for a specific duration of total ischemia (5, 10, 60, or 120 minutes) and then denatured and stored on ice. To denature brain tissue, the tissue was put into 10 ml of 20 mM Tris buffer (pH 7.2) at 100° C and

sonicated for 10 seconds, after sonication the tissue was left to boil for 10 minutes and then placed on ice (protocol from the StayBrite ATP assay kit from BioVision). ATP was quantified using the StayBrite ATP assay kit (BioVision). Protein measurements were made using a Bradford assay. Data is shown as average amount of ATP in picomoles per µg of protein. An N of 4 or 5 animals was used for each duration of ischemia for adult and neonatal mice and adult naked mole-rats. For statistical comparisons, we used a paired t-test where each ischemic half brain was paired with its matching control.

2.4 **Results**

2.4.*1* ***In vivo* tolerance to anoxia**

Respirations persisted for much longer in naked mole-rats compared to mice in 100% nitrogen anoxia (**Figure 1**). The average time to the last gasp for adult mice was 45.6 +/- 5.1 seconds, and for naked mole-rats it was more than five times as long, 250.5 +/-2.2 seconds ($t = 36.98$, $df = 6$, $p < 0.0001$). Remarkably, all four naked mole-rats recovered once in room air, even after remaining under anoxia for an additional 2 minutes beyond the last gasp for a grand total of about 6 minutes. None of the four mice recovered after being exposed for ~1 minute.

2.4.2 **Loss of ATP during total ischemia**

During complete ischemia, adult naked mole-rat brain showed a much slower loss of ATP compared to adult mouse brain.

The bar graph in **Figure 2A** shows picomoles of ATP per µg of protein in brain tissue from adult mice. The height of the first bar corresponds to the amount of ATP in control tissue. The other three bars correspond to the amount of ATP in tissue after 5, 10, and 60 minutes of

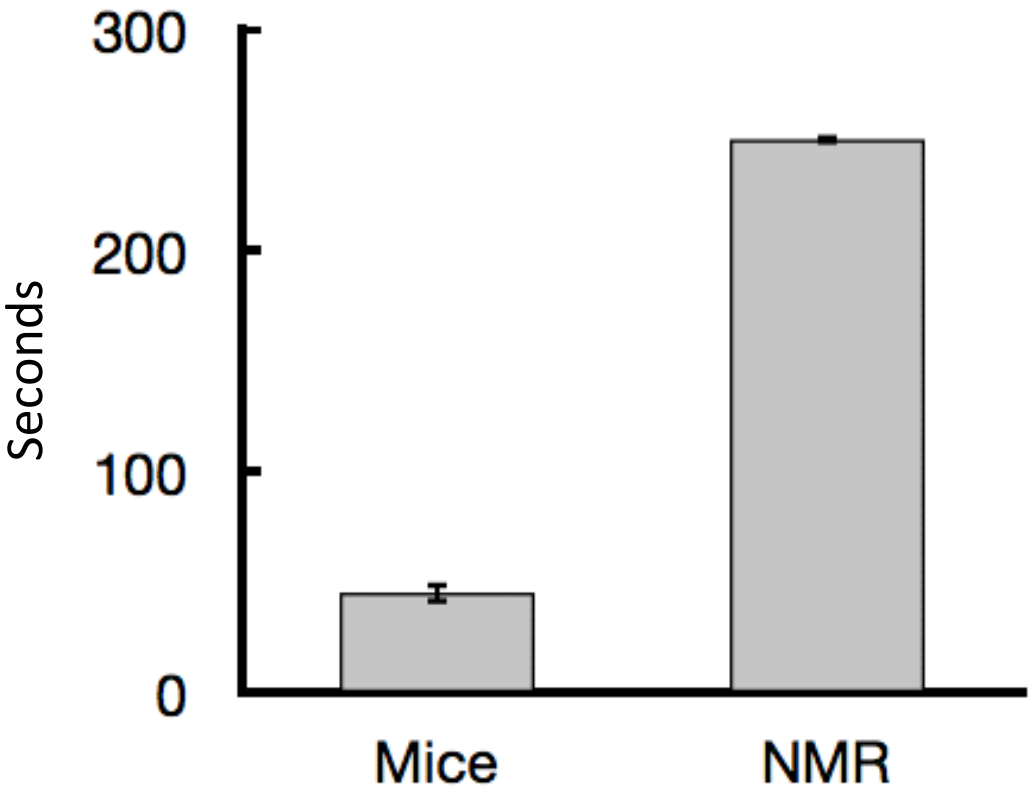
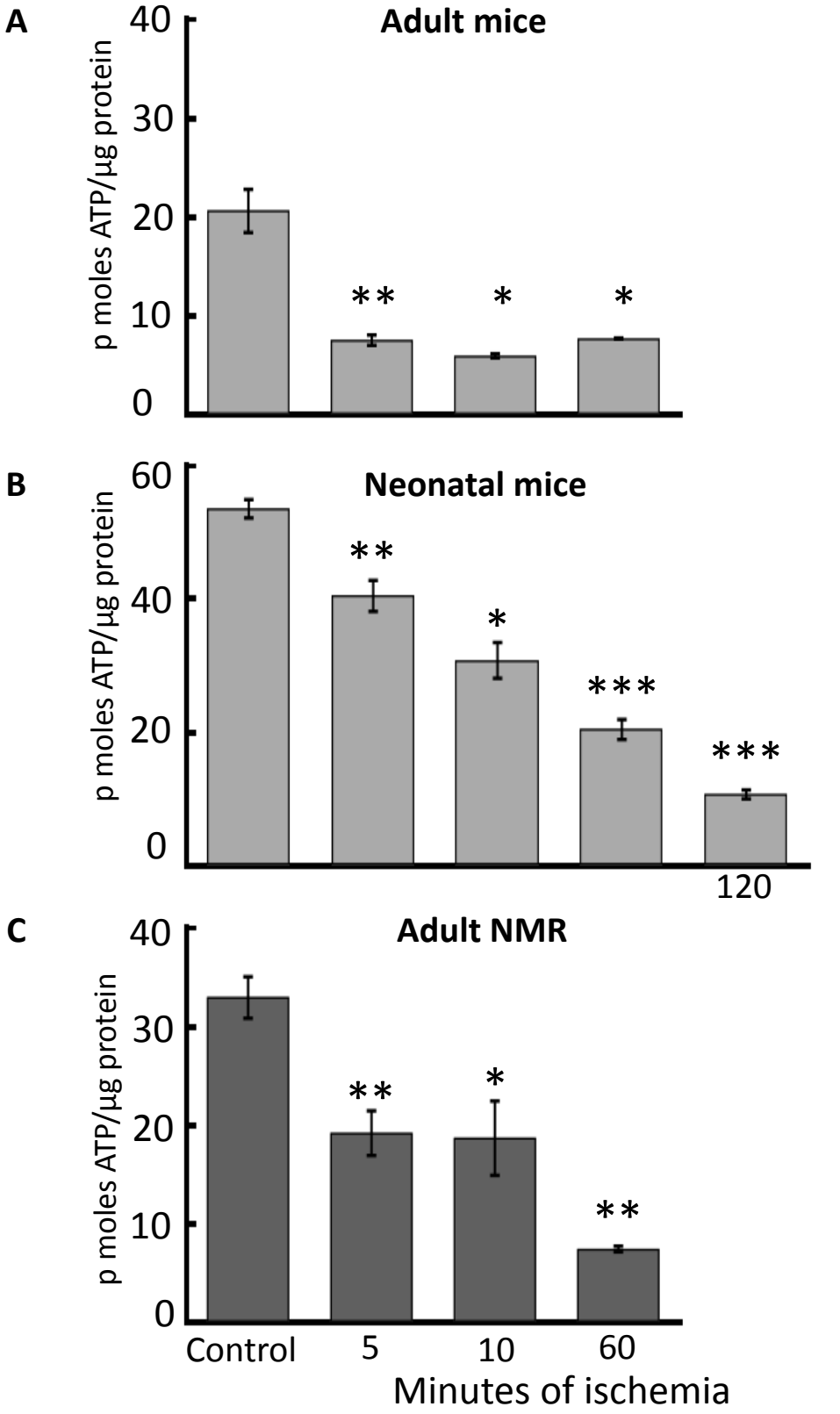


Figure 1. ***In vivo*** **anoxia, time to last gasp**. Time to last gasp in 100% nitrogen was 5 times longer for naked mole-rats compared to mice. All mice died, however, all naked mole-rats survive this treatment.

Figure 2. **ATP levels in brain tissue from mice and naked mole-rats exposed to different durations of complete ischemia**. A. Brain tissue from adult mice shows a substantial and similar loss of ATP for all durations of ischemia. B. Brain tissue from neonatal mice shows a graded decline in ATP with duration. C. Brain tissue from adult naked mole-rats shows an initial decline for 5 and 10 minutes, and then a further decline after 10 minutes. The y-axis is picomoles of ATP per μg of protein. Error bars are standard error of the mean. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$ (paired t-test, N = 4 or 5 per duration for each age/species group).


A
Adult mice
p moles ATP/µg protein
40
30
20
10
0
**
*
*
B
Neonatal mice
p moles ATP/µg protein
60
40
20
0
**
*
***
***
120
C
Adult NMR
p moles ATP/µg protein
40
30
20
10
0
**
*
**
Control
5
10
60
Minutes of ischemia

complete ischemia. There is a substantial and significant drop in ATP from control (20.7 +/- 2.4) to 5 minutes of ischemia (7.6 +/- 0.8), corresponding to a 63% decrease from control ($t = 6.41$, $df = 4$, $p < .01$). Increasing the duration of ischemia to 10 or 60 minutes did not cause further decreases in ATP suggesting that ~7 pM reflects a basal level of background label which is consistent with previous reports (Bickler et al., 1993; Thurston and McDougal, 1969).

Data from neonatal mouse brain tissue is shown in **Figure 2B**. The overall amount of ATP per μg of protein is greater in neonatal mouse control (53.5 +/- 1.7) compared to adult mouse control (20.7 +/- 2.4). But more importantly, changes in ATP from ischemia show a different pattern in brain tissue from neonatal mice compared to tissue from adult mice. For the time points we tested, tissue from neonates shows a progressive decrease in ATP with increasing durations of ischemia. The neonates continue to lose ATP even after 60 minutes of ischemia. These results are consistent with previous reports that, under hypoxic conditions, neonate brain shows a slowing of ATP loss compared to adult brain (Bickler et al., 1993).

Figure 2C shows the amount of ATP per protein from adult naked mole-rat tissue. The adult naked mole-rat control tissue has more ATP (33.0 +/- 2.4) than adult mouse control tissue, but not as much as neonatal mouse ($F=69.11$, $df=2$, $p< .0001$, one-way ANOVA). Five minutes of ischemia causes a significant loss of ATP (19.3 +/- 2.5 pM /μM of protein) compared to control ($t = 6.45$, $df = 3$, $P <0.01$). But this corresponds to a smaller percent decrease than we observed for adult mice exposed to ischemia for 5 minutes (42% decrease in naked mole-rat versus 63% decease in mouse). The amount of ATP present in adult naked mole-rat tissue exposed to 10 minutes of ischemia was similar to the amount at 5 minutes. However, after 60 minutes of ischemia the amount of ATP dropped to 7.5 +/- 0.5, very similar to adult mouse tissue exposed to 60 minutes of ischemia.

