Cannabinoid Modulation of Development and Neuroprotection in African Naked Mole-Rats

BY

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THESIS

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This thesis is dedicated to my son, Dylan McMullin, and my partner, Justin Beach, who have been a huge support and help throughout my time in graduate school.

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Contribution of Authors for Previously Published Work

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NMR	Naked Mole-Rat
CB1R	Cannabinoid Receptor 1
CB2R	Cannabinoid Receptor 2
eCB	Endocannabinoid
2AG	2-Arachidonylglycerol
AEA	Anandamide
DAG	Diacylglycerol
DAGL	Diacylglycerol Lipase
MAG	Monoacylglycerol
MAGL	Monoacylglycerol Lipase
FAAH	Fatty Acid Amide Hydrolase
vGAT	Vesicular GABAergic Transporter Protein
vGlut	Vesicular Glutamatergic Transporter Protein
GPCR	G-Protein Coupled Receptor
TM	Transmembrane
NAPE	N-acylphosphatidylethamolamines
NAPE-PLD	N-acylphosphatidylethamolamines- Phospholipase D
THC	Δ-9 tetrahydrocannabinol
CBD	Cannabidiol
WIN55	(*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

HU210	(6a*R*)-*trans*-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b*,*d*]pyran-9-methanol
LTP/D	Long Term Potentiation/ Depression
eCB-LTP/D	Endocannabinoid Dependent Long-Term Potentiation/Depression
PFC	Prefrontal Cortex
CCK+	Cholecystokinin Positive
PAG	Periaqueductal Gray Region
DSI/E	Depolarization Induced Synaptic Inhibition/Excitation
cAMP	Cyclic Adenosine Monophoshate
MGluR1	Metabotropic-Glutamate Receptor Group-1
NMDAR	N-Methyl-D-Aspartate Receptor
PLC	Phospholipase C
PKA	Protein Kinase A
MAPK	Mitogen Activated Protein Kinase
ERK1/2	Extracellular Receptor Kinase 1/2
JNK	C-Jun N-Terminal Kinase Pathway
O2	Oxygen
CO2	Caron Dioxide
OR	Olfactory Receptors
VNO	Vomeronasal Organ
OGD	Oxygen/Glucose Deprivation
CA	Cornu Ammonis
HVR	Hypoxic Ventilatory Response
GABA	Gamma Aminobutyric Acid

LVDP	Left Ventricular Developed Pressure
f(I/E)PSP	Field Inhibitory/Excitatory Post-synaptic Potential
CFA	Complete Freund's Adjuvant
NGF	Nerve Growth Factor
PPT	Preprotachiykinin
TRPV1	Transient Receptor Potential Vanilloid- 1
GAP43	Growth Associated Protein-43
PAG	Periaquiductal Gray Area
IP	Intraperitoneal
PFA	Paraformeldehyde
PBS	Phosphate Buffer Solution
DRG	Dorsal Root Ganglion
AD	Anoxic Depolarization
ko	Knock Out
PTX	Picrotoxin
pnd	Post-Natal Day

SUMMARY

The naked mole-rat (Heterocephalus glaber) displays typical subterranean features, but also has unusual characteristics even among subterranean mammals. These features may be a result of many respiring animals cramped together in unventilated burrows which elevates CO_2 levels, high enough to cause acidosis pain, and depletes O_2 concentrations low enough to kill other mammals. The naked mole-rat may be an extreme model of adaptation to subterranean life and provides insights into the complex interplay of evolutionary adaptations to the constraints of subterranean living.

Cannabis has been used for thousands of years both recreational and medicinally. Many naked mole-rat adaptations are based in systems modulated by the endocannabinoid system. The endocannabinoid system is a complex network of receptors and ligands have been found to be integral to regulation of many systems. This project characterized the three most relevant to naked mole-rat adaptations: development, pain attenuation, and neuroprotection after trauma like low oxygen.

To understand the function of the endocannabinoid system within the brain, it is important to understand the expression of the receptors within brain regions. Therefore, the introduction finishes with a quick review focusing on the hippocampus, prefrontal cortex (PFC), cerebellum, and afferent fibers of the spinal cord, due to their high expression levels of the cannabinoid receptor, CB1r, and the functionally relevant modulation of those regions in naked mole-rat physiology. Due to the pervasive expression of CB1r throughout most of the brain, the endocannabinoid system is able to modulate a variety of behaviors and cellular states. In addition to having multiple pathways for synaptic inhibition, the ability of the endocannabinoid system to up and down-regulate itself in response to the needs of the region makes this system extremely flexible in practical function. While the endogenous function of the eCB system relies on precise spatio-temporal regulation, treatments with exogenous cannabinoids

SUMMARY (continued)

often reflect a more global targeting of multiple functional areas. Understanding the complexity and integral nature of the cannabinoid system will be key to utilizing its focused modulation as a therapeutic target.

This is the first study to describe the endocannabinoid system in naked mole-rats. Naked mole-rats have retained neonatal traits for hypoxia tolerance, longer than usual post-natal brain maturation for rodents, and extremely long-life span. The endocannabinoid system participates in neurodevelopment, neuroprotection and synaptic transmission. In brain regions with higher brain functions, such as the PFC and hippocampus, naked mole-rats do not complete maturation in a similar manner as mice and rats, but rather appear to remain at an adolescent level throughout their lifespan. Brain regions with more vital functions, such as the cerebellum, do exhibit full maturation as they enter adulthood (at 1 year of age) which is developmentally similar to, though much slower than, other rodents. Additionally, cannabinoid induced motor depression/catalepsy is exhibited through 9 months of age. Surprisingly, the motor depression is abruptly extinguished in adulthood and CB1r agonist, WIN55-212,2 (WIN55), application induces no locomotor effects. This is interesting considering naked mole-rats retain a higher concentration of CB1r than expected in adulthood. Likewise, acute treatments of WIN55 impair learning in adult animals after the second dose yet adolescent animals are resistant to detriments when there are fewer than four applications. There are, however, learning detriments after chronic treatments for adolescent mole-rats that are not seen with adults even though the animals are not directly under the influence during their tests. Finally, we found developmental differences in synaptic function modulated by the endocannabinoid system in both the CA1 and dentate gyrus of the hippocampus. Importantly, the regions that exhibit abrogated maturation are integral to neuroprotection and the cannabinoid system has been shown to facilitate

SUMMARY (continued)

neuroprotection in immature mice and rats. Additionally, the cannabinoid system has developmentally dependent effects on synaptic facilitation, with paired-pulse depression observed in adolescents and facilitation in adults after treatment with cannabinoids. Therefore, like other neonatally retained traits, the retention of an immature endocannabinoid system may be due to a need for adult naked mole-rats to mediate neuroprotection through the endocannabinoid system. Examining the role of the endocannabinoid system in maintaining synaptic integrity during hypoxia could help in determining if that is indeed the case.

Naked mole-rats have adaptations within their pain pathway that are beneficial to survival in large colonies within complex tunnel systems, which are characterized by low O_2 and high CO_2 levels. These mutations ultimately lead to a partial disruption of the C-fiber pathway which allow the naked mole-rats to not feel pain from the acidosis associated with CO_2 build up. In this study we first positively established the functionality of the P2X3r C-fiber pathway, a key pathway for inflammatory pain that has yet to be described in naked mole-rats. Naked mole-rats still exhibit a phase II response after formalin application which can be greatly reduced by P2X3r antagonism, suggesting a significant portion of the response is through the P2X3r pathway. Calcium response in DRG cultured neurons to stimulation and *in vivo* behavioral response to application with ATP indicate that naked mole-rat P2X3r responds in a similar manner to mice. We also found that cannabinoids reduce phase II pain in naked mole-rats similarly to mice despite the lack of neuropeptides in their pain pathway. In the Von Frey test, P2X3r agonist α-β-met-ATP reduces naked mole-rats pain threshold. Collectively this indicates that cannabinoids reduce the sensitization of C-fibers in Phase II primarily through the P2X3r pathway in naked mole-rats.

SUMMARY (continued)

In addition to synaptic maintenance during oxygen deprivation, naked mole-rats attenuate calcium increases during oxygen deprivation and retain into adulthood subunits of NMDAr that gives protection against hypoxia to neonates. In neonatal development, the endocannabinoid system has a pivotal role in synapse formation and development, including mediating the development and maturation of PPF, axonal scaffolding, synapse formation, and reducing intracellular calcium. Therefore, the final aim of the project was to assess the role of endocannabinoids in naked mole-rat hypoxia tolerance. In an analysis of naked mole-rat expression after 5 hours of hypoxia, there is a global decrease in multiple endocannabinoids which is not seen in mice. Using hippocampal brain slice, we looked at the effect of CB1r activation and deactivation, on the CA1 region of the hippocampus when exposed to anoxia. Responses were found to be different in immature (<11 months old) vs mature (>1 year) animals. As expected from the expression data, antagonism of CB1r with AM251 was protective and could be reversed through GABA inhibition. However, pretreatment of CB1r agonist, WIN55, also exhibited protection from anoxic depolarization in mature animals and this protective mechanism is not GABA-dependent. In immature animals, WIN55 did not significantly affect the latency to depolarization; however, it did reduce the recovery time after depolarization in WIN55 pretreatment. Histology results indicate that CB1r distribution is altered from adolescence to adulthood not in intensity but rather in company, colocalization with both excitatory and inhibitory synapses increasing significantly.

This project identified multiple areas of interest for therapeutic potential in development, pain, and neuroprotection. Additionally, the initial characterization of the naked mole-rat endocannabinoid system has indicates that the unusual adaptations of this species extend into this molecular system and reinforced the dynamic potential of the endocannabinoid system to modulate a vast array of functions

Chapter I: Introduction

A. Naked Mole-Rats: Blind, Naked, and Feeling No Pain

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Abstract

Around the world and across taxa, subterranean mammals show remarkable convergent evolution in morphology (e.g. reduced external ears, small eyes, shortened limbs and tails). This is true of sensory systems as well (e.g. loss of object vision and high frequency hearing). The naked mole-rat (*Heterocephalus glaber*) displays these typical subterranean features, but also has unusual characteristics even among subterranean mammals. Naked mole-rats are cold-blooded, completely furless, very long-lived (> 30 years), and eusocial (like termites). They also live in large colonies, which is very unusual for subterraneans. Their cortical organization has reduced area for visual processing, utilizing 30% more cortex for tactile processing. They are extremely tolerant to oxygen deprivation and can recover from 18 minutes of anoxia. Their pain pathway is reduced, and they feel no pain from acidosis. They are the only rodent tested to date whose pheromone-detecting vomeronasal organ

shows no postnatal growth. These features may be a result of this species' "extreme subterranean lifestyle" that combines living underground and living in large colonies. Many respiring animals cramped together in unventilated burrows elevates CO_2 levels, high enough to cause acidosis pain, and depletes O_2 concentrations low enough to kill other mammals. The naked mole-rat may be an extreme model of adaptation to subterranean life and provides insights into the complex interplay of evolutionary adaptations to the constraints of subterranean living.

Introduction

Fossorial mammals, having found their niche in subterranean ecotopes, are faced with an array of physiological challenges that drive specific adaptations (for a thorough review see: Bennett and Faulkes, 2000, Jarvis and Bennet, 1991). Life underground lacks certain environmental properties of terrestrial life such as regular exposure to sunlight, physiologically "normal" air composition, fluctuations in ambient temperature and normal propagation of sound. Subterranean ecosystems are often resource poor with scarce food. The animals that live in these conditions have developed ways to meet these challenges head on. The African mole-rat family Bathyergidae has successfully adapted to a life underground. Evidence of the Bathyergidae family can be traced back at least 40-50 million years ago (Faulkes et al., 2004; Pickford et al., 2008). It is composed of 12 genera and about 30 species. Though they are classified in the Rodentia order, there has been some debate over their suborder classification due to the unique skull morphology and head musculature of the African mole-rat family that disallows classical distinction (Honeycutt et al., 1991).

Heterocephalus glaber or the African naked mole-rat is a member of the Bathyergidae family and is the only species in its genus. Naked mole-rats are found in the Horn of Africa in areas of Ethiopia, Kenya and Somalia. The climate in these areas is hot and dry with an average annual rainfall of 600mm (Burda, 2001). While members of the Bathyergidae family are universally subterranean (Jarvis and Bennett, 1991), their underground habitat consists of burrows of varying complexity. The burrow systems of naked mole-rat are comprised of multiple nests, toilet chambers, food storage chambers and deep tunnels for defensive retreat (Jarvis and Bennett, 1991, Brett, 1991). These burrows are dynamic and remodeled to regulate ambient temperature, moisture and to expand foraging range. The length of the burrow directly correlates to the biomass of the colony (Jarvis and Bennett, 1991) and is modulated to meet the needs of the colony.

Naked mole-rat burrows are almost completely removed from the elements of the surface. The ambient temperature of the burrows is relatively constant with little fluctuation throughout the day and throughout the year (Brett, 1991). In the instance of temperature fluctuation, naked mole-rats transition to different lamina within the system to arrive at a physiologically favorable ambient temperature (their preferred temperature being ~30 degrees C (Begall et al., 2015)). This is especially important because naked mole-rats do not thermoregulate; they are poikilothermic (Buffenstein and Yahav, 1991). However, this has not impeded naked mole-rats' ability to thrive as they have little need to self-generate heat in this habitat and, consequently, they are able to conserve much needed energy for their great foraging need. In addition to being poikilothermic, naked mole-rats are also unusual in that they have a low resting metabolic rate (Buffenstein and Yahav, 1991) they are very long lived (Buffenstein, 2008), and they are very resistant to cancer (Liang et al, 2010; Tian et al., 2013).

Adult naked mole-rats range in size from 25 grams (Figure 1), which is similar to a mouse, to 60 grams, which is similar to a small rat. Their size is dependent on factors such as food availability, colony size, and soil hardness which accounts for the broad variability in body mass (Jarvis and Bennett, 1991). Like other subterraneans, their body is cylindrical, and like other African mole-rats, naked mole-rats have enlarged incisors, which grow from above and below the lips (e.g the incisors are located permanently exterior to the oral cavity, see Figure 1c in Catania and Remple, 2002), and only 2-3 molar teeth in each jaw (Catania and Remple, 2002). Like many subterraneans, naked mole-rat have tiny eyes and no external ear pinnae (Mason, 2016). Their skin is loose and wrinkled, which allows them to easily maneuver in the tight spaces of their tunnel habitat.

Within the family Bathyergidae there is a full spectrum of sociality ranging from a solitary lifestyle to the highest degree of sociality (Bennett and Faulkes, 2000). Naked mole-rat colonies have a highly organized eusocial division of labor similar to social insects like termites. The social hierarchy of naked mole-rats is established by age and weight; the oldest and heaviest leading in rank. The

dominant female is typically the largest female in a colony and is the exclusive breeding female. The dominant female is often referred to as the queen, a nomenclature used in the description of eusocial insects (Jarvis, 1981). The queen suppresses reproductive maturation in her colony mates except for two or three males (Jarvis, 1981). The queen inhibits the circulation of sex hormones in subordinate females though physical intimidation instead of through pheromones in urine like other rodents (Margulis, 1995; Dengler- Crish and Catania, 2007). As the queen is the only female to reproduce, this generates a highly inbred population within the colony.

High sociality and large colony size in a subterranean environment may have led to unusual traits in somato-sensation, the vomeronasal organ, and responses to hypoxia and pain. The main aim of the present manuscript is to review these extreme, putative adaptations. This is important because it reveals how evolution can result in novel solutions to extreme environmental problems.

Somatosensory Adaptations

Naked mole-rats have very poor visual function (Hetling et al., 2005) and sound localization ability (Heffner and Heffner, 1993). Instead they rely heavily on their tactile senses to navigate their complex tunnel systems. They are also likely to be sensitive to vibrations (Mason and Narins, 2010). Seki and colleagues (2013) have shown that there is prenatal development of the optic nerve; however, this nerve, and additional markers for the visual system, decrease after birth leaving the eye fully developed but very small for a rodent (Hetling et al, 2005). Also, the brain's vision centers are diminished to the point where only broad shapes and variations of light are detectable by the adult (Hetling et al, 2005; Xiao et al. 2006). Naked mole-rats may also retain a diminished circadian input (Ooshuizen, 2010) though their pineal gland is significantly reduced compared to mice (Kim et al, 2011; Quay, 1981). In conjunction with a reduction in visual cortex, the somatosensory cortex appears to utilize an increased cortical volume to increase tactile representation. This is confirmed by the substantial changes in cortical structures (Catania and Remple, 2002; Seki et al., 2013). The primary

somatosensory cortex (S1 region) of the naked mole-rat is increased by up to 51% (Xiao et al., 2006). This caudal expansion projects into what is expected to be visual cortex in rats and mice. Naked mole-rats have well developed facial whiskers (Crish et al., 2003) and a weak (compared to mice) barrel pattern in somatosensory cortex representing the largest facial whiskers (Park et al., 2007). However, much of the increase in the S1 region can be considered a direct effect of the increased representation of tactile body vibrissae (Xiao, 2006) also known as somatic vibrissae (Park et al., 2003). Naked mole-rats lack fur, as the word "naked" suggests, but naked mole-rats do have regularly arranged whisker-like vibrissae on their bodies, which are well innervated, similar to guard hairs of furred species (Park et al., 2003). There are approximately 40 vibrissae on each side of the body, organized into a grid-like pattern (Figure 2A,B). The body vibrissae are arranged topographically into a sensory array, and stimulation of a given vibrissae elicits an orienting response to the point of stimulation (Figure 2C,D) (Crish et al., 2003). The structure and innervation of the body vibrissae follicles resemble those of exceptionally large guard hairs in other mammals (Park et al., 2003). Mechanically stimulating a single vibrissa causes a robust turn of the naked mole-rat's head to bring the snout to the point of contact. Importantly, when two ipsilateral vibrissae are stimulated simultaneously, the animal orients to a location midway between the two stimulated vibrissae ("averaging"; Crish et al., 2006). On the contrary, when two hairs are stimulated on opposite sides of the body, the animal responds with a full response to one of the stimulated hairs and not the other ("winner take all"; Crish et al., 2006). These findings are important because they indicate that, within a hemifield, the naked mole-rat nervous system performs computations (averaging) of multiple points of contact. It is also noteworthy that naked mole-rats, like many subterraneans, routinely locomote backwards (Lacey et al., 1991), particularly when vibrissae on the tail are stimulated (Crish et al., 2003).

The incisors are also overrepresented in the somatosensory cortex of the naked mole-rat, indicating that they are well innervated (Catania and Remple, 2002). Like most of the other African

mole-rats, naked mole-rats construct their tunnels by digging with their oversized incisors and they use their teeth to carry young, food, and debris (Jarvis and Bennett, 1991 Catania and Remple, 2002). The incisors are very conspicuous because the lips meet behind the incisors (Figure 1), allowing the animals to close their lips behind the incisors while burrowing. Nearly 25% of the total musculature of a naked mole-rat is dedicated to the jaws, including separate muscles that give naked mole-rat the ability to independently move their incisors (Catania and Remple, 2002). The cortical area associated with this large amount of muscle corresponds to 30% of the somatosensory cortex (in the lateral region), and 10% of the neocortex is associated with motor control of the jaw and incisors (Catania and Remple, 2002). While representation of the facial regions within S1 are not as distinct in naked mole-rat as in mice and rats, a barrel pattern can still be distinguished (Henry et al., 2006). Like other rodents, naked mole-rat have a dense localization of cells in layer IV of the somatosensory cortex that correspond to body parts used for burrowing- in particular, their incisors and forelimbs (Henry et al., 2006). Conversely, the secondary connections for the incisors remain very local and have little connection to the anterior cortex, instead exhibiting reciprocal connections within the somatosensory cortex (Henry and Catania, 2006).

Olfaction and the Vomeronasal Organ

Mammals have two olfactory systems, the main and the accessory. In the main olfactory pathway odorous molecules travel to the posterior nasal cavity where they are detected by the olfactory receptors of the olfactory neuroepithelium (Buck and Axel, 1991). Olfactory receptors (ORs) are seven-transmembrane G-protein coupled receptors encoded by the OR-gene superfamily, the largest gene family in the vertebrate genome (Buck and Axel, 1991; Lancet and Ben-Arie, 1993). The extent of the functional OR-gene repertoire varies significantly among vertebrates as a function of evolutionary pressure on olfactory reliance (Stathopoulos et al., 2014) with a direct association between functional OR diversity and olfactory acuity (Kishida, 2008). Animals that rely heavily on

olfaction for fitness-related tasks express a more extensive functional-OR repertoire compared to animals who rely on other sensory modalities – such as humans who rely predominately on trichromatic vision (Niimura and Nei, 2006).

African mole-rats live in dark underground burrows which has resulted in a family-wide reduction in visual acuity (Němec et al., 2008). This loss of visual abilities is compensated for by enhanced olfactory sensitivity with positive selection for functional OR-gene diversity (Stathopoulos et al., 2014). The entire family also expresses a degree of encephalization comparable to that of terrestrial rodents, which are generally characterized as having an acute sense of smell (Kruska and Seffen, 2009). Some Bathyergidae species even exhibit larger olfactory regions compared to Rattus norvegicus or the common rat (Kruska and Steffen, 2009), most likely to be a compensation for reduction of visual function. The naked mole-rat is no exception to the enhanced olfaction demonstrated by its family. The naked mole-rat exhibits well-developed olfactory structures in the brain (Hill et al., 1957; Pilleri, 1960) despite having the smallest brain within the family (Pirlot, 1990; Kruska and Steffen, 2009). The naked mole-rat relies on odors for many fitness-related tasks such as recruitment of colony members toward a food source (Judd and Sherman, 1996) as well as xenophobic behavior (O'Riain and Jarvis, 1997). The naked mole-rat demonstrates the structural, molecular and behavioral markers for acute olfactory sensitivity.

As mentioned previously, naked mole-rats exhibit xenophobic behaviors by aggressing towards non-colony members. Interestingly, this individual recognition is not mediated through pheromones as it is in other rodents (O'Riain and Jarvis, 1995). Pheromones are described as molecules released by an individual that elicit a physiological and behavioral response in another individual of the same species (Tirindelli et al., 2009). In many mammals, pheromonal detection is mediated through the accessory system's chemosensory structure - the vomeronasal organ (VNO) which is embedded at the base of the nasal septum and lined with neuroepithelium in rodents (Meredith and O'Connell, 1979).

There is rapid peri- and postnatal proliferation of the sensory neurons of the VNO in mice, rats and other mammals (Weiler et al., 1999; Wilson and Raisman, 1980). Interestingly, the naked mole-rat shows no post-natal growth of the VNO (Figure 3) (mean vomeronasal neuroepithelium VNNE volumes were 0.012 mm^3(± 0.005 mm3) for subadults and 0.015 mm^3 (± 0.007 mm^3) for adults, p>.05, Smith et al., 2007). The VNO is essential for mediating sociosexual cues in many vertebrates, however naked mole-rats appear not to rely on pheromones to relay this information (Faulkes and Abbott, 1993; Judd and Sherman, 1996; O'Riain and Jarvis, 1997). Apparently, the naked mole-rat demonstrates behavior-mediated sexual suppression instead of relying on pheromone-based urinary signals like rats, mice, and other rodents. The dominant breeding female in a naked mole-rat colony imposes her status through physical intimidation which inhibits the circulation of sex hormones (Margulis et al., 1995). It is thought that this divergence from the Rodentia "norm" is a result of the naked mole- rat's eusocial social structure eliminating the need for pheromonal-based sexual suppression (Smith et al., 2007). Overall, the naked mole-rat exhibits acute main olfaction structurally and molecularly; while the accessory pathway is extremely reduced. It is possible that pheromonal detection is occurring through the main olfactory pathway as is thought to occur in humans and some other vertebrates (e.g. Frasnelli et al., 2011).

Hypoxia Tolerance

Most subterranean mammals are solitary or live in small groups which avoids depleting the available supply of oxygen (O2). Naked mole-rats, on the contrary, are extremely social and live in colonies of as many as 300 animals. Also, they tend to spend much of their time sleeping or huddling together in deep nesting chambers. This leads to local depletion of O_2 and accumulation of carbon dioxide (CO_2) at a greater extent than that experienced by other subterraneans (Faulkes and Bennett,

2013). Naked mole-rats display dramatic adaptations to survive and even thrive in these conditions that would be deadly to most mammals.

Intrinsic Brain Tolerance

In mammals, the brain is particularly susceptible to injury from oxygen deprivation (Russell, 1964). However, naked mole-rats show extreme intrinsic brain tolerance to hypoxia and anoxia (Larson and Park, 2009; Larson et al., 2014). Hippocampal brain slices from naked mole- rats can recover from O_2 concentrations that rapidly kill slices from mice, including 0% O_2. Figure 4 shows the effects of low concentrations of O_2 on field excitatory post-synaptic potentials (fEPSPs) measured in slices from naked mole-rats and mice before, during, and after exposure to hypoxia (Figure 4A,B). Summary data show that on average, slices from naked mole-rats have less of an acute loss of function during hypoxia (Figure 4C) and a better recovery rate after hypoxia (Figure 4D) compared to slices from mice. Under nominal 0% O_2, slices from naked mole-rats continue to function much longer than slices from mice (Figure 4E). This is true at two different temperatures: at 35 degrees C, close to the physiological temperature of mice, and at 30 degrees C, the naked mole-rats' preferred temperature (Figure 4F).

When slices from rats and mice are exposed to hypoxia, there is a progressive increase in intracellular calcium levels that rapidly leads to calcium toxicity and cell death. Reduction of hypoxia-induced intracellular calcium accumulation is associated with hypoxia tolerance in many hypoxia-tolerant animals (Larson et al., 2014; Peterson, 2012a). The hippocampal CA1 region of slices from naked mole-rats shows a significant attenuation of hypoxia-induced intracellular calcium accumulation compared to mice (Peterson, 2012b). The composition of ionotropic glutamatergic NMDA receptors in naked mole-rat brain cells may play an important role in attenuating intracellular calcium accumulation during hypoxic assault. NMDA receptors are calcium channels. The efficacy of NMDA subunits to open the channel can contribute to toxicity levels within the cells, with high subunit efficacy leading to cell

death but low efficacy contributing to cell protection during hypoxia (Lee et al., 1991). NMDA receptors with GluN2D subunits exhibit the lowest opening efficacy of all of the NMDA subunits, to a sufficient level to protect against hypoxic injury (Bickler et al., 2003). The number of NMDA receptors that express the GluN2D subunit decreases greatly in mice and rats shortly after birth, reducing the hypoxia resistance that is seen during fetal development (Laurie et al., 1997). In contrast, naked mole-rats retain a large proportion of NMDA receptors with GluN2D: 66% in naked mole-rats versus 10% in mice (Peterson, 2012b).

In vivo Tolerance

In vivo experiments show that intact naked mole-rats tolerate severe hypoxia (5% O2) and anoxia (0% O_2) much longer than mice (Figure 5A,B) (Park et al., 2017). Naked mole-rats can recover from 18 minutes of exposure to 0% O_2, more than 18 times longer than mice (Figure 5C). During exposure to 0% O_2, naked mole-rats show a robust decrease in respiration and heart rates (Figure 5D,E) reminiscent of a suspended animation-like state (Park et al., 2017).

Metabolic rates decrease during hypoxia; however, because naked mole-rats are poikilothermic, they do not show significant changes in their body temperature (Park et al., 2017). Reducing metabolism during oxygen deprivation allows naked mole-rats to conserve cellular oxygen, possibly to the point of matching O_2 need with O_2 availability. Naked mole-rats show a 50% reduction in acute hypoxic ventilatory response (HVR) and hypoxic metabolic rate during acute hypoxia. Naked mole-rats exhibit metabolic O_2 extraction at 3x that of normoxic animals but no change in ventilator plasticity following chronic sustained hypoxia (Chung et al., 2016). Gamma aminobutyric acid (GABA) signaling contributes to breathing patterns and ventilatory and metabolic responses to hypoxia – specifically GABA antagonism increases breathing frequency and decreases tidal volume in naked mole-rats but does the opposite in mice – altering breathing patterns but not ventilation or

metabolism (Chung et al., 2016). Moreover, the ventilation response has been shown to be regulated by adenosine receptors; activation of these receptors blocks the reduction in ventilation (Pamenter et al., 2014). Hypoxic conditions also have serious consequences for the heart. In mice and rats extended exposure to low oxygen can lead to myocardial lesions, edema, and capillary injury (Meneely, 1974). Additionally, it is important to maintain high functioning status during hypoxic assault to maintain oxygen flow to other body parts (Abe et al., 2017). When exposed to anoxia in an atmosphere chamber, the heart rate of a naked mole-rat greatly decreases in the first 1-2 minutes and stabilizes at approximately 25% of baseline rate until oxygen is reintroduced (Park et al., 2017). Isolated heart experiments show an intrinsic ability for the organ to continue to beat during anoxia as well as to completely recover left ventricular developed pressure (LVDP) while mice can only recover to about 65% (Park et al., 2017). Anatomically, naked mole-rat hearts maintain similar cellular structure to mice, in terms of striation patterns and sarcomere architecture, but overall the size of the naked mole-rat heart is statistically larger by an average of 60mg. Furthermore, the cardiomyocyte cross-sectional area averages 40 mm^2 and diastolic wall thickness is 0.1mm^2 greater in naked mole-rat compared to mice. Naked mole-rats predominantly express MHC-β in their ventricles unlike mice who express very little MHC-β in their hearts after birth (Grimes et al., 2014). MHC-β is considered the fetal analog of MHC- α, the predominant protein in adult mice (Katsumata et al., 2017). Phosphorylation of cardiac troponin T and myosin light chain in naked mole-rats is half that of mice. Interestingly, naked mole-rats express both cardiac and skeletal troponin in their ventricular tissue, which, like the MHC-β expression, appears to be retention of neonatal traits as an adaptation for hypoxia tolerance (Grimes et al., 2017).

Fructose Metabolism

Naked mole-rats show a high expression of the GLUT5 transporter in organs that do not usually express much GLUT5 (e.g. heart and brain) (Park et al., 2017). This adaptation is paramount to one of

the most unusual adaptations to hypoxia that has been identified in a mammal. The GLUT5 transporter specifically transports fructose across cell membranes, and in the naked mole-rat it actively transports fructose into brain and heart cells. During anoxia, naked mole-rats maintain stable glucose levels because they are able to utilize fructose in an oxygen- independent manner to maintain function (Park et al., 2017). Experiments with hippocampal brain slices and isolated hearts suggest that these organs can switch from glucose metabolism to fructose metabolism (Figure 6). Figure 6A shows a schematic of the hippocampal slice preparation and example fEPSP traces on the left, and average fEPSPs amplitudes over time from mouse and naked mole-rat slices on the right. When bath glucose was switched to fructose, slices from naked mole-rats maintained greater functionality and much better recovery compared to slices from mice. Figure 6B shows that isolated hearts from naked mole-rats maintain close to baseline function during two serial episodes when bath glucose was switched to fructose. In contrast hearts from mice showed a substantial decline in function, particularly during the second episode.

Nociception

The preceding section focused on adaptations associated with living in a chronically low O_2 environment. However, naked mole-rats also are faced with living in chronically high CO_2 concentrations that would cause tissue acidosis with associated pain, as well as pulmonary edema in other mammals (Park et al., 2017). Naked mole-rats show a much higher threshold before avoiding CO_2 compared to mice (Figure 7A,B), suggesting that they are less sensitive to CO_2- induced pain (Park et al., 2017). Also, naked mole-rats are insensitive to pain from breathing acetic acid fumes (LaVinka and Park, 2012). Experiments to measure pulmonary edema from breathing high concentrations of CO_2 revealed that naked mole-rats do not get pulmonary edema, even from very high concentrations of CO_2 (Figure 7C) (Park et al., 2017).

In the upper respiratory tract, acidosis activates sensory neurons in the pain pathway. In the lungs, acidosis activates similar sensory neurons, which trigger a neurogenic inflammatory response leading to edema. Protection from CO_2-induced pain and pulmonary edema in naked mole-rats is associated with a sequence variant in the naked mole-rat voltage-gated sodium channel Nav1.7 (Smith et al., 2011). Nav1.7 channels normally propagate action potentials along the axons of peripheral receptor cells that respond to CO_2-induced acidosis. However, the variant in naked mole-rat Nav1.7 causes it to be inhibited in the presence of acid.

Interestingly, naked mole-rats are also insensitive to pain in the nasal cavity from other (non-acid) chemical pain stimuli, including capsaicin solution (the active ingredient in chili peppers), and fumes from ammonia (LaVinka et al., 2009; LaVinka and Park, 2012). Chemical pain is transduced by a specific population of peripheral pain fibers: they are unmyelinated, small caliber fibers called polymodal pain fibers or C fibers. The term polymodal derives from the ability of these fibers to respond to multiple modes of pain: chemical, inflammatory, heat, and mechanical (Beitel and Dubner, 1976). The gene variant in Nav1.7 should be specific for affecting acid pain but not pain from capsaicin and ammonia. However, the C fibers in naked mole-rats also lack the neurotransmitter, Substance P (Park et al., 2003; Park et al., 2008), which is associated with signaling chemical and inflammatory pain (Cao et al., 1998). This may explain at least part of the naked mole-rats' insensitivity to non-acid painful chemicals. The insensitivity to acid and other chemical pain stimuli described above for naked mole-rats is not restricted to the upper respiratory tract. Rather, insensitivity to chemical pain is found in the skin of the entire body. This makes sense for acid insensitivity because the gene variant for the Nav1.7 channel should make this channel inhibited by acid in all peripheral pain fibers (Smith et al., 2011; Liu et al., 2014). This also makes sense for Substance P-related insensitivity to non-acid chemical pain

stimuli if lack of Substance P is a result of dysregulation of promoter genes specific to the peripheral nervous system, as suggested by the genetic analysis of Kim et al (2011).

Consistent with an altered Nav1.7 channel and lack of peripheral Substance P, naked mole-rats demonstrate a very distinct pattern of pain behaviors. Figure 8 shows the results of a battery of pain tests on naked mole-rats and, for comparison, laboratory mice (Park et al., 2008). Naked mole-rats do not differ from mice in their response to acute mechanical pain (pinch) or acute heat, associated more with A-delta pain fibers, not C pain fibers (Figure 8A). However, naked mole-rats show virtually no response to paw injections of the chemical pain stimuli, capsaicin and acidic saline, associated with C fibers (Figure 8B). In tests of inflammatory pain, naked mole-rats do show sensitization to pressure but not heat during inflammation from complete Freund's adjuvant (CFA) (Figure 8C,D). They also show no sensitization from application of topical capsaicin or injection of nerve growth factor (NGF) (Figure 8E,F). Finally, naked mole-rats show a very reduced response to paw injection of 1% formalin solution (Figure 8G).

Interestingly, a sensitization response to topical capsaicin can be rescued in naked mole- rats using a gene therapy approach (Park et al., 2008). Figure 9 shows foot withdrawal latencies before and after application of capsaicin. One foot was treated with a recombinant herpes virus carrying the gene for Substance P, (preprotachykinin, PPT). The virus-treated foot showed a much faster withdrawal after treatment, indicative of sensitization, compared to the control foot.

It is noteworthy that naked mole-rats show no sensitization to heat after treatment with NGF or the inflammatory agents CFA and capsaicin. Sensitization to heat, called thermal hyperalgesia is mediated by the NGF receptor TrkA, which sensitizes the transient receptor potential vanilloid-1 (TRPV1) receptors. A recent study has shown that the naked mole-rat TrkA receptor has a small mutation that reduces functionality of the receptor for NGF signaling and abolishes thermal hyperalgesia while retaining other important functions of TrkA (Omerbašić et al., 2016).

Delayed or Arrested Development

Extreme adaptations are pushed by niche habitats to enable an animal to thrive, even in the most unusual places. For naked mole-rats, that habitat is deep underground, and they thrive in an environment that is actually quite familiar to most mammalian species during a distinct time in their life. Their warm, humid, and oxygen deprived habitat is very similar to the start of every mammal's life, in a womb. Aptly, many of the adaptations that naked mole-rats display appear to be associated with slowed development and retention of fetal traits. These include retention of GluN2D and MHC-β, greatly reduced calcium accumulation during hypoxia, and the lack of VNO growth past pn1. Coincidentally, Substance P (lacking in the peripheral nerves of naked mole- rats) also mediates the VNO's vascular pump. Lack of Substance P may disable the pump, which would degrade the functionality of the VNO. In the respiratory system, pulmonary neuroepithelial bodies retain neonate characteristics (Pan et al., 2014), possibly allowing naked mole-rats to respond to ambient gas concentrations as do neonates. Further evidence for the naked mole-rat's delayed maturation can be seen in the development of the central nervous system. Naked mole-rat brains continue to grow for months after birth, reaching 90% of their final volume at 3 months of age, and neuronal markers of maturation continue to be unstable for twice that time (Orr, 2016). Neoteny may also contribute to the naked mole-rats' longevity. It has also been shown that synapse distribution and firing patterns do not mature to the same level as that of mice – even after 1 year of age – and that appears related to naked mole-rats' retention of structural plasticity (Penz et al., 2015). This suggests that cellular reorganization instead of neurogenesis is the means of neuroadaptive longevity and is a potential means for naked mole- rats to adapt to the added challenges of living underground under chronic low O_2/high CO_2 conditions.

Conclusions

The naked mole-rat's large colony size and extreme social structure have been integral to their successful foraging in an environment with extremely dispersed food resources. However, this unusual lifestyle comes with major challenges: chronically low O_2 concentrations and chronically high CO_2 concentrations. We postulate that these extreme environmental pressures have driven extreme adaptations in the naked mole-rat for coping with otherwise deadly oxygen deprivation and tissue acidosis.

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Figure Legends

Figure 1A.1. A naked mole-rat in a university vivarium. Photo by Thomas Park and UIC Photo.

Figure 1A.2. Somatic vibrissae and the tactile orienting response of naked mole-rats. A. Scanning electron micrographs of somatic vibrissa compared with facial vibrissae. B. The typical distribution of somatic vibrissae. The dashed lines indicate five zones arbitrarily used to distinguish the rostro-caudal locus of vibrissae stimulated during behavioral trials. C. Two examples of orientation responses when a vibrissa was deflected. The gray form shows the animal's position just before stimulation. The red outline shows animal's maximal turning position. The black lines indicate the body and head axes every 4 frames of the video. The small blue arrowhead shows the location of stimulation. D. Topography of orienting responses following stimulation of hairs along the rostro-caudal axis. From Crish et al., 2003a.

Figure 1A.3. The vomeronasal organ (VNO) of the naked mole-rat shows no post-natal growth. A- D. The vomeronasal organs of two-week-old (A,C) and adult (B,D) naked mole-rats, illustrating that the vomeronasal neuroepithelium (*) is actually smaller in cross-sectional area in adults compared to infant pups. A and B are prepared with Gomori trichrome stain; C and D are prepared with Growth Associated Protein-43 (Gap43) which reveals growing axons. Although the neuroepithellium is approximately equal in average volume comparing infants to adults, it is neurogenic throughout life, as revealed by Gap43 immunohistochemistry (see Smith et al., this issue). Scale bars: A, B, 50 micrometers; C, D, 20 micrometers. E,F. vnne volume as a function of age in naked mole-rats and laboratory rats. From Smith et al., 2007.

Figure 1A.4. Naked mole-rats brain shows extreme intrinsic tolerance to hypoxia and anoxia in the hippocampal brain slice preparation. A. The graph shows field excitatory postsynaptic potential (fEPSP)

responses of a mouse (open circles) and a naked mole-rat (filled circles), normalized to 100% of baseline amplitude for each animal. The bar labeled "15% O_2" indicates a 30-minute period when the O_2 concentration was reduced from 95% to 15%. The traces are examples taken from before and during exposure to 15% O_2, mouse on the left, naked mole-rat on the right. NMR = naked mole-rat. B. Same as A except with 10% O_2. C. Summary data showing average maximum decrease from hypoxia at each concentration of O_2 tested. D. Summary data showing percentage of slices that recovered from each concentration of O_2 tested. E. Response to 0% O_2, the black bar indicates the duration required for the naked mole-rat slice to reach anoxic depolarization (AD), associated with loss of synaptic function. F. The bars show average time to AD, measured at two temperatures. From Larson and Park, 2009.

Figure 1A.5. Intact naked mole-rats show extreme resistance to hypoxia (5% O_2) and anoxia (0% O_2). A. Mice and naked mole-rats were exposed to 5% O_2. Mice ceased breathing attempts after about 12 minutes and did not recover. B. Exposure to 0% O_2. C. Percent of mice and naked mole-rats that recover from different durations of anoxia. D. Respiration rate and E heart rate in naked mole-rats during 18 minutes of anoxia followed by normoxia (black curve = mice). Error bars are standard errors. From Park et al., 2017.

Figure 1A.6. Effect of switching glucose to fructose on functionality of hippocampal brain slices and isolated hearts. A. Schematic of the slice preparation and example fEPSP traces (left). The graph shows average fEPSPs (as percent of control) before, during, and after bath glucose was switched to fructose for 1 hour. B. Left ventricular developed pressure (LVDP), a typical measure of cardiac function, measured from isolated, beating hearts from naked mole-rats and mice. The traces show LVDP (as

percent of control) before, during, and after two successive switches from bath glucose to fructose. From Park et al, 2017.

Figure 1A.7. Responses of mice and naked mole-rats to high concentrations of CO_2. A and B. In an avoidance test, mice (A) spent more time in room air (Air) than in 2.5%, 5%, or 10% CO_2. Naked mole-rats (B) only showed avoidance to 10% CO_2. C. Lung wet-to-dry weight ratios (a measure of pulmonary edema) as a function of CO_2 concentration. Animals were exposed for 15 minutes before lungs were weighed. From Park et al., 2017.

Figure 1A.8. Responses of mice and naked mole-rats in a variety of pain models. A. The bars show response latency to acute mechanical stimulation (pinch) and acute thermal stimulation. B. Time spent licking the injection site for capsaicin solution or acidic solution. C. Thresholds for paw withdrawal before and after inflammation with complete Freund's adjuvant (CFA). D-F. Latencies for foot withdrawal to heat before and after inflammation from capsaicin (Cap) or nerve growth factor (NGF). G. Pain scores for mice (left) and naked mole-rats (right) after foot injection of 1% formalin. From Park et al., 2008.

Figure 1A.9. Rescue of capsaicin-induced sensitization to heat one week after infection of one paw with transgenic herpes virus carrying the preprotachykinin (PPT) gene for Substance P. The paw treated with the virus (PPT Virus) shows sensitization from capsaicin (shorter latency to withdrawal from heat) compared to the paw not treated with the virus (No Virus). From Park et al., 2008.

Naked Mole-Rat in Captivity

Figure 1A.1.

Somatic Vibrissae and the Tactile Orienting Response of Naked Mole-Rats

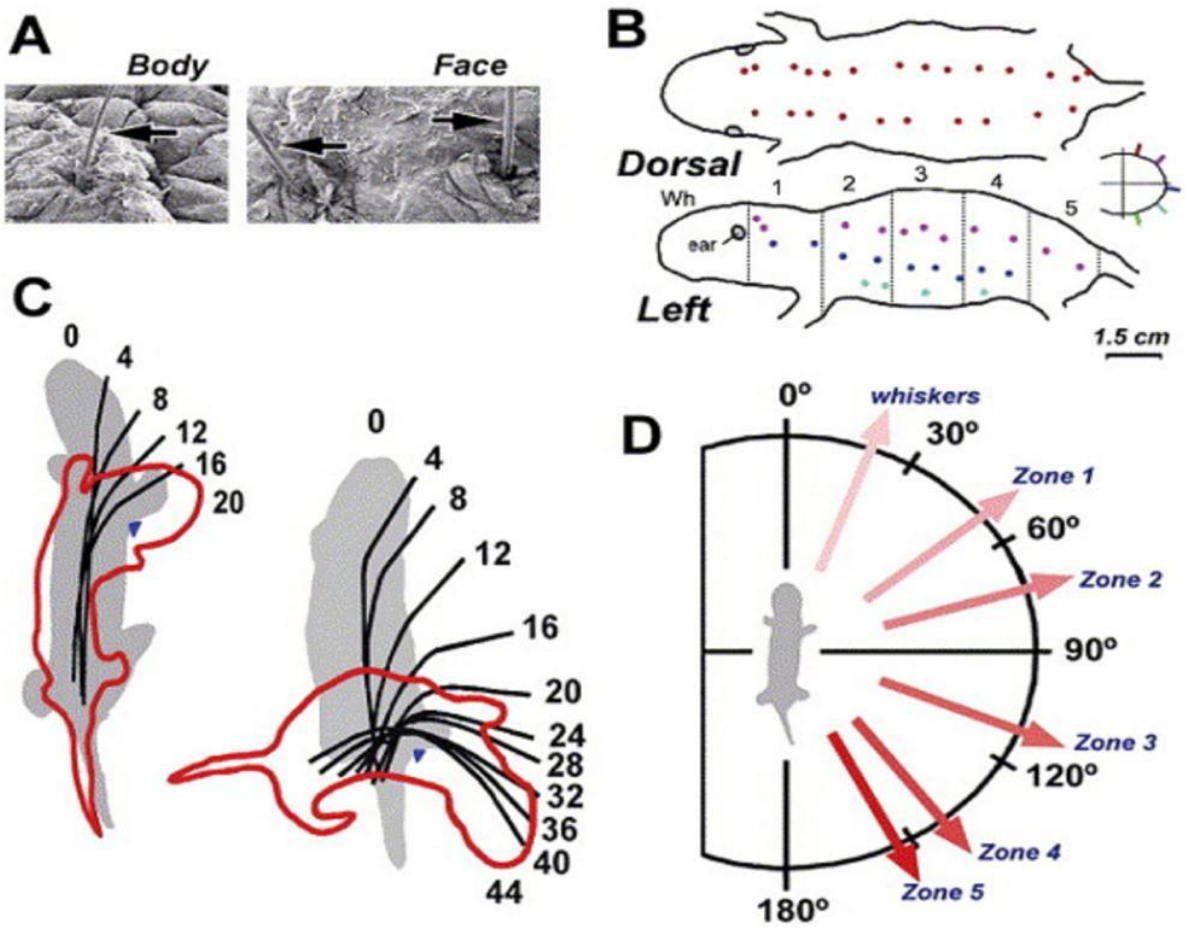

Figure 1A.2.

The Vomeronasal Organ of the Naked Mole-Rat Shows No Post-Natal Growth

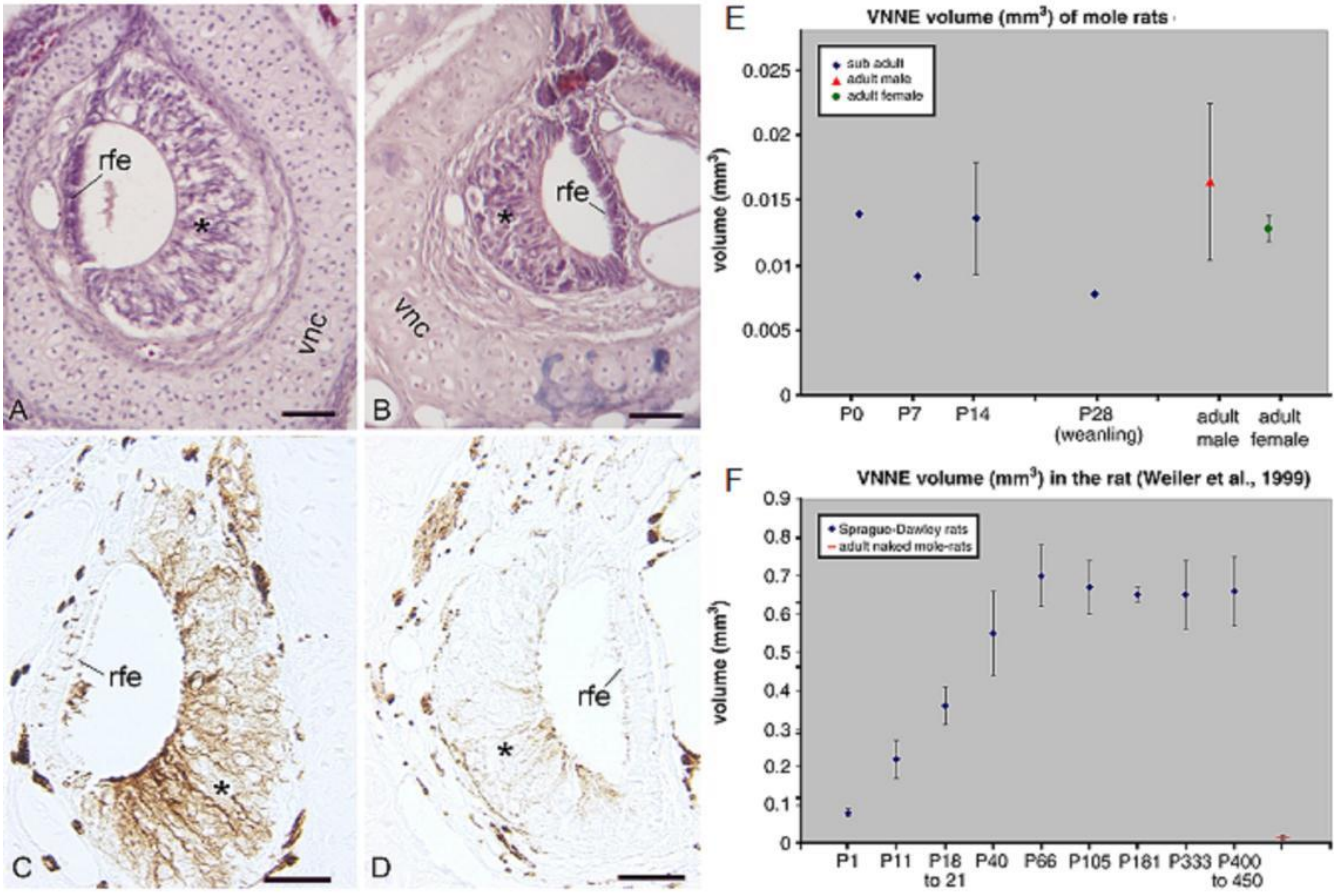

Figure 1A.3.

Naked Mole-Rats Brain Shows Extreme Intrinsic Tolerance to Hypoxia and Anoxia

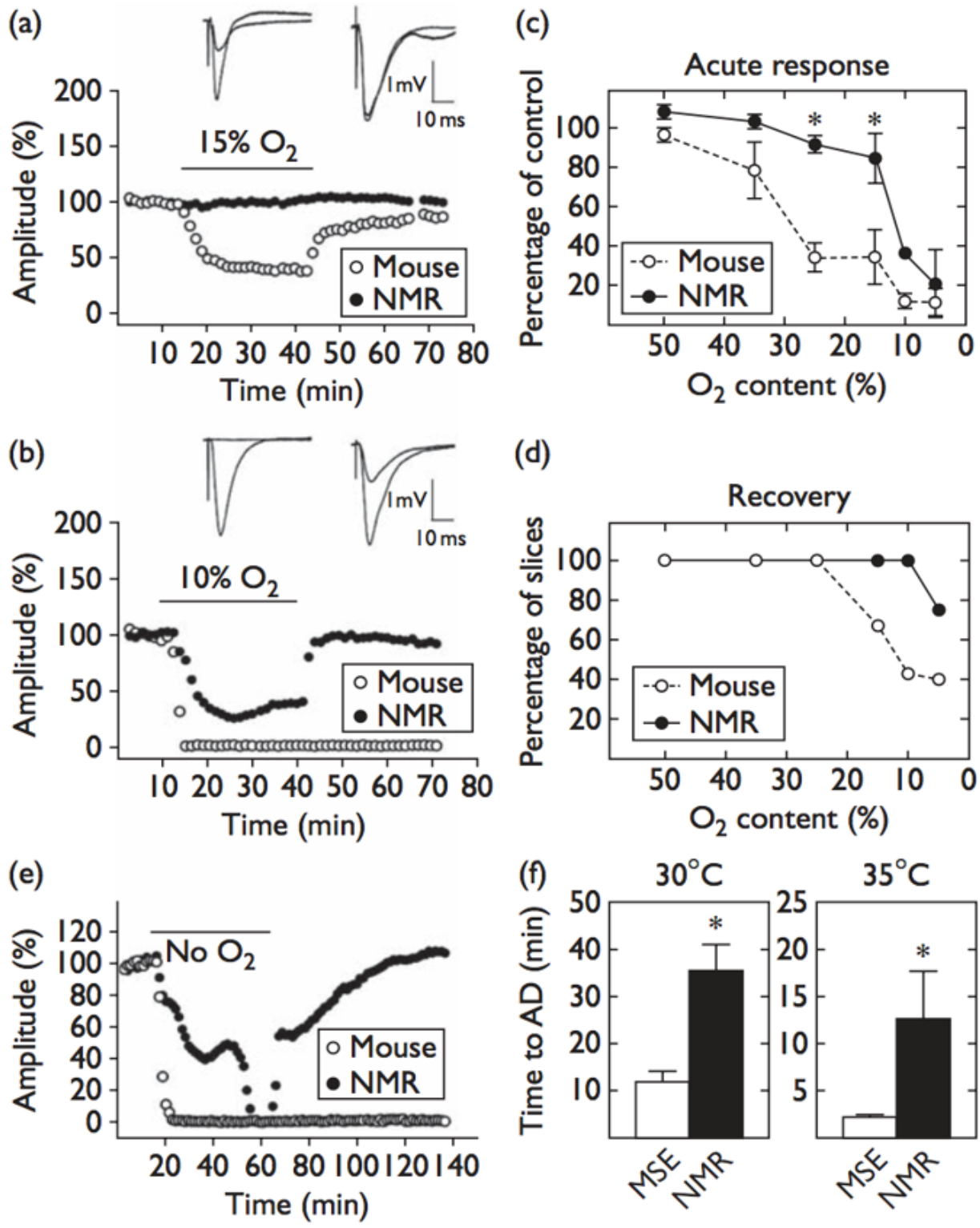

Figure 1A.4.

Intact Naked Mole-Rats Show Extreme Resistance to Hypoxia and Anoxia

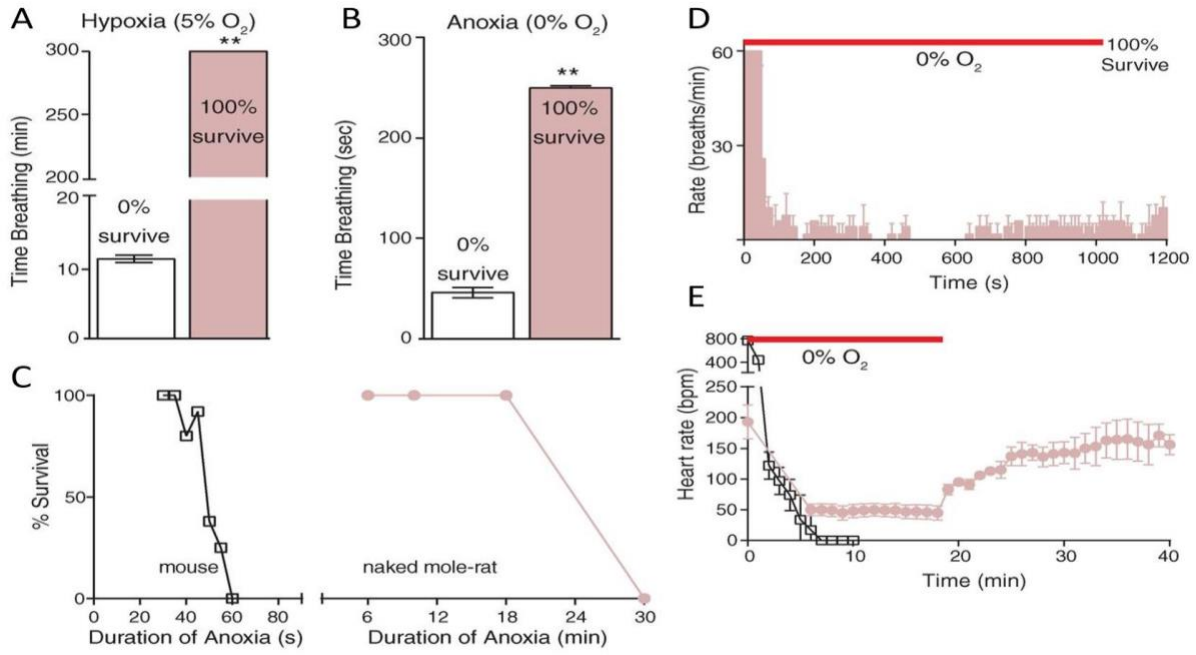

Figure 1A.5.

Functionality of Fructose in Brain Slice and Isolated Heart

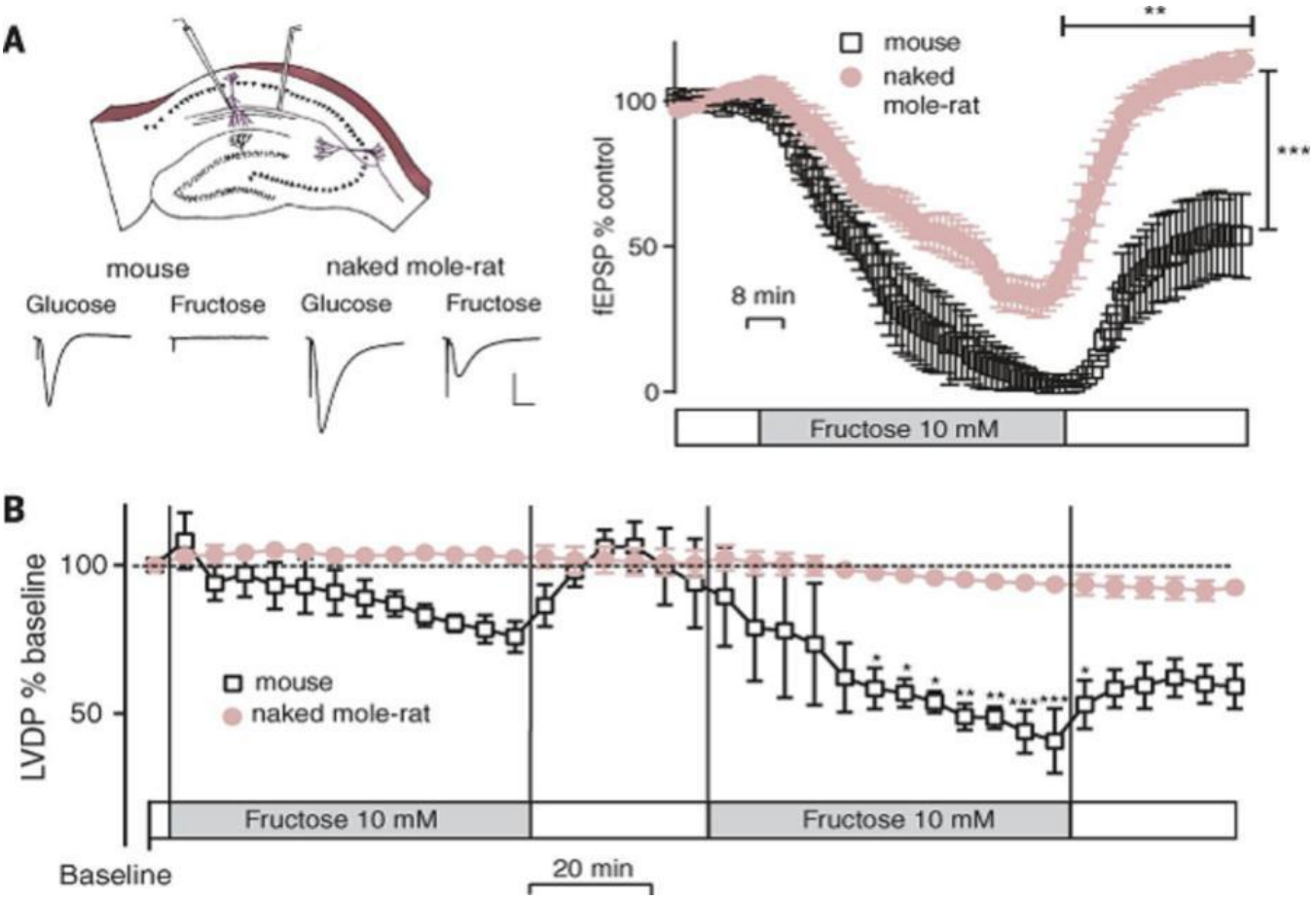

Figure 1A.6.

Response of Mice and Naked Mole-Rats to High Concentrations of CO2

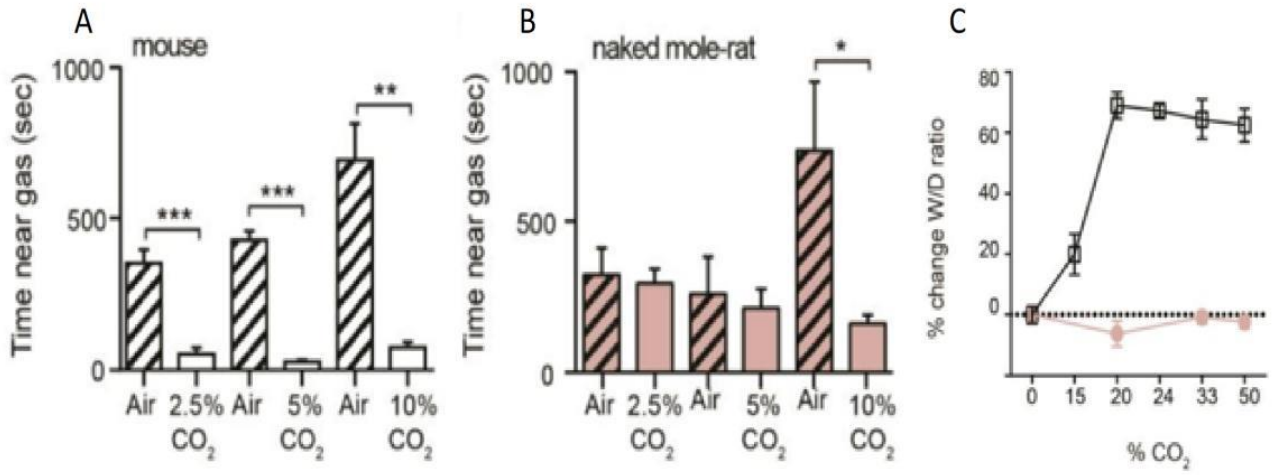

Figure 1A.7.

Response of Mice and Naked Mole-Rats in a Variety of Pain Models

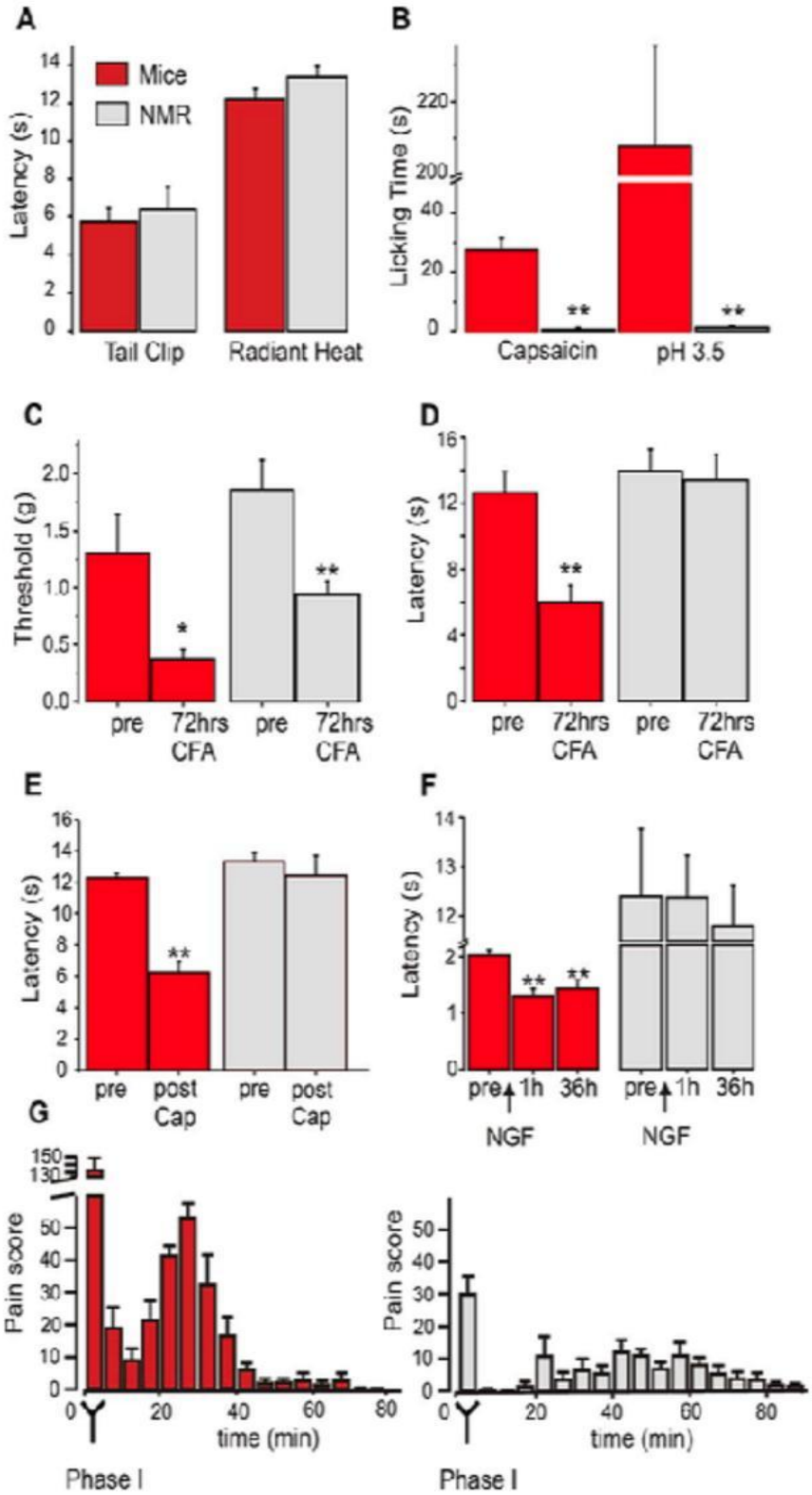

Figure 1A.8.

Rescue of Pain Sensitivity with Substance P

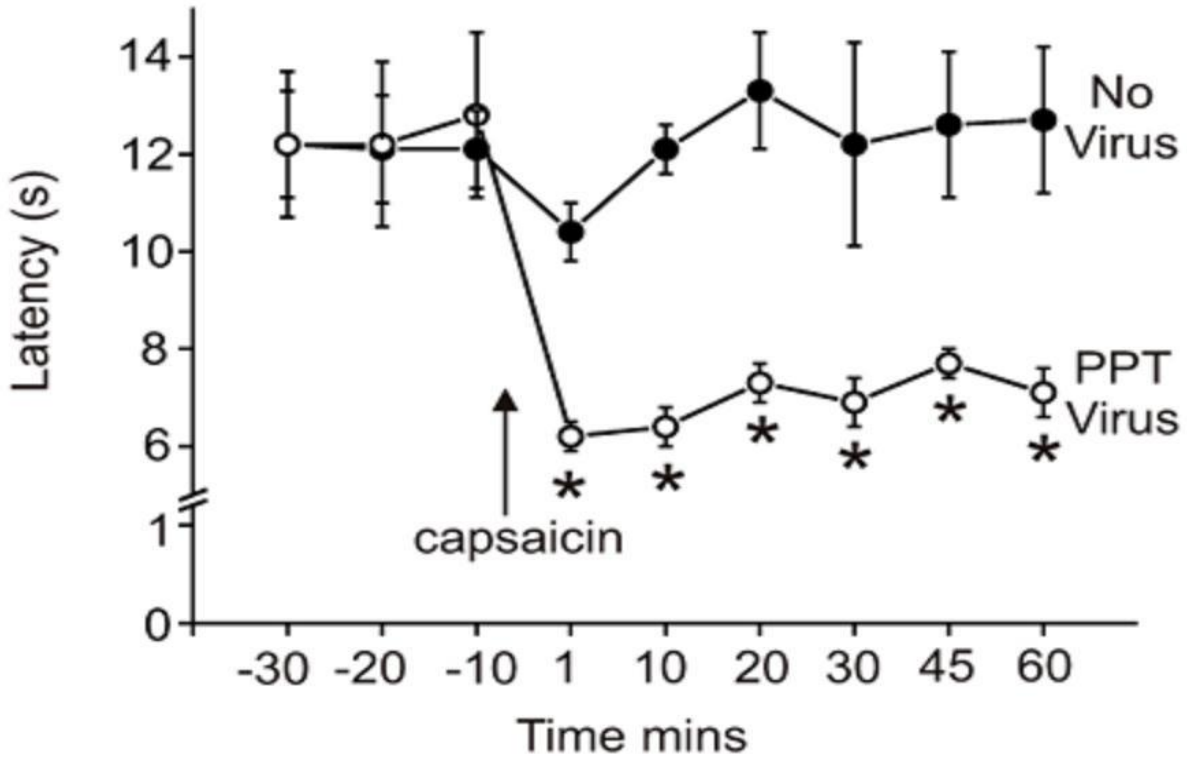

Figure 1A.9.

1.B. Review of the Endocannabinoid System in the Brain

Introduction

Cannabis has been used for thousands of years both recreational and medicinally. Only recently has the endogenous system been identified. Named the endocannabinoid system, this complex network of receptors and ligands have been found to be integral to regulation of multiple aspects of the nervous and immune system.

GPCRS

Cannabinoid receptors belong to a class of membrane proteins called G-protein coupled receptors (GPCR). Receptors in this class function to transduce extracellular stimuli into intracellular signals and are the largest known family of proteins (Yao and Mackie, 2009). When inactive, GPCRs have a GDP-bound alpha subunit and a beta-gamma subunit which once released tend to modulate different pathways (Yao and Mackie, 2009). GPCRs can have multiple active conformations that stimulate effectors through either strength of signal (strongest agonist's pathway is preferred) or biased agonism effects (stabilize one active signal over another; Hudsen et al., 2009). Post activation, GPCRs are often internalized where endosomal elimination of ligands and dephosphorylation occurs, then the receptor is either degraded or recycled back to the plasma membrane depending on receptor type, activation levels, and cellular need (Gaffuri et al., 2012). With many GPCRs, cannabinoids included, the balance between active and inactive states are very important to the overall modulatory effects of the receptors. Ligands may often only affect the balance of active states or stabilize current active states and not directly elicit a response (Hudsen et al., 2009). Cannabinoid receptors are members of the GPCR family 1a, which is characterized by amino acid signatures in Transmembrane (TM) 2 important for receptor activation, a DRY motif immediately C-terminal to TM3, a cysteine residue C-terminal to TM7 that is important for G-protein coupling and receptor desensitization, and

are generally receptors for small ligands such as odorants, histamines, and cannabinoids (Yao and Mackie, 2009).

Cannabinoid Receptors

Cannabinoid receptors consist of subtype 1 and 2, with cannabinoid receptor 1 (CB1r) being found mostly in the brain and nervous tissue and cannabinoid receptor 2 (CB2r) being found in immune cells (Basu and Dittel, 2011). This review will focus on CB1r and its effects on the brain and behavior. CB1r has been highly conserved and is found in all known vertebrate species as well as many invertebrates (Yao and Mackie, 2009). Like many GPCRs, CB1r is functionally selective and has many downstream targets (Yao and Mackie, 2009), which will be elaborated on further in this review. In addition to ligand bound activation, CB1r has a high level of constitutive activity which leads to tonic activity in many regions of the brain (Gaffuri et al., 2012). With the combination of both ligand dependent and constitutive activation, both of which lead to receptor internalization, the expression level of CB1r in selective cell types can vary greatly and can be highly influenced by the animal's physiological state (Gaffuri et al., 2012; Katona, 2009). CB1r is active in both fetal development, where it regulates the maturation of both GABAergic and glutamatergic axons, and adulthood, where it inhibits classic neurotransmitter release and regulates synaptic transmission and plasticity particularly to protect neurons from excitotoxicity (Berghuis et al., 2007; Katona, 2009; Monory et al., 2006; Lu and Mackie, 2016; Elphick and Egertova, 2001). While CB1r expression can be found in all brain regions but the rhythm generators of the brain stem, CB1r is most highly expressed in the cerebellum, hippocampus, prefrontal cortex and amygdala (Katona, 2009; Lu and Mackie, 2016). This expression is generally concentrated in the presynaptic terminals and preterminal axon segments with some expression in neuronal somata and mitochondria (Hu and Mackie, 2015). Subtle differences in CB1r expression between species can generally be explained by the lifestyle needs of the given species and synaptic protein abundance and spatial localization regulation by physiological mechanisms is very

specific (Katona, 2009; Dudok et al., 2014). Because of this, the endocannabinoid system is uniquely able to provide fine spatial modulation of synaptic transmission that is catered directly to the needs of the species and individual.

Cannabinoid Ligands

Endogenous ligands for CB1r are fatty acid-based molecules that are stored as precursor proteins in the post-synaptic membrane, they have relatively low circulatory levels *in vivo,* and are released "on-demand" through a variety of signaling pathways (Lu and Mackie, 2016; Katona, 2009; Alexander and Kendall, 2009). Two endogenous ligands have been identified for CB1r and they appear to have different functions and activate CB1r in different regions and cell types (Samson et al., 2003). Both are based on the polyunsaturated fatty acid arachidonate, the first, 2-arachidonlyglycerol (2-AG), is the more prevalent but has a similar affinity as the second, anandamide (AEA), though 2-AG has a higher efficacy at CB1r than AEA (Alexander and Kendall, 2009; Katona, 2009; Yao and Mackie, 2009).

As the most biologically relevant of the ligands, 2-AG is crucial to synapse function with its role as a negative feedback modulator. 2-AG can be released either through high frequency stimulation of neurons or elevated intracellular calcium levels on the post-synapse (Alexander and Kendall, 2009; Katona, 2009). This triggers synthesis of 2-AG by sequential activation of phospholipase C (PLC) and diacylglycerol lipase (DAGL) to hydrolyze monoacylglycerols and form 2-AG (Lu and Mackie, 2016). Once formed, 2-AG is released from the post-synaptic membrane and travels, by an as yet unidentified mechanism, to the presynapse to bind to CB1r where the inactivating enzyme monoacylglycerol lipase (MAGL) removes and degrades the ligand after use (Alexander and Kendall, 2009; Katona, 2009).

Unlike 2-AG, the formation of AEA is calcium independent, though the production of precursors may be enhanced by calcium concentrations (Alexander and Kendall, 2009). Synthesis of AEA includes a rate limiting production of phospholipid N-acylphosphatidylethamolamines (NAPES) in the post-synapse and some axonal membranes, which are then further broken down by NAPE-PLD into AEA and

phosphatidic acid (Alexander and Kendall, 2009; Castillo et al., 2012). AEA is quickly hydrolyzed by fatty acid amine hydrolase (FAAH) after production (Alexander and Kendall, 2009). Interestingly, NAPE production has only been shown in neurons and does not occur in glial cells, indicating that AEA's production is dependent on the activation of neuron synapses (Lu and Mackie, 2016; Alexander and Kendall, 2009).

The CB1r can also be activated by exogenous ligands. Phyto cannabinoids, such as Δ-9 tetrahydrocannabinol (THC) and cannabindiol (CBD) are the most well-known, being the active and therapeutic compounds found in cannabis. These compounds have either low efficacy to CB1r (THC) or are allosteric ligands (CBD), however there are synthetic ligands that have been created with much higher efficacy and specificity, such as WIN55-212,2 (WIN55) and HU210 (Yao and Mackie, 2009). Of all of the synthetic and phyto cannabinoids, WIN55 is overall the least restrictive CB1r agonist. WIN55 will activate all subtypes, has a high affinity for CB1r, and has almost no affinity for CB2r or any other GPCR in the brain (Hudsen et al., 2009; Yao and Mackie, 2009). Exogenous ligands can directly interfere with the normal function of the endocannabinoid system. Drugs that antagonized CB1r show a tetrad of phenotypes that have been described in mice and rats- catalepsy, reduced motility, analgesia, and reduction in body temperature- and are considered correlate phenotypes to the psychoactive behaviors induced in humans (Elphick and Egertova, 2001). Treatments of agonists can also affect expression levels and effectiveness of CB1r in the brain. Chronic THC treatments have been shown to downregulate membranal CB1r and, in large quantities, reduce induction of long-term potentiation (LTP) at hippocampal synapses (Hoffman et al., 2007).

Regional Expression

To understand the functions of the endocannabinoid system within the brain, it is important to understand the expression of the receptors within brain regions. This review focuses on the

hippocampus, PFC, cerebellum, and afferent fibers of the spinal cord due to their high expression levels of CB1r and the functionally relevant modulation of those regions.

Hippocampus

The endocannabinoid system of the hippocampus is one of the best described of all brain regions. During hippocampal development, CB1r expression is highest in areas that are undergoing differentiation, an indication of the role in axon guidance and synapse establishment in this brain region (Gaffuri et al., 2012; Berguis et al., 2007; Mulder et al., 2008). In the mature hippocampus, CB1r is found in every subfield and located on both GABAergic and glutamatergic terminals (Hu and Mackie, 2015; Katona, 2009; Katona, 2006). The highest regional density is in the dentate gyrus and in all hippocampal regions the terminals of the cholecystokinin (CCK)+ GABAergic interneurons have at least a 2-fold increase in expression on these neurons compared to the expression levels on pyramidal terminals (Hu and Mackie, 2015; Frazier, 2007; Dudok et al., 2014; Aguado et al., 2006; Katona et al., 1999). With the expression being heavily weighted to inhibitory neurons, the major role of CB1r is disinhibition of hippocampal circuits. Studies have shown that CB1r plays an important role in maintaining rhythmic oscillations such as for theta and gamma activity, disrupts signaling strength, shifts calcium balance at the synapse, and reduces paired-pulse facilitation; all of which may disrupt and impair spatial memory and learning (Frazier, 2007; Hu and Mackie, 2015; Katona et al., 1999). Importantly, treatments with cannabinoid agonists and antagonists may have a higher efficacy toward one cell type over another; as an example, THC reduces CB1r's efficacy on GABA release but not glutamate (Dudok et al., 2015). Because of this, it is imperative to understand the balance of reactions when using drugs to manipulate CB1r.

Prefrontal Cortex

While CB1r is heavily expressed throughout the cortex, the prefrontal area is particularly dense (Hu and Mackie, 2015). Interestingly, the frontal cortex has more diversity between species than any

other region for both CB1r expression density in particular laminar layers and between which endocannabinoid, 2-AG or AEA, are expressed in each layer being species specific (Hu and Mackie, 2015). Again, CB1r is more prevalent on CCK+ interneurons, leading to disinhibition with a lower expression level in glutamatergic neurons (Hu and Mackie, 2015). However, CB1r's inhibition of glutamate is particularly strong in synaptic transmission during adolescence (Heng et al., 2011; Hu and Mackie, 2015; Franklin et al., 2014).

Cerebellum

The cerebellum has the highest density of CB1r of all brain regions, especially in the molecular layer (Katona, 2009). Cannabinoid receptors are preferentially expressed on the basket cells surrounding the initial segments of the purkinge cell axons, as well as on climbing fibers and parallel fibers where they inhibit glutamatergic and GABAergic transmission onto purkinge neurons (Hu and Mackie, 2015).