2.5 Discussion

The present study confirms that adult naked mole-rats show extreme tolerance to anoxia *in vivo,* and that adult naked mole-rat brain retains ATP for a longer duration of ischemia compared to adult mouse brain. Slowed ATP loss during hypoxia or ischemia is consistent with what has been reported for neonatal brain tissue. This is also consistent with our working hypothesis that naked mole-rat brain is retaining neonatal-like characteristics for hypoxia tolerance into adulthood.

ATP loss in adult naked mole-rat tissue was intermediate between the neonatal mouse and adult mouse. Interestingly, the amount of ATP we measure in control tissue for adult naked mole-rats was also intermediate between neonatal mouse and adult mouse.

Slowed ATP loss in adult naked mole-rat brain is likely not the only mechanism for hypoxia tolerance in this species. Another mechanism employed by both neonatal mammals and adult naked mole-rats involves the configuration of NMDA receptor subunits. Compared to adult mammals in general, neonatal mammals and adult naked mole-rats both have a higher proportion of the type of NMDA receptor that closes during hypoxia.

To the best of our knowledge, the naked mole-rat represents the first naturally occurring model system for hypoxia tolerance that utilizes neonatal-like features into adulthood. It would be interesting to determine ATP depletion rates and NMDA receptor types in other hpoxia tolerant species such as bats and diving seals.

3. Blunted Neuronal Calcium Response to Hypoxia in Naked Mole-Rat Hippocampus

Published in PLoS ONE

3.1 **Abstract**

Naked mole-rats are highly social and strictly subterranean rodents that live in large communal colonies in sealed and chronically oxygen-depleted burrows. Brain slices from naked mole-rats show extreme tolerance to hypoxia compared to slices from other mammals, as indicated by maintenance of synaptic transmission under more hypoxic conditions and three-fold longer latency to anoxic depolarization. A key factor in determining whether or not the cellular response to hypoxia is reversible or leads to cell death may be the elevation of intracellular calcium concentration. In the present study, we used fluorescent imaging techniques to measure relative intracellular calcium changes in CA1 pyramidal cells of hippocampal slices during hypoxia. We found that calcium accumulation during hypoxia was significantly and substantially attenuated in slices from naked mole-rats compared to slices from laboratory mice. This was the case for both neonatal (P6) and older (P20) age groups. Furthermore, while both species demonstrated more calcium accumulation at older ages, the older naked mole-rats showed a smaller calcium accumulation response than even the younger mice. A blunted intracellular calcium response to hypoxia may contribute to the extreme hypoxia tolerance of naked mole-rat neurons. The results are discussed in terms of a general hypothesis that a very prolonged or arrested developmental process may allow adult naked mole-rat brain to retain the hypoxia tolerance normally only seen in neonatal mammals.

3.2 **Introduction**

Naked mole-rats (*Heterocephalus glaber*) initially received a great deal of attention when scientists discovered that they had a eusocial lifestyle similar to that of bees and termites (Jarvis, 1981). Since then, a number of additional remarkable characteristics have been identified in this species (Edrey, et al, 2011). Naked mole-rats are the only known poikilothermic mammals (Buffenstein and Yahav, 1991), and they live an extraordinarily long life (~ 30 years, Buffenstein, 2005). Also, they lack a sense of inflammatory pain and pain from chemical irritants including capsaicin and acid (Park, et al, 2008; LaVinca, et al, 2009), and they show a profound resistance to cancer (Seluanov, et al, 2009; Liang, et al, 2010). The present study was designed to explore yet another remarkable trait of this species: extreme brain tolerance to hypoxia (Larson and Park, 2009).

Naked mole-rats are mouse-sized rodents that naturally live in large colonies of up to 290 individuals in sealed subterranean burrows in northern East Africa (Brett, 1991). Subterranean animals, in general, must cope with low ambient oxygen levels, due both to poor gas exchange from the surface through soil and to competition for oxygen with microorganisms and respiring plant roots (Arieli 1979; Buffenstein, 1996; Bennett and Faulkes, 2000). Oxygen depletion (and carbon dioxide accumulation) is even more pronounced for naked mole-rats since large groups of con-specifics huddle together in nests 1.5-2.5 m underground, competing for the same poorly-ventilated air (Brett 1991; Bennet and Faulkes, 2000).

Consistent with this environmental challenge and a long subterranean evolutionary history dating to the Miocene (Lavocat, 1978), naked mole-rats display several physiological adaptations for survival in a chronically hypoxic environment. Notably, their hemoglobin has a higher affinity for oxygen than most other mammals (Johansen et al., 1976), and their weight-specific metabolic rate is about one-third less than that of other rodents (Buffenstein and Yahav,

1991). We recently reported another characteristic consistent with evolving in a hypoxic environment (Larson and Park, 2009). We found that hippocampal brain slices from adult naked mole-rats maintained synaptic transmission at low oxygen concentrations that caused transmission to decrease or cease altogether in slices from laboratory mice. Also, in nominally zero oxygen, naked mole-rat slices maintained electrophysiological function more than three times as long as slices from mice, and frequently recovered even after an anoxic depolarization lasting several minutes.

Oxygen deprivation triggers a cascade of cellular processes in neurons, including alterations in metabolic enzymes and ion channels, release of neurotransmitters such as glutamate and adenosine, and activation of receptor-coupled signaling mechanisms (Lipton, 1999; Erecinska and Silver, 2001). A key element of the hypoxia cascade that determines whether the cellular response is reversible or, alternatively, leads to cell death is the accumulation of free intracellular calcium ions, which triggers cytotoxic mechanisms (Deshpande et al, 1987; Lee et al, 1991; Bickler, 2004). Therefore, the present study was undertaken to determine if the calcium response to hypoxia in hippocampal neurons is different in brain slices from naked mole-rats and mice. Mice were chosen as representatives of typical terrestrial mammals since they are not known to be specially adapted to hypoxia and have similar body sizes to naked mole-rats. fura-2 was used to image intracellular calcium in slices from relatively mature (postnatal day 20, P20) naked mole-rats and mice during episodes of hypoxia.

We also made measurements of calcium accumulation in slices from early postnatal (P6) mice and naked mole-rats because age is an important factor in hypoxia tolerance. It has been known for decades that embryonic and early postnatal mammals are relatively tolerant to hypoxia. This is also of interest because other data suggest that several possibly unrelated

electrophysiological processes in adult naked mole-rat brain resemble those of neonatal mice or rats (Larson and Park, 2009). Furthermore, factors identified as protective in neonatal rat brain generally act to limit intracellular calcium accumulation; such factors include levels of glutamate release (Szatkowski and Attwell, 1994; Bickler and Hansen, 1996; Rossi and Brown, 2000), NMDA receptor subunit composition (Laurie et al., 1997; Bickler et al., 2003), ATP consumption rates, and other metabolic adaptations (Bickler et al., 1993; Hochachka et al., 1996; Bickler, 2004).

3.3 **Materials and Methods**

3.3.1 **Animals**

Experiments were performed on male and female C57BL/6 mice (bred from stock obtained from Charles River Laboratories, Wilmington, MA) and naked mole-rats of both sexes (born in colonies maintained in our laboratories) housed under normoxic laboratory conditions. Most experiments were conducted on mice and naked mole-rats at P5-7 (early postnatal) or P18-22 (late postnatal). For some experiments, we used older naked mole-rats, up to three weeks after weaning (P37-42). The total number of animals and the total number of slices for each age group, species, and experimental treatment is listed in table 1. Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

3.3.2 **Slice preparation**

Hippocampal brain slices from mice and naked mole-rats were prepared for fura-2 imaging as described previously (Takahashi, et al, 1999; Beierlein et al., 2002; Maclean and Yuste, 2005). Briefly, animals were anesthetized with isoflurane, decapitated, and brains were

quickly removed and put into a high sucrose (70 mM) solution on ice. The chilled and submerged brain was cut on a vibratome into 300 μm slices. The slices were incubated in the high sucrose solution at 34°C for 35 minutes, then transferred to normal artificial cerebral spinal fluid (ACSF) at 34°C for 20 minutes. The ACSF contained 125 mM NaCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 1.5 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM glucose, and had a pH of 7.4. Solutions were aerated with 95% O_2/5% CO_2. The slices were allowed to recover at room temperature for 10 minutes before staining. **Figure 3A and B** show low and high magnification images of the CA1 target area.

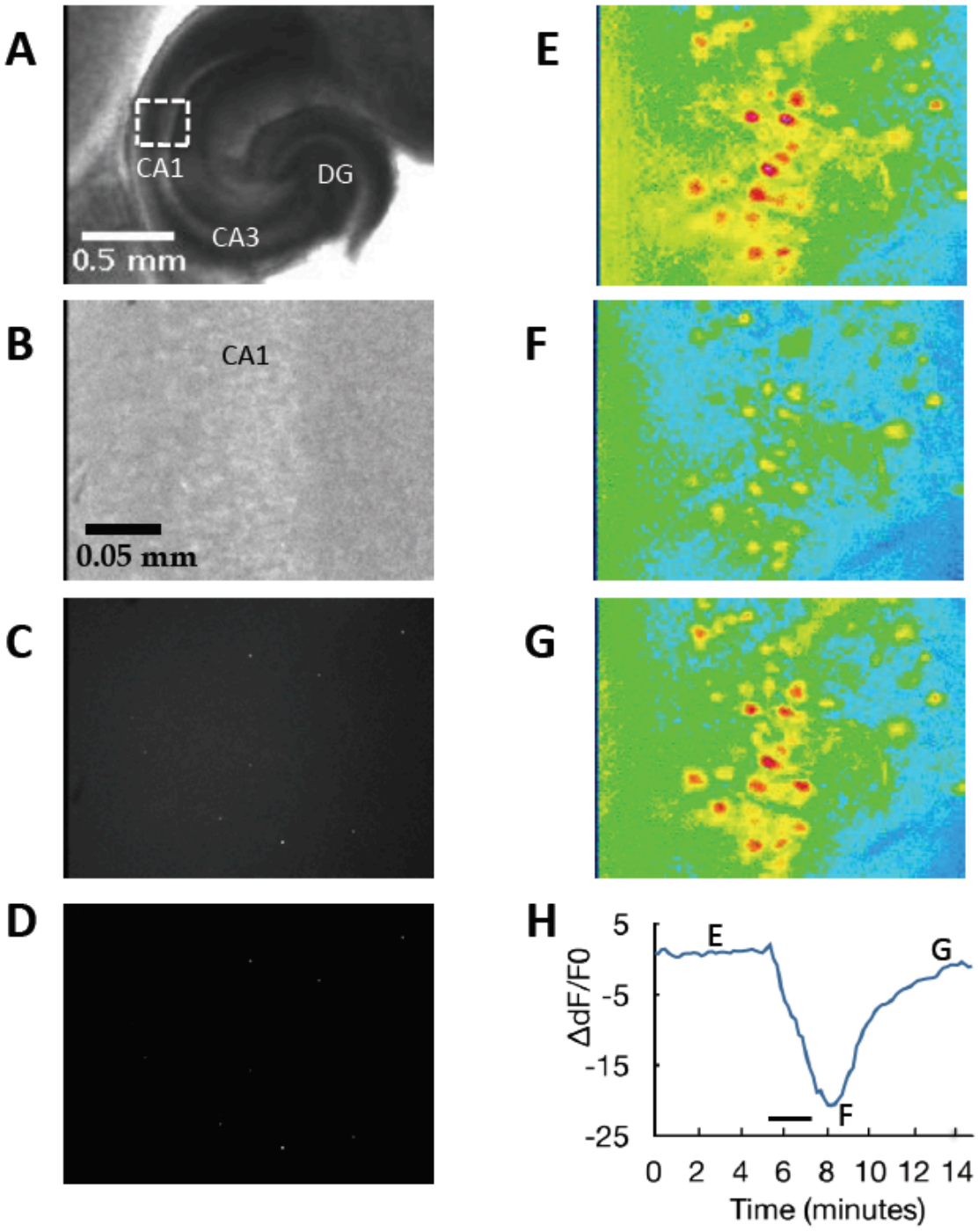
3.3.3 **Fura-2 AM Staining**

Slices were loaded with 5 μM membrane-permeable fura-2 AM (acetoxymethyl ester) dye (Biotium, Inc., Hayward, CA) in ACSF for 30 minutes at room temperature, while resting on a thin membrane of oxygen-permeable PDMS, oxygenated from below with 5% CO_2/ 95% air. The slices were then incubated in ACSF at 34°C for 10 minutes and allowed to recover in ACSF at room temperature for 15 minutes. All experiments were conducted at room temperature. Except for the 30 minute loading time, ACSF solutions were aerated with 95% O_2/5% CO_2.

3.3.4 **Imaging**

A stained slice was transferred into a physiological chamber mounted on an epifluorescence microscope with a 40x objective (Olympus, Center Valley, PA). The slice was submerged in ACSF bubbled with a 95% O_2/ 5% CO_2 gas mixture. The bath solution was perfused into the 1 ml chamber at a rate of 2 ml/min. The slice was positioned such that the

Figure 3. Examples of tissue and calcium imaging. A. Low magnification, bright field image of a slice with a box indicating the typical target area for imaging within the CA1 field of the hippocampus. DG = dentate gyrus. Top is posterior. **B.** High magnification, bright field image of a slice of the CA1 region. **C.** Same slice as shown in B, exposed to 365 nm wavelength, this slice was not loaded with fura-2 and the image reflects a 10 second integration time. **D.** Same slice as B and C, exposed to 365 nm wavelength, again not loaded with fura-2. The image reflects a 1 second integration time during exposure to hypoxia. **E-G**. Representative data from a P6 mouse slice tested with 25 mM potassium. Images show CA1 cells loaded with fura-2 before, during, and after application of 25 mM potassium for 2 minutes. The decrease in fluorescence in F corresponds to an increase in internal calcium due to application of potassium. These representative images are at 380 nm wavelength (not ratiometric). **H**. Curve showing the ratiometric data for the slice in E-G over 15 minutes. For this example, 4 minutes of data are shown prior to switching the bath solution to 25 mM potassium to illustrate the stability of typical baseline responses. For all group analyses, we collected data for 1 minute prior to switching solutions. The black bar indicates when the 25 mM solution was in the recording chamber (there was about a 1 minute time lag after switching solutions due travel time to the recording chamber).


A
CA1
DG
CA3
0.5 mm
B
CA1
0.05 mm
C
D
E
F
G
H
5
-5
-15
-25
ΔdF/F0
E
G
F
0 2 4 6 8 10 12 14
Time (minutes)

visual field through the microscope was centered on cell bodies in the CA1 region of the hippocampus (**Figure 3B**). Measurements were made of relative internal cytosolic calcium by measuring the fura-2 fluorescence emission at 510 nm using a Cooke Sensicam CCD camera. Ratiometric data were made with 365/380 nm wavelength excitations using a software-controlled (Imaging Workbench, Santa Clara, CA) fast wavelength changer (Sutter, Inc., Novato, CA) coupled to a metal halide source lamp (Exfo, Inc., Quebec, Canada). For group comparisons and figures, ratiometric data (380 nm intensity divided by 365 nm intensity) were converted to percent change in florescence by dividing the ratios obtained from each image by the average intensity ratio during the baseline recording period and multiplying the result by 100.

To determine if there was an appreciable background fluorescence signal we took images of slices (n = 4) that had been prepared as described above except that they had not been loaded with the fura-2 dye. We found a negligible amount of fluorescence even with a long integration time of 10 seconds (1 second integration time was used for the experiments), and there were no structural features distinguishable **(Figure 3C)**.

3.3.5 **Potassium Application**

In one set of experiments, slices were challenged by perfusion of ACSF containing elevated concentrations of potassium. Baseline images were recorded every 20 seconds for 20 minutes prior to application of potassium to ensure that the slice was healthy and that movement was negligible. For potassium application, the bath solution was switched to one with a high concentration of potassium (equimolar replacement of NaCl with KCl). In pilot tests with potassium concentrations ranging from 5 to 30 mM, we found that 15 and 25 mM were well on the dynamic part of the concentration/response curve for slices from P6 mice (which had the

maximum response) and did not saturate the fura-2 indicator response. Based on that, we collected a complete data set for both 15 and 25 mM potassium. Images were collected every 20 seconds for 15 minutes beginning 1 or 4 minutes before switching to one of the high potassium solutions. After 2 minutes, the solution was switched back to normal bath solution and the slice was allowed to recover. Ten minutes after recovery, the other high potassium solution was applied for 2 minutes followed by recovery. The order of potassium solutions was alternated between slices. Pseudocolor example images of fluorescence at 380 nm taken before, during, and after application of 25 mM potassium are shown in **Figure 3E, F, G**. The curve showing ratiometric data for all 15 minutes of testing is shown in **Figure 3H**. Note that in this particular example slice, we collected data for 4 minutes prior to switching to a high potassium solution. This duration of baseline illustrates the stability of the response. Data collected for group analyses used a baseline duration of 1 minute.

3.3.6 **Hypoxia**

In another set of experiments, slices were challenged with ACSF depleted of oxygen (hypoxia). After collecting baseline images, hypoxia was induced by switching the bath solution from the one saturated with 95% O_2/ 5% CO_2 to one saturated with 95% N_2/ 5% CO_2 for 10 minutes.

Ten minutes of hypoxia was chosen based on pilot experiments. For durations of hypoxia lasting longer than 10 minutes, most mouse slices did not show an appreciable recovery and our aim was to induce a reversible effect. A second issue with longer periods of hypoxia was that the fluorescent signal saturated. Our aim was to avoid saturation because there is no

way to distinguish saturation of calcium concentration versus saturation of the fluorescent signal (the fluorescent signal can saturate before the calcium concentration saturates).

A series of images was recorded every 20 seconds for 20 minutes beginning 1 minute before switching to the hypoxic solution. After 10 minutes, the bath solution was switched back to the one saturated with 95% O_2.

In this type of hypoxia experiment, the actual O_2 concentration in the bath is typically not reported. However, to assure ourselves that the bath was indeed becoming hypoxic, we measured percent saturation of O_2 in slices before and during 10 minutes of hypoxia exposure. We measured percent saturation of O_2 using an Ocean Optics Foxy-PI200 probe with a 200 μm diameter fiber optic fluorescence sensor and Neofox oxygen sensing system software (Ocean Optics, Dunedin, FL). At baseline (normoxia), percent saturation of O_2 measured 100 μm into the slice was 46.95 +/- 1.5% (357 Torr), and during hypoxia it was 11.42 +/- 0.63% (87 Torr) (1 slice each from 4 mice at P20; 16 measurements were made from each slice in normoxia and in hypoxia). We arbitrarily selected 100 μm so that we could get a measure in the tissue that was substantially below the surface but not in danger of hitting the bottom of the chamber. It took 3-4 minutes exposure to hypoxic solution to reach the lowest O_2 concentration, and then during re-oxygenation it took ~7 minutes exposure with oxygenated bath solution to reach the highest post hypoxia O_2 concentration.

To determine if autofluorescence increased during hypoxia exposure, for instance from increased NADH production, we subjected unstained slices (n = 4) to the hypoxia exposure protocol. We found a negligible amount of fluorescence and there were no structural features distinguishable **(Figure 3D)**.

3.3.7 **Statistical Analysis**

We used a 2-way ANOVA (GB STAT, Dynamic Microsystems, Inc., Silver Spring, MD) for comparisons involving more than two groups (e.g. species and age), followed by the Newman-Keuls test for multiple comparisons. The values used for statistical analyses were from the last data point collected under exposure to hypoxia or high potassium. In addition, we used a t-test (Excel) to compare the late postnatal (P18-22) naked mole-rat slices with slices from a group of even older naked mole-rats (P37-42).

Table 1. Number of animals and slices used to collect data for hypoxia and high potassium. NMR = naked mole-rat.

		Mouse P5-7	Mouse P18-22	NMR P5-7	NMR P18-22	NMR P37-42	NMR P5-7 (15min)
Hypoxia	Total # animals	6	6	5	3	2	2
	Total # slices	11	11	14	10	6	7
High K^+	Total # animals	4	5	4	4	---	---
	Total # slices	9	8	10	15	---	---

3.4 **Results**

3.4.1 **Hypoxia-induced increase in internal calcium is reduced in naked mole-rat compared to mouse**

As predicted, hippocampal CA1 pyramidal cells of both naked mole-rats and mice responded to hypoxia with a decrease in fura-2 fluorescence, corresponding to an increase in intracellular calcium. However, there were statistically significant differences in the