Spinal Cord

The spinal cord is the only region outside of the brain that will be discussed. It is an important region for the endocannabinoid system due to CB1r's ability to produce analgesia at this relay point in the afferent pain pathway (Hu and Mackie, 2015). CB1r can be found in the superficial layers of the dorsal horn, the dorsolateral funiculus, and lamina X, all of which are associated with analgesia (Hu and Mackie, 2015). In these regions, CB1r can be found in primary afferent C fibers, large myelinated Ab and Ad fibers, and on postsynaptic interneurons (Hu and Mackie, 2015). The main function of CB1r in the spinal cord is to inhibit the strength of the pain signal to the periaqueductal gray and other sensory regions of the brain (Chiou et al., 2013). Despite C-fibers expressing CB1r to a lesser extent than A-fibers, removal of CB1r from C-fibers significantly reduces the analgesic effects of cannabinoids (Hu and Mackie, 2015). During inflammation, the endocannabinoid system is upregulated, presumably to reduce added pain input (Hu and Mackie, 2015). However, an auxiliary complexity of CB1r's function in

the spinal cord is that increased levels of AEA can activate the peptidergic transient receptor potential vanilloid 1 (TRPV1) receptor to increase the pain signal (Alexander and Kendall, 2009) and therefore the upregulation of CB1r during inflammation may actually be indicative of increased pain and not increased analgesia.

Cannabinoid Function

Neural Plasticity

CB1r activation, in mature neurons, leads to either transient or persistent suppression of vesicular release which it modulates by acting as a retrograde messenger system (Kano et al., 2009; Wilson and Nicoll, 2002). Retrograde signaling refers to the inhibition of synaptic transmission in a post-synaptic to presynaptic manner (Fig. 1B.1). This signal is almost exclusively mediated by CB1rs with a $G_{i/o}$ subunit and inhibits neurotransmitter release through two mechanisms (Castillo et al., 2012). This plasticity is mediated by 2-AG and does not involve AEA (Alexander and Kendall, 2009). The first is fast acting inhibition of voltage gated calcium channels, which is referred to as depolarization-induced synaptic inhibition or excitation (DSI/E) and the second is long-term inhibition characterized by down regulation of the cAMP/PKA pathway called endocannabinoid-dependent long-term depression (eCB-LTD; Lu and Mackie, 2016; Castillo et al., 2012). Cannabinoids have long been suspected as detrimental to neural plasticity but phenotypic description of what these detriments are, as well as when they occur developmentally, have been difficult to determine. Damage to plasticity can be illustrated by reduced learning and memory phenotypes. Some studies show detriment in reference memory from adolescence injections of cannabinoids, while others show only long-term detriment in verbal memory after chronic adolescent use (Rubino et al., 2009; Solowij et al., 2011; Steel et al., 2011; Broyd et al., 2015). Cannabinoid receptor activation here has been found to modulate cellular models of synaptic plasticity through the CCK+ GABAergic inhibitory interneurons that express high levels of

CB1r in the hippocampus (Marisco et al., 2002; Chevaleyre et al, 2006; Herkenham et al., 1990; Katona et al., 1999; Tsou et al., 1999).

Intracellular Transcription

Cannabinoid receptors with Gi/o subunits have other functional pathways that elicit modulation to cellular transcription. Already alluded to as a mechanism for retrograde signaling, the alpha subunit of the Gi/o complex inhibits adenylyl cyclase to reduce the production of cyclic adenosine monophosphate (cAMP) which leads to prolonged inhibition of neurotransmitter release (Castillo et al., 2012; Yao and Mackie, 2009). When neurotransmitter binding in the post-synapse causes a large enough depolarization in the post-synapse, internalized post-synaptic calcium levels will elicit formation and release of eCB ligands to travel presynaptically to bind to CB1r, causing Cannabinoid-based transient suppression of GABA release (depolarization induced synaptic inhibition, DSI; Alexander and Kendall, 2009; Katona, 2009; Lenz et al., 1998). Activated receptors release Gi/o subunits to inhibit N-type voltage gated calcium channels, inhibiting depolarization to stop vesicular release (Pitler and Alger, 1992; Diana and Marty, 2003; Varma et al., 2002; Cheveleyre and Castillo, 2003). Persistent synaptic suppression is modulated by eCB-LTD. Here, post-synaptic rise of calcium is often aided by metabotropic-glutamate receptor group-1 (MGluR-1) activation or N-methyl-D-aspartate receptor (NMDAR) mediated calcium influx to produce larger amounts of endocannabinoids and stimulate long-term depression (LTD) of transmission (Varma et al., 2002; Chevaleyre and Castillo, 2003; Malenka and Bear, 2004). This calcium influx activates PLC converting diacyglycerol (DAG) into 2-AG which is released into the synaptic space and transported to the CB1r on the presynapse (Chevaleyre and Castillo, 2003). Both LTD and DSI are invoked by the same neurons. In the presynaptic neurons, CB1r are GPCRs with Gi/o proteins that are coupled negatively to adenylate cyclase and activation of the CB1r inhibits vesicle release through these mechanisms (Chevaleyre and Castillo, 2003; Pertwee, 2006). CB1r calibrates excitatory synaptic balance in the mouse hippocampus, as well

as the cortex and cerebellum and this synaptic plasticity is driven by the control CB1r has on GABA and glutamate transmission balance (Monory et al., 2006; Heng et al., 2011; Hu and Mackie, 2015; Diana and Marty, 2003).

In mature cannabinoid circuits, PKA and voltage gated calcium channels are inhibited as well, the inhibition of calcium in particular is considered a major function of the endocannabinoid system (Yao and Mackie, 2009; Lu and Mackie, 2016; Gaffuri et al., 2012). CB1r also activates inwardly rectifying potassium channels, further aiding in the inhibition of neurotransmitter release (Gaffuri et al., 2012; Yao and Mackie, 2009; Lu and Mackie, 2016). As well as the direct effects on synaptic release, CB1r can also activate the transcriptional activity of MAPKs (Gaffuri et al., 2012). The most well described of the MAPK functions is the activation of the RK1/2 signaling cascade which can be activated in either a G-protein or b-arrestin dependent manner and forms complexes that regulate long-term activation and cell survival (Franklin et al., 2014; Chang and Karin, 2001). Activation of multiple MAPKKKs by CB1r can also activate both JNK and p38. CB1r's effect on JNK is to enhance the ability of Jun proteins to activate transcription (Chang and Karin, 2001). Both JNK and p38 may also induce apoptosis after activation by CB1r (Chang and Karin, 2001). The actual role of CB1r after activation is dependent on the physiological state and cell type, which means that exogenous exposure to cannabinoids has the possibility to make long term changes to the balance created by the endogenous cannabinoid system (Castillo et al., 2012; Chang and Karin, 2001).

Function in the Immature Brain

Importantly, there are developmental differences in this transmission. Embryonically, CB1r and the endocannabinoid's main function is to guide axonal growth cones, whereas DSI induction is weak in rats of less than 2 weeks of age and LTD is nearly impossible to induce before this age (Zhu and Lovinger, 2010; Bolshakov and Siegelbaum, 1994). Instead, at this age CB1r has a greater role in the modulation of glutamatergic LTP and in neonates CB1r activation decreases hyperactivity through a

PKA dependent pathway leading to neuroprotection at the synapse (Yasuda et al., 2008; Shouman et al., 2006). The magnitude of CB1r's control of synaptic plasticity increases during post-natal development to peak during adolescence in mice and rats, reduced expression of CB1r in adulthood correlates with reduced plasticity modulation (Yasuda et al., 2008; Kawamura et al., 2006). Because the eCB system activation inhibits calcium intake, it is also the mechanism for neonatal neuroprotection, reducing the damage caused by low oxygen induced over activation of NMDAR and is most likely the reason for the difference in developmental induction of synaptic modulation by CB1r.

Neuroprotection

Though the major signal of the endocannabinoid system is inhibition of GABA release, CB1r continues to act in a similar manner on mature glutamatergic neurons, and over activation of presynaptic vesicular release is inhibited. Over activation of NMDAR with glutamate can lead to excitotoxicity in the post-synapse due to large increases in calcium (Monory et al., 2006). CB1r activation inhibits the release of glutamate vesicles presynaptically to reduce the damage of calcium in the cell (Monory et al., 2006). Treatment with both synthetic and endogenous cannabinoids have been shown to decrease necrotic areas after multiple types of excitotoxic assault such as ischemia, oxygen/glucose deprivation, and kainate acid induced seizures (Martinez-Orgado et al., 2003; Degn et al., 2007; Fernandez-Lopez et al., 2013; Monory et al., 2006).

Additional Functions

In addition to these traditional functions, there is evidence that the endocannabinoid system works in other less well-defined pathways. These include a possible anterograde mechanism of release for AEA, tonic signaling that mediates tone in the hippocampus, and early acquisition of LTP and potentiation of synaptic transmission by CB1rs coupled to Gq/11 in astrocytes (Alexander and Kendall, 2009; Lu and Mackie, 2016; Castillo et al., 2012; Robin et al., 2018). The full influence of these alternative cannabinoid pathways has only just recently been examined and is therefore still relatively

unknown, particularly with regards to the effects of exogenous treatments. While still in the early stages of definition, it is important to keep these alternate pathways in mind during any consideration of the endocannabinoid system and its mechanisms.

Conclusions

Due to the pervasive expression of CB1r throughout most of the brain, the endocannabinoid system is able to modulate a variety of behaviors and cellular states. In addition to having both direct and long-term pathways for synaptic inhibition, the ability of the endocannabinoid system to up and down-regulate itself in response to the needs of the region makes this system extremely flexible in practical function. While its endogenous function is primed to focus on direct spatial targets and individual areas, treatments with exogenous cannabinoids often reflect a more global targeting of multiple functional areas. Understanding the complexity and integral nature of the cannabinoid system will be key to utilizing its focused modulation as a therapeutic target.

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Figure Legends

Figure 1B.1: Model of retrograde signaling in a network including excitatory and inhibitory axon terminals. Postsynaptic modulations, including increased calcium concentrations, activation of MGluR5 and NMDAR, elicit hydrolysis of the membrane bound DAG by DAGL to form and release 2-AG into the synaptic cleft. 2-AG is then trafficked to CB1r which may be on either the presynaptic glutamatergic and/or GABAergic presynaptic terminal (cell type, brain region, and physiological state are all determining factors in the availability of CB1r on presynaptic membranes). Activation of CB1r by 2-AG leads to a reduction of synaptic transmission through a multitude of signals depending on the coupled G-protein subunit. $G_{i/o}$ coupling is the most common and generally elicits an inhibition of voltage-gated- N-type calcium channels, activation of GIRK potassium channels, down regulation of cAMP/PKA, and an upregulation of pERK1/2. Receptors are deactivated by internalization after activation and 2-AG is degraded by MAGL. (Schematic creation was a collaboration between Brigitte Browe and Emily Vice)

Model of Retrograde Signaling by the Endocannabinoid System

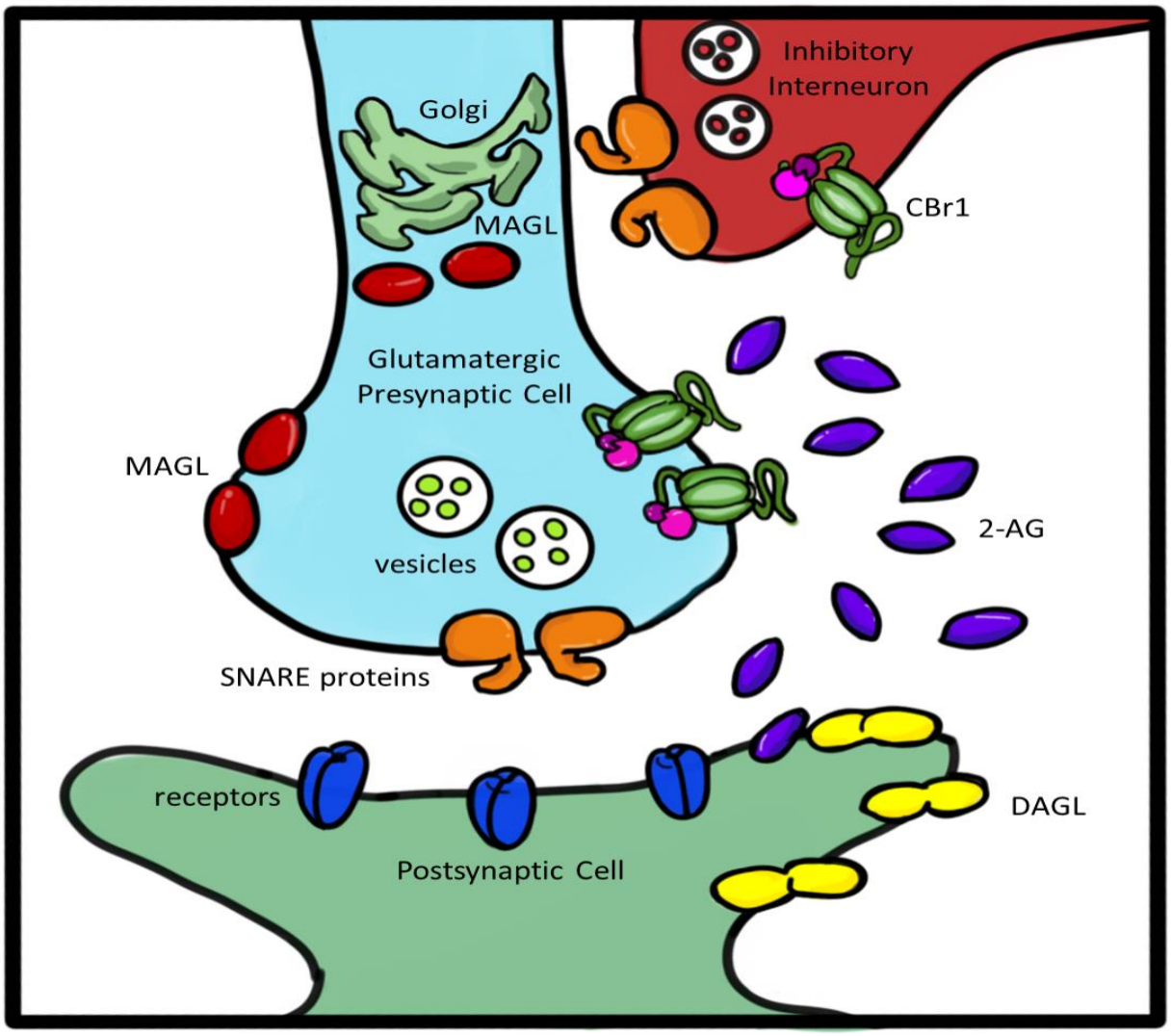

Figure 1B.1

Chapter II: Development of the Endocannabinoid System in the African Naked Mole Rat

Abstract

The African naked mole-rat (*Heterocephalus glaber)* has been established as an exceptional model for development, due to retention of neonatal traits for hypoxia tolerance, longer than usual post-natal brain maturation for rodents, and extremely long-life span. Yet much about their development has yet to be explored. Determining the regulatory mechanisms that underlie these adaptations is an important step in understanding the overall effects of delayed development as well as further comprehension into the causes of aging. One aspect of neuronal development and regulation that has yet to be explored in naked mole-rats is the endocannabinoid system. The endocannabinoid system mediates neurodevelopment, neuroprotection and synaptic transmission. We have previously shown that adult naked mole-rats exhibit immature separation of their excitatory and inhibitory cell layers of the hippocampus, extended brain growth for at least one year post-natal, slowed neurogenic turnover, and retention of cortical guide scaffolding: all of which are modulated by the eCB. Here we tracked the development of endocannabinoid expression and phenotypes. When considering expression, we had two key results. The endocannabinoid system's development is delayed compared to mice and rats, but the extent of that delay is regionally specific. In brain regions with higher brain functions, such as the PFC and hippocampus, naked mole-rats do not complete maturation in a similar manner as mice and rats, but rather appear to remain at an adolescent level throughout their lifespan. Brain regions with more vital functions, such as the cerebellum, do exhibit full maturation as they enter adulthood (at 1 year of age) which is developmentally similar to, though much slower than, other rodents. Naked mole-rats also exhibit developmentally dependent changes in behavioral responses to exogenous cannabinoid exposure. In three separate tests, naked mole-rats generate responses that are delayed age-wise compared to mice and rats yet track with the expected developmental stage (neonatal, juvenile, adolescent, adult). Application of WIN55 disrupts the righting reflex for weeks after birth,

compared to days in mice. Like adolescent mice learning deficits from chronic exposure to WIN55 were demonstrated in 4-6-month-old naked mole-rats. Additionally, motor depression/catalepsy is exhibited through 9 months of age. Surprisingly, the motor depression is abruptly extinguished in adulthood and WIN55 application induces no locomotor effects. It is interesting that despite retaining a high concentration of CB1r, naked mole-rats do not continue to exhibit motor depression from CB1r agonists. Likewise, acute treatments of WIN55 only impair learning in adult animals. Finally, we found developmental differences in synaptic function modulation by the endocannabinoid system in both the CA1 and dentate gyrus of the hippocampus. Importantly, the regions that exhibit abrogated maturation are integral to neuroprotection and the cannabinoid system has been shown to facilitate neuroprotection in immature mice and rats. Additionally, the cannabinoid system has developmentally dependent effects on synaptic facilitation, with paired-pulse depression observed in adolescents and facilitation in adults after treatment with cannabinoids. Therefore, like other neonatally retained traits, the retention of an immature endocannabinoid system may be due to a need for adult naked mole-rats to mediate neuroprotection through the endocannabinoid system. Examining the role of the endocannabinoid system in maintaining synaptic integrity during hypoxia could help in determining if that is indeed the case.

Introduction

In spite of living in a harsh hypoxic environment, naked mole-rats are the longest-lived rodent known to date (Edrey et al., 2011). Low oxygen environments are uninhabitable for most adult mammals, hypoxic conditions are pervasive during embryonic development due to a variety of developmentally different expression patterns of certain genes and proteins. Interestingly, many of the naked mole-rats' evolutionary adaptations can be categorized as neotenous (retaining neonatal features and delayed development, through adulthood; Browe et al., 2018; Horder, 2006; Larson & Park, 2009; Larson, et al., 2014). Many aspects of naked mole-rat neural development take years to mature compared to the days or month time scale of traditional laboratory rodents (Penz et al., 2015). The most striking of these is their ability to survive extremely hypoxic conditions vastly longer than most mammals which includes a near 10-fold increase of time that hippocampal neurons are able to maintain basic membrane homeostasis and synaptic function (Larson and Park, 2009). Much of the neoteny on naked mole-rats research has focused on the hippocampal traits that remain juvenile such as: lack of Paired-Pulse Facilitation, retention of axonal scaffolding, years long elongation of dendritic segments, and minimal separation of GABAergic and glutamatergic neuron layers (Larson and Park, 2009; Penz et al., 2015). We have found that in addition to synaptic maintenance during oxygen deprivation, naked mole-rats attenuate intracellular calcium increases during hypoxic assault and inherently retain the NMDAr subunit GluN2D that gives protection against hypoxia at neonatal levels into adulthood (Peterson et al., 2012a; Peterson et al., 2012b; Bickler et al., 2003). We also know that naked mole-rat have extremely long lifespans (greater than 30 years) and experience very little deterioration in senescence (Buffenstein, 2008). How their retention of neonatal traits is initiated and sustained has yet to be fully clarified. Identifying additional regulatory mechanisms that underlie these adaptations is important in understanding the overall effects of delayed development as well as further comprehension into the progression of aging.

In this regard, the endocannabinoid (eCB) system is an important modulator of neuronal development and regulation that has yet to be explored in naked mole-rats. The eCB system plays an important role in regulating neuron and glial growth and maturity during fetal and neonatal development and in the mature neuron becomes an essential modulator of synaptic transmission (Berghuis et al., 2007; Chevaleyre et al., 2006). The eCB system in the brain revolves around the most prominent G-protein coupled receptor (GPCR): Cannabinoid Receptor 1 (CB1r), and its endogenous ligands, namely arachidonylglycerol (2-AG) and anandamide (AEA; Alexander and Kendall, 2009). Receptors can be found highly concentrated in the hippocampus, cerebellum, amygdala, and the prefrontal cortex (PFC) with distinct developmentally associated expression levels (Katona, 2009; Lu and Mackie, 2016). In traditional laboratory rodents, receptor expression peaks during adolescence whereas their ligands have biphasic expression with peaks during late embryonic development and adolescence, and low expression during neonatal development and adulthood (Kano et al., 2009). Endocannabinoid ligands, being lipophilic, are not able to be stored in vesicles and are created “on demand” from post-synaptic membrane bound precursor proteins (Stella and Piomelli, 2001). CB1r activation is known to affect behaviors, synaptic plasticity, memory, neurite proliferation and differentiation, and energy metabolism. Once synaptogenesis has been completed, CB1r switches gears to most prominently directly regulated neurotransmitter release presynaptically by inhibition of voltage gated calcium channels, activation of potassium channels, and modulating downstream pathways such as cAMP, ERK1/2 (Wilson and Nicoll, 2002; Gaffuri, 2012; Yao and Mackie, 2009; Chang and Karin, 2001; Castillo et al., 2012). This changing developmental role can have drastically different effects on their downstream targets. For example, the phosphorylation of ERK1/2, is inhibited by AEA release during neuronal differentiation and maturation but upregulated in most brain regions in adulthood (Compagnucci et al., 2013). There is still much to be determined about the underlying mechanisms and potential of the eCB within the mammalian nervous system. The eCB mediates

neurodevelopment, neuroprotection, and synaptic transmission (Tortoriello et al., 2014; Aguado et al., 2006; Alger 2002).

Adult naked mole-rats exhibit neoteny in functions modulated by the endocannabinoid system, including: separation of their excitatory and inhibitory cell layers of the hippocampus, extended brain growth for at least one year post-natal, slowed neurogenic turnover, and retention of cortical guide scaffolding (Penz et al., 2015; Martinez-Orgado et al., 2003; Harkany et al., 2007; Hatzimanikatis and Lee, 1999). To understand the role of the endocannabinoid system in naked mole-rat neoteny, we focused mainly on the hippocampus, due to its well described neotenous features in naked mole-rat physiology, but also briefly examined some aspects of the cortex and the cerebellum due to the endocannabinoid system's pervasive high expression in these regions in many animal species.

Methods

Animals

All Mice were C57BL/6 males, which were bred from stock, original obtained from Charles River Laboratories, Wilmington, Massachusetts, USA. Mice were kept in a temperature-controlled environment of 72°F with a 12-hour light-dark cycle. Naked mole-rats of both sexes were born in colonies maintained at the Park laboratory and housed under similar conditions as found in the wild. Naked mole rats were kept at a controlled temperature (80°F) and humidity (%) with a 12-hour light-dark cycle. All procedures were conducted according to the animal protocols approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

Sequence alignment:

Known human CNR1 gene sequence and predicted naked mole-rat sequence was acquired from NCBI. Alignment was done by hand.

Histology:

Mice and naked mole-rats were decapitated and brains were quickly removed and drop fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer (PBS, pH 7.4) overnight, and subsequently cryoprotected in 30% sucrose (in PBS). Brains were sectioned coronally (26 µm thickness) on a Leica CM1850 cryostat microtome and directly placed on slides. Non-specific immunoreactivity was suppressed by incubating our specimens in a cocktail of 5% normal donkey serum (Jackson Laboratories), 1% bovine serum albumin (BSA, Sigma) and 0.3% Triton X-100 in PB for an hour at 22–24 °C. Sections were then exposed to primary antibodies: guinea pig anti-CB_1R (1:15000; Frontier-Science) (Kawamura et al., 2006); diluted in PBS (48–72 h at 4 °C) to which 0.5% normal donkey serum and 0.3% Triton X-100 had been added. After extensive rinsing in PBS, immunoreactivities were revealed by carbocyanine (Cy) 3-tagged-antiGp secondary antibodies raised in donkey (1:200 (Jackson), at 22–24 °C, 2 h) and neuronal nuclei were stained with Hoechst (1:1000). Glass-mounted sections were cover slipped with Aquamount (Dako, Glosstrup, Denmark). Images acquired on a Fluoview FV10i confocal laser-scanning microscope (Olympus) and analyzed with ImageJ.

Preparation of Tissue for Mass Spectrometry

Naked mole-rats aged 2 weeks, 4 months, 1 year, and 5-15 years old and mice aged 2 weeks and 3 months (n=3 for each species and age group) were quickly decapitated for tissue collection. Brains were removed from the animals and regions of interest, hippocampus, prefrontal cortex, posterior cortex, and cerebellum, were quickly dissected and immediately frozen on dry ice until completely frozen. After freezing, tissue was stored at -80C until ready to be shipped. All tissue was sent out for analysis in a dry shipper cooled with liquid nitrogen to the University of Aberdeen, Scotland UK Mass Spectrometry Core Facility to measure levels of endocannabinoids by liquid chromatography tandem mass spectrometry (LC/MS/MS).

Behavior

Righting Reflex

Drug naive naked mole-rats and mice between the ages of post-natal day (pnd) 3-168 (naked mole-rats)/pnd7-28 (mice) were tested for the righting reflex. Each animal was given a 10uL IP dose of the CB1r agonist, WIN55-212,2 (WIN55) at concentration of either: 0mg/kg (control), .5mg/kg, 1mg/kg, 2.5mg/kg, or 5mg/kg. Experiments were performed by blinded researchers. Each animal was tested 3 times per time point and their scores (seconds) were averaged per time point. To test, each animal was flipped supine with a finger gently holding them in place. The finger was removed as the timer was started. When the animal righted itself, as indicated by being fully on their stomach, the time was stopped and recorded. Each animal was tested at 8 time points: 5 minutes prior to injection, directly after injection, 10, 20, 30, 45, 60, and 120 minutes after injection. All time points were recorded in order to account for any discrepancies in the activation of WIN55 in animals of different ages and species. Drug effects were stable at all doses by the 30-minute time point, therefore, most of the data displayed is based on the 30-minute time point to correlate with other experiments (Supplemental Figure 2.1).

Plus Maze

The plus maze apparatus consists of four arms connected to a central compartment (arm dimensions: 55x10x15 cm, central box dimensions: 10x10x15 cm). The animals were placed into the central compartment of the maze and allowed to explore. The maze was cleaned with 70% EtOH before and between each animal. The sequence and number of the arms visited were recorded by a blinded observer for 10 minutes. The criterion for a visit was entry into a compartment with all four paws visibly crossing the marked threshold of the arm. Animals were tested with a randomized dose of either saline, WIN55-212,2 at .5mg/kg, 1mg/kg, 2.5mg/kg, 5mg/kg, or 10mg/kg, or CB1r antagonist, AM251 at 1mg/kg, 2.5mg/kg, or 5mg/kg. Each animal received each dose once with at least 48 hours

between tests to avoid developing a tolerance which can occur with WIN55 after 9 consecutive days of treatments (Nealon et al., 2019).

Hebb-Williams Maze

Acute effects of WIN55 were tested using a modified Hebb Williams Maze Protocol (Rabinovitch and Rosvold, 1951). A standard Hebb-Williams maze consisting of a closed field (60x60 cm) with a start box and goal box at opposite corners was used. The field was marked into thirty-six (10cm) squares which served to define the errors and also to act as marker for the placement of barriers. To this standard maze (see Rabinovich and Rosvold (1951) for further details) a guillotine door was added at the exit to the start box to inhibit return to the starting area once the test began. Video recording was done from a GigE Behavioral tracking camera which was mounted approximately 2 meters above the center of the maze.

Method. The animals were food restricted beginning 24 hours prior to introduction into the maze. Under food restriction, the mice received 10g food pellets/litter/day and the naked mole-rats received 10g sweet potato/litter/day. Animals were adapted to and pretrained in the maze. This consisted of the following sequences:

1) The subjects explored the field in groups of 2-4 for about 30 minutes on 3 consecutive days.
2) The subjects were run individually for 4 trials on two different practice problems each day (2 trials/practice problem) until a criterion of completing 4 trials in a total of less than 60 sec consecutively was reached. Any trial which lasted more than 5 minutes was stopped, and the animal was removed without receiving a reward. When an individual reached the criterion, pretraining continued until all subjects were ready for testing. Apple and protein mash were provided in the goal box throughout all stages: acclimation and testing.

Completion of this section is referred to as **Simple Maze Learning** throughout the remainder of the text.

3) One cohort of animals were tested in completion of the Simple Maze Learning task after chronic cannabinoid treatments. Animals in this cohort were treated daily for 4 weeks and continually throughout the experiment with 1mg/kg of WIN55, 1mg/kg AM251, or saline.

All animals in the second testing phase were drug naive prior to the start of this phase. Animals were tested in 8 consecutive trials of one maze configuration per day of testing. To assure there were no drug interactions from previous testing days, animals were given 48 hours between testing days. Testing was modified from the full 12 mazes described in Rabinovitch and Rosvold (1951) to utilize only 6 maze configurations. These configurations were paired by difficulty: [3, 4]- simple [6,7]- moderate, [10, 11]-difficult (difficulty levels described and assessed by Meunier et al., 1986). For each paired set of tests, the animal was randomly assigned either the control (Saline) or experimental (WIN55) condition to be tested first then subsequently followed by the opposite for the following day. In other words, for each maze set, animals were given either WIN55or vehicle for one of the mazes and then the other drug condition for the matching maze in the set. Errors were defined based on segments outside of the most direct path to completion of each problem. Both errors and time were tracked for each maze. The subjects were allowed approximately 10 sec/trial to eat the reward in the goal box. Trials that were greater than 15 minutes were stopped, and the animal did not receive a reward for that trial. This portion of the test is referred to as **Complex Maze Learning** throughout the remainder of the text.

All trials were recorded, and analysis was done using Noldus Ethovision software. Analysis averaged the number of trials in which the animal reached one of two criteria. Criteria 1: reaching the goal box in <30 seconds, Criteria 2: reaching the goal box with 2 or fewer mistakes (entering pre-

mapped areas divergent from the direct path to the goal box; Rabinovitch and Rosvold, 1951).

Analysis was done by comparing control vs WIN55 trials per difficulty with a paired t-test (vassarstats.com) in adolescent (4mo) and young adult (1yr) naked mole-rats or young adult mice (2-4 mo).

ELISA: Receptor Activation

Using the Phospho-ERK ELISA kit from Sigma, pERK1/2 expression levels were compared among naked mole-rat at age's 2wk, 4mo, 1yr, and 5yr and Bl6 mice at age's 2wk, 4wk, 4mo and 1yr. Efficacy of phosphorylation was examined between drug treatments at each age. Animals were decapitated and brains were rapidly removed and divided into prefrontal cortex, hippocampus, and cerebellum then rapidly frozen on dry ice and stored at -80C until use. Tissues were then homogenized in HEPES buffer and proteins were isolated and quantities assessed with Bradford assay. The ELISA protocol was performed per instructions of Sigma, with 80ug of proteins used per assay. Wells were split and either 5uL of .09% saline or 5uL of .5ng WIN55 (agonist, equivalent to 2.5ug/kg *in vivo*) were added to protein aliquots and set on ice for 30 minutes. This dose was chosen because, as shown in fig. 8, it demonstrated a significant behavioral change between the species at all ages and the naked mole-rat at 2wk and 4mo compared to 1 yr.

Brain Slice Preparation

Brain slices were prepared as described by Larson and Park (2009). Experiments were performed on 4-9 month or 1-3-year naked mole-rats. The lifespan of the naked mole-rat is greater than 30 years; the animals of the younger cohort are considered to be adolescent animals and the older cohort are considered to be young adults.

Animals were treated with either: 2.5mg/kg of cannabinoid agonist WIN55, 2.5mg/kg of cannabinoid antagonist AM251, or .9% saline by intraperitoneal injection 30 minutes prior to

dissection. Transverse hippocampal slices were prepared in the conventional manner. Briefly, naked mole-rats were decapitated, and the brains were rapidly removed into ice-cold ACSF containing: 124mM Na Cl, 3mM KCl, 1.2mM KH2PO4, 26mM NaHCO3. 2.5mM MgSO4, 3.4mM CaCl2, 2mM Na-ascorbate, and 10mM D-glucose. ACSF was gassed with 95% O2 and 5%CO2. The tissue was sliced at 400um on a tissue chopper. Slices were placed in an interface chamber and constantly perfused (1.0ml/min) with ACSF at 34C. Paired recordings were performed in two brain regions per experiment. In the first, stimulation electrode 1 (S1) was placed in the stratum radiatum of subfield CA1 to activate Schaffer-commissural fibers. The second stimulation electrode (S2) was placed in the Lateral Perforant pathway (LPP) of the dentate gyrus. Population recordings of synaptic field potentials (fEPSPs) were made with micropipettes positioned in the stratum radiatum of CA1 and LPP of the dentate gyrus respectively. Evoked responses were digitized by computer and analyzed online using custom software. fEPSPs were evoked at 10sec intervals of alternating stimulations throughout the experiments. Baseline stimulus intensity was set to evoke a half-maximal fEPSP in each slice. Baseline recordings were taken for at least 10 minutes before manipulations. Initial slope and peak amplitude were calculated for each fEPSP and normalized to the baseline average in each slice.

Paired-pulse response was measured by averaging the difference between 5 paired responses at 50, 100, 200, 400, and 800ms inter-pulse intervals. Theta burst stimulation (TBS) of 1 train containing 10 bursts with IBI of 200ms with 4 pulses at 100 Hz in each burst in the stratum radiatum of the hippocampus and 2 TBS trains (20 sec apart) for the dentate gyrus LPP was used to electrically induce potentiation. All experiments were allowed to recover for 45 minutes after induction of long-term potentiation (LTP) to determine the extent of potentiation.

Drug effects were analyzed at TBS, 10 minutes and 30 minutes post-TBS and compared to control responses by t-test (vassarstats.com).

Results

Cannabinoid Receptor 1 is highly conserved in naked mole-rats

To our knowledge, the gene expression of the CB1r and sequence conservation has never been examined in naked mole-rats. Therefore, we used the NCBI database to confirm that the gene CNR1 was present in the genome (Kim et al., 2011) and included in predicted protein sequences listed on the database and the Naked Mole-Rat Database ((Naked Mole Rat Database, mr.genomics.org.cn). The predicted protein is 473 amino acids in length and is more similar to the human CNR1 gene than to that of traditional laboratory rodents like mice. In our analysis of the human CNR1 gene compared to the predicted naked mole-rat CNR1 gene, we found 91% homology within the nucleotide sequence, with 118 nucleotide substitutions (Figure 2.1, red base pairs). When converted to their amino acid sequences, the conservation increased to 96% homologous, with 13 amino acid substitutions (Figure 2.1, highlighted regions).

Expression of CB1r indicates a delay in post adolescent reduction of receptor expression

Anatomical expression of CB1r was performed in the hippocampus because this is the area known to have one of the highest levels of CB1r expression and it has been thoroughly examined on an anatomical and physiological basis in naked mole-rats (Katona, 2009; Larson and Park, 2014, Penz et al., 2015). We looked at three different age groups of naked mole-rat as well as adult mice. Immunohistochemistry fluorescence (IF) CB1r expression is 135% greater (Fig. 2.2E) in young adult (1 year NMR; 3.874(±.3)*10^6; Fig. 2.2C) compared to juvenile naked mole-rats (2 week NMR; 1.914(±.2)*10^6; Fig. 2.2A). Instead juvenile naked mole-rats showed similar expression levels to adult mice(1.687(±.2)*10^6; Fig. 2.2D). As expected for the adolescent naked mole-rats (4month NMR; Fig. 2.2B), there was an increase in expression (4.105(±.6)*10^6) over juvenile naked mole-rat ($p<.01$), but not significantly different between the adolescent and young adult naked mole-rat ($p>.05$; Fig. 2.2E).

Endocannabinoid ligand expression is reduced through adulthood in the naked mole-rat

Endocannabinoids, due to their lipid-based structure, are not held in vesicles and are released 'on-demand' for signaling needs (Lu and Mackie, 2016). To analyze the developmental trajectory of naked mole-rat endocannabinoid expression we compared the expression levels and pattern of expression levels to neonatal/juvenile mice (low expected expression) and adult mice (high expected expression) with LC/MS/MS in four brain regions: early developing regions, cerebellum and posterior cortex; and late developing regions, anterior/prefrontal cortex and hippocampus.

The cerebellum and posterior cortex (including the somatosensory and visual cortices) do not exhibit neotenous expression and a significant increase during adolescence and trend toward a decrease in adulthood is seen, tracking with previously published mouse expression levels for development (2-AG expression shown, Fig. 2.3A,B). In the cerebellum (Fig. 2.3A) there is a significant increase from 2-week-old naked mole rats to 1year old ($p<.01$) and a less pronounced decrease after early adulthood into mature adulthood ($p<.1$). In the posterior cortex (Fig. 2.3B), the most significant change is from adolescence to early adulthood ($p<.05$), but there is again a less pronounced decrease from young adulthood into mature adulthood as would be expected in a complete developmental cycle of mammalian 2-AG expression.

The PFC additionally exhibits low endocannabinoid expression throughout the lifespan (2-AG shown, Fig. 2.3D). In mice the increase in adult expression is very stark ($p<.001$), but there is no significant change in expression over any of the naked mole-rat developmental stages quantified in this study ($p>.2$). In the hippocampus (Fig 2.3C), the oldest cohort of naked mole-rats did not express 2-AG at significantly higher levels than the neonatal mice nor at significantly lower levels than adult mice but did trend to being closer to the younger mouse cohort. The mouse expression for this endocannabinoid trended upward in adults but was also not significantly different in our measurements ($P=.269$) though it has been in previous publications (Long et al., 2012; Meyer et al.,

2018). We did not find a significant difference between naked mole-rat age groups (p=.36), suggesting that the neonatal eCB expression levels are consistent throughout the naked mole-rat lifespan.

We next looked at expression levels of AEA in the hippocampus over the same developmental time points, the naked mole-rat expression levels in general are similar to neonatal mice throughout their lifespan and are lower than those of adult mice (Fig. 2.3E). Naked mole-rat AEA levels were often below the detection level sensitivity for our analysis, therefore, we analyzed the young animals: 3 day, 2 week, and 4 month as a cohort and the adult animals: 1 year and >5 years as a separate cohort and used a one-way ANOVA to determine variance (F= 19.45). The young mice expressed hippocampal AEA at a significantly lower level than older mice ($.0196 \pm 7.5 * 10^{-4}$: $.036 \pm 1.0 * 10^{-3}$, $p<.05$). Both cohorts of naked mole rats had significantly lower expression of AEA than adult mice ($p<.01$ younger and $p<.01$ older). There was not a significant difference in the two age groups of naked mole rats (older $.0007 \pm 7.5 * 10^{-4}$: younger $.0146 \pm 8.5 * 10^{-3}$). There was also a significant difference between the expression of younger mice and both age groups of naked mole-rats ($p<.05$), indicating that there is an overall reduction in circulating AEA in naked mole-rats compared to mice.

The trend found in hippocampal AEA expression is similar for the other derivative of arachidonic acid, PEA and OEA (Fig. 2.3E). There are two exceptions to this trend in the hippocampus. OEA in 3-day animals has a significantly greater level of expression than the juvenile mice ($p<.05$) and older naked mole-rat cohorts ($p<.05$ Tukey, T-test: vs 4mo: $p<.01$; vs 1yr and 5y: $p<.05$); coincidentally, this particular cannabinoid is highly associated with neuroprotection and could also indicated that the hippocampus is still expressing some endocannabinoids at the expected elevated embryonic level (Sun et al., 2007).