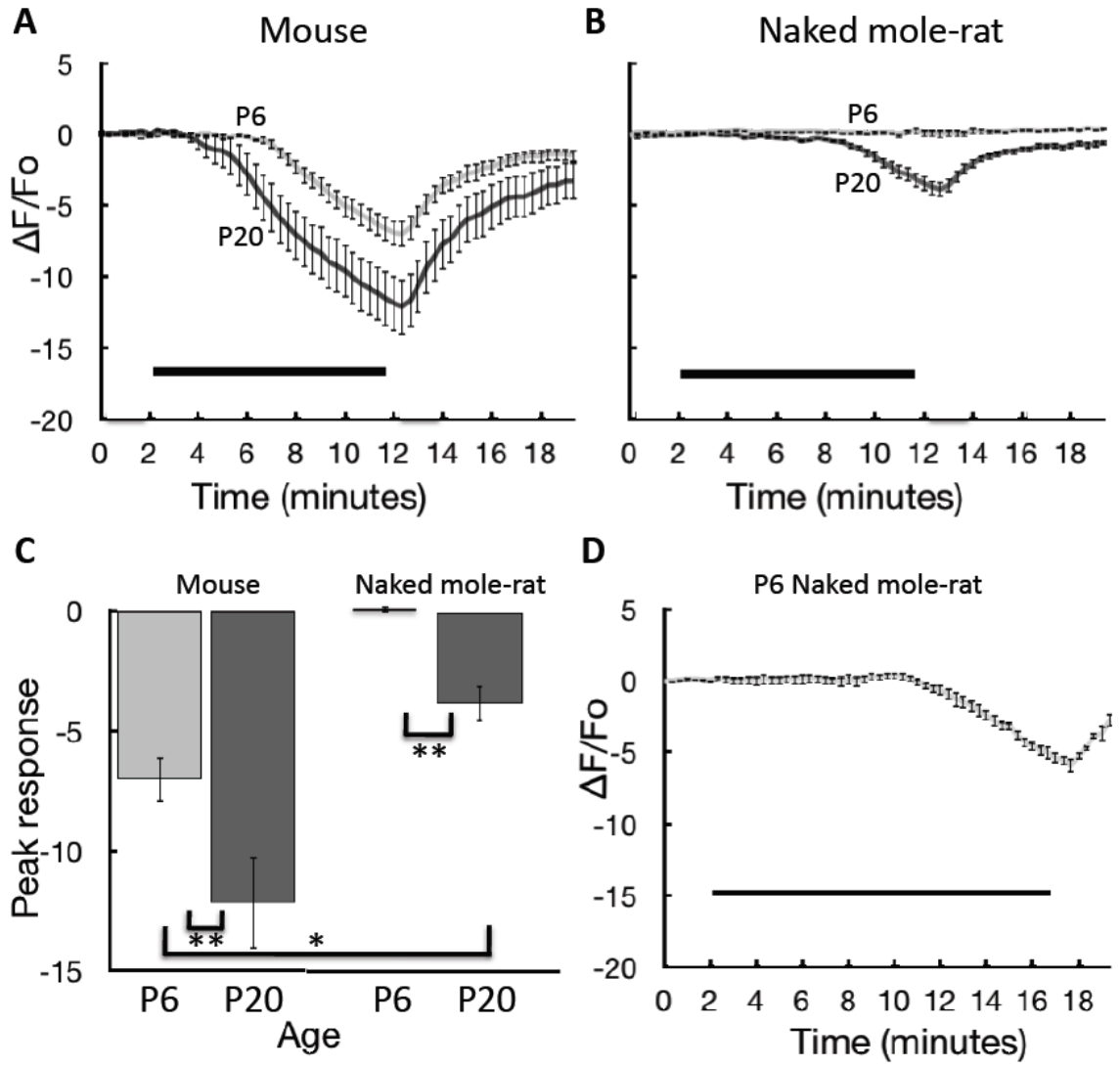
fluorescence response due to main effects of species ($\mathbf{F}_{1,45}$=52.61, p<.0001) and age ($\mathbf{F}_{1,45}$= 18.96, p<0.0001) (2-way ANOVA). There was no significant interaction between age and species ($\mathbf{F}_{1,45}$=0.37, p=.55).

The data from mouse slices were consistent with previous studies of mouse, rat, and gerbil (Mitani et al., 1990; Friedman and Haddad, 1993; Shimazaki et al., 1998; Diarra et al., 1999; Bickler et al., 2009). Mouse slices showed a progressive increase in internal calcium (decrease in fluorescence signal) beginning shortly after application of hypoxic bath solution and continuing for the entire 10 minutes of hypoxia exposure (**Figure 4A**). Calcium then decreased after re-oxygenation. As expected, slices from neonatal mice showed a weaker increase than slices from older mice. The maximal percent change in fluorescence signal for slices from older mice (P18-22) was -12.1% (mean) +/- 1.96% (SE), whereas the maximal percent change for slices from younger mice (P5-7) was -6.9 +/- 0.93% (p<.01, Newman-Keuls test). Note that both of these values could have probably reached higher values if we had used a longer exposure to hypoxia, but that would have precluded an appreciable recovery (see materials and methods). Therefore, it is the differences between groups that are important, not the absolute values. It took the younger mouse group 100 seconds to reach the half-time recovery point, whereas it took the older mouse group 160 seconds.

Naked mole-rat slices tested with the same procedure showed a much smaller change in intracellular calcium-mediated fluorescence (**Figure 4B**). The slices from older naked mole-rats had a maximal percent change of -3.8 +/- 0.75%, which was significantly less than both the older (p<0.01, Newman-Keuls test) and younger (p<0.05, Newman-Keuls test) mouse groups. Remarkably, the slices from neonatal naked mole-rats showed no detectable change in internal calcium during the entire 10 minute hypoxia exposure. The summary data for both age groups of

naked mole-rats and mice are presented in **Figure 4C**. It took the older naked mole-rat group 100 seconds to reach the half-time recovery point, and this could not be calculated for the younger group.

Figure 4: Increase in internal calcium from exposure to hypoxic bath solution. **A**. Data from P6 (11 slices, 6 animals) and P20 (11 slices, 6 animals) mouse hippocampal slices. Values on the y-axis indicate the percent change in calcium-mediated fluorescence within CA1 neurons in the field of interest with negative values corresponding to an increase in calcium (calcium decreases the fluorescent signal). Images were collected every 20 seconds over 20 minutes. The black bar indicates the 10 minutes when hypoxic bath solution was in the recording chamber. Error bars are +/- S.E.M. **B.** Data from P6 (14 slices, 5 animals) and P20 (10 slices, 3 animals) naked mole-rat slices. **C.** Summary data showing the change in maximal calcium with age for mice and naked mole-rats for a 10-minute exposure. * and ** correspond to significance at $p<0.05$ and $p<.01$, respectively according to the Newman-Keuls test. **D.** Data from P6 (7 slices, 2 animals) naked mole-rats slices with an extended hypoxia exposure (15 minutes). Note that in all panels, animals in the P6 groups actually ranged in age from P5 to P7, and animals in the P20 groups actually ranged in age from P18 to P22.


A
Mouse
P6
P20
ΔF/Fo
Time (minutes)
B
Naked mole-rat
P6
P20
Time (minutes)
C
Mouse
Naked mole-rat
Peak response
**
**
*
P6
P20
P6
P20
Age
D
P6 Naked mole-rat
ΔF/Fo
Time (minutes)

Historically, the age groups we tested are considered to be representative of neonatal (P5-7) and mature (P18-22) animals in regard to responses to hypoxia in hippocampal slices (Friedman and Haddad, 1993; Bickler et al., 2003). We tested both mice and naked mole-rats at the identical chronological ages. However, it should be noted that the maximum lifespan in naked mole-rats (30 years) is much longer than that in mice (3 years), raising the possibility that chronological and biological age diverge in the two species. On the other hand, both naked mole-rats and mice reach a major developmental milestone (weaning) at about the same age (3-4 weeks) suggesting a comparable level of overall maturity at this time point. In any case, we addressed the issue of biological maturity by testing a group of naked mole-rats at P37-42, well after weaning. The responses to hypoxia for these naked mole-rats were not significantly different from the P20 group (maximal percent change for P37- 42 = -3.82 +/- 0.49%, maximal percent change for P18-22 = -3.80 +/- 0.75%, p=0.959, t-test). These results suggest that the difference in hypoxia tolerance between mice and naked mole-rats at P20 is not simply due to a difference in rate of maturation. Both mice and naked mole-rats show hypoxia tolerance in the neonatal period (P5-7), but hypoxia sensitivity reaches adult levels by P18-22. The calcium response to hypoxia is significantly reduced in naked mole-rat neurons relative to mouse neurons both early and late in development.

It is also notable that the uptake of the fura-2 AM dye was not qualitatively different in slices from P37-42 and P18-22 naked mole rats, whereas staining of mice older than P22 was poor and inconsistent.

The dramatic results for slices from neonatal (P5-7) naked mole-rats warranted further testing on this age group. In our experiments, the 10 minute duration of hypoxia was selected based on pilot studies with mouse slices and was somewhat arbitrary. Therefore, we tested slices

from neonatal naked mole-rats with a longer exposure to hypoxia (15 minutes). During this longer period of hypoxia, slices from naked mole-rats began to show an increase in calcium, but not until much later than the other groups (**Figure 4D**).

3.4.2 **Potassium-induced increase in internal calcium is similar in older groups of naked mole-rats and mice**

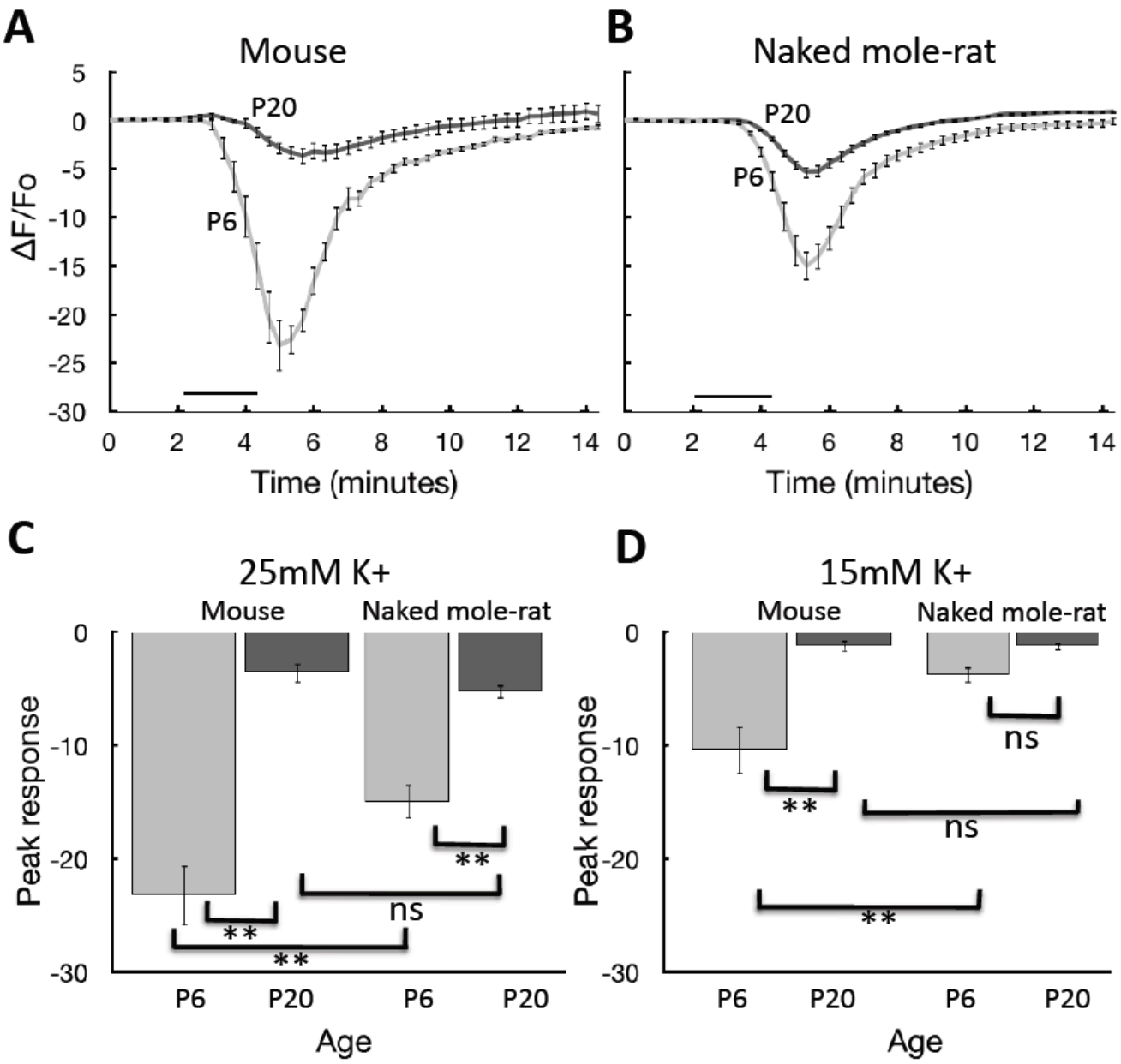
The blunted calcium response to hypoxia in naked mole-rat slices could be due to a number of factors (calcium channel density, buffering capacity, etc.), not related to hypoxia *per se*. In order to explore this issue further, calcium signals in naked mole-rat and mouse slices were measured during challenges with elevated extracellular potassium concentrations. **Figure 5A-C** presents the data for experiments in which slices from mice and naked mole-rats (both at P6 and P20) were exposed to 25 mM potassium. A two-minute exposure to 25 mM potassium triggered an increase in intracellular calcium in both mice and naked mole-rats (**Figure 5A,B**). In both species the response was much greater in the younger age group: 6 times greater in mice and 3 times greater in naked mole-rats (**Figure 5C**). For mice, the maximal percent change in fluorescence signal for slices from older mice (P18-22) was -3.64 +/- 0.88%, whereas the maximal percent change for slices from younger mice (P5-7) was -23.19 +/- 2.6% ($p<.01$, Newman-Keuls test). For naked mole-rats, the maximal percent change in fluorescence signal for slices from older naked mole-rats (P18-22) was -5.29 +/- 0.65%, whereas the maximal percent change for slices from younger naked mole-rats (P5-7) was -15.0 +/- 1.51% ($p<.01$, Newman-Keuls test). Using a 2-way ANOVA, we found significant differences between age groups ($\mathbf{F}_{1,38}=93.69$ $p<0.0001$), and between species ($\mathbf{F}_{1,38}=4.72$, $p<.05$) and a significant interaction between age and species ($\mathbf{F}_{1,38}=10.49$, $p<0.01$). With a lower dose of potassium (15

mM), all responses were reduced but the same patterns among age groups and species remained (**Figure 5D**): significant differences were found between age groups ($\mathbf{F}_{1,38}$=30.67,p<0.0001), and between species ($\mathbf{F}_{1,38}$=10.17, p<.05) as well as a significant interaction between age and species ($\mathbf{F}_{1,38}$=10.22, p<0.01).

There are several noteworthy aspects of these data. The potassium challenge demonstrated a concentration-dependent increase in fluorescence that was greater in some cases than the responses due to hypoxia, suggesting that the hypoxia measurements were well within the dynamic range of the fura-2 indicator. In addition, the responses to potassium challenge were much more similar for naked mole-rats and mice than the responses to hypoxia; this was particularly evident for the older age groups where the potassium responses were not significantly different. This is important because it shows that the blunted calcium response to hypoxia in naked mole-rats cannot be entirely accounted for by a generalized reduction in responsiveness to all stimuli. Furthermore, it suggests that the differences we found were not due to species differences in dye uptake which in our experience is most problematic in older mice.

Finally, the older age groups had a smaller calcium accumulation response to potassium challenge than the younger age groups in both mouse and naked mole-rat, which is the opposite pattern from the one we observed with hypoxia. This is important because it suggests that the age differences we found for hypoxia were not due to poor slice health in older animals.

Figure 5: Increase in internal calcium from exposure to high K+ bath solutions. **A.** Data from P6 (9 slices, 4 animals) and P20 (8 slices, 5 animals) mouse hippocampal slices. Images were collected over 15 minutes. The black bars indicate the 2 minute time course that 25 mM potassium bath solution was applied. **B.** Data from P6 (10 slices, 4 animals) and P20 (15 slices, 4 animals) naked mole-rat slices tested under the same conditions as A. **C.** Summary data showing the change in maximal calcium with age for P6 and P20 mice and naked mole-rats during the 2 minute exposure to 25 mM K+. **D.** Summary data showing the change in maximal calcium with age for P6 and P20 mice and naked mole-rats during a 2 minute exposure to 15 mM K+. * and ** correspond to significance at $p<0.05$ and $p<.01$, respectively according to the Newman-Keuls test.


A
Mouse
P20
P6
ΔF/Fo
5
0
-5
-10
-15
-20
-25
-30
0
2
4
6
8
10
12
14
Time (minutes)
B
Naked mole-rat
P20
P6
0
2
4
6
8
10
12
14
Time (minutes)
C
25mM K+
Mouse
Naked mole-rat
Peak response
0
-10
-20
-30
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ns
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P6
P20
P6
P20
Age
D
15mM K+
Mouse
Naked mole-rat
Peak response
0
-10
-20
-30
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ns
**
P6
P20
P6
P20
Age

3.5 **Discussion**

The main finding of this study is that hippocampal neurons in naked mole-rats show a blunted intracellular calcium response to hypoxia when compared to neurons in mice. The attenuated calcium accumulation response in naked mole-rat hippocampus was highly significant compared to the response in mouse hippocampus, whether assessed in animals at an early stage of postnatal development (P6) or in weanlings (P20). Technical issues did not permit a comparison of calcium responses in adult mice and naked mole-rats because AM staining of adult tissue is impaired as the neuropil develops; however a previous electrophysiological study demonstrated that adult (> 1 year old) naked mole-rat hippocampus is extremely tolerant to two related effects of hypoxia: namely, the suppression of synaptic transmission during partial hypoxia and the collapse of membrane potentials (anoxic depolarization) that accompanies severe oxygen deprivation (Larson and Park, 2009).

We chose to examine hypoxia responses in acute brain slices rather than cell culture because the slice represents more closely the neuronal circuits and neural-glial ensembles present *in situ* and we wanted to compare responses in tissue from animals at different ages. However, we are limited to inferring relative changes in calcium, in this case in an ensemble of neurons in our region of interest, in slices bulk-loaded with the calcium indicator. Absolute calibration of indicator-based calcium measurements is technically difficult under optimal conditions (isolated cells, individually loaded) and even then interpretation can be problematic (Neher, 2005). It was therefore not possible to calibrate absolute calcium changes accurately in our bulk-loaded slices. We are very interested to know whether resting calcium levels differ in neurons from naked mole-rats and mice and attempts to measure intracellular calcium in individually-loaded cells are underway.

The attenuation of calcium response in naked mole-rat hippocampus appears to be specifically related to hypoxia tolerance because the calcium response to potassium challenge was much more similar for naked mole-rats and mice. First, both mice and naked mole-rat neurons showed an age-dependent *reduction* in calcium response to potassium rather than the age-dependent *increase* in calcium response to hypoxia. Second, at P20, naked mole-rat neurons showed a highly significant reduction (68%) in calcium response to hypoxia compared to mouse neurons, but no significant difference in calcium response to potassium compared to mouse neurons.

A diminished accumulation of intracellular calcium during hypoxia is a common end-point in hypoxia tolerance under a variety of conditions (Bickler, 2004; Drew, et al, 2004). For example, during hibernation, Arctic ground squirrels have less phosphorylation of the NR1 subunit of NMDA receptors; this effect decreases the activity of the receptor, thereby limiting calcium accumulation (Ross et al., 2006; Zhao et al., 2006). Similarly, western painted turtles survive long periods of anoxia (months) using a variety of mechanisms, including down-regulation of ion channels and suppression of glutamate release. These adaptations limit calcium accumulation in neurons (e.g., Buck and Bickler, 1998; Bickler and Donohoe, 2002). In hypoxia-tolerant neonatal rats, hippocampal neurons have elevated levels of the NMDA receptor subunit, NR2D, compared to adult rats; this subunit is less sensitive to hypoxia than the other NMDA receptor subunits (Bickler et al.; 2003; Bickler, 2004). Metabolic differences (Hochachka et al., 1996) may also indirectly tend to limit calcium accumulation during hypoxia in neonates compared to adults.

Naked mole-rats share two important features with these other model systems: resistance to hypoxia *in vivo* and an attenuated hypoxia-induced neuronal calcium response *in vitro*. At

present we do not yet know the underlying mechanism(s) behind the extreme tolerance to hypoxia in naked mole-rat neurons. However, it appears that, unlike the turtle (Buck, 2004), an increase in adenosine does not contribute to the diminished increase in calcium. In a previous study, we showed that hippocampal cells in adult naked mole-rats were less sensitive to adenosine as compared to cells from mice (Larson and Park, 2009). We suggested that the adult naked mole-rat brain resembles the neonatal rat (and mouse) brain in terms of response to adenosine, resistance to hypoxia, and lack of paired-pulse facilitation (Muller et al., 1989). (The robust staining of naked mole-rat neurons with fura-2 AM at P42, an age when staining of mouse or rat neurons is poor and inconsistent, may also reflect a difference in neuronal maturation.) Furthermore, preliminary data indicate that adult naked mole-rat brain retains more of the (neonatally abundant) NMDA receptor subunit, NR2D, compared to mice (Unpublished observations). The calcium imaging results from the present study are not inconsistent with the notion that slowed or arrested brain development may endow the naked mole-rat brain with extreme hypoxia tolerance. Even at P42 (the most advanced age tested), naked mole-rat brain showed an attenuated calcium accumulation response to hypoxia, compared to neonatal (P6) mice.

Even though naked mole-rats showed a blunted response to hypoxia compared to mice at all ages, the naked mole-rats also showed a significant age-related increase in the response from P6 to P20. It is possible that naked mole-rats undergo a much attenuated change with age (compared to mice), retaining a substantial, although incomplete, degree of "neonatal" hypoxia tolerance into adulthood. Alternatively, the naked mole-rat and mouse may have a comparable age-related *change* in hypoxia sensitivity, but the naked mole-rats have such an extremely diminished response to begin with (at P6) that the change is not large enough to bring older

animals into the response range of even the P6 mice. In any case, the calcium accumulation response to hypoxia was significantly reduced in naked mole rats at P20 even compared to P6 mice, and did not show further changes from P20 to P40.

Our working hypothesis is that naked mole-rats have evolved an extreme tolerance to hypoxia as a consequence of their unusual lifestyle which combines subterranean living with a proclivity for living in great numbers (up to hundreds of individuals per colony). Hence, even compared to other fossorial mammals, naked mole-rats are challenged by unusually high levels of hypoxia due to many individuals sharing the same poorly ventilated air (Bennett and Faulkes, 2000). The current thought on what has driven the naked mole-rat to live the way it does is based on its habitat - hard soil which makes burrowing costly, and patchy food resources (roots and tubers) which make foraging by a small number of animals risky (food-aridity hypothesis; Jarvis, et al, 1994). Consistent with the notion that naked mole-rats are hypoxia tolerant even among fossorial mammals, a previous study found that hippocampal slices from another fossorial mammal, the blind mole-rat (*Spalax*) which primarily lives a solitary life, responded to severe hypoxia much more similarly to slices from mice than slices from naked mole-rat. Under severe hypoxia, hippocampal slices from naked mole-rats maintained synaptic function for 12.63 +/- 5.06 minutes whereas slices from mice maintained function for 2.16 +/- 0.23 minutes and slices from blind mole-rats maintained function for 1.58 +/- 0.17 minutes (Larson and Park, 2009). This was an interesting finding because the blind mole-rat is considered to be a hypoxia tolerant animal *in vivo*, with a variety of physiological and anatomical adaptations in blood, respiratory organs, and a number of gene products which are consistent with living under hypoxic conditions (see Avivi, et al, 2010 for review). Apparently, this fossorial species achieves hypoxia tolerance via a variety of mechanism that do not include intrinsic brain tolerance.