Naked mole-rats exhibit an extended developmental period of cannabinoid-based sensitivity to the righting reflex

The latency to righting reflex is commonly used as an assay of locomotor coordination and sedative drug effects in neonatal rodents. To corroborate the apparent retention of an adolescent cannabinoid system in adult naked mole-rats, we looked at the naked mole-rat's response to standardized behavioral phenotypes associated with the eCB system. If the eCB system is associated with delayed development, it would make sense that the naked mole-rats would display neonatal mouse-like characteristics for an extended period when testing their kinetic (movement/activity level) response to agonists which can be tested with locomotor assays. For extremely young mice and naked mole-rats, we analyzed the effect of WIN55 on righting reflex (Fig. 2.4). Rather than developing sensitivity soon after infancy, as is seen in mice, naked mole-rats show dose-dependent hypokinesis as young as post-natal day (pnd) 3 (data not shown; Orr et al., 2016). For mice, there is a short window from approximately pnd 5-10 where CB1r agonists will exhibit a dose dependent loss of righting when the dose of WIN55 is .2mg/kg or greater ($P<.05$; Fig. 2.4A,C). Interestingly, neonatal naked mole-rats still show a dose-dependent attenuation of the reflex at pnd 7 ($P<.05$; Fig. 2.4A) through at least pnd 56 ($p>.05$; Fig. 2.4B).

Adult naked mole-rats lose cannabinoid-based sensitivity in locomotor assays

For older animals, we used a standard 4-arm plus maze to analyze locomotion. Mice between 2-4-month-old showed a dose-dependent suppression of maze activity after WIN. Juvenile and adolescent naked mole-rats continue to display hypomobility ($p<.01$) as observed in the righting reflex test and a dose-dependent suppression of activity in response to WIN55 ($p<.05$-$p<.001$; Fig. 2.5A). Paradoxically, after 9 months of age naked mole-rats then become completely insensitive to 2.5 and 5mg/kg doses of WIN55 despite stability in ligand and receptor levels (<1 year: $p<.05$; > 1year: $p>.1$; Fig. 2.5A). Even when differences in mobility were accounted for by normalization to the control

locomotor response of the drug-matched age group, there was still a significant reduction in locomotion in naked mole-rats less than 1 year old and no change in animals greater than 1 year of age (Fig. 2.5B). Treatment with AM251 also elicited no changes in locomotion (Supplemental Fig. 2.2). Though spontaneous alternation is not a trait normally displayed by naked mole-rats (Deacon et al., 2012), we analyzed the effects of WIN55 on spontaneous alternation because it is reduced in mice and rats after CB1r activation (Basavarajappa and Subbanna, 2014). We found no significant difference after treatment (data not shown, $p>.1$).

The efficacy of the CB1r on ERK phosphorylation in the hippocampus is significantly different in 1-year naked mole-rats compared to mice

Since our behavioral data indicates that the naked mole-rat stops responding to cannabinoid treatments after 1 year, it is important to examine the viability of the CB1r throughout development to determine if it is still functional after the behavioral change. Neuronal CB1r most often activates the G_{io} subunit, therefore, the receptor's viability can be determined by examining this subunit's known downstream effects. We looked at the phosphorylation of ERK1/2 with ELISA. Phosphorylation of ERK1/2 is coupled to CB1r activation in mice and rats (Schulte et al., 1996; Sugden and Clerk, 1997). We examined the cerebellum, hippocampus, and PFC as the effects of CB1r are often region and cell specific. The PFC (Fig. 2.6A) and cerebellum (Fig. 2.6B) did not show significant changes in pERK levels after WIN55 application in naked mole-rat compared to mice at the adult age groups (Rubino et al., 2005). In general, there was an increase in pERK1/2 in the PFC across age groups, and there was a reduction in pERK in the cerebellum of adult mice and naked mole-rats (Fig. 2.6A,B). The hippocampus, however, did show a significant decrease in pERK1/2 after WIN55 application in the naked mole-rats ($p<.05$; Fig. 2.6C) while the mouse shows a nonsignificant trend toward reduction of phosphorylation after treatment. Additionally, the younger adolescent naked mole-rats show an increase in ERK1/2 phosphorylation ($p>.05$; Fig. 2.6C) in opposition to the naked mole-rat young adult. The only significant

difference in phosphorylation of ERK1/2 at this dose that was shown in the mouse occurred in the 2-week-old juvenile mice ($p<.05$; Fig. 2.6C).

Chronic but not acute exposure to cannabinoids in adolescence reduces maze learning acquisition

Our anatomical and molecular data indicate there is an extended time period of adolescence compared to mice and rats. We used the Hebb-Williams maze (Supplemental Fig. 2.3) to look at WIN55's effect on learning and memory in complex mazes. In brief, the Simple Maze Learning paradigm (Fig. 2.7A) assessed the effects of daily pretreatments of WIN55 that were given chronically for 4 weeks prior to testing in the 6 practice maze configurations. Animals were then run in 2 trials of 2 separate practice maze configurations (for a total of 4 mazes) each day until reaching the time criterion of 4 consecutive trials reaching the goal box in less than 60 seconds. During the testing, chronic treatments were continued, however, the treatments were given after completion of daily trials. Therefore, the Simple Maze Learning paradigm assessed the compounded effects of chronic treatments on learning. We found that chronic injections of WIN55 for 4 weeks slightly increased the time for adolescent naked mole-rats (32 ± 11 days) to reach the criterion (4 consecutive trials where the animal reached the goal box in under 60 seconds) compared to naked mole-rats that were chronically treated with either AM251 (16.75 ± 6 days, $p=.14$) or saline (16 ± 4.8; $p=.07$). There was no difference in simple maze learning for mice of any treatment group.

When testing the Complex Maze Learning paradigm, we examined the performance of adolescent naked mole-rats and young adult mice and rats using two criteria. The first criterion examined the number of maze trials that the animal reached the goal box in under 30 seconds. In this paradigm, animals were drug naive until testing in the complex maze configurations. Animals were treated with WIN55 or vehicle 30 minutes prior to testing in the daily 8-trial maze configuration (see Table 2.1 for drug treatment protocols). We found that for criterion 1: Medium complexity (Fig. 2.7B) there was a significant reduction in completed mazes in trials when adult naked mole rats were

pretreated with WIN55 (p=.03) compared to their complexity matched control trials (Fig. 2.7B). There were no significant differences in either adolescent naked mole-rats or young adult mice, though the mice did show a trend of reduced performance in criteria 1 (Fig. 2.7B) after treatment with WIN55 with all complexity levels which was not present in the adolescent naked mole-rats (Fig. 2.7B)**.** The second criterion examined the number of maze trials that the animal reached the goal box with 2 or fewer mistakes. There was no significant difference in any of the animal groups for either treatment or maze complexity for this criterion (Fig. 2.7C).

Cannabinoid modulation of facilitation and potentiation is developmentally dependent

Finally, we examined the developmental differences in the eCB system's ability to modulate paired-pulse response and long-term potentiation. CB1r activation, in mature neurons, leads to either transient or persistent suppression of vesicular release which it modulates by acting as a retrograde messenger system (Wilson and Nicoll, 2001; Kano et al., 2009). Here we focused on the effects within the hippocampus because they are one of the best described, there is anatomical evidence of delayed development in the NMR, and this is the region with the most significant differences in WIN55-stimulated ERK phosphorylation in NMRs over mice (Fig. 2.6C; Penz et al., 2015**)**. We used standardized hippocampal slice evoked synaptic potentials to examine any developmental modifications in naked mole-rats in adolescence (4-9 months of age) and early adulthood (1-3 years of age). These ages have been chosen to specifically examine how the effects of CB1r expression remains similar in these developmental states yet the behavioral response to WIN55 in these developmental ages is significantly different in motility and CB1-dependant regulation of ERK1/2 phosphorylation (adolescent versus young adult).

The endocannabinoid system is well known as a regulator of intracellular calcium in presynaptic terminals. We looked at paired-pulse responses in two areas of the hippocampus to determine the effect of cannabinoids on adolescent and adult naked mole-rat synaptic function. Animals were

pretreated with either saline, WIN55, or AM251, 30 minutes prior to sacrifice for hippocampal slice preparation. We found that cannabinoids only affected the CA1 region in adolescent naked mole rats, by depression of the second of paired responses (Fig. 2.8A,B) in animals with AM251 pretreatment. WIN55pretreatment had no effect in adolescent animals and neither drug had effects in adults. In the LPP of the dentate gyrus, there was no change in paired-pulse responses with cannabinoids in adolescents, but both AM251 and WIN55 enhanced paired-pulse facilitation in adults (Fig. 2.8C,D).

We measured in LTP in hippocampal field CA1 and the DG of slices from adolescent and adult NMRs. Animals were pretreated with the CB1r antagonist AM251 or agonist WIN55 before slice preparation. LTP was induced using Theta Burst Stimulation (TBS). Potentiation after TBS was quantified at three time points: immediately (<1 min) after TBS, and at 10 and 30 minutes post-TBS. The initiation of LTP was nearly doubled in strength from adolescence to adult naked mole-rats (Fig. 2.9A,D). In adult slices, both WIN55 (Fig. 2.9B) and AM251 (Fig. 2.9C) inhibited the induction of LTP. We analyzed the difference potentiation in responses 30 minutes after TBS (Fig. 2.10A) in adults and found inhibition of LTP induction in WIN ($p<.05$; Fig. 2.10A) and trending toward induction lasting 30 minutes for the AM251 response ($p=.07$; Fig. 2.10B). In adolescent naked mole-rats, there was much more variation in LTP induction in control slices (Fig. 2.9D), which appeared to be stabilized by WIN55 treatments with no appreciable reduction in LTP (Fig. 2.9E). Inhibition of CB1r with AM251, however, did not induce a noticeable induction or sustained potentiation (Fig. 2.9F). The reduction of potentiation did not reach significance compared to controls (Fig. 2.10B), though it did trend downward, and the lack of significance may be a result of the variability in the control response.

Discussion

This research sought to examine the relationship between the eCB system and naked mole-rat delayed development. It is possible that the development of the endocannabinoid system is responsible for guiding the observed neotenous features of naked mole-rats or conversely, the

developmental delay of the endocannabinoid system may be driven by other neotenous features. Previously, researchers have drawn connections between increases in CB1r expression and an increased role in the eCB system for mediating developmental advancement, particularly during synaptic structuring (Tapia et al., 2017; Meyer et al., 2017). We were particularly interested in the role of the endocannabinoid system in the development of the hippocampus, which in naked mole-rats is protected from hypoxic insult by retention of neonatal characteristics. We were also curious to examine if the brain regions associated with vulnerability to hypoxia and higher brain function, such as the hippocampus and PFC, would show exaggerated developmental delay compared to regions necessary for vital functions like mechanical control, such as the cerebellum (Paulin, 1993; Martinez-Orgado et al., 2003; Dhandapani et al., 2005). We expected the hippocampus and PFC to express the most significant changes from other rodents due to their late maturation during development and being regions of higher-level functions of cognitive reasoning and memory retrieval. The cerebellum was examined as an internal control due to there being no known or observable changes in function for this region in naked mole-rats, its maturation occurring rather early in the development of the nervous system of naked mole-rats (Orr, 2016; personal correspondence with Lech Kiedrowski).

The early stages of development have been linked to many neuroprotective aspects of the endocannabinoid system, including resistance to hypoxia, the activation of the fetal CB1r influences progenitor cell proliferation, and neuronal commitment/survival (Aguado et al., 2006; Galve-Roperh et al., 2013;Guzman et al., 2002; Harkany et al., 2007;Martinez-Orgado et al., 2003). The eCB system is also responsible for determining the neuronal/astroglial balance in the brain during development and affects neurite growth and synapse formation (Kano et al., 2009; Harkany et al., 2007; Berguis et al., 2007).

We showed that delayed development of the CB1r occurs in both the hippocampus and PFC, both of which are late developing regions of the brain and control higher brain functions. Our data

indicate that both receptor and ligand expression do not develop past a juvenile or adolescent state. The dentate gyrus is known to have the highest density of CB1r receptors in the brain; additionally, these receptors are more heavily co-localized with GABAergic neurons (Katona, 2009). Based on these characteristics, we chose the dentate gyrus to analyze for both receptor density and proximity relationships. We previously observed that adult naked mole-rat have immature separation of GABAergic and glutamatergic cell layers in the dentate gyrus (Penz et al., 2015); however, we had not yet examined the distribution of the CB1r within this system or elsewhere in the hippocampus. The hippocampus is particularly sensitive to excitotoxicity and we have shown previously that retention of neonatal features is key to the naked mole-rat's retention of neuroprotective abilities into adulthood. The cannabinoid system also plays a role in both neuroprotection in neonates and mediates the maturation of neuronal circuits in the hippocampus. It is possible that increasing CB1r while maintaining low levels of eCBs allows the eCB system to quickly recognize and respond to imbalances to hippocampal circuits to maintain proper function even with chronic bouts of low oxygen.

Certain brain regions show increases in endocannabinoids during distinct developmental stages that are heavily modulated by the endocannabinoid system. These elevations occur during embryonic development and begin again in late adolescence to adulthood. The distribution in higher brain regions are similar to CB1r expression regarding neoteny; naked mole-rats maintain similar eCB properties as neonatal mice into adulthood.

It is intriguing that the delayed development of endocannabinoid expression is so regionally specific, though cannabinoid expression is known to be differentially depending on the needs of a species or pathophysiological changes within an individual animal. Examples include increased CB1r in the human basolateral amygdaloid complex compared to rodents, cortical expression of CB1r being concentrated in different layers in humans, rats, and monkeys, and CB1r expression being elevated in GABAergic neurons but not glutamatergic neurons in epileptic samples (Hu and Mackie, 2015; Katona,

2009; Dudok et al., 2015). Our data indicates that increases in endocannabinoid ligand expression may be necessary for maintaining function in the cerebellum and posterior cortex. Since naked mole-rats have evolved to live in dark tunnels with poor acoustic integrity, the precise maturation of their motor functions and somatosensory system is critical to their way of life more so than other mammals. Modulation of those circuits by the endocannabinoid system is an important aspect of fine circuitry control.

In regard to the lack of adult locomotion effects, it is possible that some aspects of the endocannabinoid system have adapted to the neonatal expression in order to maintain proper functions. It is important to explore how this system is controlled in the naked mole-rat to gain a greater understanding of how, as a neuro-regulator, an altered eCB system functions within the nervous system. Older naked mole-rats appear to be completely insensitive to the agonist, which is similar to neonatal (< pnd 3) mouse behavior. Neonatal mice at this age do not display any motor defects from eCBs, which is considered due to the neonatal GABAergic neurons being excitatory at this age (Cherubini et al., 1991). In other words, the naked mole-rats develop a neonatal-like behavioral response at 1 year of life and then retain that response throughout their lifespan. In early development, CB1r is almost exclusively on excitatory axons, only in later stages of mouse embryonic development do they begin to be expressed in GABAergic neurons of the hippocampus. In most regions of the mature brain of traditional laboratory rodents, there is very little CB1r expression on excitatory neurons, leading to a limited ability to mediate the activity of these neurons. It is possible that an adultlike structural architecture, heavily reliant on inhibition of GABA activity, is in place early on in naked mole-rat development only to evolve a shift in expression balance from being almost exclusively functioning on inhibitory interneurons (functioning as synaptic modulator) to being more heavily expressed on excitatory neurons (functioning as a modulator of neuroprotection; Berguis et al., 2007; Yasuda et al., 2008). The level of CB1r expression is still elevated at 1 year of age, indicating that

neural synapses in the hippocampus are not yet set (Gaffuri et al., 2012). Additionally, we have shown previously that the neural circuitry of GABAergic and glutamatergic neurons of the hippocampus are still developing in the age groups studied here and that there are significant differences in the biophysical properties of granule cells at 1 year of age (Penz et al., 2015). Taken together, the protracted development of the naked mole-rat brain allows plasticity that could functionally alter the key purpose of the endocannabinoid system in adulthood.

Activation levels of CB1r were examined in the cerebellum, hippocampus, and PFC. These structures were chosen to account for regional differences in tolerance to continuous activation of CB1r. The increased expression of CB1r into adulthood could over-activate system and have caused functionality to be reduced as a counter measure to protect naked mole-rats. Research has shown that the cerebellum is prone to develop tolerance to CB1r activation over time while the prefrontal PFC and hippocampus show stable activation phenotypes despite prolonged exposure to eCBs (Rubino et al., 2005). The cerebellum is not only susceptible to long-term tolerance effects; it is also one of the regions responsible for motor coordination and therefore could be a target in the hypokinesia- or lack thereof- effects of WIN55 (Manto et al., 2012). However, in both species, the PFC exhibited a robust level of phosphorylation in all but the youngest age group, while the hippocampus exhibited very little effect from the dose given. We expected the cerebellum would be the region to deviate from the mouse and therefore explain our behavioral experiments via cerebellar motor systems becoming tolerant, and therefore impervious, to cannabinoids. The cerebellum, however, also showed similar effects to mice. Instead, we found that the naked mole-rat hippocampus has the most divergence from the mouse in terms of regulation of ERK1/2 phosphorylation. Though the difference in receptor efficacy were only seen in the young adult cohort, the hippocampus remains an important target for exploring the developmental changes in cannabinoid maturation.

CB1r activation here has been found to modulate synaptic plasticity through the cholecystokinin-positive GABAergic inhibitory interneurons that express high levels of CB1r in the CA1 region and in the dentate gyrus (Frazier C, 2007; Tsou et al., 1999; Marsicano et al., 2002; Katona et al., 1999; Herkenham et al., 1990; Chevaleyre et al., 2006). Since receptor tolerance to WIN55 in the cerebellum was not found in the 1-year naked mole-rat, it was important to see if there are developmentally based changes in the molecular eCB-signaling pathway in the naked mole-rat hippocampus compared to adolescent naked mole-rats. We looked at changes to the eCB molecular pathway that could explain how the naked mole-rat becomes insensitive to cannabinoid application after 1yr as well as to verify the age specific changes seen with IF imaging. Because the eCB system modulates both excitatory and inhibitory vesicle release in the CA1 and the dentate gyrus, shifts in balance between these receptors' expression levels can affect the balance of eCB-mediated transmission, this could ultimately affect the behavioral phenotypes expressed after CB1r activation.

Previous research studies in mice have established the CA1 region of the hippocampus to have a well-defined pattern of effects on neurons in the hippocampus (Chevaleyre and Castillo, 2003; Hajos et al., 2000). Additionally, since CB1r expression in mice is denser on GABAergic neurons than any other in the hippocampus balancing their effects to be more heavily involved in modulation of synaptic inhibition, leading to disinhibition of the principal neurons (Katona et al., 1999; Aguado et al.,2006; Zhu and Lovinger, 2007). However, a smaller population of CB1r is also found on pyramidal neurons and can directly inhibit vesicular release at excitatory synapses as well (Kano et al., 2009; Katona and Freund, 2012). We found that adolescent naked mole-rat LTP induction was not as strong or as stable as that of adult animals. Additionally, cannabinoids were able to strong inhibit the induction of LTP in adults but not in adolescent animals. This was surprising as in other animals, such as mice and rats, the modulation of potentiation is much stronger in younger animals (Chevalyre and Castillo, 2003; Chevaleyre et al., 2006; Yasuda et al, 2008; Kawamura et al., 2006). While there was stronger

modulation of potentiation in adult naked mole-rats, inhibition of CB1r in adolescents induced paired-pulse depression in adolescents but not in mice. Also, interesting was the similar inhibition of potentiation in adults with both WIN55 and AM251, as they have been shown to have opposing effects on initiation of potentiation in studies with other rodents (Kawamura et al., 2006). Further exploration of the balance in CB1r's function between interneurons and pyramidal cells is needed to understand the mechanisms behind this phenomenon.

In mice the LPP of the dentate gyrus is modulated by cannabinoid treatment to a greater extent than the CA1 region (Wang et al., 2016; Navarrete and Araque, 2010; Gomez and Gonzalo et al., 2015). In the case of paired-pulse response, this was correct for naked mole-rats as well, both WIN55 and AM251 were able to induce PPF in adults, indicating a strong effect on intracellular calcium in the presynapse, but again showing a similar response in both the agonist and antagonist. There was no effect on facilitation in adolescents. The dentate gyrus also showed very little effect on potentiation, which was overall much smaller than the potentiation induced in the CA1 region. There was a small trend of WIN55 increasing the induction of adult LTP, particularly in the initiation and early sustained time points, but it was not significant.

Since cannabinoid mediation of the induction of potentiation in naked mole-rats that was age dependent, it was interesting to see a similar developmental difference with the naked mole-rat's performance in learning assays with cannabinoid treatments. We found sustained treatments of WIN55 to be detrimental in learning simple maze configurations in adolescent naked mole-rats. However, single treatments had no effect on adolescent learning and only caused a small detriment in young adults. Current rodent models of cannabis use during adolescence have produced contradictory results (Gorey et al., 2019) which are most likely due to the short period of adolescence and inability to create a chronic state of use within rat adolescence that is analogous to human usage. In fact, studies have shown that it is imperative to treat rodents for at least 21 days, however, traditional laboratory

rodent adolescence is only 14 days (Agoglia et al., 2017; O'Shea et al., 2004). With the increased adolescent window of naked mole-rats, including retention of adolescent endocannabinoid features in the hippocampus and PFC, this developmental issue could be resolved. Of particular translational concern is how these synaptic formations are maintained during the pruning stage during adolescence if cannabis is used. Recent findings indicate that endogenous cannabinoid lipid expression in the hippocampus is differentially affected by THC administration in early adolescence, late adolescence, and adulthood indicating the endogenous circuitry may be disturbed with recreational use of cannabis (Leishman et al., 2018). Utilizing the naked mole-rat model, chronic treatments could be studied at each stage of adolescence in isolation, reducing the confounding variables associated with overlapping developmentally dependent cannabinoid functions in mice.

CB1r activation by endogenous and exogenous cannabinoids is known to affect behavior, synaptic plasticity, memory, neurite proliferation and differentiation, and energy metabolism. It is also known that the endocannabinoid system plays a prominent role in regulating neurodevelopment and neuroprotection (Martinez-Orgado et al., 2003; Harkany et al., 2008). There is still much to be determined about the underlying mechanisms and potential of the eCB within the mammalian nervous system and how use of synthetic and phyto cannabinoids disrupt or enhance neural scaffolding. In particular, with the use of the naked mole-rat model system, we can examine periods of development that have traditionally been difficult. Moreover, we can use our understanding of naked mole-rats' unique evolutionary needs to further understand the capabilities of the endocannabinoid system's targeted specificity.

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Figure Legend

Figure 2.1: Predicted gene sequence of NMR CB1r (CNR1) compared to the Human CB1r Sequence NCBI/ NMR Genome (2014). The human sequence is on the upper row and the naked mole-rat sequence is on the lower row of the pair. Nucleotide mutations are indicated as red base pairs. Amino acid changes are indicated as highlighted groups with the aa>aa change listed below. Nucleotide mutations that are silent mutations are indicated with an *.

Fig 2.2: Hippocampal Expression of Cannabinoid Receptors in the Dentate Gyrus. Immunolabeled CB1r (Red) in the dentate gyrus of the 2wk NMR (A), 4mo NMR(B), 1yr NMR (C), and adult mouse(D). CB1r is co-expressed with Hoechst (Cyan) to show cell layer. Relative fluorescent density of CB1r expression at each age-group (E). N=4. ** p<.01

Figure 2.3: LC/MS/MS was performed to analyze the expression of the endocannabinoid 2-AG from early neonatal naked mole-rats through adulthood (light blue through dark blue) in the cerebellum (top left panel), posterior cortex (middle left panel), hippocampus (top right panel), and prefrontal cortex (middle right panel).The data indicates that regions necessary for vital functioning have fully matured expression of 2-AG that is similar to the developmental milestones of other animals (left panels). Regions of higher brain function exhibit developmental delay through adulthood compared to other animals (right panels). Hippocampal expression of AEA and similar NAPE derivatives also indicate delayed developmental expression levels into adulthood (bottom panel). The naked mole-rat data is shown in comparison to juvenile (light green) and adult mice (dark green). N=3, ND = expression levels too low to detect, no data.

Fig 2.4. Latency to righting was measured in seconds between mice (green) and naked mole-rats (blue) at 7 days post-natal. Both species show a dose dependent increase in time to righting after treatment with WIN55. N=4 mice, N=3 naked mole-rats. The WIN55 reduction of the righting reflex was tested at different ages in naked mole-rats (B) and mice (C). Control= Saline, Medium= 2.5mg/kg, High= 5mg/kg. N=4 mice, N=3 naked mole-rats.

Fig 2.5. Dose-dependent effects of eCB agonist, WIN55,212-2, on locomotion and sedation using a standard 4-arm radial maze. Animals were placed in the center of the maze and allowed to move freely for 10 minutes. Naked mole-rats that were immature (2 and 6 months old) had a dose dependent decrease in locomotion similar to mice, but mature naked mole-rats showed no dose dependent response (A). NMR age 1mo, 6mo, and 9mo all display increased hypokenesia when given increased doses while older than 1yr naked mole-rats shows no effect on number of arm crossings regardless of dose even when normalized for differences in control performance (B). N=4 per age group.

Fig 2.6: Phosphorylation of ERK1/2 was analyzed in total proteins with no drug (light green/blue) or .3mg/kg of WIN55based on total animal volume (dark green/blue) to determine the downstream effect of CB1r activation in the prefrontal cortex (A), hippocampus (B), and cerebellum (C). Developmental changes were compared between mice (green, left panels) and naked mole-rats (blue, right panels) at juvenile (2wk:2wk), adolescent (4wk:4mo), young adult (4mo:1yr), and mature adult time points(1yr:5yr). N=3. (* $p<.05$, ** $p<.01$, *** $p<.001$)

Fig 2.7: A) Adolescent naked mole-rats (blue, 4 months of age) and mice (green, 4 weeks of age) were treated with WIN55 for 24 days prior to testing in the Hebb-Williams format of complex maze learning. Animals were considered to have reached the learning criteria when they could complete 8

consecutive trials of at least 2 separate maze configurations in under 60 seconds. N=4 naked mole rat and mouse WIN55and AM251, N=6 naked mole-rat and mouse control. $P < 0.1$. B) Animals were tested in 8 consecutive mazes per configuration at Easy, Medium, and Hard complexity levels. The number of mazes in each configuration that reached: B) time criteria (reaching the goal box in less than 30 seconds) or C) mistake criteria (reaching the goal box with 2 or fewer mistakes) were averaged for adolescent (4moNMR), young adult (1yNMR), and young adult mice (<5 months at test completion) and their performance with pretreatment of WIN55 was compared to saline control performance in the same difficulty maze. (mice, n=9. 4-month NMR, n=8. 1-year NMR, n=7). $P<.05$.

Figure 2.8: Properties of adolescent and adult naked mole-rat neurophysiology. Graphs show excitatory postsynaptic potential (EPSP) initial amplitude for second response as a percentage of the first response of the pair with interpulse intervals (IPIs) of 50-800ms. Recordings in the CA1 region show depression in shorter intervals after CB1r antagonism with AM251 (A). The CA1 region is unaffected by cannabinoids in adult naked mole-rats (B). The lateral Perforant pathway (LPP) of the dentate gyrus is not affected by cannabinoids in adolescent naked mole-rats (C), but both AM251 and the agonist WIN55 increase facilitation, particularly at the shorter intervals 50-200 ms (D). (n=5)

Figure 2.9: Theta Burst Stimulation of hippocampal brain slice to induce LTP. Adult and adolescent naked mole-rat slopes were recorded normalized (100%= Average of Baseline) from baseline through 40 minutes of recovery after LTP induction in the CA1 region. Comparison of induction and recovery in control (A), treatment with WIN55 (B), and AM251 (C).

Figure 2.10: Cannabinoids Effect of LTP After TBS in the CA1 Region of the Hippocampus in Adolescent and Young Adult Naked Mole-Rats. Normalized slopes were averages for adult (A) and adolescent (B)

brain slices recorded in the CA1 region of the hippocampus. Analysis was done 30 minutes after TBS.

Table 2.1: Drug treatment protocol for animal groups (n=2/group).

Supplemental Figure S2.1. Time course for WIN55 in the righting reflex of mice (A) and naked mole-rats (B), to determine the optimal length of time before WIN55 has taken maximal effect. N=4 mice, N=3 naked mole-rats.

Supplemental Figure S2.2. Dose dependent effect of AM251 in comparison to previously described mouse and adult naked mole-rat WIN55 data in the same paradigm. n=4.

Supplemental Figure S2.3. Hebb Williams maze with representative configurations.

Supplemental Figure S2.4. Ability of Adolescent and Adult Naked Mole-Rats to Perform in the Hebb Williams Maze Compared to Mice. Easy and Hard Configuration Difficulties were assessed using a criteria of maze completion within 30 seconds (Criteria 1, A) and a criteria of maze completion with less than 3 mistakes (Criteria 2, B).

Comparison of Naked Mole-Rat Predicted CNR1 Gene Sequence to Human CNR1 Known Sequence

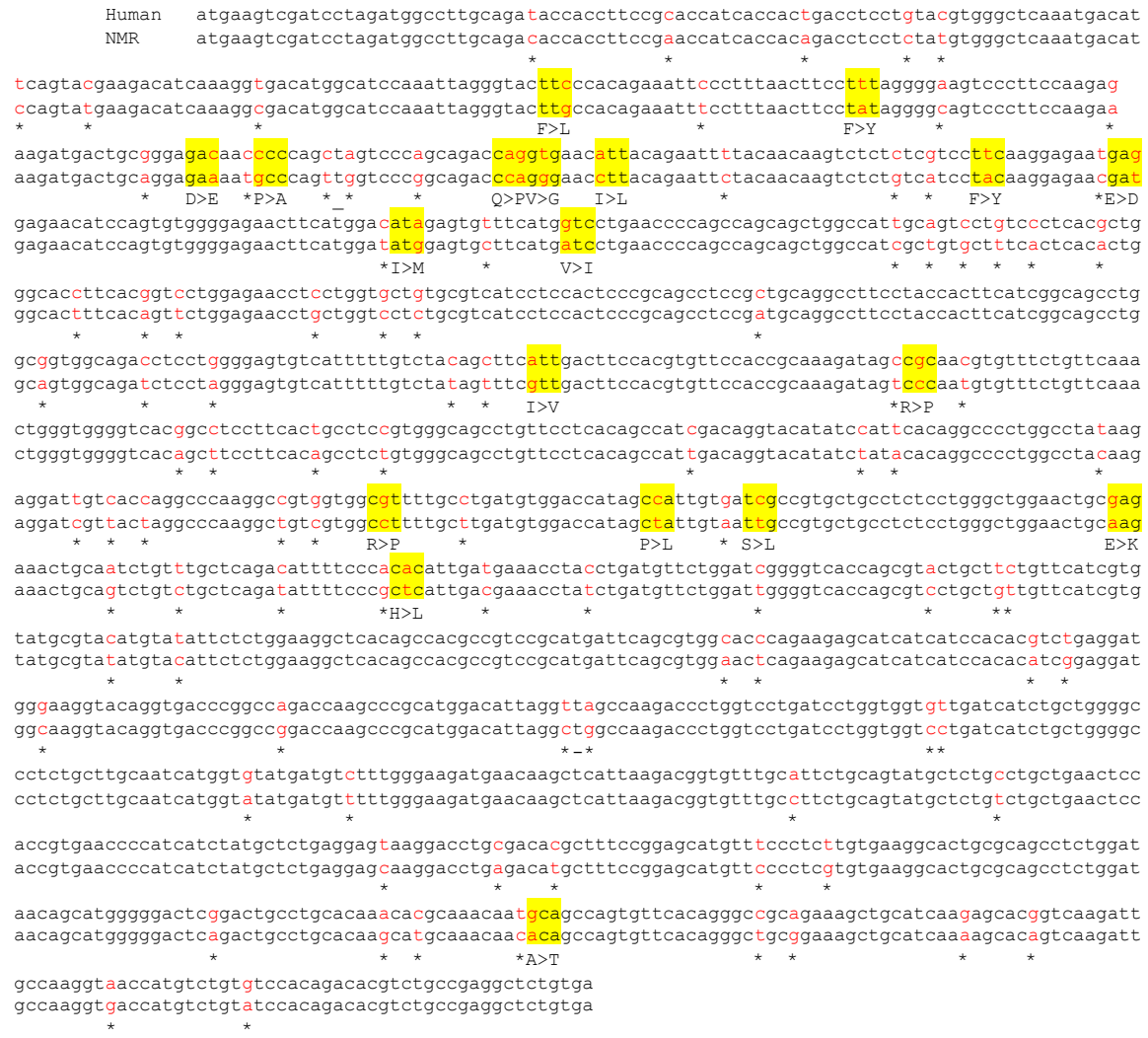


Figure 2.1.

Developmental Changes in Naked Mole-Rat Cannabinoid Receptor 1 Expression

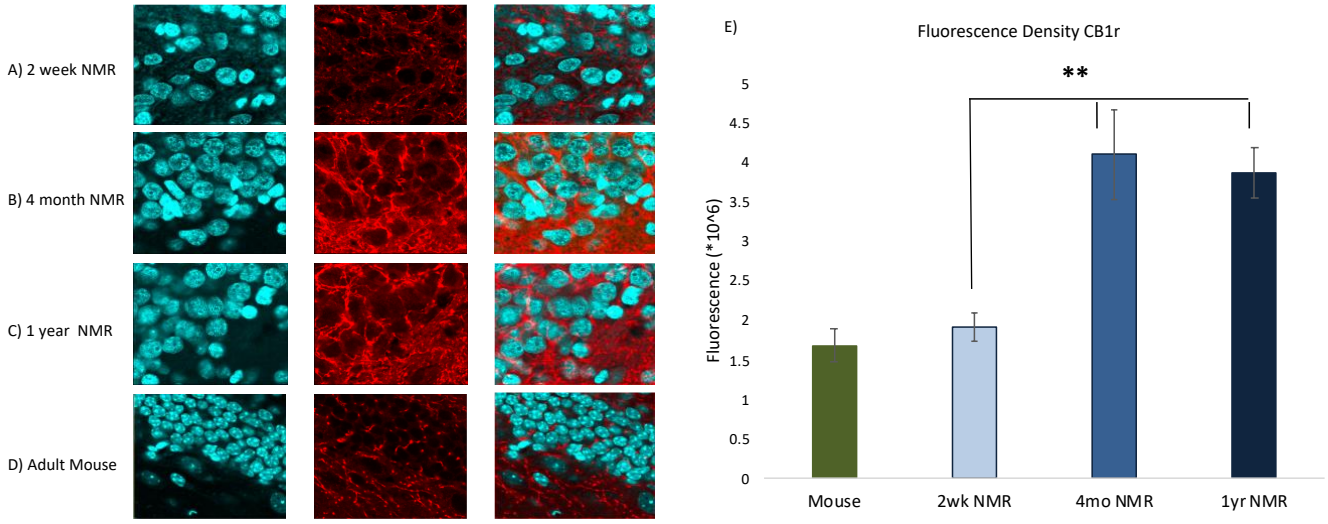


Figure 2.2.

Endogenous Cannabinoid Expression in Various Brain Regions

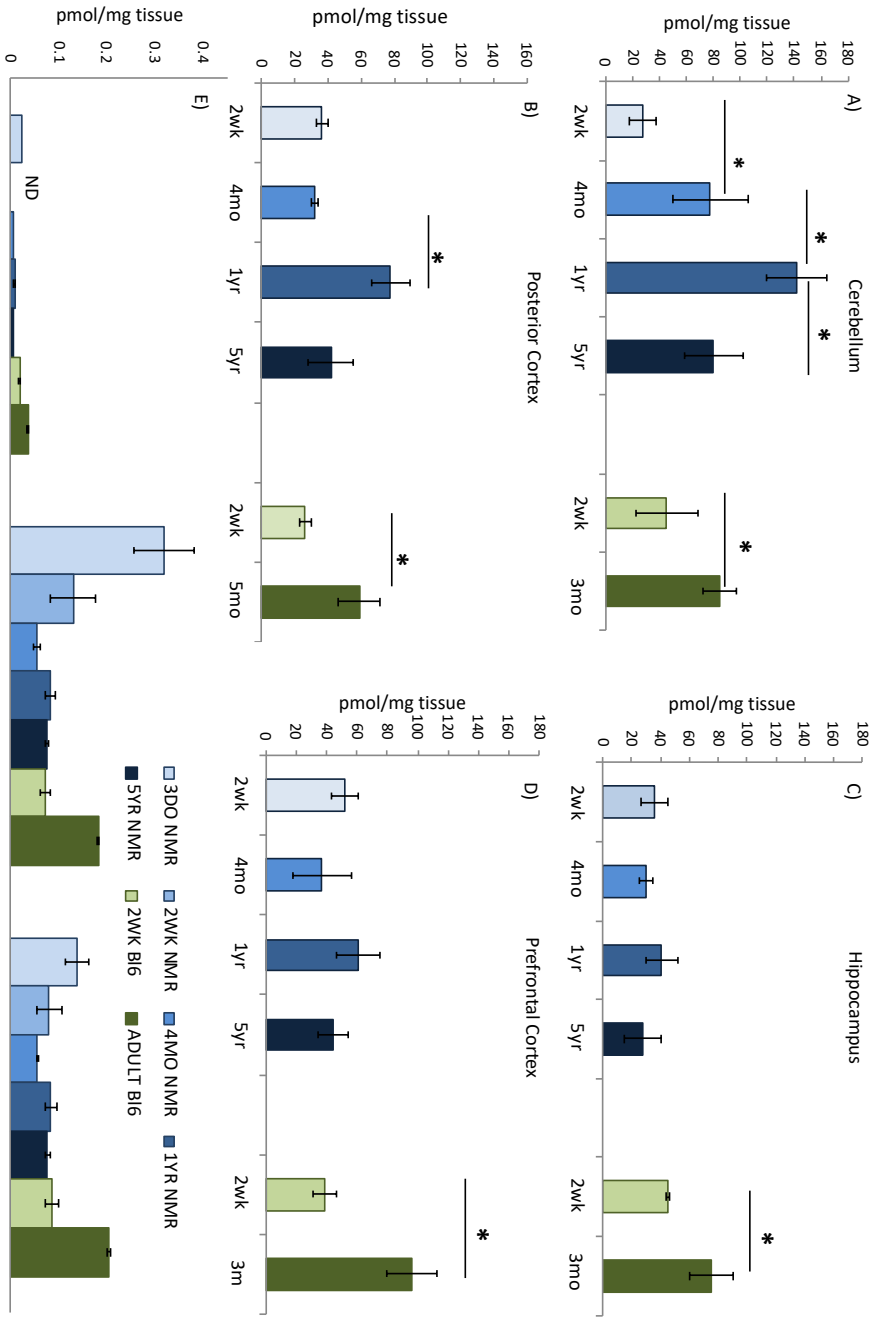


Figure 2.3.

Locomotor Response to Cannabinoids in Neonatal and Juvenile Mice and Naked Mole-Rats

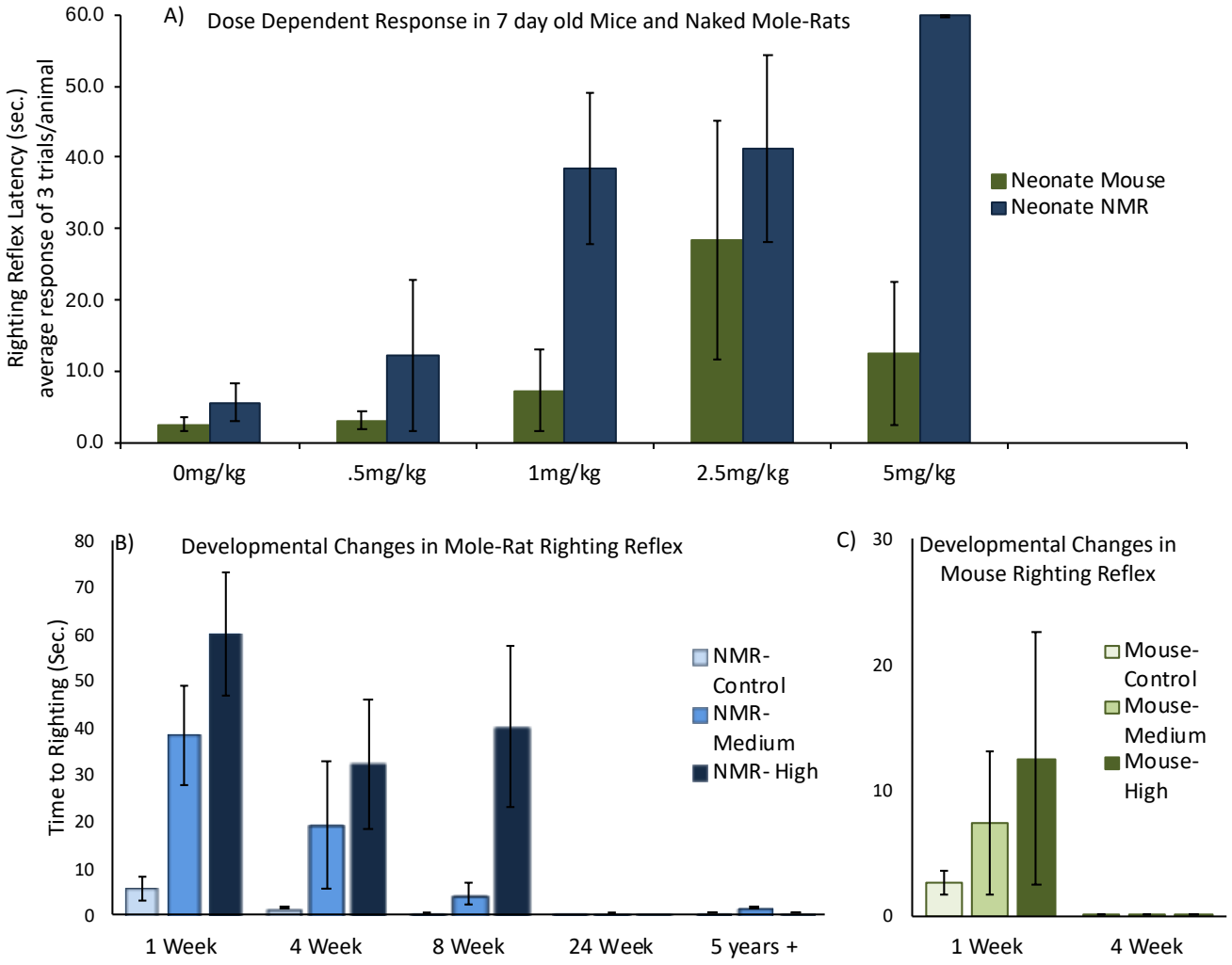


Figure 2.4.

Adult Naked Mole-Rats Do Not Exhibit Motor Depression After Treatment with WIN55

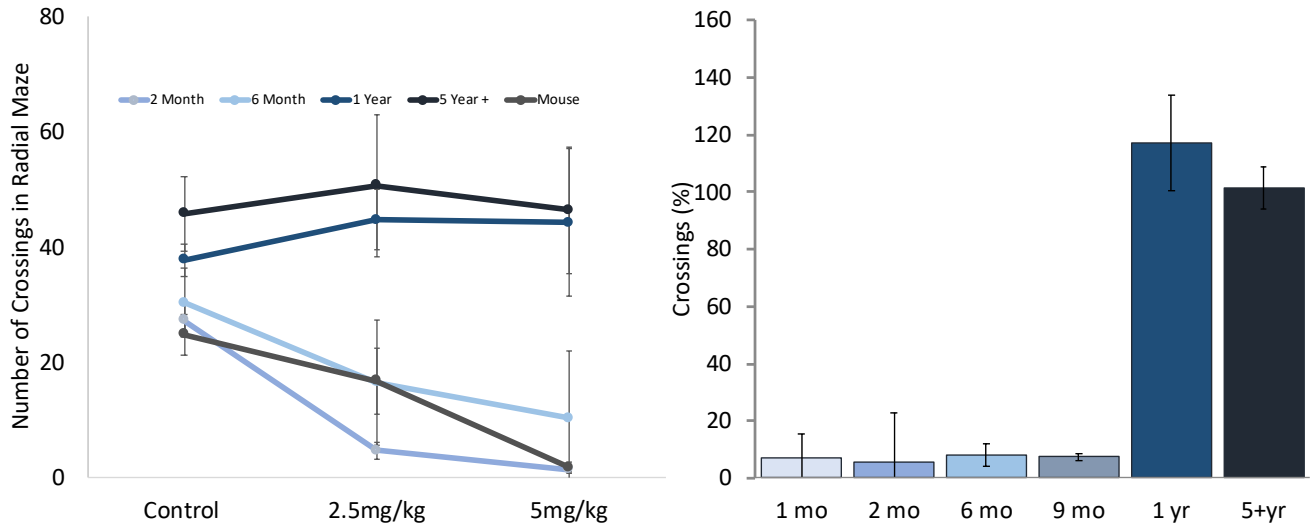


Figure 2.5.

WIN55 Regulates ERK1/2 Phosphorylation in Naked Mole-Rats

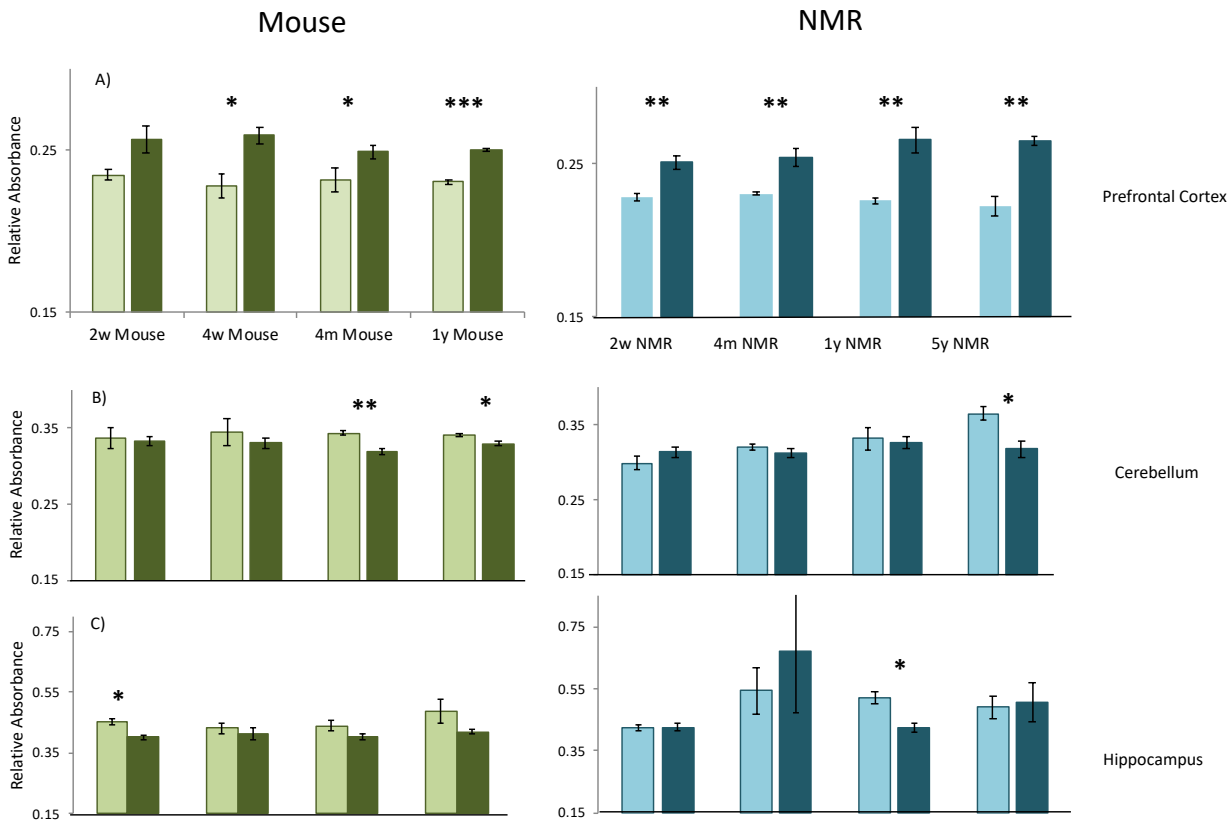


Figure 2.6.

The Effects of Chronic and Acute Treatments of Cannabinoids on Maze Learning

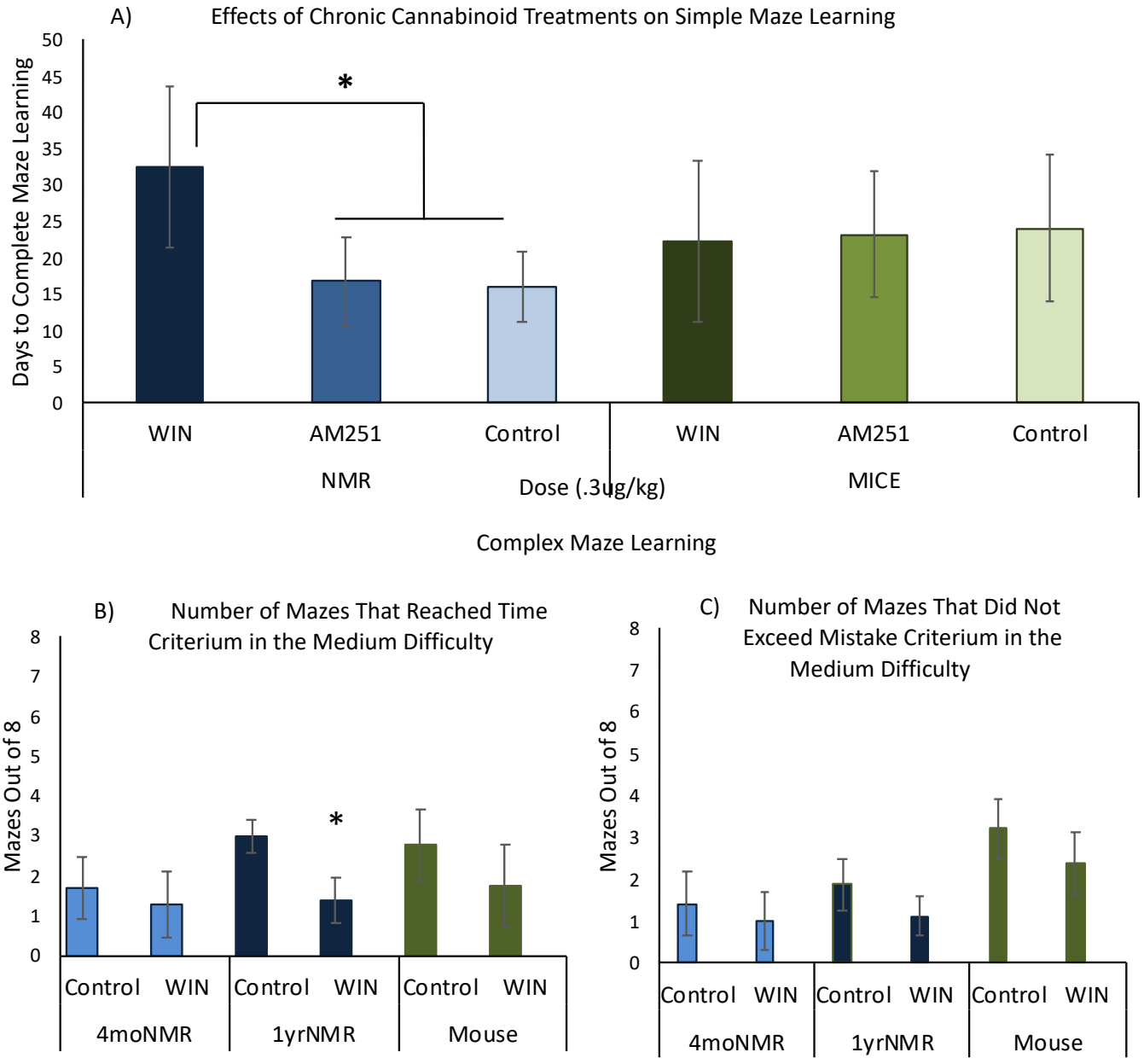


Figure 2.7.

Facilitation in Adolescent and Young Adult Naked Mole-Rats

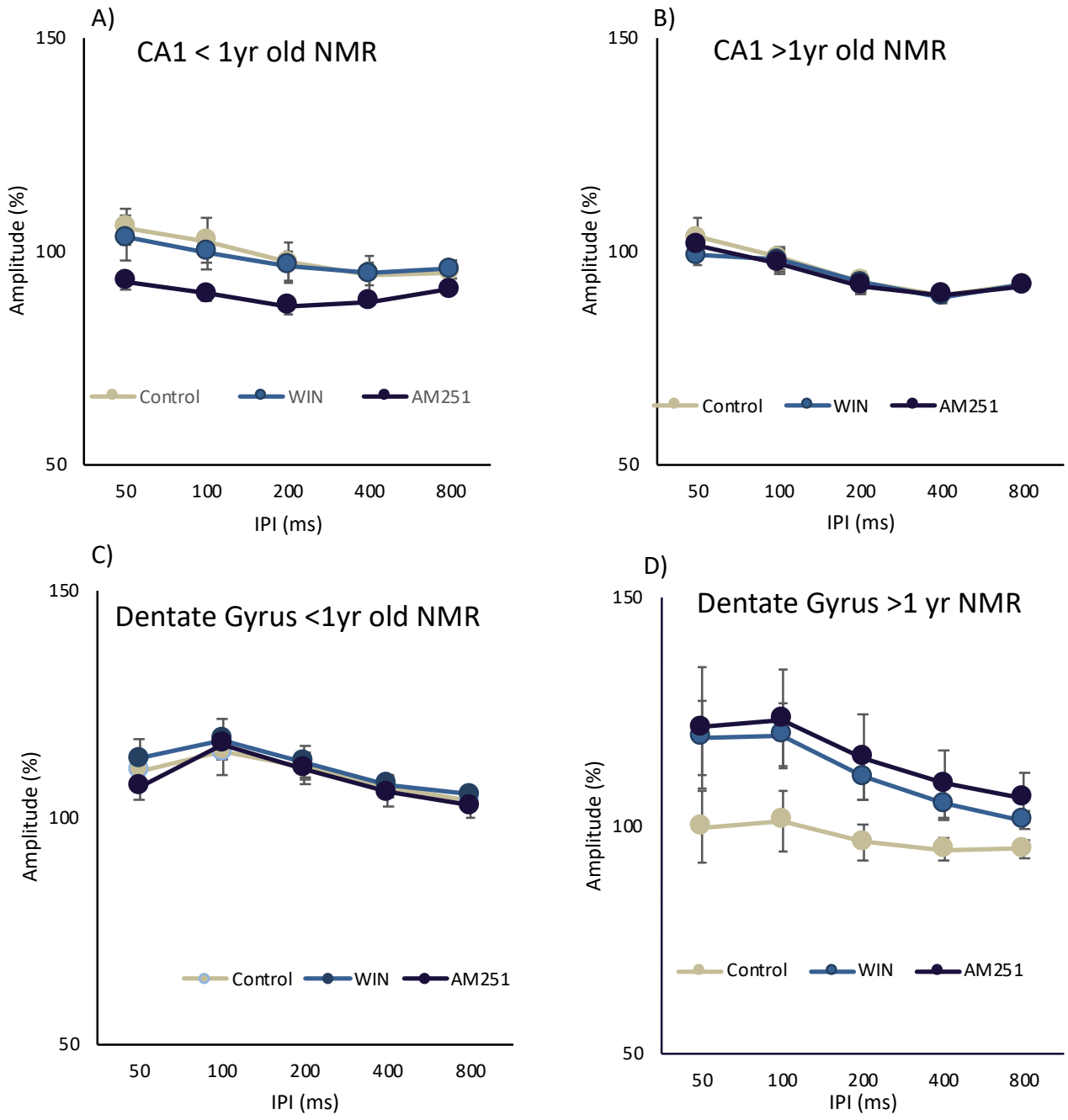
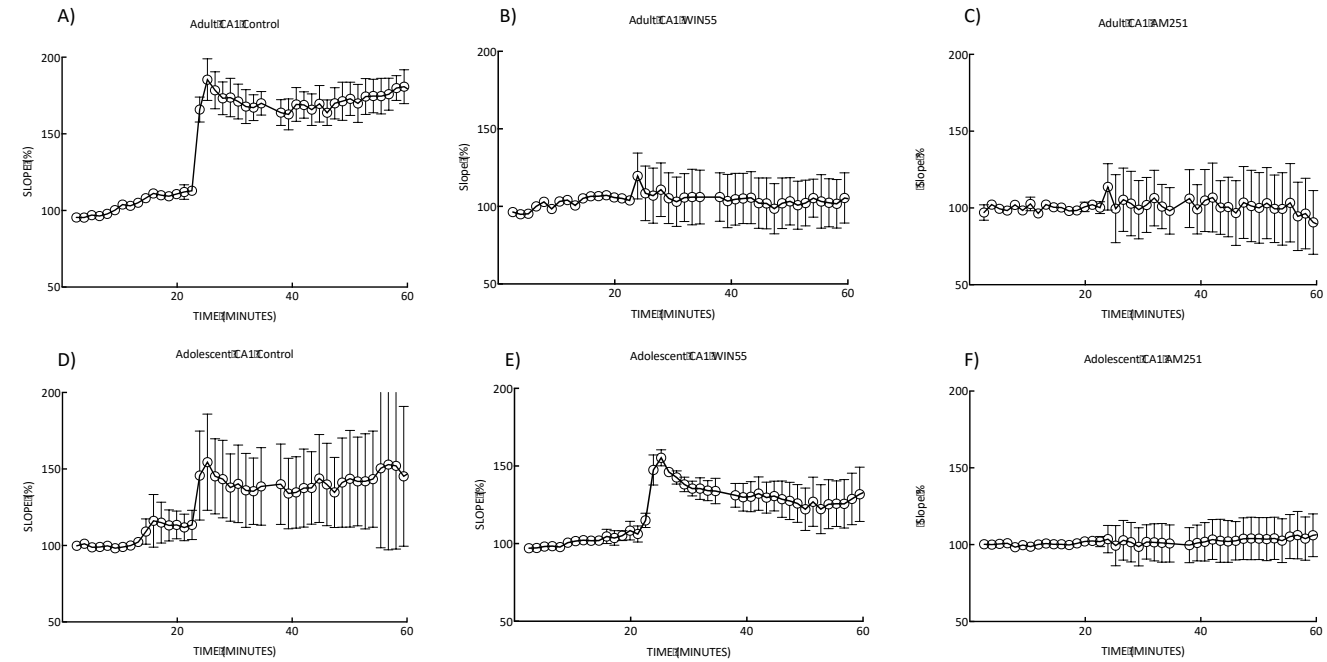


Figure 2.8.

Induction of LTP with TBS in the CA1 Region of the Hippocampus in Adult and Adolescent Naked Mole-Rats



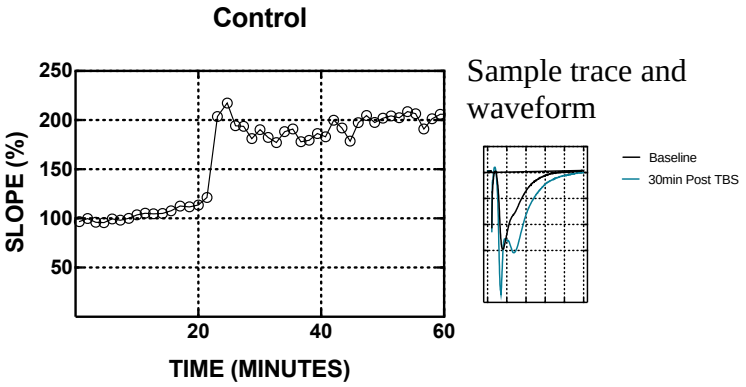


Figure 2.9.

Cannabinoids Effect on TBS-Induced LTP in the CA1 Region of the Hippocampus in Adolescent and Young Adult Naked Mole-Rats

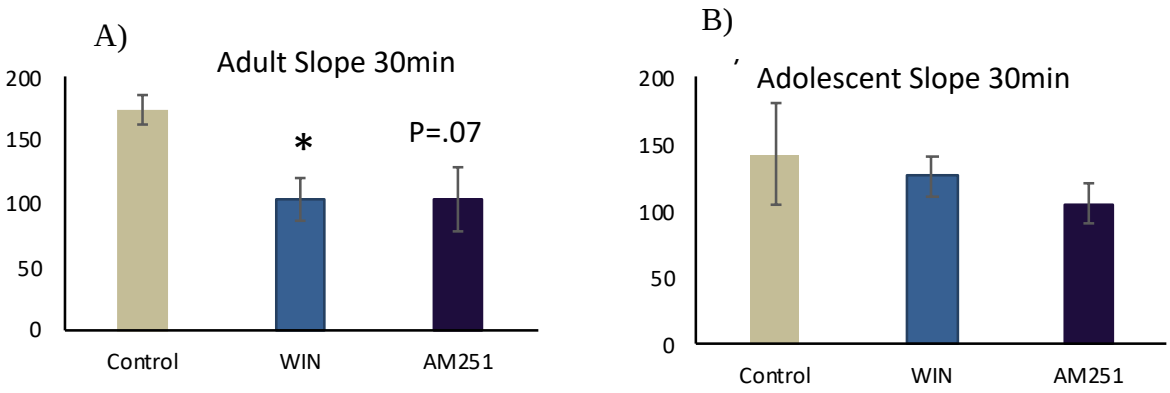


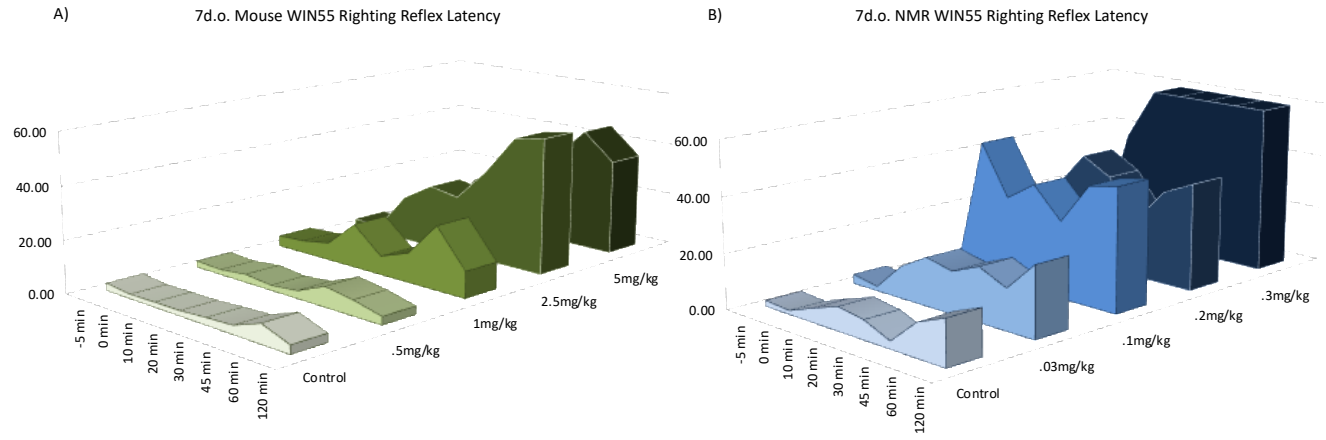
Figure 2.10.

Drug treatment paradigm for Complex Maze Learning

Drug Condition Protocol:						
Animal:	Maze Difficulty	Easy		Medium		Hard	
	Maze Number	3	4	6	7	10	11
	1	WIN	SALINE	WIN	SALINE	WIN	SALINE
	2	SALINE	WIN	SALINE	WIN	SALINE	WIN

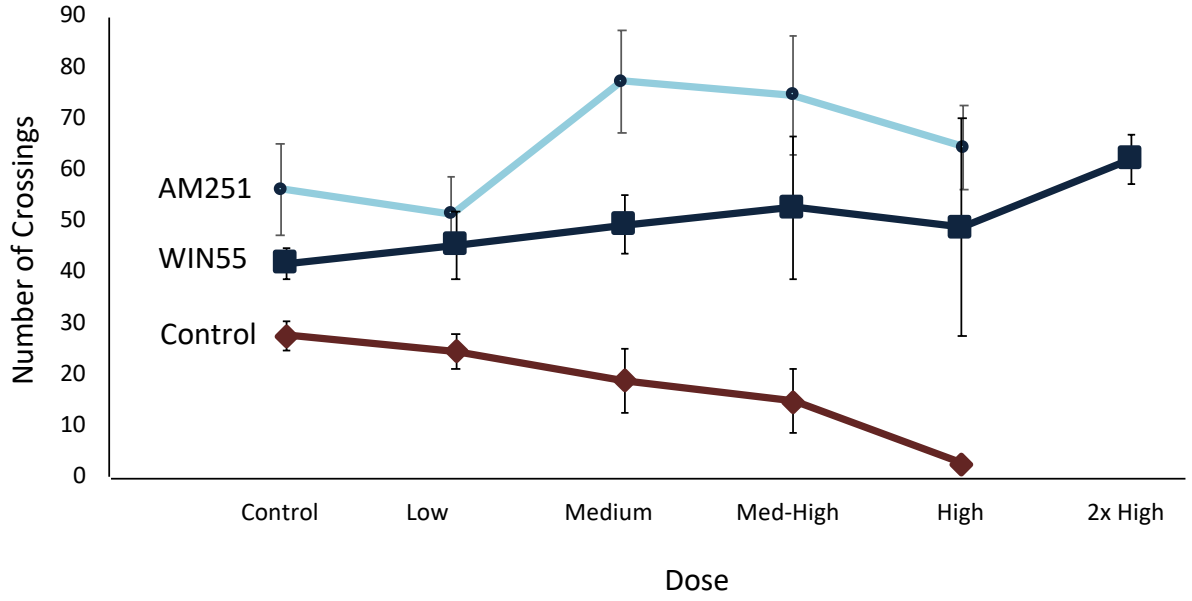
Table2.1.

Time Course of Effectiveness for WIN55 in the Righting Reflex Test in Mice and Naked Mole-Rats



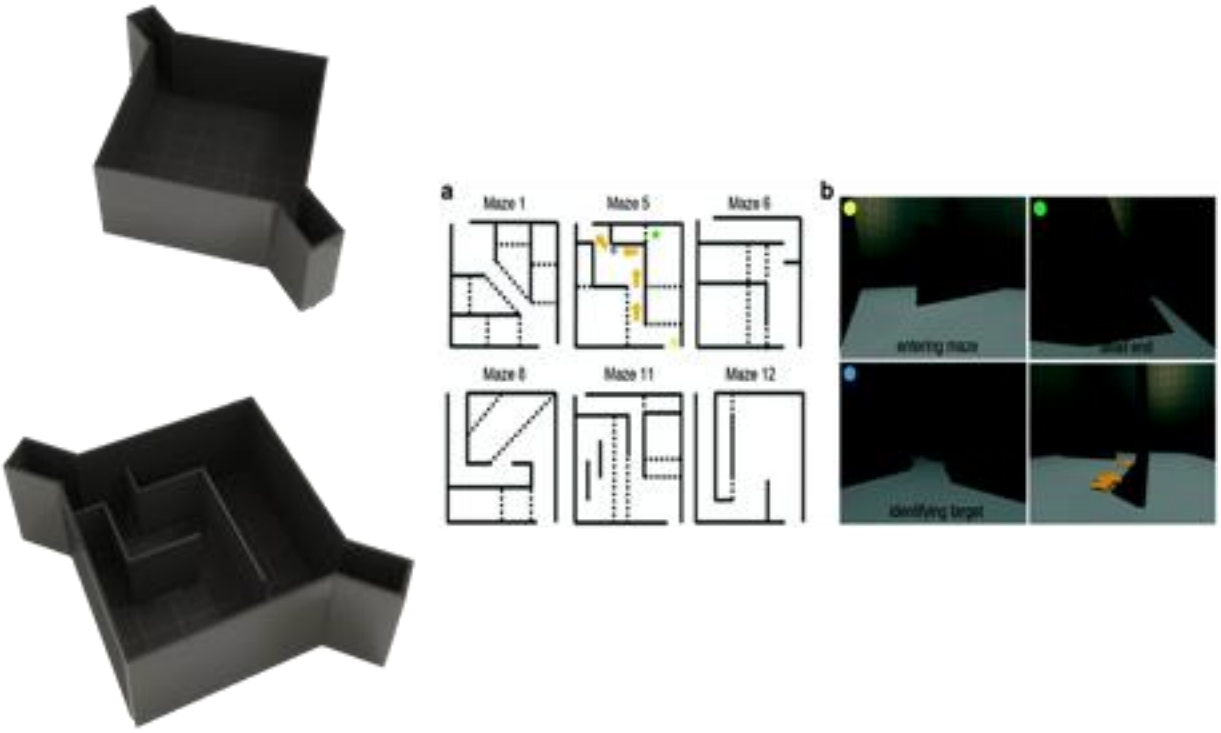
Supplemental Figure S2.1.

AM251 Does Not Depress Locomotion in Adult Naked Mole-Rats



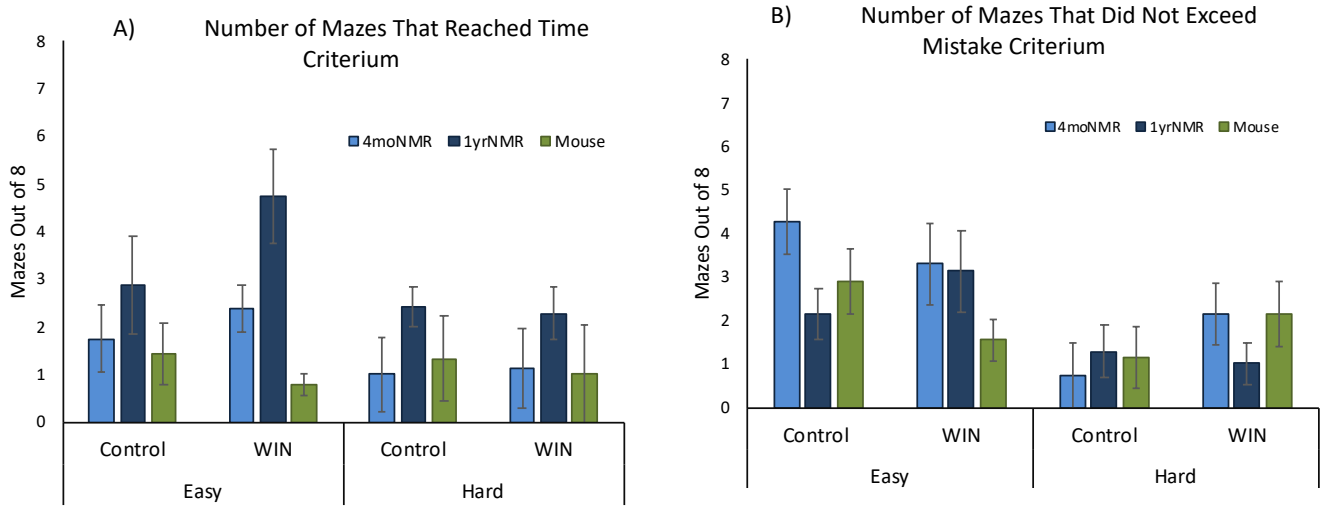
Supplemental Figure S2.2.

The Hebb Williams Maze and Sample Configurations



Supplemental Figure S2.3.

Results of Complex Maze Learning in Easy and Hard Configurations



Supplemental Figure S2.4

Chapter III: The Inflammatory P2X3r Pathway is functional in African Naked Mole-Rats and Can Be Targeted by Cannabinoids

Abstract

Naked mole-rats have adaptations within their pain pathway that are beneficial to survival in large colonies within unventilated tunnel systems. This results in the tunnel environments maintaining lower O_2 and higher CO_2 levels than above ground environments. These adaptations ultimately lead to a partial disruption of the C-fiber pathway which allow the naked mole-rats to not feel pain from the acidosis associated with CO_2 accumulation. The hallmark of this disruption is that naked mole rats do not express neuropeptides, such as Substance P and CPRG, therefore, effectively making the C-fiber's peptidergic pain pathway inoperative. However, there are currently no studies that have examined the remaining aspects of the C-fiber pathway, namely the purinergic pathway, in naked mole-rats, despite this being a key pathway for inflammatory pain. This study aimed to establish the functionality of the remaining purinergic C-fiber pathway by looking at the P2X3 receptor. Additionally, this study examined the effectiveness of cannabinoids in attenuating inflammatory pain through the P2X3r pathway in naked mole-rats. Cannabinoids have been found to help manage chronic pain due to inflammation in both human and mouse models and studies suggest a major role for P2X3r in this treatment. First, we utilized calcium imaging of dorsal root ganglion cultured neurons and *in vivo* behavioral methods to demonstrate that the purinergic C-fiber pathway is functional in naked mole-rats. We found that naked mole-rats still exhibit a phase II response after formalin application, suggesting a significant response through the P2X3r pathway. Calcium response to stimulation indicate that naked mole-rat P2X3r responds to agonism by ATP in a similar manner to mice. Next we examined the ability of cannabinoids to attenuate purinergic pain responses. Cannabinoids did reduce phase II pain in naked mole-rats similarly to mice despite the lack of neuropeptides in their spinal cord. In the

Von Frey test, P2X3r agonist α-β-met-ATP increases naked mole-rats pain sensitivity. This can be rescued by *in vivo* application of cannabinoid agonists, validating that cannabinoids directly inhibit P2X3r. Importantly, cannabinoid agonist WIN55 only reduced the pain response to formalin in phase II, and not phase I of formalin tests, while P2X3r antagonism reduced the pain response in both phases of the formalin test. Collectively this indicates that cannabinoids reduce the sensitization of C-fibers in Phase II primarily through the P2X3r pathway in naked mole-rats. However, naked mole-rats show no inflammatory response to ATP. This study establishes that naked mole-rats purinergic C-fiber pathway is present and functional. This study adds supportive evidence for the naked mole-rat model to be used in studying the detailed response of the P2X3r pathway in an isolated system due to the lack of peptidergic functioning in the C-fiber pain pathway.

Introduction

The African naked mole-rat is a eusocial subterranean rodent with adaptations that have evolved to be resistant to a multitude of painful stimuli. This includes immunity to the burning pain associated with capsaicin, the spicy compound in chili peppers (Park et al., 2008). Normally, capsaicin activates transient receptor potential vanilloid 1 (TRPV1) channels, which are localized to a particular population of unmyelinated sensory neurons called C-fibers. When activated, $TRPV1^{+}$ neurons release neuropeptides, including Substance P and calcitonin gene-related peptide, at the synapse in the spinal cord, as well as extra-synaptically in the periphery (Eberhardt et al., 2017). The $TRPV1^{+}$ peptidergic C-fibers synapse in lamina I which is important for relaying the pain signal from ascending pathways to the periaqueductal gray area (PAG) in most mammals (Todd, 2012). While TRPV1 is expressed in naked mole-rats at similar quantity to other rodent models, the distribution of receptors at laminar layer I is reduced (Park, 2008). However, their insensitivity to capsaicin can be attributed to their lack of peripherally expressed neuropeptides, therefore, effectively making the TRPV1 pain pathway inoperative (Park, 2003, Park et al., 2008).

Lacking neuropeptides reduces naked mole-rats' overall response to C-fiber induced pain, including that of the formalin test. The naked mole-rats have a longer yet significantly less intense response to the irritant formalin, particularly during the second pain phase (Phase II; Park et al., 2008). Phase II is considered to be inflammatory pain that is predominantly aroused by central sensitization of the spinal dorsal horn neurons as a result of the initial activation of a barrage of inputs from C-fiber nociceptive afferents during the early phase and a model for chronic pain (Corderre, 1993, Dickenson and Sullivan, 1987; Raboisson et al., 1995; Shibata et al., 1989, Park et al 2008, Dulu, 2014). Importantly, though naked mole-rat response to formalin is reduced, it is not completely eliminated, indicating that there is still a portion of the inflammatory pain pathway that is intact. Currently, it is unknown which receptors mediate the remaining pain response in naked mole-rats.

Here we consider another major receptor subtype on C-fibers, the non-peptidergic, purinergic family of receptors which are activated by ATP (Pan et al., 2009). Of the purinergic receptors, homomeric P2X3r is the predominant subtype followed by heteromeric P2X2/3r with other subunits having negligible effect (Zhong et al., 2001). Together, these receptors can elicit a response in nearly 70% of all C-fibers of the dorsal root ganglion (Ueno et al., 1999). Previously, we have shown that naked mole-rats express isoelectin B4 (IB4), which is expressed in fibers containing P2X3r, in laminae II of dorsal horn of naked mole-rat spinal cord (Park et al., 2008) where non-peptidergic C fibers innervate ascending pathways (Todd, 2012). Together, this indicates that P2X3r activation may be causing the remaining pain response in naked mole-rat C-fibers. Therefore, the first objective in this study was to examine the functionality of the P2X3r pathway in naked mole-rats and determine its role in formalin-induced pain.

As mentioned above, P2X3r are activated by extracellular ATP and this activation has been implicated as a significant source of inflammatory pain (Cauwels et al., 2014; Oliveira et al., 2009). Inflammation can cause a marked increase to the Phase II formalin response (Hunskaar and Hole, 1987). Cannabinoids have long been used as pain relievers and evidence shows that they are effective in reducing inflammatory pain (Kim et al., 2015). Indeed, cannabinoids have been found to help manage chronic pain due to inflammation in both human and mouse models (Bruce et al. 2018; Baron, 2018). The potential reliance of naked mole-rats on P2X3r to discern peripheral pain can be a valuable tool in analyzing how the endocannabinoid (eCB) system influences pain perception through this pathway. The eCB system is a retrograde synaptic signaling pathway with analgesic effects that have been identified in the dorsal horn of the spinal cord and mediate pain through cannabinoid receptor 1 (CB1r; Tsou et al, 1996; Hohmann et al, 1999; Agarwal et al, 2007; Herkenham et al, 1991; Martin et al, 1995; Martin et al, 1996; Nackley et al, 2003). Inflammation increases CB1r expression in C-fibers but not A-fibers, indicating inflammatory sensitivity to cannabinoids is primarily through the C-fiber route

(Amaya et al, 2006; Evans, 2004). Attenuation of pain by cannabinoids has been described in the C-fiber pathways of both peptidergic (TRPV1r) and purinergic (P2X3r) receptors, through CB1r activation on the axon terminals in the dorsal horn of the spinal cord (Nerandzic et al., 2018; Starowicz et al., 2013).

However, the mechanisms of cannabinoid pain reduction are not well understood. While the role of cannabinoids in the TRPV1 pathway has been well studied, little is known of the role of cannabinoids in the P2X3r pathway. Cannabinoid inhibition of TRPV1 was originally thought to be a good target for pain therapeutics, but results were mixed. For instance, it is unknown if neuropeptides exclusively mediate the mGluR-dependant stimulation of 2-AG production *in vivo* for pain attenuation (Drew et al., 2009; Mitchell et al., 2011; Mitchell et al., 2009; Rosen et al., 2004; Kalivas et al., 1982; Chen et al., 1998). Additionally, TRPV1r activated release of neuropeptides can be inhibited by CB1r activation and also directly activated by the endocannabinoid AEA, indicating that there are both pro- and anti- nociceptive pathways for this receptor (Carey, et al., 2016). Because of this, a new target for therapeutics would be preferable. And while TRPV1ko mice are available and have been used to elucidate the response of P2X3r, TRPV1r has recently been shown to be responsible for more than just pain (Martins et al., 2014), particularly in the brain, and therefore confounding effects of the knock-out are possible.