Currently there are relatively few comparative studies on intrinsic hypoxia tolerance in brain slices in mammals. However, in one such study, Folkow, et al (2008) measured membrane potentials from visual cortex slices from diving seals and mice. They found that under severe hypoxia, slices from seals maintained synaptic function approximately 4 times longer than slices from mice (19 minutes versus 5 minutes). This is similar to what we found previously when comparing hippocampal slices from naked mole-rats and mice (12.63 minutes versus 2.16 minutes; Larson and Park, 2009). Using cell survival as a metric, Frerichs and Hallenbeck (1998) showed that CA1 cells in hippocampal slices from both hibernating and active 13-lined ground squirrels survived longer than CA1 cells in slices from rats. Consistent with this finding, a variety of hypoxia tolerant adaptations have been found in brain cells of hibernating species (see Drew, 2004 for review). In our present study, we did not look at cell survival because our protocol was designed to measure recovery. However, our previous study which measured physiological responses to hypoxia clearly showed that hippocampal slices from naked mole-rats were able to survive and/or recover from hypoxia applications that slices from mice could not recover from (Larson and Park, 2009). Non-mammalian models of hypoxia tolerance include some fishes, frogs, and turtles (Bickler and Buck, 1998). Interestingly, forebrain cells from tadpoles (Hedrick, et al, 2005) and cortical slices from turtles (Bickler and Buck 1998) show increases in internal calcium during severe but survivable hypoxia without showing the cell damage characteristic of mammalian neurons exposed to high calcium.

In the present study, the animals that we used were maintained under normoxic conditions prior to slicing. It is intriguing to speculate about the possible effects of maintaining the animals under moderately hypoxic conditions that might simulate conditions within a naked mole-rat burrow. Periods of moderate hypoxia are well known to increase hypoxia tolerance in a

variety of species (Gidday, 2006 for review). Future experiments using this type of preconditioning may reveal an even more pronounced blunting of the neuronal calcium response to hypoxia in naked mole-rat neurons.

In summary, hippocampal neurons from naked mole-rats show an attenuated intracellular calcium accumulation response to hypoxia, as compared with mouse hippocampal neurons. The blunted calcium response may represent an important adaptive response to a chronic hypoxic environment that is achieved by retarding or arresting a developmental process that normally limits hypoxia tolerance in adult mammals.

4. Adult Naked Mole-Rat Brain Retains the NMDA Receptor Subunit NR2D Associated with Hypoxia Tolerance in Neonatal Mammals

Published in Neuroscience Letters

4.1 Abstract

Adult naked mole-rats show both a neonatal-like tolerance to hypoxia *in vivo* and *in vitro*, and an attenuated calcium response to hypoxia in brain slice. Other similarities to neonatal mammal brain include lack of paired-pulse facilitation and insensitivity to adenosine. An important component of neonatal tolerance to hypoxia involves the profile of NMDA receptor subunits. Neonates have a high proportion of NMDA receptors with an NR2D subunit which is protective during hypoxia because it slows calcium entry into the cell during hypoxic episodes. We hypothesized that adult naked mole-rats retain a protective, neonatal-like NMDA receptor subunit profile. If this were true, we should observe less prominent age-related changes in subunits compared to the changes observed in mice. We used Western blot methods to quantify age-related change in NMDA subunits. The results show that naked mole-rat brain retains a much greater proportion of the hypoxia protective NR2D subunit compared to mice. However, age-related changes in other subunits (NR2A and NR2B) were comparable to those in mice. mRNA results were consistent with the immunoblot data. Hence, naked mole-rat brain only retains the neonatal subunit that is hypoxia tolerant.

4.2 Introduction

Brain tissue is especially vulnerable to a deprivation of oxygen in almost all species. However brain slices from naked mole-rats (*Heterocephalus glaber*) show an exceptional resistance to hypoxia's negative effects (Larson and Park, 2009). This is consistent with

evolving in a chronically low oxygen environment. Naked mole-rats are indigenous to East Africa where they live in sealed subterranean burrows in colonies of up to around 300 individuals (Brett, 1991). Since many animals are breathing the same poorly ventilated air, especially when huddled together in their nests, oxygen becomes depleted while CO2 becomes elevated (Bennett and Faulkes, 2000).

Naked mole-rats have many peripheral adaptations to survive in their hypoxic environment. Their hemoglobin has a higher affinity for oxygen than most other mammals (Johansen et al., 1976), and their weight-specific metabolic rate is about one-third less than that of other rodents (Buffenstein and Yahav, 1991). Also, their peripheral nerves are insensitive to acidosis, which is useful in their environment high in CO2 (Park et al., 2008).

As previously mentioned, naked mole-rats also have a central resistance to hypoxia. We have reported that synaptic transmission in hippocampal slices from naked mole-rats was maintained in low oxygen concentrations that caused transmission to decrease or cease in laboratory mice (Larson, and Park, 2009). Moreover, we recently found that slices from naked mole-rat brain had an attenuated increase in internal calcium concentration during a 10 minute hypoxia exposure compared to mice (Peterson et al., 2011). This is important because too much internal calcium accumulation leads to irreversible damage or cell death.

These characteristics, hypoxia tolerance and an attenuated calcium response to hypoxia, resemble neonatal brain characteristics. Other similarities to neonatal mammals include lack of paired-pulse facilitation and insensitivity to adenosine (Bauman et al., 1998). These similarities between naked mole-rats and neonates prompted us to suggest that naked mole-rats retain neonatal brain characteristics into adulthood.

Mammalian neonatal brains display a number of hypoxia tolerant mechanisms (Bickler, 2004). An important one involves NMDA receptors and intracellular calcium (Bickler et al., 2003). Neurons experience a cascade of cellular processes when they are oxygen deprived. High levels of ATP are needed to retain transmembrane ion concentration gradients. When ATP levels drop, concentration gradients are lost and the cells depolarize which leads to a reversal of the sodium-dependent glutamate transporters causing a rise in extracellular glutamate (Bickler and Donohoe, 2002; Hochachka et al., 1996; Rossi et al., 2000). This rise in glutamate over-activates glutamate receptors, importantly NMDA receptors, allowing toxic amounts of cations, especially calcium, to enter the cells. Calcium then over activates calpains, calcium-sensitive proteases that cause neuronal degeneration (Lee et al., 1991).

Therefore, NMDA receptors play a large role in hypoxic injury. These receptors are made up of 4 subunits. Two of the subunits are of the NR1 type. The other two subunits vary among individual receptors, and most include members of the NR2 type. There are four different NR2 subunits: NR2A, NR2B, NR2C, and NR2D (Collingridge and Watkins, 1994).

The NR2 subunit determines the biophysical and pharmacological activity of the receptor (Waters and Machaalani, 2004). Importantly, receptors that include NR2D subunits are particularly resistant to hypoxia. Under hypoxia these receptors have a greatly reduced open time compared to receptors without NR2D subunits. Consequently, less calcium enters the cells through NMDA receptors with NR2D subunits. The resulting lower internal calcium concentration could prevent calcium mediated cell injury, and is thought to be an important mechanism of hypoxia resistance in neonatal mammals (Bickler et al., 2003).

The subunit profile of NMDA receptors changes as an animal develops and ages. NR2B and NR2D are highly expressed in neonatal brains while NR2A and NR2C are relatively scarce.

As the animal matures the expression pattern changes, NR2B and NR2D decrease while NR2A and NR2C increase (Laurie et al., 1997). During the developmental change in the NR2 subunit profile, there is a corresponding decrease in tolerance to hypoxia (Vannucci and Hagberg, 2004). In mice, these changes occur during the first postnatal month. Thus neonatal mice (e.g. P6) show a relatively high proportion of NR2D, and during hypoxia, a relatively attenuated accumulation of intracellular calcium *in vitro*, and a robust tolerance to hypoxia *in vivo*. In contrast, postnatal 3-4 weeks and older mice show a relatively low portion of NR2D, a much greater accumulation of intercellular calcium during hypoxia, and a much lower tolerance to hypoxia *in vivo*.

Since adult naked mole-rats show both a neonatal-like attenuated calcium response and tolerance to hypoxia, we hypothesized that they retain a protective, neonatal-like NMDA receptor subunit profile. If this were true, we should observe less prominent age-related changes in subunits compared to the changes observed in mice. We used Western blot methods to quantify age-related change in NMDA subunits. The results show that naked mole-rat brain retains much more of the hypoxia protective NR2D subunit compared to mice. However, age-related changes in other subunits (NR2A and NR2B) were comparable to those in mice. Hence, naked mole-rat brain only retains the neonatal subunit that is hypoxia tolerant.

4.3 **Materials and Methods**

4.3.1 **Animals**

Experiments were performed on male and female C57BL/6 mice (bred from stock obtained from Charles River Laboratories, Wilmington, MA) and naked mole-rats of both sexes (born in colonies maintained in our laboratories) housed under normoxic laboratory conditions. Experiments were conducted on mice and naked mole-rats at postnatal day 6 and adults (1-3

years). Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

4.3.2 **Western Immunoblotting**

Whole brains (without cerebellum) were freshly excised from both mouse and naked mole-rat at P6 and adult P>60. The younger brains were homogenized with pestle and adult brains are homogenized with glass-glass homogenizer, in ice-cold buffer containing 50mM Hepes, 125mM NaCl, 100mM sucrose, 4 mM KAcetate, .5M EDTA, 1M N-Em, 10mg/mL pepstatin A, 2μg/mL aprotinin, and 200mM PMSF. The homogenate was centrifuged at a low speed (1000 X g, 10 min) then the supernatant was re-centrifuged at a high speed (20000 X g, 10 min) and the pellet was re-suspended in homogenate buffer. 200μl was then taken from that and methanol precipitated at 4C, the remaining was used to calculate protein concentration or stored. Each sample was re-suspended with SDS-PAGE with 1% DTT for a final concentration of 2μg protein/ μl and boiled for 5 min. Samples were resolved on 7.5% Tris HCL gels. Proteins were transferred (40 V, 20 min, 4°C then 15 V, overnight, 4°C) to membranes in transfer buffer (12mM Tris HCL, 6mM NaAcetate, 0.3 mM EDTA disodium, pH 7.5). Blots were blocked for 1 h at room temp in 1% skim milk blotting buffer, then incubated overnight at 4C with the antibody. NR1 antibody (1:500; Antibodies Incorporated), NR2A antibody (1:5,000; Chemicon Internation), NR2B (1:500; Antibodies Incorporated), and NR2D antibody (1:1,000; Chemicon Internation) was used for detection. After incubation, blots were washed with PBS for two cycles of a quick wash then 5 min repeated twice, then incubated with corresponding secondary antibody in 1% skim milk blotting buffer. After the blot was again washed with PBS, the blot was incubated for 5 min in a chemiluminescence reagent mixture (ECL plus, GE Healthcare) and

exposed to film for 5 to 10 min depending on signal strength. Four different blots were run for each subunit, each blot contained a sample from a neonate and an adult mouse and a neonate and an adult naked mole-rat. All blots were re-blotted with β-actin as a loading control.

Western blots were scanned and analyzed quantitatively by densitometry with Image J software. Data was statistically analyzed using an unpaired t-test. For NR2A, which increased in an age-dependent manner, we designated the density of the band from adult as 1.0. We then determined relative density as a percentage of the band from neonate relative to the band from adult. For NR2B, NR2D and NR1, which decreased in an age-dependent manner, we designated the density of the band from neonate as 1.0 and determined relative density as a percentage of the band from adult to the band from neonate.