Previous research suggests that activation of CB1r directly inhibits inflammatory pain through P2X3r, as shown by inhibition of the hyperalgesic response to mechanical stimuli and calcium influx in DRG small diameter neurons after α,β-met-ATP (ATP, a P2X3r specific agonist) administration both *in vivo* and in culture (Oliveira-Fusaro, et al., 2017). Cannabinoids can activate TRPV1r, it is difficult to parse out how CB1r affect analgesia in the peripheral system (the opposing effects may cancel each other out; Zygmunt et al, 1999; De Petrocellis et al., 2001). Using a model system that does not have peripheral neuropeptides can elucidate their necessity in cannabinoid mediation during analgesia.

Therefore, the second objective of this study was to examine cannabinoid effects on P2X3r responses directly. We looked at the naked mole-rat's response to C-fiber mediated inflammatory stimuli with cannabinoid treatments. While cannabinoid receptor 2 has been implicated in the immune response, we focused on the response of CB1r due to previous studies which have shown CB1r agonist WIN55 to directly affect P2X3r (Kristal et al., 2006). Additionally, cannabinoids mediate the response of P2X3r through calcium modulation, which is CB1r-dependant (Long et al., 2018; Guo and Ikeda, 2004). Oliveira-Fusaro et al. (2017) further exposed CB1r to be the direct target of P2X3r and also the target for reduction of P2X3r induced hyperalgesia from inflammatory bradykinins.

METHODS

Animals

All Mice were C57BL/6 males, which were bred from stock, originally obtained from Charles River Laboratories, Wilmington, Massachusetts, USA. Mice were kept in a temperature-controlled environment of 72F with a 12-hour light-dark cycle. Naked mole-rats of both sexes were born in colonies maintained at the University of Illinois at Chicago and housed under similar conditions as found in the wild. Naked mole rats were kept at a controlled temperature (80F) and humidity with a 12-hour light-dark cycle. All procedures were conducted according to the animal protocols approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

Drug Preparation

Formalin Preparation. 37% stock Formaldehyde solution was purchased from Sigma-Aldrich and diluted in water to 2% by volume. *WIN-55, 212-2 CB1r Agonist Preparation.* WIN55, 212-2 mesylate salt (WIN55) was obtained from Sigma-Aldrich and suspended at 1 mg/mL in 49.5% TEG, 49.5% Saline, .5% DMSO, and .5% Cremaphor and ready to be diluted to injection concentrations with saline. Dilutions were injected at either 1.5mg/kg or 3mg/kg or saline vehicle. *Substance P Preparation.* Used

dilutions in accordance with Smith et al., 2010. Briefly, 100uM of Substance P was dissolved in .9% Saline. *A-317491 P2X3r Antagonist Preparation.* A-317491 was diluted in .9% Saline and given at a dose of 50μg/20μL. *α-β-metATP (ATP) P2X3r Agonist Preparation.* ATP was diluted to 50μM in .9% Saline.

Drug Conditions

An insulin syringe was used to administer all of the drug conditions. Cannabinoids were injected via intraperitoneal (IP) administration. Injections contained either a 1.5mg/kg, or a 3mg/kg dose of WIN55, 212-2 or a .9% dose of saline. Animals were injected 30 minutes prior to the commencement of behavioral tests. Substance P was injected by intrathecal administration between the L4 and L5 vertebrate at 20uL volume 30 minutes prior to testing. A-3117491 was injected into the dorsal paw prior to testing in the same manner as the formalin 5 minutes prior to the injection of formalin. 10μL of ATP was injected to the plantar side of the hind paw 10 minutes prior to the first von Frey test.

Formalin Test

Each mouse and naked mole-rat received 15-20 μL of formalin (2%) subcutaneously into the dorsal side of the hind paw with an insulin syringe. The animal was placed into an empty mouse cage without a lid and observed for 90 minutes. Licking, biting, and lifting of the formalin injected foot were operationally defined as nociceptive behavior. The time spent performing nociceptive behaviors was recorded for all animals in intervals of 5 minutes for the entire 90-minute duration. The total time observed was divided into 0-10 minutes (Phase I) for both naked mole-rats and mice. However, the late phase was defined as 10-60 minutes for mice and 10-90 minutes for naked mole-rats due to species differences in reaction to formalin (Eigenbrod et al., 2019). The formalin test was performed and scored by an observer blinded to experimental conditions.

Tail Flick

Mice and naked mole-rats were acclimated to a plastic restraint cone where they were place in it and given a sugar treats daily for 1 week prior to testing. Animals were given a treatment of either

Saline (control), 1.5mg/kg WIN55, or 3mg/kg WIN55 by IP injection 30 minutes prior to testing. Each animal was tested at all three experimental conditions with at least 48 hours between treatments. To test thermal analgesia, a water bath was prepared with water held at 48C. For testing animals were placed in a restraint cone with the tail remaining free from the restraint. When the animals were restrained and calm, the experimenter lowered the tail into the water and recorded the time in seconds it took for the animal to attempt to remove the tail from the water.

Von Frey

Mice and naked mole-rats were injected with saline or WIN55 by IP injection 30 mins prior to injection of α-β-met-ATP in the plantar surface of the left hind paw. Sensitivity to mechanical stimulus was assessed using calibrated von Frey filaments (Stoelting, Wood Dale, IL). Animals were placed in 10 cm diameter wire bottom restraint apparatus. The von Frey filaments (0.4–15.1 gm) were applied to the mid-plantar surface of each hind paw. The mechanical stimulus producing the 50% paw withdrawal threshold was determined using the up–down method (Chaplan et al., 1994). Behavioral tests using mechanical stimuli were performed on both the ipsilateral and contralateral paw at 10 and 180 min after intraplantar injection of saline or α-β-met-ATP.

Immunohistochemistry Spinal Cord Dorsal Horn

Mice and naked mole-rats were decapitated, and brains were quickly removed and drop fixed with a fixative containing 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer (PBS, pH 7.4) overnight, and subsequently cryoprotected in 30% sucrose (in PBS). Brains were sectioned coronally (26 µm thickness) on a cryo-microtome and directly placed on slides. Non-specific immunoreactivity was suppressed by incubating our specimens in a cocktail of 5% normal donkey serum (Jackson), 1% bovine serum albumin (BSA, Sigma) and 0.3% Triton X-100 in PB for an hour at 22–24 °C. CB1r was stained using CB1 anti-Rabbit (Synaptic signaling 1:1000) and P2X3 was stained using P2X3r anti-Guinea Pig (Sigma, 1:50). Neuron nuclei were stained using Hoechst (1:1000). Primary antibodies were

counterstained with AlexaFluor 488 Anti-Rb (1:1000), and Cy3 anti-Gp (1:1000). Glass-mounted sections were cover slipped with Aquamount (Dako, Glosstrup, Denmark). Images acquired on a Fluoview FV 10i confocal laser-scanning microscope (Olympus). Colocalization was analyzed using the FV 10i internal software to determine Pearson's Coefficient and Overlap in CB1r and P2X3r.

Calcium Imaging

Animals

Both male and female adult C57BL/6J (Envigo) mice (8-12 weeks) and naked mole-rats (9-24 months) were used in this study. Mice were kept under 12-hour dark/light cycle in a temperature-controlled (21 °C) holding room, with food and water available *ad libitum*. Naked mole-rat colonies were housed in a bespoke caging system consisting of mouse and rat cages connected by tunnels containing standard bedding and nesting material. Naked mole-rats were maintained in a temperature-controlled (28-32 °C) room and kept under red lighting (08:00-16:00). All animal protocols were conducted under a Home Office Project License (P7EBFC1B1) that had been reviewed by the University of Cambridge Animal Welfare and Ethical Review Body, and experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012.

Primary culture preparation

Mice were killed by cervical dislocation and naked mole-rats were immobilized using a rising concentration of CO2 and then decapitated. For each animal, a primary DRG culture was prepared using a mixture of TL DRG. The spinal column was dissected and T1-L6 DRG were collected, trimmed of connective fibers, and placed in ice-cold dissociation media containing L-15 Medium (1X) + GlutaMAX-1 (Life Technologies), supplemented with 24 mM $NaHCO_3$. media (Thermo Fisher Scientific, UK). Harvested DRG were then incubated in 3 mL type 1A collagenase (1 mg/mL with 6 mg/mL bovine serum albumin (BSA in dissociation media; Sigma-Aldrich) for 20 minutes at 37 °C (mouse) or 32 °C (NMR) in an incubator with 5 % CO_2; NMRs are cold- blooded and are maintained at 28 – 32 °C and

hence this temperature was used for preparing and maintaining NMR DRG neuron cultures. This was followed by a 30-minute incubation in 3 mL trypsin solution (1 mg/mL with 6 mg/mL BSA in dissociation media; Sigma-Aldrich) at respective temperatures. After removing the enzymes, the DRGs were placed in 2 mL of DRG culture media composed of L-15 Medium (1X) + GlutaMAX, 10 % fetal bovine serum (Sigma-Aldrich), 24 mM $NaHCO_3$, 38 mM glucose, and 2 % penicillin/streptomycin (Life Technologies, UK). DRG neurons were dissociated via gentle mechanical trituration using 1 mL Gilson pipette tips, by pipetting up and down approximately 8 times. Following a 30 s centrifugation at 160 x *g* (Biofuge primo, Heraeus Instruments; Hanau, Germany) the supernatant containing dissociated cells was collected in a fresh tube, and the remaining pellet was resuspended in 2 mL of media. This process of trituration, centrifugation and collection was repeated a total of five times. Following a 5-minute centrifugation at 160 x *g*, the pellet was resuspended in media and plated onto poly-D-lysine-coated 35 mm glass- bottom dishes (MatTek Corporation, USA) coated with laminin (20 µg/mL; Life Technologies) and were incubated at 37 °C (mouse) and 32 °C (NMR) in 5 % CO_2 for 20-24 hours. The cells were flooded with fresh media after 3 hours.

Calcium Imaging

A day after the preparation of the primary DRG culture, neurons plated on coverslips were loaded with the fluorescent calcium indicator Fluo-4 (10 µM; Thermo Fisher Scientific) in extracellular solution (ECS; 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM glucose; adjusted to pH 7.4 with NaOH and 300-310 mOsm with sucrose) for 45 minutes at room temperature. Coverslips were washed once with ECS and placed in an imaging chamber (RC-26, Warner Instruments, UK) and incubated for 5 minute with either 0.0102% DMSO (highest concentration used for vehicle/control for WIN55,212-1), 3 µM ACEA (Tocris), 1 nM or 100 nM (*R*)-(+)-WIN55. Cells were imaged using a Nikon Eclipse T*i* microscope and a 10x objective. Fluo-4 was excited using a 470 nm LED

(Cairn Research, UK) and fluorescent images were acquired every second using a Zyla sCMOS camera (Andor, UK) and Micro-Manager software (v 1.4; National Institute of Health). For all conditions, neurons were initially perfused with whichever prior solution they were incubated in for the final 30 seconds of the 5 minute incubation, then perfused with αβ-met-ATP (30 μM, 20 s) and washed with ECS (240 s) before a 20 s KCl (50 mM) stimulus which was applied at the end of every experiment to check for cell viability. Unless otherwise specified, all chemicals and drugs were obtained from Sigma-Aldrich.

Immunohistochemistry of Dorsal Root Ganglion

Mice and naked mole-rats were euthanized as described in "Primary culture preparation". Thoracolumbar (TL) dorsal root ganglion (DRG) were dissected and fixed in Zamboni's fixative (2% paraformaldehyde and 15% picric acid in sodium phosphate buffer; 1 hour). DRG were then cryoprotected overnight in 30 % sucrose (in PBS) at 4 °C, before embedding in ShandonTM M-1 Embedding Matrix (Thermo Fisher Scientific) and snap frozen in liquid nitrogen before being then stored at -80 °C. Embedded DRG were sectioned (12 μm) using a Leica Cryostat (CM3000; Nussloch, Germany), and mounted on Superfrost Plus microscope slides (Thermo Fisher Scientific). Sections were stored at -20 °C until further use. Slides were defrosted, washed with PBS-tween and blocked in antibody diluent solution (1% BSA, 5% donkey serum (Sigma-Aldrich), and 0.2% Triton X-100 (Sigma-Aldrich) in PBS; 1 hour) at room temperature. All sections were incubated overnight (4 °C, humid atmosphere) with primary antibodies prepared in diluent. Primary antibodies used were Rabbit anti-P2X3 polyclonal (1:1000, Alomene) and Guinea pig anti-TRPV1 polyclonal (1:500, Alomone). Slides were washed three times using PBS-T before incubation in the appropriate species-specific fluorophore-conjugated secondary antibodies (donkey anti-rabbit IgG-AF350 (Invitrogen); donkey anti-guinea-pig IgG-AF594 (JacksonLab) for 2 hours at room temperature (20-22 °C). All secondary antibodies were used at a 1:1000 dilution. For each antibody, a negative control experiment excluding primary antibody

incubation was also performed and showed no staining. Slides were washed in PBS-T, mounted using Mowiol-based mounting media. Sections were imaged with an Olympus BX51 microscope (Tokyo, Japan). Brightfield and fluorescent images were acquired at 10x and 20x magnification using a Q-Imaging camera and software (Surrey, Canada). Exposure levels were kept constant for each slide and the same contrast enhancements were made to all slides.

Statistical Analysis

All images from Calcium-imaging and dorsal root ganglion immunohistochemistry experiments were processed and analyzed using Fiji/ImageJ (National Institute of Health).

P2X3 and TRPV1 receptor expression in DRG sections:

To determine the expression of P2X3 and TRPV1 receptors in immunostained DRG sections, 2-5 DRG sections were randomly chosen using a random number generator for analysis per each imaged DRG. ROIs were drawn around the borders of all neuron cell bodies that were identified morphologically using the brightfield image. The criteria for determining a DRG neuron cell body where they had to have a clear circular outline and a nucleus. Mean fluorescent intensities of the ROIs were measured for the fluorescent channels corresponding to P2X3 and TRPV1. For each section, background fluorescence was subtracted from ROI fluorescent intensities. For each section, F_{min} and F_{max} were determined from ROIs with the lowest ("negative") and highest background-subtracted fluorescent intensities, respectively. For each experiment, the average F_{min} and F_{max} was determined by taking the mean of all F_{min} and F_{max}. Normalized fluorescence was calculated as (F - average F_{min})/(average F_{max} - average F_{min}). For each animal group, the threshold used for scoring a neuron as positive for a stain was set as the normalized minimum grey value across all sections +1 times SD. Each ROI with a normalized fluorescence above the threshold was counted as being immunoreactive

and the percentage of P2X3 and TRPV1-positive cells was calculated for each section. Size distribution of P2X3 and TRPV1-positive cells were plotted using R.

Calcium-imaging analysis:

Neurons were identified morphologically and regions of interest (ROIs) were drawn around them for each coverslip. Background fluorescence was subtracted from fluorescent intensities of ROIs. Baseline fluorescence was calculated by taking the average fluorescence of 20 seconds before the application of the first stimulus in each experiment, and background- and baseline-subtracted fluorescence (F) was calculated for all cells from each coverslip. Maximum fluorescence (F_{max}) was calculated from the baseline- and background- subtracted fluorescent intensity following the application of 50 mM KCl at the end of each experiment. Normalized fluorescence intensity ($\Delta F/F_{max}$) was calculated by dividing the difference between F_{max} and F by F_{max}. Cells were determined as being αβ-meATP/KCL- responsive if the average $\Delta F/F_{max}$ at pulses were above 0.05 noise threshold (determined from control data) of the baseline. Cells that did not respond to KCl or had an unstable baseline, were excluded from analysis. The peak of each pulse was calculated as the max within the time period of response duration. Proportions of cells responding to different stimuli were calculated using Excel. The peak data were plotted in GraphPad Prism. The size distribution of all cells analyzed was plotted using the ggplot2 package of R.

All calcium imaging and DRG data was produced by the laboratory of Ewan St. John at Cambridge University as part of an ongoing collaboration.

Statistics: The statistical analysis was carried out using GraphPad Prism version 4 (GraphPad Software, San Diego, CA) or Microsoft excel. Data from calcium-imaging, and immunohistochemistry experiments were analyzed and plotted using R and Python. Statistical test used are referred to where appropriate.

Results

As shown in previous studies (Park et al., 2008, Dulu, 2014), naked mole-rat's reaction to formalin was less intense and longer lasting than the response from mice (Fig 1A). However, when the total phase response for phase I: 0-10 minutes and Phase II: 10-90 minutes were binned, only phase I showed a significant reduction in naked mole-rat response compared to mice ($p<.01$; Fig. 1B). Using the longer time point of 90 minutes for phase II made it possible to compare more accurately between the two species responses and this extended binned time was used for the remainder of the formalin tests. Time course of responses differ across rodent species (Eigenbrod et al., 2019), therefore we utilized the appropriate time for formalin to complete the pain response in mice and naked mole-rats.

We first examined whether or not a rescue of the peptidergic response to formalin could be accomplished by replacement of the peptide Substance P into the spinal region. This was previously shown to be possible thermal response latencies (Park et al., 2008). Indeed, phase I responses after intrathecal substance P addition were no longer significantly different from mice (Fig. 2A). However, they were also not significantly different from naked mole-rat control responses though the response trended toward an increase in pain ($p=.06$ for naked mole-rat Substance P vs Control; Fig. 2A). In phase II, naked mole-rats treated with substance P had a significantly greater response compared to both control mice and control naked mole-rat ($p<.05$; Fig. 2B), and the majority of that increase is in the 10-60-minute range (Fig. 2C), exhibiting a greater intensity in the earlier part of phase II compared to control naked mole-rat.

Next, we observed how direct inhibition of the P2X3r with A-317491 would affect the behavioral response of naked mole-rat to formalin. There was a significant reduction of phase I ($p<.05$; Fig. 3A) response, with a reduction of 46% compared to control. Phase II had an even greater reduction of 70% in response ($p<.001$; Fig. 3B), indicating that the majority of the remaining phase II response is P2X3r dependent.

The expression pattern of P2X3r was assessed in DRG in both mice and naked mole-rats and quantified by the number of small fiber neurons expressed P2X3r, TRPV1r, P2X3r + TRPV1r, or neither receptor. Mice had more small diameter neurons (n=2747; Fig. 4A,B) than naked mole-rats (n=1016; Fig. 4C,D); however, the ratio of expression remained similar (Fig. 4B,D,E). Importantly, in calcium imaging studies the responsiveness of naked mole-rat P2X3r to stimulation was similar to mice (Fig. 4F) and the responsiveness correlated with the percentage of neurons expressing the P2X3r antibody (Fig. 4E,F).

Cannabinoid relationship with P2X3r in naked mole-rats was assessed in the Von Frey assay of mechanical pain sensitivity after application of ATP (Fig. 5). Short term pain sensitivity (10 minutes after ATP application), as determined by pressure needed to elicit paw withdrawal, was initiated by injection of ATP (.09$\pm$.02 grams of force) when compared to control response to filaments (Contra: .494$\pm$.15; $p < .05$). The short-term pain was rescued by pretreatment of WIN55 (.29$\pm$.07; $p<.05$). Short-term response to filaments was not significantly different in control and WIN55 trials ($p > .1$).

Inflammatory response to ATP activation of P2X3r was tested by reassessing animals at 180 minutes post ATP application. There was no significant difference in response to filaments in any cohort tested ($p>.05$; Fig. 5).

Since the cannabinoid system has been shown to attenuate P2X3r-dependent pain response in the von Frey test, we looked at the response of naked mole-rats to the CB1r agonist WIN55 in the formalin test to see if there was a similar pain reduction as seen with A-317491. As far as we know, there are no studies to date that have looked at separating the effect of cannabinoids on the different pain pathways in the formalin test in naked mole-rats. There was no significant pain reduction from WIN55 in the naked mole-rats in phase I, though there was a significant reduction in mice ($p<.01$; Fig. 6A), indicating that cannabinoids attenuate the peptidergic response (which is non-functioning in naked mole-rats) in phase I ($p=.96$). Presumably not attenuating the initial P2X3r or A-fiber response. In

phase II (Fig. 6B) there was a significant reduction in pain response in both mice and naked mole-rat ($p<.05$), indicating that the P2X3r inflammatory response may be the target for WIN55. To solidify that the WIN55 effect was due to P2X3r and not alternate undescribed pathways, we also assessed the response of WIN55 in naked mole-rat thermal pain. Naked mole-rats do exhibit a pain response from thermal stimuli (Park et al., 2008), which is independent of the P2X3r pain pathway (Ramer, Bradbury, and McMahon, 2001; Souslova et al., 2000). We found that the time to tail flick was not reduced after either dose of WIN55 in the naked mole-rat, compared to control animals ($p>.05$; Fig. 7). The doses of WIN55 were high enough to elicit a small reduction in pain response from mice that trended toward a significance (all mouse data ANOVA: $p=.2$; control vs. 3mg/kg, t-test: $p=.11$; Fig.7) and is probably due to a hypomobility response from the WIN55.

The dorsal horn was examined as this is where peripheral nociceptors synapse with neurons in the spinal cord and are implicated in both acute and inflammatory pain (Donvito et al., 2017). Both species expressed CB1r and P2X3r in lamina II of the dorsal horn (Fig. 8). We analyzed the proximity of CB1r to P2X3r to determine if there was colocalization between the receptors. We found no significant difference between overlap in mice ($.95\pm.007$) compared to naked mole-rats ($.969 \pm .004$) though the individual indices for naked mole-rats (Index 1-CB1r: $1.04\pm.13$; Index2- P2X3r: $.977\pm.14$) were closer to a 1:1 ratio than that of mice (Index 1-CB1r: $.745\pm.05$; Index2- P2X3r: $1.24\pm.07$). Individual Pearson Coefficients were also calculated for each dorsal horn imaged. In naked mole-rats, the coefficients all showed at least a moderately positive correlation with a range from .37-.81. The percentage of images with a relatively strong positive correlation (>.5) were 69.23% and with a strong positive correlation were 38.46% (n=13). The range of Pearson coefficients in mice was from .32-.88, which showed greater variation than in naked mole-rats. There were also fewer images that were in the relatively strong (42.85%) and strong (14.28%) correlation ranges.

Discussion

This study aimed to determine the functionality of the purinergic pain pathway in naked mole-rats. Naked mole-rat have been shown to express IB4 in the dorsal horn of the spinal cord (Park et al., 2008), which is an indication that they express P2X3r purinergic receptors. However, naked mole-rats also express TRPV1r, yet due to a lack of neuropeptides, have a non-functional peptidergic profile (as indicated by no pain response to capsaicin). Therefore, we first showed that the P2X3r is functional in naked mole-rats and can be stimulated by application of ATP. Additionally, we showed that the endocannabinoid system, namely through CB1r, is able to inhibit the pain induced by P2X3r activation. Currently, no studies have looked at the functionality of P2X3r or at separating the effect of cannabinoids on individual pain pathways in naked mole-rats.

When neuropeptide Substance P was applied to the naked mole-rat spinal column there were two interesting results. First, the reduction of Phase I pain was completely recovered to mouse levels, indicating that the entire reduction of Phase I pain is due to a lack of response in the peptidergic C-fiber pathway. The second result showed a more nuanced effect of Substance P in Phase II. If the entire pain response for the second phase is measured, there is no significant difference between mice and naked mole-rat in control formalin tests. Where there is a marked difference is in the response during the 10-60-minute portion of recording. Mice spend a significantly greater portion of this time reacting to the formalin-induced pain than naked mole-rat. This significantly larger response to the early portion of Phase II pain is mirrored in naked mole-rats after Substance P injections, indicating a rescue of the pain response lost from the neuropeptide pathway. However, there is still a significant response in the 60-90 minute later portion of Phase II which demonstrates that there is an increased response from the other inflammatory pain circuits compared to mice.

We found that, unlike the nonfunctioning peptidergic pathway of C-fibers, naked mole-rats do have a functional purinergic pathway. Not only did we find that the receptor can be stimulated directly

in cultured neurons, but its activity was also confirmed *in vivo* by both activating and antagonizing the pathway. The strong response to ATP application as well as the significant reduction in formalin induced pain after application of the P2X3r antagonist indicates that this pathway is a robust relay center for peripheral nociceptors to central pain regions of the brain.

If cannabinoids are able to reduce pain despite the lack of Substance P in the spinal cord but not after P2X3r inhibition that would establish that the cannabinoid system is reducing inflammatory pain through the P2X3 pathway exclusively. We found that cannabinoids reduce pain in naked mole-rat similarly to mice despite the lack of neuropeptides in their spinal cord. Furthermore, cannabinoids directly inhibit P2X3r, as shown by rescuing the increased pain response from ATP. However, there was not a full recovery to baseline, so WIN55 is not able to fully modulate the entire P2X3r response. Therefore, we concluded that WIN55 is directly inhibiting the response of P2X3 receptors to inflammatory stimuli such as formalin. Collectively this indicates that cannabinoids reduce the sensitization of C-fibers in Phase II primarily through the P2X3r pathway in naked mole-rat.

Interestingly, there was no effect of ATP or WIN55 on naked mole-rats 3-hours after ATP application. Previous studies indicated that the 3-hour time point was indicative of an inflammatory response to ATP mediated by P2X3r and was more responsive to ATP (sensitization) and WIN55 (attenuation) than at earlier time points (Oliveira-Fusaro et al., 2017). Further investigations into the response of naked mole-rats to inflammatory pain and the role of P2X3r in the induction of inflammation are necessary. Importantly, WIN55 only reduced the pain response to formalin in Phase II, and not Phase I of the formalin tests in naked mole-rats. P2X3r antagonist A-3117491 reduced the pain response in both phases of the formalin test. This indicates that CB1r is only reducing the chronic pain production caused by P2X3r activation and may not attenuate the direct pain response of these receptors of naked mole-rats. This agrees with previous research that proposed endocannabinoid 2-AG is released post-synaptically after P2X3r initial activation to down-regulate the cAMP/PKA signal and

turn off the pain response (Long et al., 2018). Naked mole-rats appear to only have a central response to chronic pain from inflammation. This model could prove useful in understanding the effects of peripheral inflammatory response compared to central inflammation in chronic pain patients.

Overall, we found that naked mole-rat purinergic C-fibers are still able to functionally receive and relay pain signals. Additionally, the eCB system regulates this pain signal through inhibition and pain can be attenuated with treatments of CB1r agonists. Finally, we did not find an inflammatory response to ATP, as reported in mice, indicating further research is needed to determine if the naked mole-rats peripheral inflammatory response is altered. It is possible that ATP does not stimulate downstream inflammatory signals in naked mole-rats. Alternatively, the peptidergic pathway may be necessary to stimulate the ATP-dependant inflammatory response.

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Figure Legends

Figure 3.1: Mice and NMR reaction to formalin pain (A). NMR have a significantly reduced response to formalin in Phase I, this is generally agreed to be due to the lack of neuropeptide release from TRPV1r in the spinal cord (B). NMR have a less intense yet longer response to formalin in Phase II than mice; however, when the time of response is binned to account for the time increase, the response of the two species is similar (B). (* $p < .05$)

Figure 3.2: Substance P increases response to formalin in NMR. NMR show a significant increase in both phase I, minutes 0-10 (A), and phase II, minutes 10-90 (B, * $p < .05$). The time point graph (C) shows that the increase in phase II is primarily caused by an increase in the 20-60-minute portion of the phase. Note that one effect of IT Substance P is that there is an extended pain response, therefore, Phase II was measured from 15-105 mins after formalin injection.

Figure 3.3: Inhibition of P2X3 receptors decreases pain response from formalin in naked mole-rats in both Phase I (A) and almost completely for Phase II (B). (* $p < .05$, *** $p < .001$).

Figure 3.4: Functionality of P2X3r was assessed with IHC and Calcium Imaging Assays. Expression of P2X3r and TRPV1 in DRG neurons of mice (A) and naked mole-rats (C). Quantification of neurons per fiber type in mice (B) and naked mole-rats (D). Percentage of all neurons in the imaged DRG that expression P2X3r (E). Responsiveness of mouse and naked mole-rat DRG neurons to P2X3r agonist αβ-meATP (F).

Figure 3.5: NMR response to Von Frey stimuli following application of 5ug α-β-met-ATP to the plantar surface of the hind paw. This increased sensitivity can be attenuated by .3mg/kg pretreatment of WIN55. N=9. (* p<.05)

Figure 3.6: Mouse and NMR response to formalin (light green and light blue) and attenuation of pain reaction caused by .3mg/kg of WIN55 (dark green and dark blue) in Phase I (A) and Phase II (B). (* p< .05, ** p<.01)

Figure 3.7: Thermal pain was assessed by immersion of the animal's tail into water at 48C and recording the latency to tail flick. NMR response latencies with control, 1.5mg/kg WIN55, and 3mg/kg WIN55were compared to mice responses at the same dose. n=24.

Figure 3.8: Example confocal images of immuno-expression in the hippocampus in mice (A) and naked mole-rats (B). Figure shows co-expression of P2X3r (green) with CB1r (red, 1), P2X3r independently (2), and CB1r independently (3).

Supplemental Figure 3.1: Proposed model of pain attenuation in the dorsal horn of the NMR. Due to the lack of Substance P expression, CB1r is only effective in laminae 2 of the dorsal horn, where it inhibits the purinergic signal of P2X3r to reduce pain.

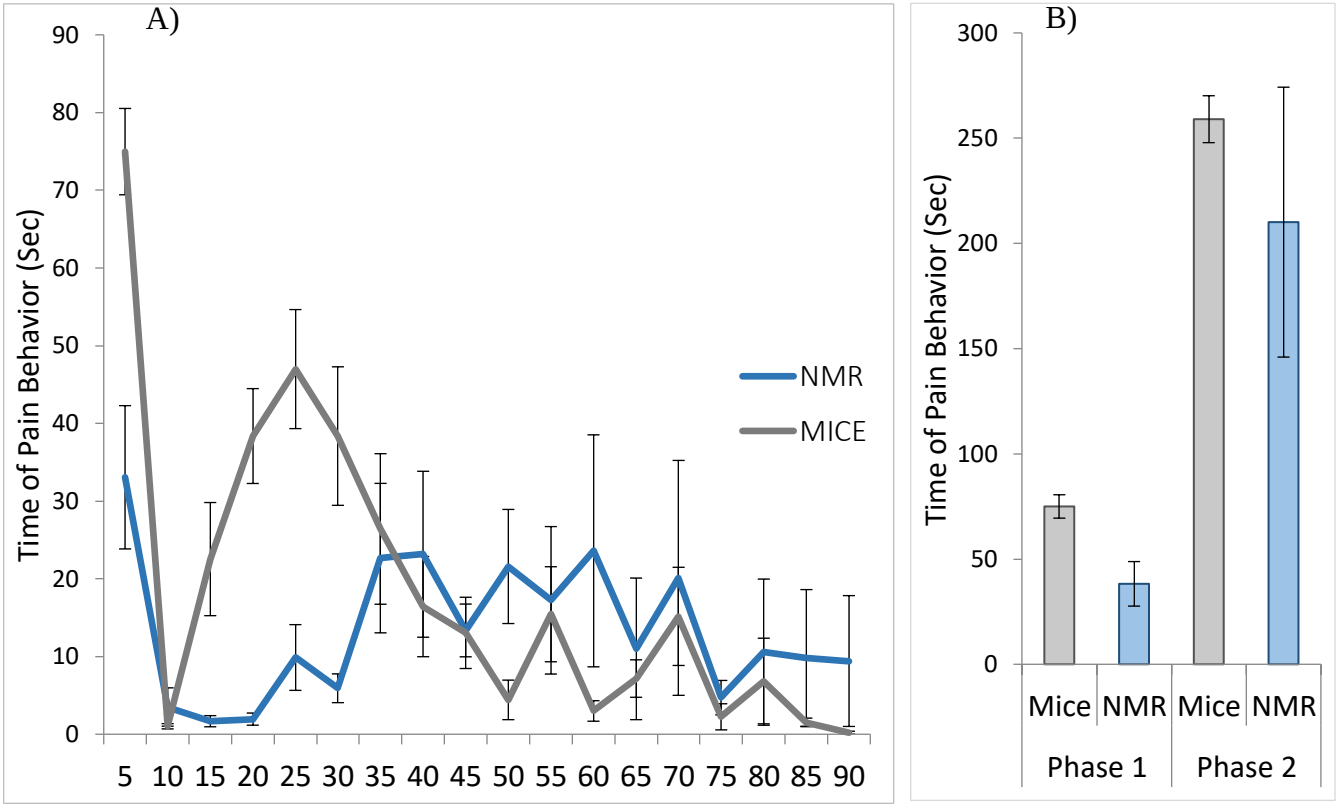

A)
Time of Pain Behavior (Sec)
90
80
70
60
50
40
30
20
10
0
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
NMR
MICE
B)
Time of Pain Behavior (Sec)
300
250
200
150
100
50
0
Mice
NMR
Mice
NMR
Phase 1
Phase 2

Figure 3.1.

Pain Attenuation in Naked Mole-Rats Can Be Reduced with Intrathecal Substance P

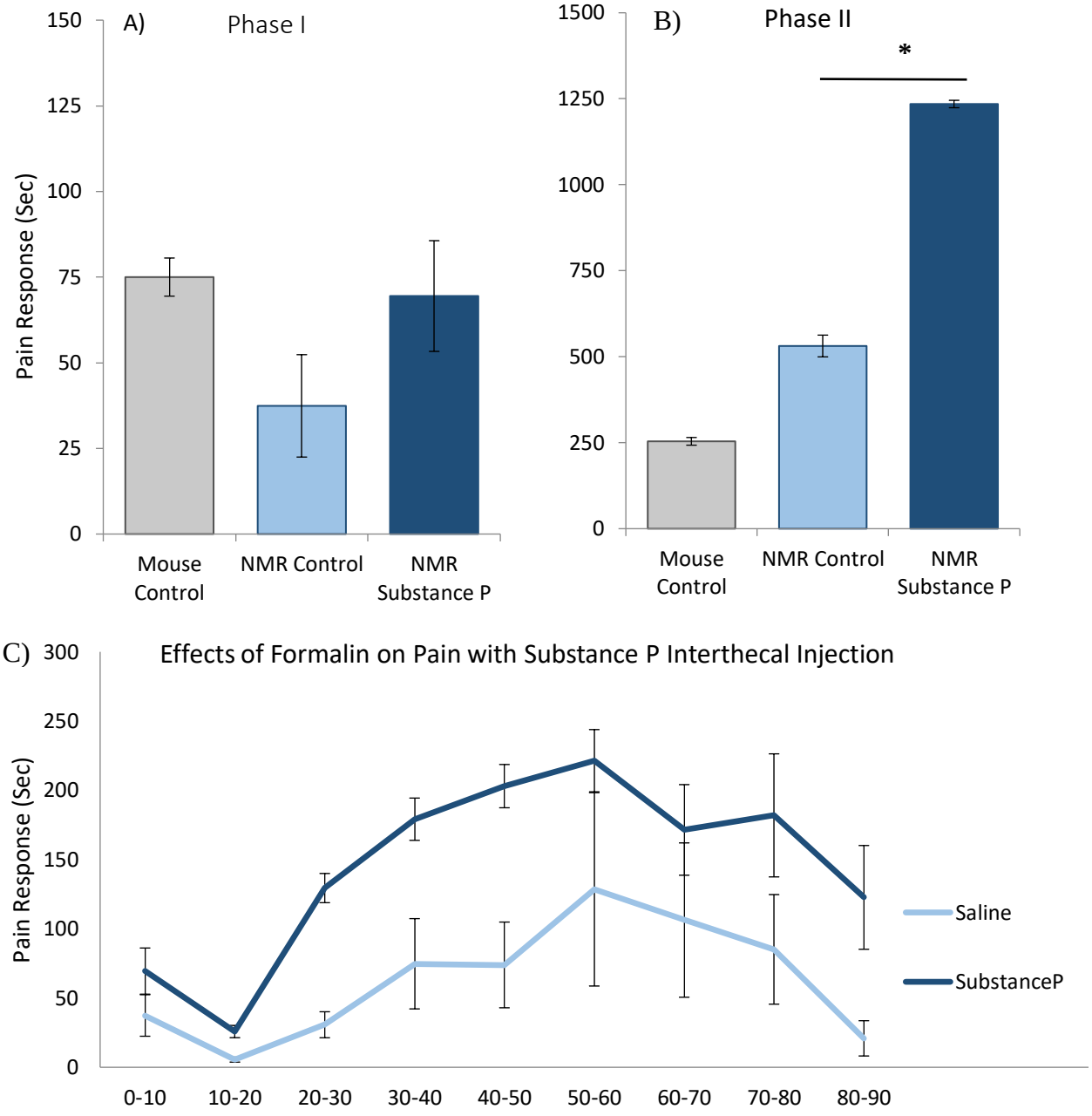


Figure 3.2

Inhibiting P2X3R Reduces Naked Mole-Rat Pain Response to Formalin

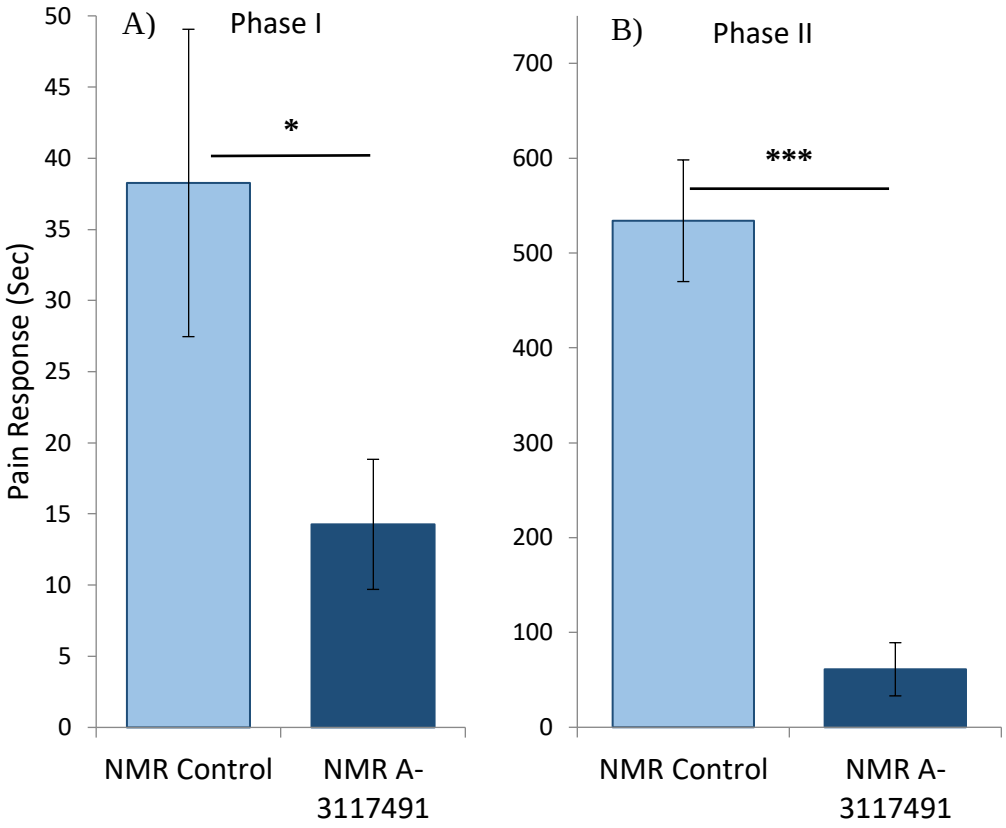


Figure 3.3.