4.4 **Results**

We used Western immunoblot and mRNA analyses to quantify age-related changes in the NMDA receptor subunits NR2A, NR2B, NR2D, and NR1 in mice and naked mole-rats. Previous studies have shown that in mice NR2B and NR2D decrease with age whereas NR2A increases and NR1 remains relatively stable (Laurie et al., 1997). Our results for mice were consistent with this. For naked mole-rats, we also found a variety of age-related changes. The most robust species difference we found was for NR2D with the mice showing a decrease in expression of ~90% but naked mole-rat showing a decrease of only ~33%. There were no statistical differences in age-related changes for NR2A or NR2B.

Figure 6 shows examples of Western blots for each receptor subunit that we examined for both species and for both age groups. The important comparisons were within subunit and species. The absolute density of bands cannot be compared across subunits or species because of

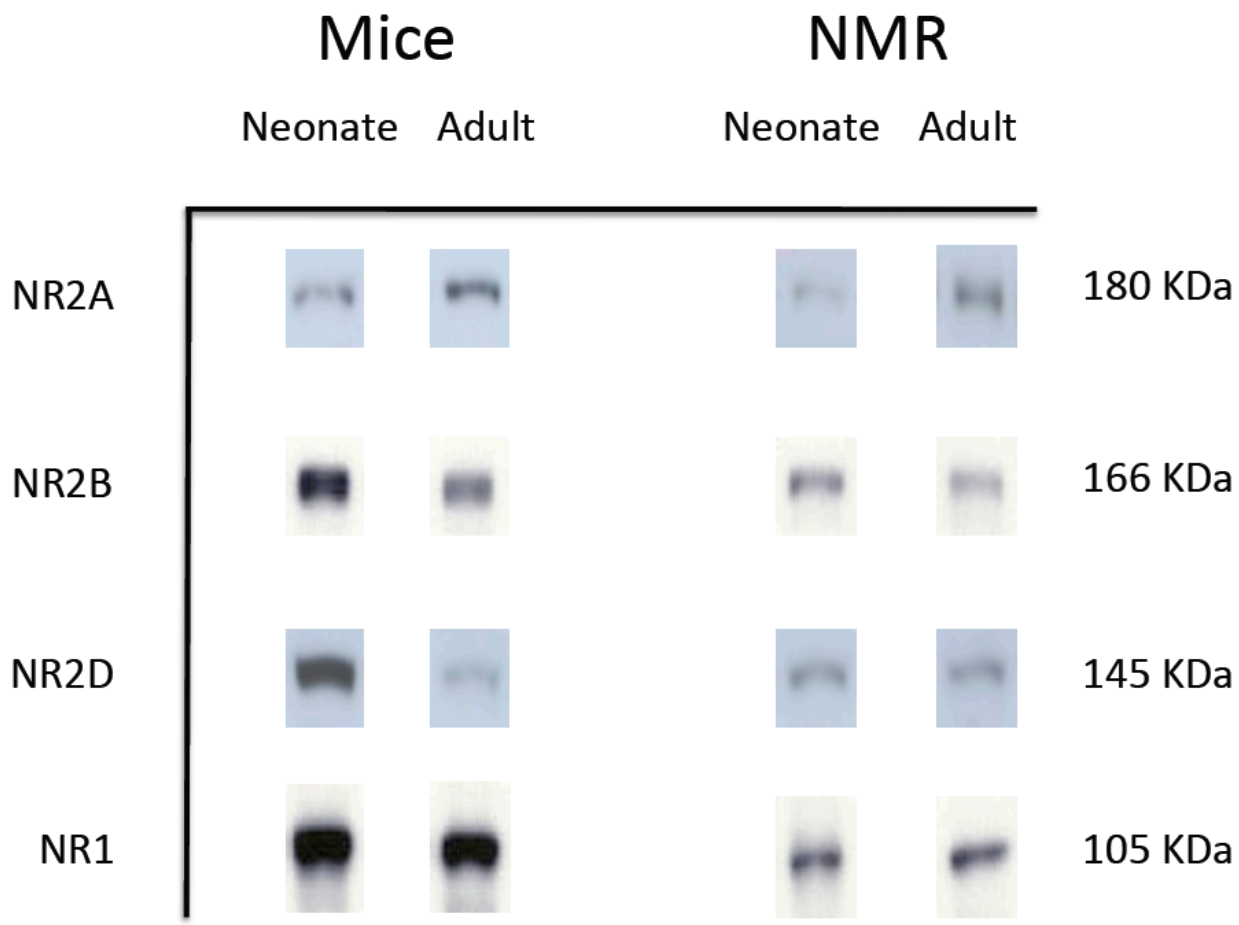


Figure 6. Example western blots from the four NMDA receptor subunits examined. For each subunit an example from whole brains without cerebellums from a neonate and an adult is shown for both mice and naked mole-rats (NMR). A change in the density of the blots between neonate and adult corresponds to an age-related change in expression of that subunit. Due to difference in antibody affinity across species and subunit, the only valid comparisons that can be made are between neonate and adult within a given subunit.

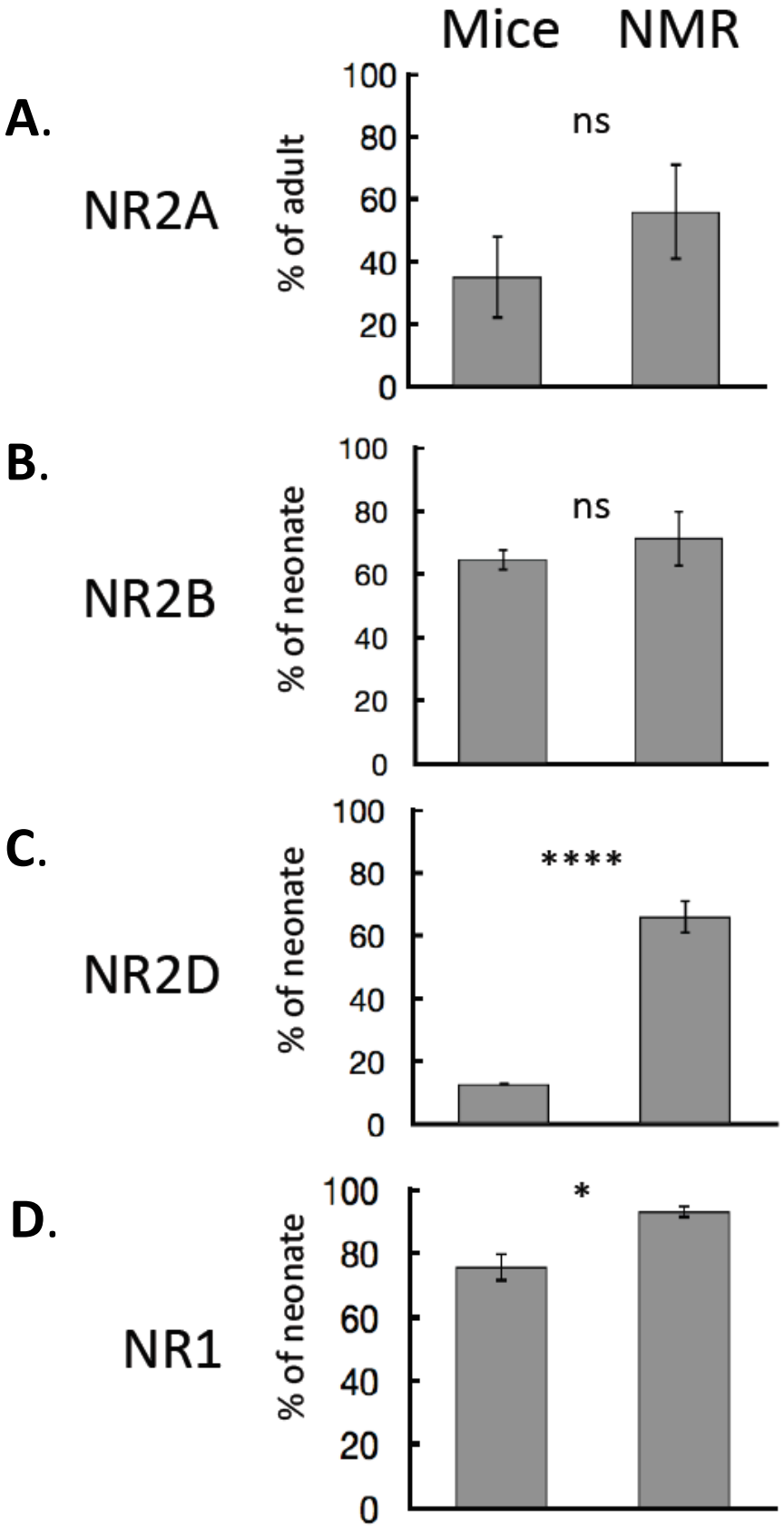
differences in antibody affinity. As the examples in the top row of **Figure 6** show, both adult mice and adult naked mole-rats show a higher density for NR2A compared to their neonate counterparts. In contrast, the other three receptor subtypes show a decrease in density between neonates and adults for both species (**Figure 6**).

To make quantitative comparisons of these age-related changes, we calculated the average percentage difference in the density of blots from neonates and adults within subunit type and species. For NR2A in mice, we calculated that the density of bands from neonates was 35.2 +/- 13.5% that of adults (**Figure 7A**). Similarly, the density of bands from neonatal naked mole-rats was 56.0 +/- 15.55% that of adults (**Figure 7A**). These quantitative results correspond to the age-related increases shown for the examples in **Figure 6**. We used a t-test to compare the age-related change between species (35.2% mice and 56.0% naked mole-rats) and found no significant difference ($t = 1.02$, $df=6$, $p = 0.35$). In **Figure 7b** the NR2A data has been reconfigured to be presented in percent of neonate to be similar to the other receptor data.

The remaining subunits that we tested showed age-related changes in the opposite direction from NR2A. For NR2B (**Figure 7B**), the density of the bands decreased from neonates to adults for both species, and there was not a significant species difference ($t = 0.67$, $df=6$, $p = 0.53$). The adult mice decreased to 64.6 +/- 3.6% that of neonates and the adult naked mole-rats decreased to 71.4 +/- 9.0% that of neonates.

The density of bands for NR2D also decreased from neonates to adults in both species. However, as mentioned above, NR2D showed the most robust species difference (**Figure 7C**). For adult mice, the density of the bands decreased, such that adults were 12.7 +/- 0.6% that of neonates whereas in naked mole-rats, the density decreased to only 66.1 +/- 5.5% that of neonates and this species difference was significant ($t = 9.74$, $df=6$, $p=.0001$).

Figure 7. Quantitative analysis of age-related changes in blot density. A. NR2A increase from neonate to adult in both species. Hence, in Image J we designated the adult band as having a density of 1.0. The bars indicate the average percentage of adult density shown in neonates. **B., C., and D.** NR2B, NR2D, and NR1 decrease from neonate to adult in both species (opposite from NR2A). Therefore, in Image J we designated the neonatal band as having a density of 1.0. The bars indicate the average percentage of neonatal density shown in adults. Each bar in Figure 2 was derived from an N =4, except for the bar corresponding to NR1 for naked mole-rat, which was N= 3. Error bars are standard error. * = $p< 0.05$, **** = $p< 0.0001$, ns = not significant.