P2X3R is Functional in Cultured Naked Mole-Rat Dorsal Root Ganglion

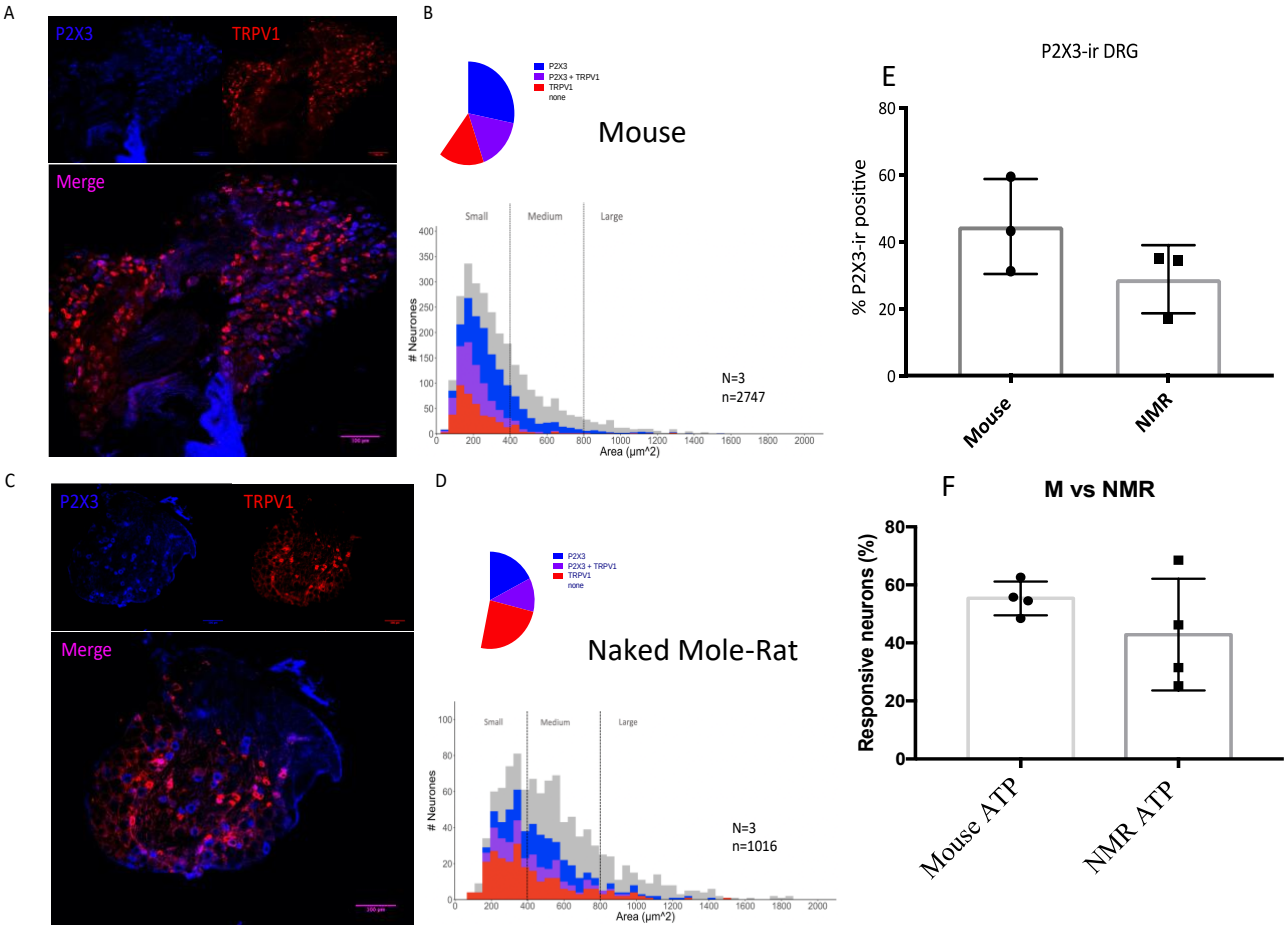


Figure 3.4.

Mechanical Pain Response to ATP in Naked Mole-Rats

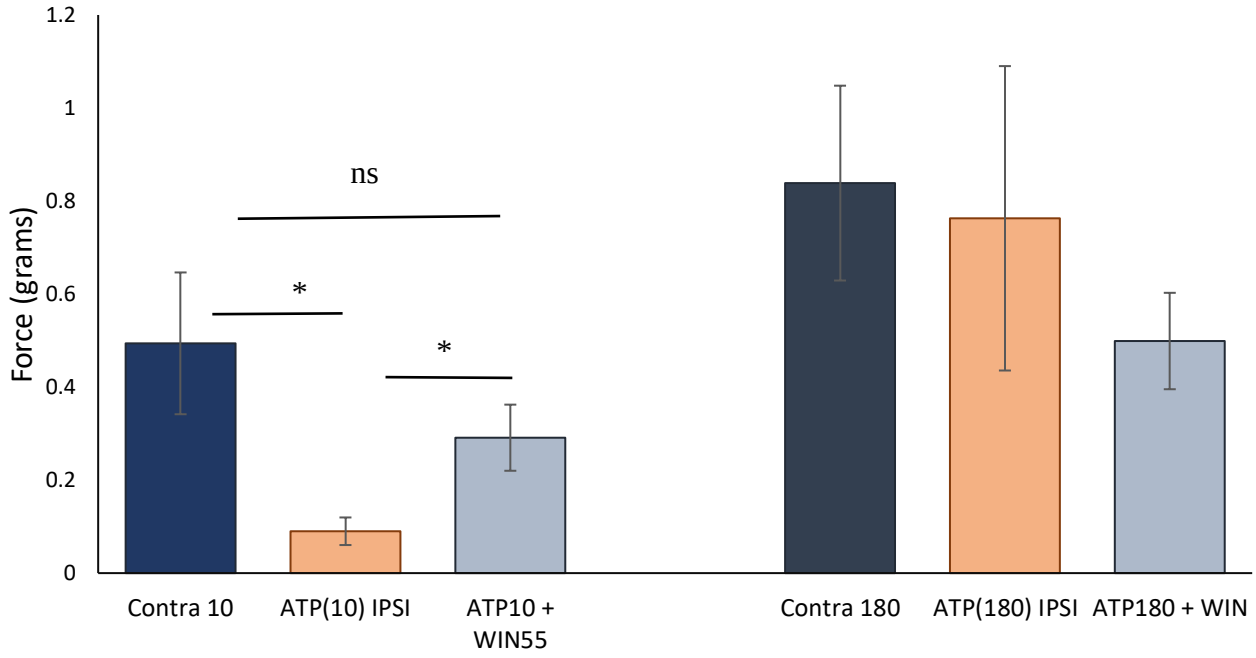


Figure 3.5.

WIN55 Attenuates Pain in Phase II of the Formalin Test

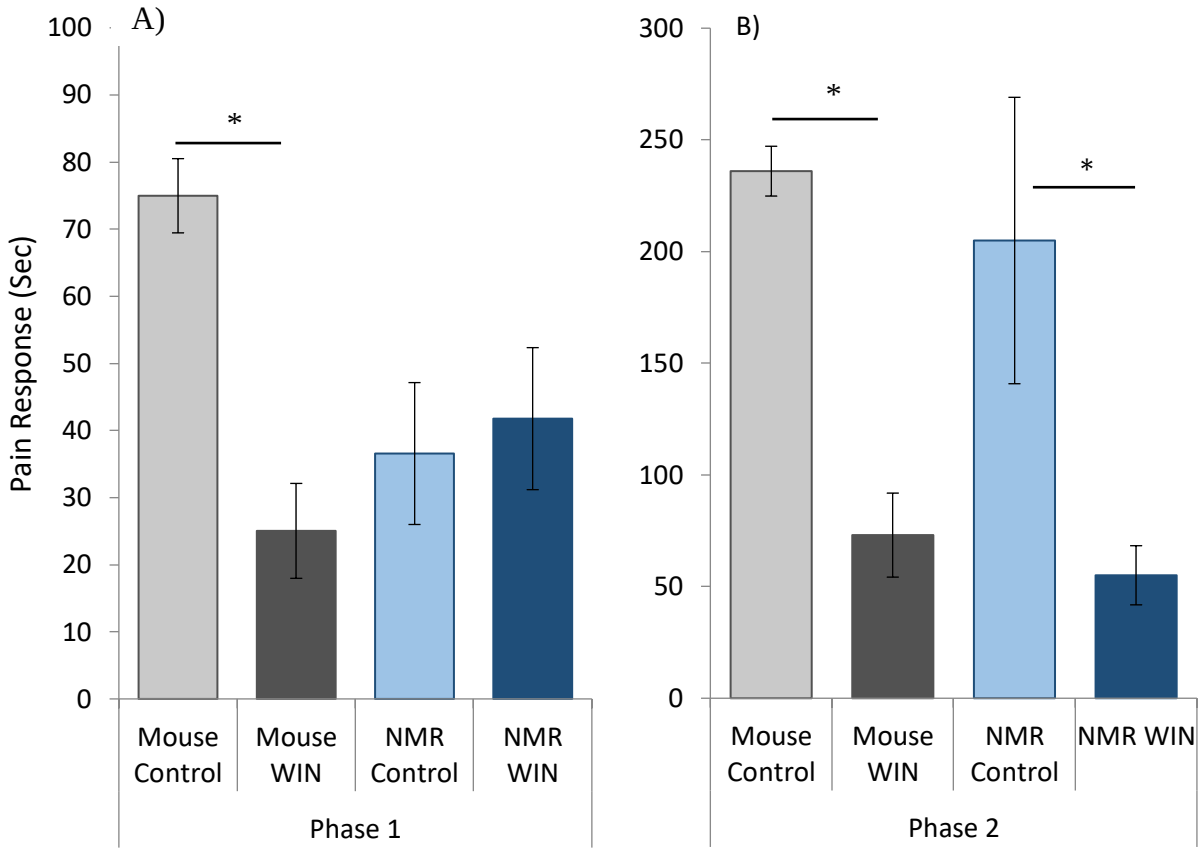


Figure 3.6.

WIN55 Does Not Reduce Thermal Pain

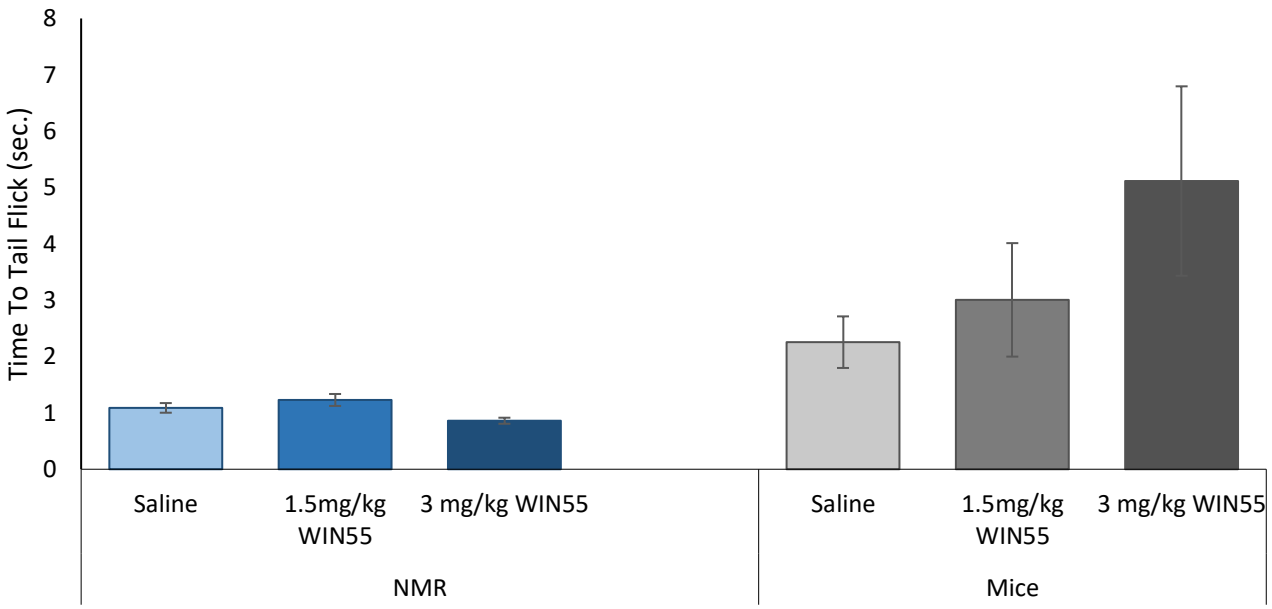
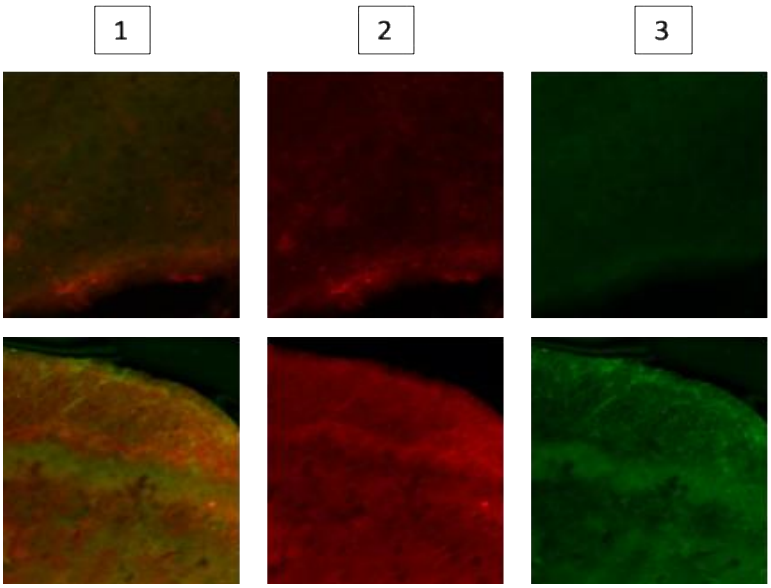


Figure 3.7.

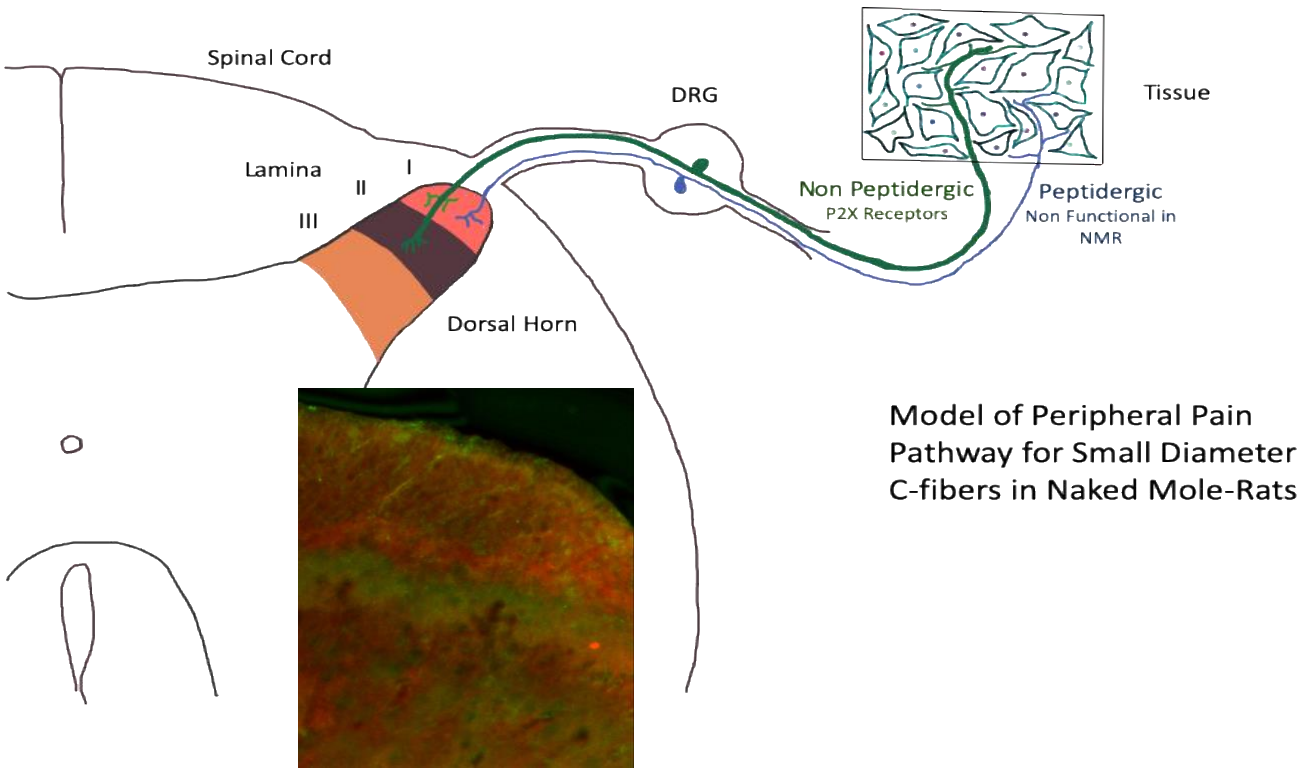
CB1r and P2X3r are Co-expressed in Lamina II of the Dorsal Horn of Mice and Naked Mole-Rat Spinal Cords



A) Mouse Dorsal Horn.
1: Composite
2: P2X3r
3: CB1r

B) NMR Dorsal Horn.
1: Composite
2: P2X3r
3: CB1r

Figure 3.8.



Supplemental Figure 3.1.

Chapter IV: The Endocannabinoid System of African Naked Mole Rats Mediates Protection Against Hypoxic Insult in a Dynamic Manner

Abstract

Naked mole-rats have been shown to retain multiple neonatal traits and these adaptations have been pivotal for the animal to survive in large subterranean colonies. The most striking of these traits is their ability to survive extremely hypoxic conditions vastly longer than most mammals. This includes innate cellular protection in the hypoxia sensitive hippocampus. Using a variety of techniques, we have found that the hippocampus exhibits neonatal facets including: a lack of Paired-Pulse Facilitation, retention of axonal scaffolding, and minimal separation of GABAergic and glutamatergic neuron layers. We have also found that in addition to synaptic maintenance during oxygen deprivation, Naked mole-rats have an attenuated calcium increase during oxygen deprivation and that they retain into adulthood the NMDAr subunit GluN2D that gives protection against hypoxia to neonates. Recently, we found that naked mole-rats also retain an immature endocannabinoid system in certain brain regions, particularly the hippocampus. In neonatal development, the endocannabinoid system has a pivotal role in synapse formation and development, including mediating the development and maturation of PPF, axonal scaffolding, and synapse formation. While evidence for the endocannabinoid system's ability to protect against oxygen deprivation in adult animals has been conflicting, it has been established as a protective system in neonatal rodents. In a mass spec analysis of naked mole-rat expression, there is a global decrease in endocannabinoid which is not seen in mice. Using hippocampal brain slice, we looked at the effect of WIN55 and antagonist AM251, on the CA1 region of the hippocampus when exposed to anoxia. Responses were found to be different in immature (<11 months old) vs mature (>1 year) animals. As expected from the expression data, antagonism of CB1r with AM251 was protective and could be reversed through GABA inhibition. However, pretreatment of WIN55 also exhibited protection from anoxic depolarization in mature animals, increasing the latency to depolarization by

16.7±3.1 minutes and this protective mechanism is not GABA-dependent. In immature animals, WIN55 did not significantly affect the latency to depolarization; however, it did reduce the recovery time after depolarization in WIN55 pretreatment (reduced by 25.13±5.3 minutes). Histology results indicates that CB1r distribution is altered from adolescence to adulthood not in intensity but rather in company, colocalization with both excitatory and inhibitory synapses increasing significantly. Taken together, a developmentally dependent change in protective mechanisms could be caused by a few different pathways. For instance, brain regions, such as the cerebellum and medulla, that express CB1r equally amongst all of the neuron types have been shown to exhibit a bidirectional control of synaptic function that is fine tuned to suppress each neuron individually as needed. On the other hand, the reduced colocalization with vGAT and VGluT in younger animals may indicate that CB1r is activating astrocytes to enhance synaptic recovery.

Introduction

The reduction of oxygen availability to the brain during stroke and heart attack, is one of the leading causes of brain damage resulting from these diseases (Ferdinand and Roffe, 2016). Following the loss of cerebral blood flow, the ischemic cascade, a series of biochemical reactions to the assault, leads to the processes of neuronal cell death in a stepwise manner that begins at the epicenter of assault and radiates outward. Early interventions to halt the procession of damage, is considered to be important in therapeutic outcomes, yet thus far treatments utilizing calcium channel and membrane receptor antagonists- two major players in the induction of the cascade- have not had translational success from animal to human trials (Felberg et al., 2000). Utilizing a model which has been able to reduce harm from the key insults that induce the ischemic cascade has the potential to elucidate aspect of the pathway that are more susceptible to damage and control of cell death. African naked mole-rats have evolved to live without neurological damage in oxygen levels far below what would be survivable for most other mammals (Browe et al., 2018; Larson et al., 2014). Importantly, naked mole-rats are able to reduce intracellular calcium accumulation during low oxygen and therefore reduce damage from the early stages of excitotoxicity, particularly in the hippocampus (Peterson et al., 2012a). We have found that naked mole-rats account for some of this calcium modulation by retention of a fetal N-methyl-D-aspartate receptor (NMDAr) subunit that has a higher threshold for activation than traditional adult NMDAr subunits, NR2D (Peterson et al., 2012b). While an increase in this subunit induces protection during hypoxia, reduced excitability in the synapse from consistent blockade of the NMDAr circuitry would be restrictive to many necessary behavioral functions. In particular, induction of potentiation in the CA1 of the hippocampus, an important aspect of spatial learning, would be severely limited (Regehr and Tank, 1990). However, naked mole-rats' navigational abilities appear to be not only fully intact, but quite advanced as the animals excavate and blindly navigate burrow systems that are both complex and widespread- often extending many meters out from the main dens

(Browe et al., 2018). We therefore were interested in determining how naked mole-rats balance the need for hypoxic protection and potentiation of synapses.

The endocannabinoid system is able to directly modulate synaptic function based on individualized needs of the neurons involved. In neonatal development, the endocannabinoid system has a pivotal role in synapse formation and development, including mediating the development and maturation of PPF, axonal scaffolding, and synapse formation (Crepel, 2007; Tortoriello et al., 2014; Berguis et al., 2007). The release of endocannabinoids, such as 2-arachidonylglycerol (2-AG) and anandamide (AEA), is tightly regulated so that postsynaptic release targets only the cannabinoid receptors on presynaptic terminals of neurons in direct communication with the post-synapse (Dudok et al., 2014). We've previously shown that cannabinoids alter both presynaptic release potential in a calcium dependent mechanism and in both short- and long-term potentiation (Chapter 2). In mice and rats, the endocannabinoid system has been shown to directly modulate both induction of potentiation and inhibition in hippocampal neurons (Lu and Mackie, 2016) and also has been implicated in protection in multiple steps of the ischemic cascade, but in particular in modulation of calcium accumulation (Ohno-Shosaku et al., 2007). In fact, during embryonic development in mice and rats, there is an increase of cannabinoid receptor 1 (CB1r) expression in glutamatergic neurons and that expression has been shown to reduce excitotoxicity during hypoxia in a similar and parallel manner to NMDA's NR2D (Gaffuri et al., 2012; Rodriguez-Munoz et al., 2016). Neuroprotection by mouse and rat cannabinoid receptors after hypoxic/ischemic insult has been shown in an abundance of studies (Kolb et al., 2019). However, the direct effects of cannabinoids in maintenance and protection during hypoxic assault has only been shown in a few, mostly neonatal mouse models which have a longer survival time during extreme hypoxic/ anoxic insult. These neonatal mouse and rat studies share similarities with the mechanisms naked mole-rats use for neuroprotection against hypoxia. Specifically, *in vivo,* Cb1r agonist WIN55-212,2 (WIN55) induces increased protection against anoxia in neonatal

mice that is undistinguishable from an untreated naked mole-rat response to anoxia (Martinez-Orgado, et al., 2003). Additionally, CB1r reduces the necrotic area after hypoxic-ischemia assault, while CB1r knockout (CB1ko) animals have increased levels of injury after hypoxic assaults (Fernandez-Lopez et al., 2013; Marsicano et al., 2002; Martinez-Orgado et al., 2003; Panikashvili et al., 2005).

Previously, we have used the hippocampal slice model with hypoxia and anoxia induction to analyze the physiology of naked mole-rat low oxygen tolerance (Larson and Park, 2009). While neonatally the endocannabinoid system appears to exhibit protective functions in the hippocampus, changes in expression during maturation decrease the role of CB1r in direct reduction of calcium accumulation and instead appear to exhibit neuroprotection only after ischemic assault (Youssef et al., 2007; Martinez-Orgado et al., 2003). Accordingly, CB1r antagonist, AM251 improves recovery from 15 minutes of oxygen/glucose deprivation (OGD) in adult rat hippocampal slice due to the majority of CB1r disinhibiting the excitatory circuits in adults, yet in cultured hippocampal pyramidal neurons CB1r is able to reduce excitotoxicity (Youssef et al., 2007; Zhuang et al., 2005). This indicates that there are functionally available CB1 receptors available to reduce the excitotoxic mechanisms induced by ischemic assault.

Our goal was to determine whether there was direct neuroprotective action by the cannabinoid system in the event of hypoxia and anoxia.

Methods

Animals

All Mice were C57BL/6 males, which were bred from stock, original obtained from Charles River Laboratories, Wilmington, Massachusetts, USA. Mice were kept in a temperature-controlled environment of 72°F with a 12-hour light-dark cycle. Naked mole-rats of both sexes were born in colonies maintained at the Park laboratory. Naked mole rats were kept at a controlled temperature (80°F) and humidity with a 12-hour light-dark cycle. All procedures were conducted according to the

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

Preparation of Tissue for Mass Spectrometry

Naked mole-rats aged 1-3 years and mice aged 5 months were randomly assigned to control or hypoxic conditions. Control animals (3 mice, 3 naked mole-rats) were housed separated from the colony in ambient air for 5 hours prior to tissue collection. Hypoxic mice (n=3) were placed in a chamber with 8% Oxygen (O2):92% Nitrogen (N) and hypoxic naked mole-rats (n=3) were placed in a chamber with 5% O2: 95% N for 5 hours prior to tissue collection. Hypoxic animals were removed from the chamber and quickly decapitated taking special care that the decapitation occurred prior to taking a breath in ambient air. Control animals were also quickly decapitated. Brains were removed from all animals and regions of interest, hippocampus, prefrontal cortex, posterior cortex, and cerebellum, were quickly dissected and immediately frozen on dry ice. Tissues were stored at -80C until ready to be shipped. All tissue was sent out for analysis in a dry shipper cooled with liquid nitrogen to The University of Aberdeen, Scotland UK Mass Spectrometry Core Facility for analysis with liquid chromatography tandem mass spectrometry (LC/MS/MS).

Electrophysiology

Experiments were performed on 8-10 month or 1-3-year naked mole-rats. The lifespan of the naked mole-rat is greater than 30 years; the animals of the younger cohort are considered to be adolescent animals and the older cohort are considered to be young adults. Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

Animals were treated with either: 5mg/kg WIN55, 5mg/kg AM251, or .9% saline by intraperitoneal injection 30 minutes prior to dissection. Transverse hippocampal slices were prepared in the conventional manner. Briefly, naked mole-rats were decapitated, and the brains were rapidly removed into ice-cold ACSF containing: 124mM Na Cl, 3mM KCl, 1.2mM KH2PO4, 26mM NaHCO3.

2.5mM MgSO4, 3.4mM CaCl2, 2mM Na-ascorbate, and 10mM D-glucose. ACSF was gassed with 95% O2 and 5%CO2. The tissue was sliced at 400um on a tissue chopper. Slices were placed in an interface chamber and constantly perfused (1.0ml/min) with ACSF at 34C. Stimulation electrodes were placed in the stratum radiatum of subfield CA1 to activate Schaffer-commissural fibers. Population recordings of synaptic field potentials (fEPSPs) were made with micropipettes positioned in the stratum radiatum of CA1. Evoked responses were digitized by microcomputer and analyzed online using custom software. fEPSPs were evoked at 20sec intervals throughout the experiments. Baseline stimulus intensity was set to evoke a half-maximal fEPSP in each slice. Baseline recordings were taken for 20 minutes before manipulations. Initial slope and peak amplitude were calculated for each fEPSP and normalized to the baseline average in each slice.

Episodes of hypoxia were induced by replacing the O2 content of the chamber atmosphere and perfusion ACSF with 100% N for nominal anoxia. In some experiments, 2.5mM picrotoxin (PTX) was perfused with the ACSF for the duration of the experiment. The time required to completely eliminate fiver volleys evoked by stimulation was determined as an estimate of anoxic depolarization (AD). In experiments that measured time to AD, the slices remained in nominal anoxia for 2 minutes after AD before returning to 95%O2 and 5%CO2. In some experiments, nominal anoxia was applied for a 10-minute period. All experiments were allowed to recover in 95%O2 and 5%CO2 for 60 minutes after the bout of anoxia to determine the degree of recovery after reinstatement of oxygen.

Histology:

Mice and naked mole-rats were decapitated and brains were quickly removed and drop fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer (PBS, pH 7.4) overnight, and subsequently cryoprotected in 30% sucrose (in PBS). Brains were sectioned coronally (26 µm thickness) on a Leica CM1850 cryostat microtome and directly placed on slides. Non-specific immunoreactivity was suppressed by incubating our specimens in a cocktail of 5% normal donkey serum (Jackson

Laboratories), 1% bovine serum albumin (BSA, Sigma) and 0.3% Triton X-100 in PB for an hour at 22–24 °C. Sections were then exposed to select combinations of primary antibodies: Mouse monoclonal anti-VGLUT1 (1:1000; Synaptic Systems), rabbit anti-VGAT (1:1000, Synaptic Systems), guinea pig anti-CB_1R (1:15000; Frontier-Science) (Kawamura et al., 2006); diluted in PBS (48–72 h at 4 °C) to which 0.5% normal donkey serum and 0.3% Triton X-100 had been added. After extensive rinsing in PBS, immunoreactivities were revealed by carbocyanine (Cy) 3-tagged-antiGp, AlexaFluor488-antiRb, and AlexaFluor 647-antiMu secondary antibodies raised in donkey (1:200 (Jackson), at 22–24 °C, 2 h). Glass-mounted sections were cover slipped with Aquamount (Dako, Glosstrup, Denmark). Images acquired on a Fluoview FV10i confocal laser-scanning microscope (Olympus) and analyzed with ImageJ.

Results

It is well established that naked mole-rats have an intrinsic resistance to hypoxic and anoxic insult in the hippocampus (Larson and Park, 2009; Larson et al., 2014) and that their protection is related to retention of neonatal features of hypoxic resistance. In neonatal mice, increasing the expression of 2-AG and AEA have been shown to be protective against hypoxia and ischemia (Lara-Celador et al., 2013) by reducing calcium inducible excitotoxicity and reducing necrotic injury. We wanted to see if naked mole-rats' upregulated their expression of endocannabinoids when in hypoxic conditions. We measured the expression of 2-AG, AEA, OEA, and PEA with mass spectrometry in control animals and compared it to the expression in animals exposed to 5 hours of hypoxia (5% for naked mole-rats, 8% for mice). With few exceptions, naked mole-rats decreased circulating endocannabinoids by 25-50% after hypoxic exposure in all brain regions examined (Fig. 4.1A,B,C,D). Mice also had an overall trend toward decreased circulatory levels, however, not to as great of an extent- most endocannabinoids decreased in the 10-25% range. The exceptions to this in the naked mole-rat were anterior cortex AEA levels (9.58%; Fig. 4.1B), hippocampal PEA (-3.8%; Fig. 4.1C), and hippocampal OEA levels (45.49%; Fig. 4.1D). Mice expressed nearly double the amount of 2-AG in the

posterior cortex (Fig. 4.1A) after hypoxia exposure as well as a small increase in OEA (13.42%; Fig. 4.1D) in the same region.

In adult mice WIN55 increases the hippocampus' susceptibility to anoxic insults (Youssef et al., 2007). Therefore, naked mole-rats may decrease the role of CB1r during hypoxic exposure to protect against damage from low oxygen. Conversely, the reduction in circulating endocannabinoids could be due to an increase in binding to CB1r, indicating that CB1r is protective in naked mole-rat adults in a similar manner to neonatal mice and rats. To test whether naked mole-rats showed a further increase in protection, like neonates, or an increased susceptibility, like in adults, we tested hippocampal brain slices under periods of anoxia in adolescent and adult naked mole-rats by recording fEPSPs (Fig. 4.2 C,D). In adolescent naked mole-rats (<1 year old), CB1r activation does not significantly affect the time to loss of fiber volley, or anoxic depolarization (AD), though there is a trend toward WIN55 reducing the time to AD (Control time to AD: 452.33±122 secs.; WIN55 time to AD: 373.17±307 secs.; Fig. 4.2A**)**. There is, however, a significant improvement in the time to recovery after anoxia, with the time for the amplitude to return to baseline reduced by nearly half (Control time to 100% baseline:40.99±7 mins.; WIN55 time to 100% baseline: 15.86±3.6 mins.; Fig. 4.3A). Conversely, when CB1r is antagonized in the adolescent mole-rats, there is an increase in AD time over control (AM251 time to AD: 885±132 secs.; Fig. 4.2A).

In naked mole-rat adults (> 1year old), CB1r is able to protect from anoxic depolarization both by activation of the receptor with WIN55 (Control time to AD: 509.67±110 secs.; WIN55 time to AD: 1083±265.42 secs.; Fig. 4.2B), without any appreciable change to recovery time after AD compared to control animals (Control time to 100% baseline: 43.88±7.9 mins.; WIN55time to 100% baseline: 37.18±9.6 mins.; Fig. 4.3B). Interestingly, adult naked mole-rats also show protection when the CB1r receptor is antagonized by AM251 (AM251 time to AD: 701±18 secs.; Fig. 4.2B; AM251 time to 100%

baseline: 41.67±8 mins.; Fig. 4.3B**)**, indicating a dynamic system that has two forms of protection, one that is CB1r dependent and one that requires the blockage of CB1r activity. Since the majority of CB1r activity is at GABAergic synapses, we further explored the protective effects of CB1r during anoxia after GABA was inhibited by picrotoxin (PTX). To see if we could separate the duel mechanisms of protection with WIN55and AM251 in the adult animals, we focused on that age group. The results with GABA inhibition in WIN55 treated animals was highly variable, one animal's brain slice survived for over 90 minutes. The enhanced protection of WIN55 appeared unaffected by PTX application (WIN+PTX time to AD: 2560±1719 secs.; Fig. 4.4A; statistical analysis was not run as high survivability outliers of more than two standard deviations were removed). Time to AD in adult naked mole-rats treated with AM251 was reduced with the PTX bath and there was no longer a significant difference between AM251+PTX (time to AD: 473±169 secs.) and control slices ($p>.05$) or control + PTX slices (Control time to AD 670.7±183.8; $p>.05$; Fig. 4.4A,B). Recovery time also appeared to be improved in AM251+PTX (time to 100% baseline: 29.27±12 mins.) slices compared to Control+ PTX (time to 100% baseline: 32.24±18.54*) and WIN+PTX (time to 100% baseline: 46±6.8*), however, post hypoxic recovery could not be followed in 33% of Control and WIN55 slices as they did not recover to 100% of baseline in PTX bathed slices and differences could not be determined statistically (* indicates averages for recovered slices only).

The eCB system is a modulator of neurotransmitter release on both excitatory and inhibitory neurons. In neonatal hippocampal networks, the CB1r has a greater influence on principle excitatory neurons than in adults where the inhibitory neurons have more than 30 times the response to CB1r compared to glutamatergic neurons (Chevalyre and Castillo, 2003; Chevaleyre et al., 2006; Yasuda et al, 2008; Kawamura et al., 2006; Hu and Mackie, 2015; Katona et al., 1999). We imaged the expression levels of GABAergic and glutamatergic vesicle transport proteins (vGAT and vGlut3, respectively) with

immunofluorescence and assessed their colocalization patterns with CB1r to determine if the location of CB1r could explain the duel protection with agonism and antagonism seen in adult naked mole-rats. While there appears to be no significant change in the vGAT levels between species (Fig.4.5A,B,C), there is an obvious shift in region of expression in naked mole-rats (Fig. 4.5B,C) to have higher concentrations in the cellular layer, a distribution similar to what we reported in Penz et al. (2015) suggesting that the cellular layering of vGAT has not matured. The images also suggest an increase in co-localization between vGAT and CB1r in naked mole-rats (Fig. 4.5B,C). However, like CB1r, the level of vGlut fluorescence appear to have increased expression and high levels of co-localization with CB1r in adolescent and adult naked mole-rats (Fig.4.5B,C). This also coincides with published neonatal distributions; CB1r and glutamate have high co-localization until post-natal day 5 (Gaffuri et al., 2012). Interestingly, the distribution of vGlut is almost exclusively in the axonal layers which indicates mature synaptic development (Fig. 4.5B,C).

Discussion

In the brain there is a vital necessity to delicately balance protecting tissue from the pathophysiological issues associated with low oxygen and retaining proper synaptic function for other systems. While many aspects of the naked mole-rat's adaptations to chronic hypoxia have been described, the role of the endocannabinoid system has yet to be defined. We previously showed that one-way naked mole-rats protect against low oxygen is by switching to metabolism that is not oxygen dependent. Fructose almost completely explains the heart's use of alternative metabolism during hypoxia/anoxia whereas brain still showed detriment with fructose alone (33% functionality after 60 mins of fructose replacement but the heart barely dropped 5%), indicating that fructose-based glycolysis is not the primary or at least not the only source of protection in the brain/hippocampus (Park et al., 2017). One of the most damaging responses to low oxygen is an influx of calcium intracellularly, releasing glutamate in large quantities into the synaptic space (Weber, 2012). Low

oxygen reduces the production of ATP which glycolysis cannot fully accommodate and is necessary for the reuptake of glutamate (O'Donovan et al., 2017). We have found that one method naked mole-rats use in reducing overactivation of NMDAr by retention of the predominantly neonatal GluN2D NMDAr subunit which needs increased stimuli to open, reducing the impact of excess glutamate (Peterson et al., 2012b). We also know that naked mole-rats blunt the influx of calcium (Peterson et al., 2012a). In other neonatal mammals, the cannabinoid system is also integral to mediating calcium. Activation of presynaptic CB1r at excitatory neurons also directly inhibits voltage gated calcium channels causing a reduction of glutamate release (Pitler and Alger, 1992; Diana and Marty, 2003; Varma et al., 2002; Chevalerye and Castillo, 2003). Further, increased NMDAr activity itself can provoke eCB release (from the post-synaptic membrane) and CB1r stimulation which will diminish NMDAr activity and therefore prevent excitotoxicity (Rodriguez-Munoz el al., 2016). Therefore, this study examined the role of eCBs in the tolerance o naked mole-rats to hypoxia/anoxia.

We found that the level of 2-AG was universally decreased after 5 hours of hypoxia in all four of the brain regions that were examined, which is in direct contradiction to a previously study which showed an increase of 2-AG after 4 hours of hypoxia in mice (Degn et al., 2007). That study however, analyzed whole brain tissue and did not account for region specific changes. While our mice data did show a small decrease in the anterior cortex, there was no significant expression change in either the hippocampus or cerebellum and there was a much larger increase in posterior cortex expression, which could coincide with the previously reported 2-AG increase after hypoxia in mice.

Interestingly we found that the adult naked mole-rat can induce protection from AD through both CB1r activation and inhibition. It is not uncommon for the eCB system to modulate both excitation and inhibition within the same circuitry causing seemingly contradictory responses depending on the physiological state. In fact, this has been well described in the mechanisms of CB1r in growth cone guidance. CB1r repulses growth cone migration in neurodevelopment. This attraction is

initiated by CB1r's activation of RhoA: Downstream, RhoA activates ROCK which phosphorylates myosin light chains causes a contraction of the cytoskeleton on the activated side. When the signal to ROCK is inhibited, not only is the repulsion stopped, but also CB1r agonist WIN55 becomes a chemo attractor and initiates cone growth on the activated side (Berguis, et al., 2007). There is some precedence for the duel protection observed in ischemia as well, where a CB1/CB2r agonist and CB1r antagonist in combination have been shown to reduce cellular damage, improve behavioral outcomes post-insult, and downregulate apoptotic proteins in an additive manner compared to either treatment alone (Shiasi et al., 2018). Though the mechanisms of attraction have not yet been elucidated, the eCB system has been shown to utilize multiple actions of the receptor with some factors being sufficient to suppress others even if they are opposing actions. The same duel mechanistic actions could be present in the hypoxia tolerance pathway of naked mole-rats, which would explain how an antagonist and agonist can both be protective toward fiber volley maintenance.

There is one mechanism of the adult naked mole-rat cannabinoid mediation of hippocampal circuits during anoxia that was determined by this study. When the GABAergic response was inhibited in the hippocampus, AM251 no longer increased the time to AD in adult naked mole-rats. This indicates that inhibition of CB1r on the GABAergic presynapse elicits a protective response. This is similar to the protective response shown in other adult rodent slices and has been explained as protection through disinhibition of GABA or protection that is caused by allowing GABA to produce negative feedback at pyramidal excitatory synapses which would reduce glutamate release (Youssef et al., 2007).

The mechanism behind protection by WIN55 in adult naked mole-rats is still elusive. However, his could be an important target for therapeutics as there does not appear to be any adverse consequences in the recovery phase in adults and increases cellular recovery in adolescents. We propose two targets for Win55 in protection. The first is direct inhibition of glutamate release at

pyramidal neurons, which would reduce overall calcium influx in the post-synapse (Kimura et al., 1998). Astrocytic release of ATP may be an alternative pathway, as Win55 has been shown to increase ATP release (Rasooli-Nejad et al., 2014). This would aid in the reuptake of glutamate in the synaptic space to prevent accumulation (O'Donovan et al., 2017).

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Figure Legends

Figure 4.1: Percent change of endogenous cannabinoid expression in hypoxic animals relative to control animals after 5 hours of 5% hypoxia in NMR and 8% hypoxia in mice (the respective lowest maintainable atmospheric oxygen reduction that does not induce death in mice). Measured using LC/MS/MS. N=3.

Figure 4.2: After establishment of a steady baseline, oxygen to the chamber and ACSF was replaced with nitrogen. Hippocampal slices were timed while recording stimulus response in the CA1 region. Anoxic depolarization (AD) was marked by the visible loss of fiber volley in the slice. Time to AD was recorded in seconds and averaged in naked mole younger than 1 year of age (A) and rats older than 1 year of age (B). Animal's AD times were compared in animals that were pretreated with AM251 or WIN55 to control animals in the same age category. (C) Example of trace from one control experiment with baseline, anoxia until 2 minutes post AD, and 60 minutes of recovery. (D) Waveforms from trace experiment taken from Baseline, AD, and at 100% recovery. Control >1y N=6, <1y N=7, AM251 >1/<1 N=3, WIN55 >1y N=5, <1y N=6. * p<.05 compared to control.

Figure 4.3: After 2 minutes of anoxia in a depolarized state, oxygen was returned to the slice bath and the time to recovery was measured in minutes. Recovery was defined as time for the amplitude of response to return to the baseline level. Control >1y N=6, <1y N=7, AM251 >1/<1 N=3, WIN55 >1y N=5, <1y N=6. * p<.05 compared to control.

Figure 4.4: After establishment of a steady baseline, oxygen to the chamber and ACSF was replaced with nitrogen. Hippocampal slices were timed while recording stimulus response in the CA1 region. Anoxic depolarization (AD) was marked by the visible loss of fiber volley in the slice. Time to AD was

recorded in seconds and averaged in naked mole rats older than 1 year of age (A) and younger than 1 year of age (B). Animal's AD times were compared in animals that were pretreated with AM251 or WIN55 to control animals in the same age category. C) After 2 minutes of anoxia in a depolarized state, oxygen was returned to the slice bath and the time to recovery was measured in minutes. Recovery was defined as time for the amplitude of response to return to the baseline level (33% of control and WIN55 treated slices did not recover to baseline, these results are a combination of the remaining samples). n=3: AM251; n=2: Control and WIN55.

Figure 4.5: Example confocal images of immuno-expression in the hippocampus in adult mice (A), adolescent naked mole-rats (4mo NMR; B), and young adult naked mole-rats; C). Figure shows a composite with Hoechst stained nuclei (1), the co-expression of vGat (blue) with vGLUT (green; 2), vGlut independently (3), co-expression of CB1r (red) and vGLUT (4), CB1r independently (5), co-expression of CB1r and vGat (6), and vGat independently (7).

Effects of Hypoxia on Endocannabinoid Expression in the Brain of Mice and Naked Mole-Rats

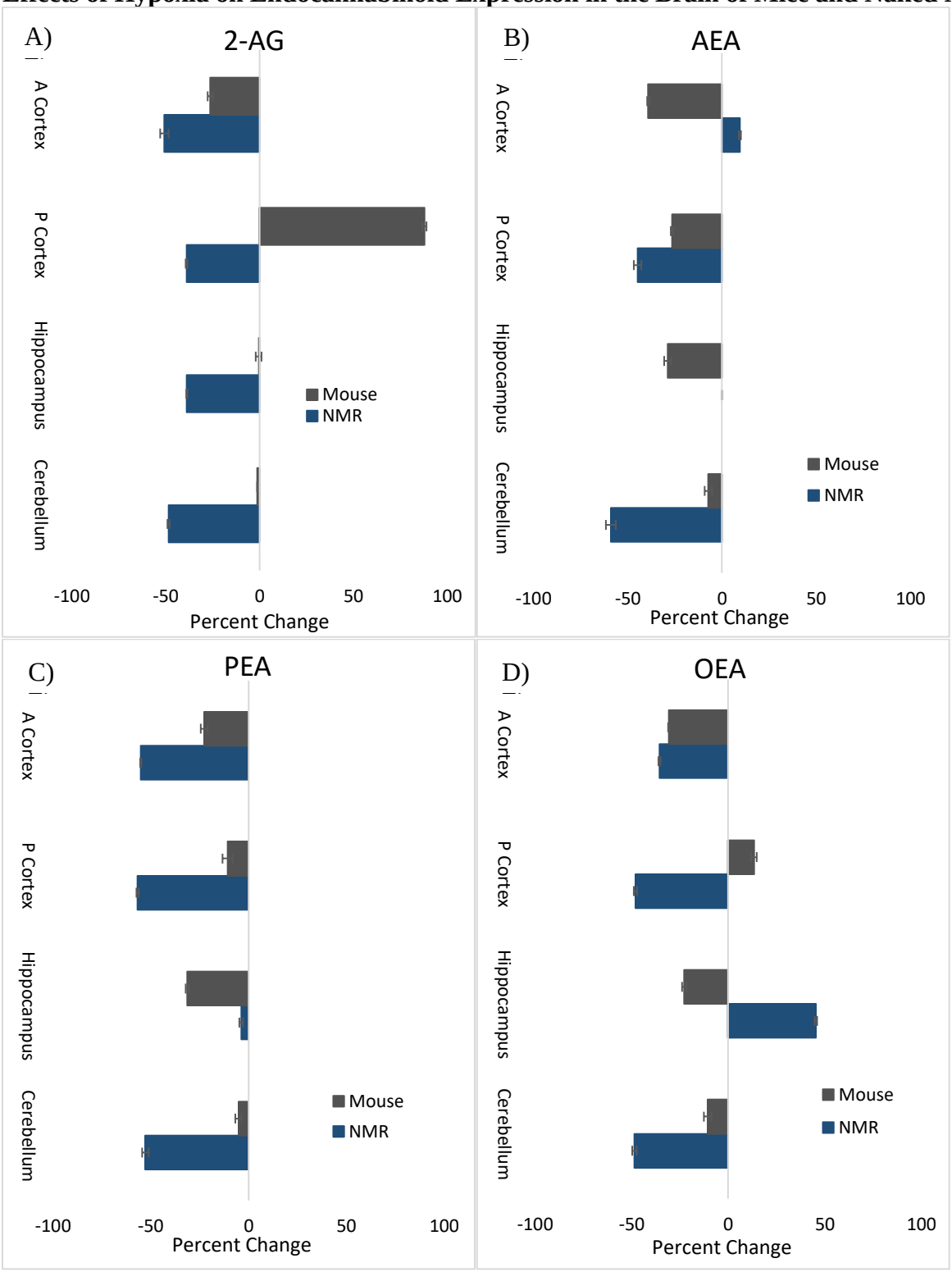

Figure 4.1.

Cannabinoid Modulation of Intrinsic Tolerance to Anoxia in Naked Mole-Rat Brain

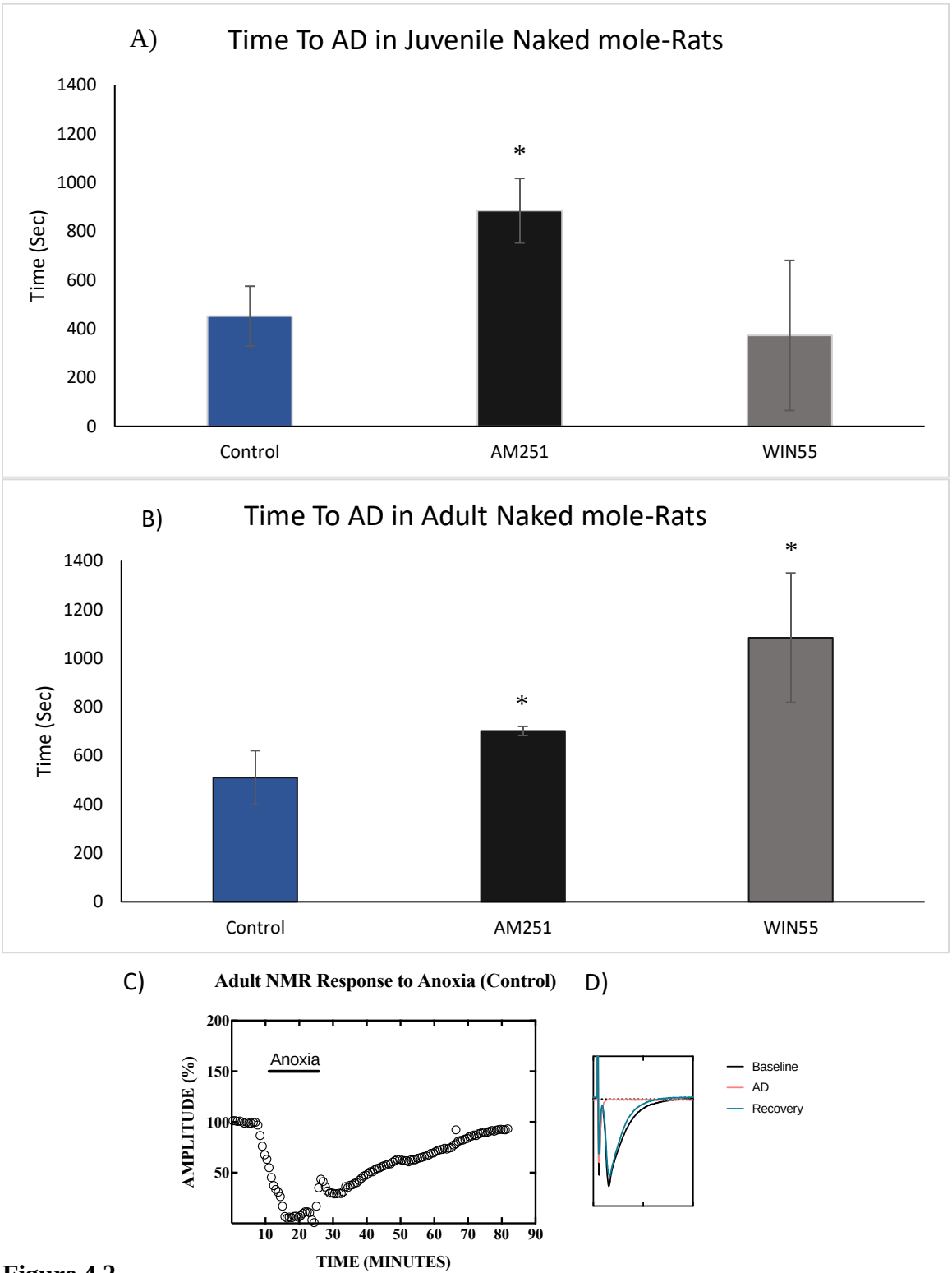

Figure 4.2.

CB1r Mediation of Recovery After Anoxic Depolarization

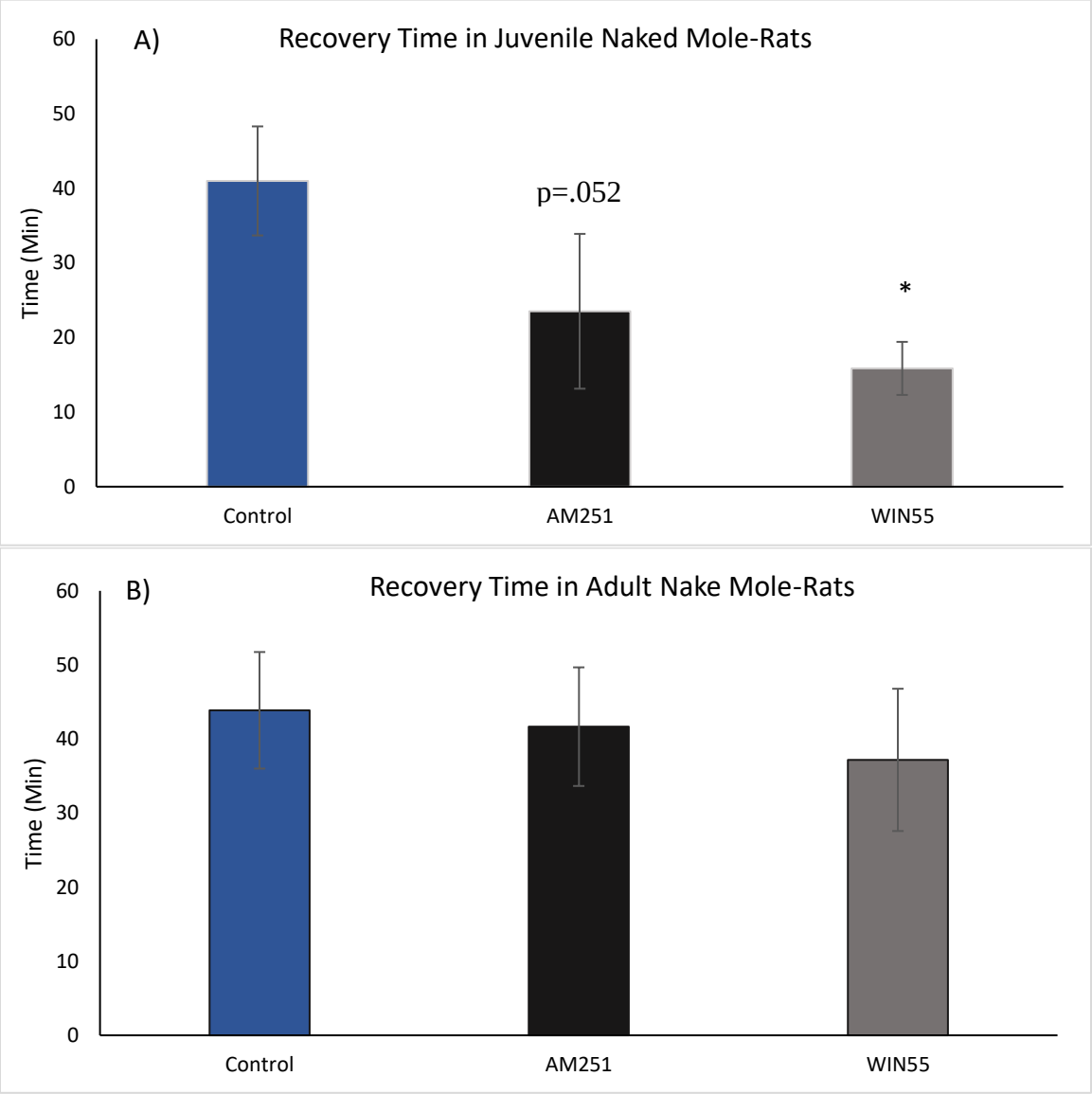

Figure 4.3.

AM251-Induced Protection Reduced by GABA Inhibition in Adult Naked Mole-Rats

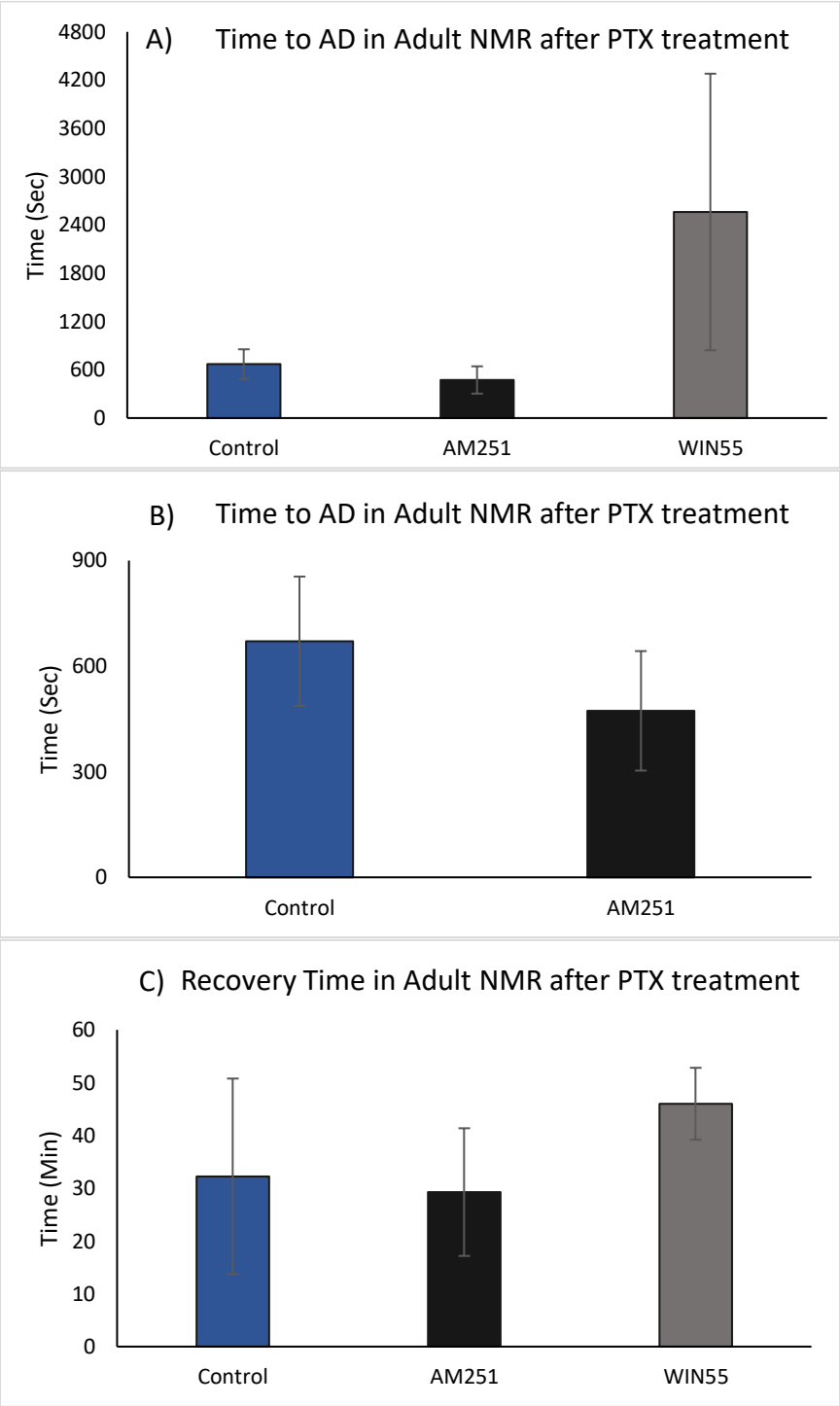

Figure 4.4.

Naked Mole-Rat Expression of CB1r Compared to GABA and Glutamate Transporter Protein Expression in the Hippocampus

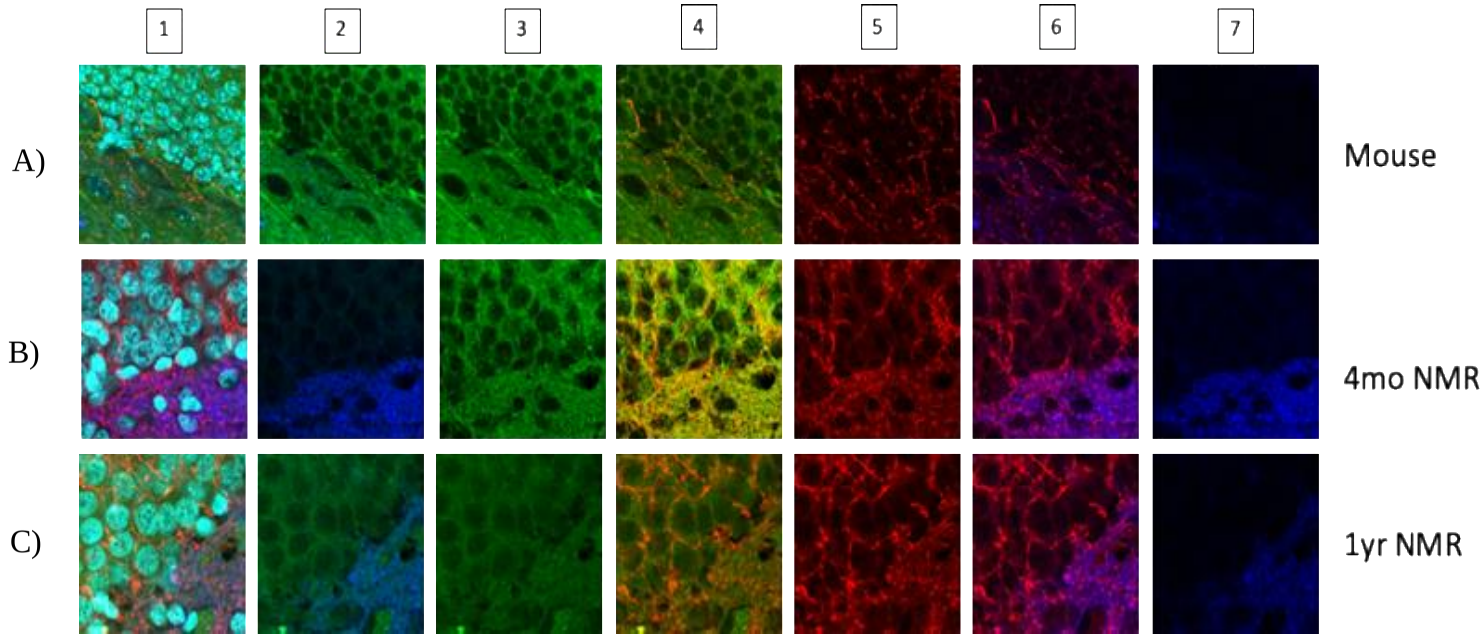

Figure 4.5.

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Chapter V: Conclusions

Summary

The purpose of this project was to examine how the endocannabinoid system modulates aspects of naked mole-rats' adaptations to their extreme environment. Through this goal, I characterized how the endocannabinoid system is involved in a wide range of functions in the naked mole-rat. The key findings of this project describe three of the naked mole-rats' most unusual adaptations. They revolve around naked mole-rats' neotenous developmental delays, extreme pain tolerance, and tolerance to low oxygen.

I found that the developmental profiles of the endocannabinoid system are delayed in higher brain regions, such as the hippocampus and prefrontal cortex, but not in regions of vital functions. In addition to delayed expressions, young adult naked mole-rats have CB1r with reduced receptor function in the hippocampus and that may lead to reducing psychotropic effects such as hypomobility. Synaptic function and plasticity are altered by cannabinoids in an age-dependent manner that indicates a shift in function from adolescence into early adulthood.

Second, I found that endocannabinoids are still able to modulate C-fiber pain, despite an inoperable peptidergic signaling pathway. The P2X3r pathway of C-fibers is functional in both cultured neurons and *in vivo.* Cannabinoids attenuate pain directly elicited by activation of P2X3r. However, no inflammatory response was found after P2X3r activation and there was no long-term pain attenuation with cannabinoids.

Finally, I found that the endocannabinoid system of naked mole-rats modulates synaptic function during hypoxia. Interestingly, this modulation is different in juveniles compared to adults and implicated a dual mechanism for prevention of damage in adult animals but post-hypoxic/anoxic recovery protection in juveniles. The protection elicited by inhibition of CB1r in adults is reversed by

PTX, indicating that the mechanism is protective through disinhibition directed through GABAergic interneurons.

Assessment of Contributions

This project is the first known research to describe the endocannabinoid system in African naked mole-rats. The focus was to assess if three naked mole-rat adaptations that have implications in health and wellness, slowed developmentally based longevity, pain attenuation, and hypoxia tolerance were modulated differently in naked mole-rats compared to traditional laboratory rodents.

In the developmental project, I identified naked mole-rats as a new model system for chronic cannabis studies on adolescent development. Additionally, I identified an anomalous discontinuation of psychoactive behavior phenotypes in adult naked mole-rats. Further exploration into the mechanisms that underlie this could result in more targeted cannabinoid-based therapeutics without psychotropic side-effects. This also lays a strong foundation for using naked mole-rats to assess the dynamics of the endocannabinoid system in extreme adaptations.

The second project is the first to identify that the purinergic C-fiber pathway elicits a pain response in naked mole-rats. This is due to having a functional P2X3r, which I measured with direct activation. However, this work also indicates that P2X3r does not increase sensitivity with a long-term inflammatory response as is seen in other rodents. This may indicate that naked mole-rats have mutations in the inflammatory system that include attenuation of inflammatory response to ATP.

The final project focused on the innate hypoxia tolerance of naked mole-rats. This research indications the eCB system is an additional regulatory mechanism for hypoxia tolerance in naked mole-rats. First, I identified that naked mole-rats downregulate endocannabinoid expression to reduce available 2-AG and arachidonic acid derivatives during hypoxic assault. The developmental slice physiology described in the development project indicated that the cannabinoid system modulates the functions of the hippocampus to protect synaptic circuitry with multiple mechanisms. This makes the

endocannabinoid system a more dynamic mediator than GluN2D. It is possible that the duel mechanisms work in tandem to protect and reduce excitatory function during hypoxic assault but then upregulate GABAergic inhibition to maintain necessary synaptic functions.

Recommendations for Future Work

There are many aspects of the endocannabinoid system in development that were not addressed in this project. Especially intriguing is the molecular mechanisms that underlie the locomotor changes in adulthood. The lack of hypomotility is especially perplexing considering cannabinoid modulation is intact in other areas, such as neuroprotection, learning and memory, and pain. The behavioral effects of THC and other CB1r agonists have been associated with the downregulation of cAMP (Elphick and Egertova, 2011). Looking directly at cAMP production in particular cell types would be interesting to understand how one behavioral assay can be so starkly changed without effecting other functions of the cannabinoid system. There is an important need for understanding the balance of how cannabinoid disinhibition affects multiple neurotransmitters. In some brain regions, in particular the prefrontal cortex, large doses of THC increase circulating glutamate but not GABA levels. This would indicate that THC is inhibiting GABAergic transmission and therefore increasing the amount of excitatory response in these regions (Pistis et al., 2002). The combination of multiple neuronal types all being separately modulated by CB1r is a hallmark of the cannabinoid system yet is not well understood. The naked mole-rat model may prove useful in disseminating how developmental changes in receptor distribution contribute to physiological issues with cannabinoid use. Further evidence shows that the regulation of neurotransmitter release by cannabinoids is developmentally linked, with GABA and dopamine being highly regulated in adolescence compared to adulthood (Pistis et al., 2002; Mason et al., 2018). The protracted development of naked mole-rats, especially with the expression of the cannabinoid system also being

delayed, has a lot of implications for understanding cannabinoid modulation throughout this period of development.

This research confirmed that the P2X3r pathway in naked mole-rats is functional. P2X3r can be inhibited by CB1r's downregulation of cAMP in traditional laboratory rodents (Long et al., 2018). Therefore, we explored the relationship of CB1r and P2X3r in naked mole-rats. The aspects of cannabinoid modulation in the P2X3r pain pathway are interesting considering that we showed a significant decrease in early stages of ATP-induced pain but not after 3-hours when the inflammatory response should be measurable. In other mammalian species, cannabinoids have been shown to have a greater effect on long term inflammation by specifically decreasing the response of C-fiber neurons in the DRG (Oliveira-Fusaro et al., 2017). There are two directions which this project logically leads to. The first is to look directly at CB1r's response to other inflammatory proteins in the naked mole-rat. AEA has been shown to suppress proinflammatory cytokines and TNF-alpha to reduce pain more efficiently in inflammatory states (Fine and Rosenfield, 2013; Donvito et al., 2017). The second is that this project only examined the response of cannabinoids in the peripheral ascending pathway and spinal cord. To fully understand how CB1r attenuates pain and inflammation in naked mole-rats, it is important to look further up the ascending pain pathway. Targets that are the most important to look at include, periaqueductal grey region, dorsal raphe nuclei, and the thalamus which are all involved in modulation of the pain response prior to its relay into the somatosensory cortex and have exhibited CB1r-dependant attenuation of pain signal (Chiou et al., 2013).

The mechanisms that underlie protection from hypoxia are still mostly unknown. It is critical to elucidate whether naked mole-rat protection with WIN55 is due to CB1r directly modulating pyramidal principle cells to reduce calcium release when activated or if there is another mode of circuit protection through other mechanisms such as astroglia in the triparate system (Rasooli-Nejad et al., 2014). Another aspect of the unusual role of CB1r in naked mole-rat anoxia protection is that juvenile

animals do not exhibit protection from anoxic depolarization but does speed up recovery after normal oxygen levels have been reestablished. Further exploration of the physiological properties of CB1r protection in slice experiments should include examining the endogenous effect of increasing 2-AG and AEA production through MAGL and FAAH inhibitors, respectively, during anoxic insults to determine if there is an endogenous drive to modulate synaptic functionality.

To elucidate the mechanisms of action, it is imperative to examine the downstream molecular pathways activated and inhibited by CB1r. There are multiple kinase pathways that may regulated by the endocannabinoid system which are region and cell type specific. Cell survival signaling can be regulated by CB1r stimulation of p38 which is required for hypoxia induced stabilization of erythropoietin mRNA (Chang and Karen, 2001). CB1r can also upregulate JNK signaling to promote apoptosis or ERK1/2 signaling to promote cell survival (Yao and Mackie, 2009; Chang and Karin, 2001). These two pathways are prime targets to determine naked mole-rat eCB-dependent hypoxia response. Furthermore, the determinant of which pathway the endocannabinoid system promotes may be regulated by translational changes determined by open reading frame variants in CNR1. Gene variants of CNR1 have been shown to directly mediate CB1r expression in response to hypoxia and low glucose stress, these variants can rapidly respond to cellular anoxia and current CB1r conditions (such as an increase or decrease of activity) and translate the appropriate variant to react to conditions (Barbosa et al., 2013; Eggert et al., 2015).

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APPENDIX

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EDUCATION

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PUBLICATIONS/PRESENTATIONS

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Browe, B.M., Vice, E.N., And Park, T.J. (2018). Naked mole-rats: Blind, naked, and feeling no pain. *The Anatomical Record.* Early View https://doi.org/10.1002/ar.23996

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Browe, B. M., et al. (2009). Long-term hypoxia and adipose tissue structure in the near-term fetal sheep. *Supplement to Reproductive Science 16 (3).*

Scientific Presentations:

Society for Neuroscience Meeting, 2019. *Chicago, Il "*Naked mole-rats, neuroprotection, and the endocannabinoid system: Upregulation of Cannabinoid Receptor 1 in naked mole-rats leads to increased control of neuroprotective characteristics during hypoxic and anoxic insult."

Wonder and Skepticism, April 2019. *Chicago, Il* "Naked mole-rats, getting high and living forever: Lessons in endocannabinoid control of development."

Society for Neuroscience Meeting, 2018. *San Diego, Ca* "Short-term inflammatory pain in the African naked mole-rat is driven by P2Xr and can be attenuated by the endocannabinoid system."

UIC Biological Colloquium, 2018. *Chicago, Il.* "Naked mole-rats: Lessons in evolutionary potential from extreme environments."

UIC Spring Symposium, 2017. *Chicago, Il.* "Chicago Scientists march for awareness."

'Sense'ational Neuroscience Symposium, 2017. *Chicago, Il* "Peripheral to Central and Back." *Chicago, IL.* "Endocannabinoid pain attenuation at different developmental states of the African naked mole-rat."

Sid Simpson Spring Symposium, 2016. "Characterization of naked mole-rats' endocannabinoid system."

NIH Marijuana and Cannabinoids: A Neuroscience Research Summit, 2016. *Washington DC.* Panel participant for: Legal restrictions to medical marijuana roundtable.

Society for Neuroscience Meeting, 2016. *San Diego, Ca* "The unusual structure and function of the African naked mole-rat endocannabinoid system."

American Association of Anatomists, Annual Meeting at Experimental Biology, 2013. "Many faces of Anatomy." *Boston, Ma.* "Innovative technology expands student laboratory experience during medical gross anatomy course: Addition of iPads in lab revolutionizes how anatomy is taught."

Association of American Medical Colleges, Annual Meeting, 2012. *San Francisco, Ca "*Innovative approach to the gross anatomy teaching lab."

Society for Gynecologic Investigation, Annual Meeting, 2009. "Science and Women's Health: The Impact of Genes, Hormones and Environment." *Glasgow, Scotland* "Long-term hypoxia and adipose tissue structure in the near-term fetal sheep."

GRANTS

Dean's Scholar Fellowship. 2019-2020. $25000 stipend plus tuition and fees. Competitive fellowship for PhD candidates at UIC.

Excellence in Teaching Award. 2019. $100. For Bios 486- Neuroethology and Animal Behavior.

Excellence in Research Award. 2018. $100. For publications in Science and The Anatomical Review.

Travel Award-Biological Sciences Award. 2018. $600. For travel expenses incurred at the Society for Neuroscience 2018 meeting.

Travel Award- Graduate Student Council. 2016. $275. For travel expenses incurred at the Society for Neuroscience 2016 meeting.

Travel Award- LAS Award. 2016. $350. For travel expenses incurred at the Society for Neuroscience 2016 meeting.

Travel Award-Biological Sciences Award. 2016. $600. For travel expenses incurred at Marijuana and Cannabinoids: A Neuroscience Research Summit.

Grant- Faculty of Science Award. 2015. $30,570. Co-authored with Thomas Park.

Excellence in Teaching Award. 2016-2017. $100. Bios 486- Neuroethology and Animal Behavior.
2014-2015. $100. Bios100- Cell and Organisms.

Provost/Deiss Scholarship. 2014. $2500.Student research money for cannabinoid project.

OUTREACH

Organizer, UIC Spring LIN (Laboratory of Integrative Neuroscience) Symposium, Spring 2018/2019 (ongoing). Working with 4 other graduate students to organize annual symposium that includes

choosing and inviting two speakers, organizing student data blitzes, hosting and judging poster sessions, and organizing 2 receptions for the speakers.

Treasurer, Science Policy Outreach Team, UIC. 2018. Inaugural year, organized this group with the intent of uniting researchers across disciplines who are interested in advocating for science, evidence-based reasoning, and scientifically-sound policy to the voting-aged public and policymakers. Responsible for managing and arranging all financial interests.

Volunteer, Expanding Your Horizons 2018/2019
Conference day volunteer for day-long symposium that introduces middle school age girls to STEM through multiple workshops.

Committee Member, March for Science Chicago 2017
In charge of planning the March for Science Expo, outreach at UIC, and dealing with diversity issues for the April 22nd March for Science event. Lead organizer for family event planning and head of family/youth science outreach during the science expo.

Host- Upward Bound Tour. 2015. Hosted 25 students from the University of Nevada Reno group. Organized Q&A with graduate students and faculty, animal facility tour, and lunch with undergrads.

Mentoring. 2013-Present. I have mentored/am currently mentoring 14 undergraduate students. Responsibilities included teaching laboratory techniques, organizing research schedules, providing feedback with proposals, applications, and other academic reports. Most students represent at least one underrepresented minority group. 5 have received LASURI awards, 3 have received Honors College research grants, 4 are Honors college students, and 3 are work study students.

PROFESSIONAL EXPERIENCE

University of Illinois at Chicago, Chicago, IL **2015-2018**
Instructor/ Teaching Assistant Neuroethology, Biological Sciences Dept.
Team-taught course, with three instructors. Original position was as a teaching assistant aiding with laboratories that focused on behavioral adaptations in naked mole-rat and mouse pain response, avian migration and evolution, and c. elegans mating and social interactions. In 2018 after promotion to replace one instructor, the avian section was adapted and expanded to include bird song development and labs involving planarian light aversion and Tardigrade extreme survival adaptations.

University of Illinois at Chicago, Chicago, IL **2017-2018**
Instructor Advance Mammalian Physiology, Biological Sciences Dept.
Lectured 3 hours per week for 8 weeks of 16-week course. Revised curriculum for four systemic modules to integrate cellular-based physiological processes with clinically relative examples. The lecture series was also organized to align with the laboratory course.

University of Illinois at Chicago, Chicago, IL **2015-2018**
Instructor Advance Mammalian Physiology Lab, Biological Sciences Dept.

Lab instructor for two sections in 2015 and 2016, one section in 2017 and 2018. Revised curriculum for 16-week course in physiology to present more focus on clinical techniques and create more appropriate labs to correspond with lecture material.

University of Illinois at Chicago, Chicago, IL **2014/2016**

Teaching Assistant Comparative Vertebrate Anatomy Lab, Biological Sciences Dept.

Lab instructor for two sections of 16 students in 2014, aided with practical exams in 2016. Aided students in dissection and distinction of anatomical characteristics of organisms from tunicates to true vertebrates. Extensive dissections of lamprey, shark, cat, and sheep brains were a major portion of the course. Maintenance and use of an extended index of vertebrate specimens was required.

University of Illinois at Chicago, Chicago, IL **2013-2016**

Teaching Assistant Biology of Cells and Organisms Lab, Biological Sciences Dept.

Lab instructor and discussion leader for two sections of 16 students. Aided students in an array of introductory biology concepts including dissection of fetal pigs, genotyping, and proper laboratory techniques.

East Tennessee State University, Johnson City, TN **2011-2013**

Laboratory Coordinator, Anatomy/ Biomedical Sciences Dept.

Instructed first year medical students and physical therapy students in Medical Human Gross Anatomy and Physiology. Dissected prosection to give students a model for each class period. Taught summer course to CRNA that included full dissection of four cadavers for the students as well as teaching the nervous system-based curriculum. Taught suturing and joint aspiration workshops for students of a variety of levels, from middle school to third year medical students. Prepared and maintained skeletal and tissue (organ, plastinated limbs, cross sections, and microscopic) specimens for teaching purposes.

East Tennessee State University, Johnson City, TN **2009-2010**

Research Assistant, Biochemistry Dept.

Researched antibiotic effects on bacterial ribosome assembly; used methods such as ribosomal reconstitution, radioactive binding activity and subunit separation.

East Tennessee State University, Johnson City, TN **2009**

Laboratory Technician, Molecular Biology Core Facility

Aided researchers by performing electrophoresis, real-time qPCR, DNA sequencing, and phosphor imaging.

LOMA LINDA UNIVERSITY MEDICAL CENTER, Loma Linda, CA **2008-2009**

Research Intern

Researched the effect of long-term hypoxia on the leptin pathway in fetal sheep. Focused on the adrenal system's histological changes due to the increased levels of leptin in the tissue.