Mice NMR
A.
NR2A
% of adult
100
80
60
40
20
0
ns
B.
NR2B
% of neonate
100
80
60
40
20
0
ns
C.
NR2D
% of neonate
100
80
60
40
20
0
****
D.
NR1
% of neonate
100
80
60
40
20
0
*

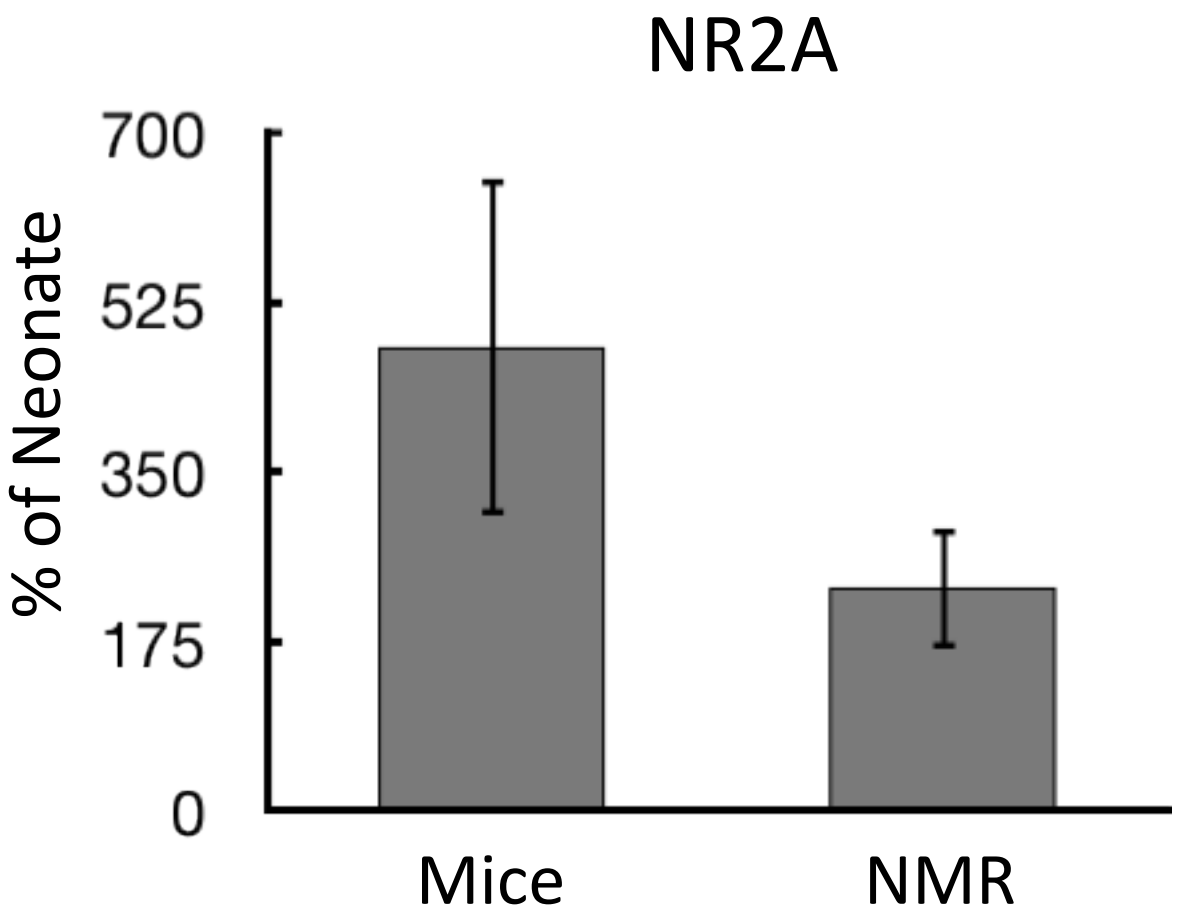


Figure 7b. NR2A represented in percent of neonate. This shows the NR2A data again represented in percent of neonate so that it can be compared to the other data.

We also measured age-related changes in the NMDA receptor subunit NR1. NR1 is found in all NMDA receptors and thus gives a measure of overall changes in the quantity of NMDA receptors. NR1 showed the smallest age-related change among subunits for both mice and naked mole-rats as expected (**Figure 7D**). However, the difference was significant. Adult mice showed a decrease to 75.6 +/- 4.7% that of neonates and naked mole-rats showed a decrease to 93.1 +/- 2.1% ($t=2.60$, $df=5$, $p=.048$).

4.5 **Discussion**

The present study shows that adult naked mole-rats retain relatively high levels of NR2D, an NMDA receptor subunit associated with hypoxia tolerance in neonatal rats and mice. To the best of our knowledge this is the first demonstration of this phenomenon. Because the animals we tested were maintained under normoxia, this characteristic is not induced by hypoxia but is inherent to this species (much like their high affinity hemoglobin and low metabolic rat).

It is already known than some other hypoxia tolerant animals alter their NMDA receptors in a variety of ways that are induced by hypoxia (acute hypoxia). In turtle neurons, it has been shown that NMDA receptors are removed from the cell membrane after they are exposed to hypoxia for several days (Bickler et al., 2000). NMDA receptors in the hibernating Arctic Ground squirrel had significantly less phosphorylation on the NR1 subunit of the NMDA receptor during hibernation. Phosphorylation of the NMDA subunit NR1 is known to enhance NMDA receptor activity (Zhao et al., 2006a; Zhao et al., 2006b; Ross et al., 2006). As already mentioned, neonatal rats have more NR2D which has a higher percentage of close time during hypoxia exposure. All of these model system work to prevent the toxic increase of internal calcium entry through NMDA receptors. Therefore, it is not surprising that naked mole-rats

would also show alterations in their NMDA receptors. It is possible that naked mole-rats living under chronic hypoxia would show additional modulation of NMDA receptors resulting in an even greater attenuation of the calcium response.

Naked mole-rats do not retain the entire neonatal NMDA profile into adulthood. NR2A increased and NR2B decreased with age as in mice. However, compared to mice, naked mole-rats do retain much more of the important neonatal subunit, NR2D, that is protective during hypoxia. The retention of NR2D in adult naked mole-rats is consistent with other brain features that suggest a slowed or arrested maturation in this species.

5. DISCUSSION

5.1 **Hypoxia tolerance summary**

All animals need oxygen in order to make ATP, which is an energy source for cells. When an animal is deprived of oxygen its cells can no longer make enough ATP to meet the energy demand of the animal. Since neurons have a very high metabolism they are extremely vulnerable to this ATP loss. Maintaining ion gradients is one of the major demands for high levels of ATP in neurons. The sodium/potassium pump, which keeps the neuron polarized, is ATP dependent. Therefore when ATP begins to decrease it can no longer function and ion are allowed to move down their concentration gradient ultimately depolarizing the cell. This starts a cascade effect that will release glutamate into the extracellular space activating the glutamate receptors AMPA and NMDA. Once NMDA receptors over activated they let toxic levels of calcium into the postsynaptic cell that can kill the neuron of excitotoxicity.

In an effort to learn more about how to prevent this type of injury in patients many hypoxia tolerant animals have been studied. Some of the model systems, such as turtles, are able to prevent lose of ion gradients by lower the energy demand and maintaining ATP levels. All of the model systems are able to prevent the toxic levels of calcium from accumulating. A man-made model system of drosophila raised in very low levels of oxygen demonstrated that the tolerant flies could regulate their genes to promote their survival. Even though much knowledge has been gained by studying these animals, we still do not have a way to prevent hypoxic injury in humans. Naked mole-rats are a new hypoxia tolerant model system that differs from the others because it is a naturally occurring animal that lives in a chronically low oxygen environment. Since this animal has to tolerate low oxygen while maintaining function throughout its life there is potential to learn about hypoxia tolerance in a whole new way.

5.2 **Current study summary**

The main purpose of these experiments was to explore the mechanisms underlying the extreme hypoxia tolerance of naked mole-rat brain tissue. We found that naked mole-rats have several protective characteristics to reduce or prevent injury from lack of oxygen. These characteristics are similar to the protective characteristics in the hypoxia tolerant neonatal mammal.

We found that the adult naked mole-rat *in vivo* can withstand anoxia at least 5 times longer than the adult mouse. When the brain tissue of adult naked mole-rats was further examined, we learned that they have a reduced rate of ATP utilization during hypoxia compared to adult mice, however, it was not as slowed as neonatal mice. Interestingly, adult naked mole-rat brain slices had a much-reduced increase in internal calcium in CA1 pyramidal cells during a ten-minute hypoxia exposure than both adult and neonatal mice brain slices. Moreover, the neonatal naked mole-rat brain slices did not show a measureable increase in internal calcium during hypoxia until a 15-minute hypoxia exposure time. The reduced increase in internal calcium during hypoxia exposure could be due in part to our finding that brain tissue from adult naked mole-rats retain more of the NMDA receptor subtype NR2D (which closes during hypoxia) into adulthood. This receptor subtype is usually found in higher quantities in neonatal animals, then is greatly reduced as the animal matures.

5.3 **Future Directions**

The findings from the current study will help steer this project's future experiments. Even though the naked mole-rats in these experiments showed remarkable tolerance to hypoxia, the animals tested were maintained under normal room air, which is different from their natural

environment. It would be interesting to see if hypoxia tolerance were not even more pronounced in animals raised in an environment where the oxygen content is more similar to what they experience in the wild. It would be reasonable to hypothesize that all of the reported results would be more extreme in naked mole-rats living in chronic hypoxia for an extended period of time.

The *in vivo* study that determines how long naked mole-rats can withstand anoxia compared to adults could be expanded. It would be insightful to determine if and how much brain damage naked mole-rats had following their time in the anoxic chamber. In continuing with the hypothesis of naked mole-rats being similar to neonatal mice, it would also be noteworthy to test neonatal mice to see how long they can survive during the anoxic exposure.

A series of patch-clamping experiments on naked mole-rat brain tissue would be very beneficial to learn more about the physiology of this animal during hypoxia exposure. It needs to be determined if and by how much the membrane potential is depolarized in the presynaptic neuron during hypoxia. If it is depolarizing as much as the mouse, then the mechanism of tolerance must be focused in the postsynaptic neuron. It would also be appealing to use patch-clamp to characterize the NMDA receptor subtypes in the adult naked mole-rat. This could affirm our finding that naked mole-rats continue to use NMDA receptors with the NR2D subunit into adulthood.

There is still a lot more to be discovered about how naked mole-rats are able to survive in a chronically low oxygen environment. This is a worthy topic of study because it has the potential to assist in minimizing brain damage in stroke victims and premature infants.

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VITA

NAME:	Bethany Leanna Peterson
EDUCATION:	B.S., Biology, University of Arkansas, Fayetteville, Arkansas, 2005
	Ph.D., Biological Sciences, University of Illinois at Chicago, Chicago, Illinois, 2011
TEACHING:	Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois: General Microbiology, 8 semesters.
	Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois: Biology of Cell and Organisms Laboratory, 5 semesters.
	Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois: Microbiology Laboratory, 2 semesters.
	Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois: Cellular Biology, 1 semester.
	Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois: Neurobiology I, 1 semester.
	Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois: Biology of the Brain, 1 semester.
	Individual mentor for 1 master's student and 2 undergraduates
PROFESSIONAL MEMBERSHIP:	The New York Academy of Sciences - 2009
ABSTRACTS:	Peterson BL, Larson JR, Fall CP, Park TJ (2011) Extreme Hypoxia Tolerance in the Brain of Naked Mole-Rat. Society for Neuroscience, Chicago Chapter meeting.
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