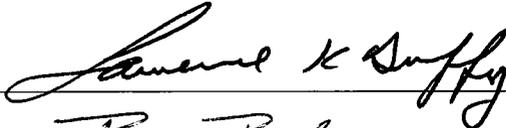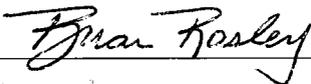# CENTRAL NERVOUS SYSTEM REGULATION OF METABOLIC SUPPRESSION

IN ARCTIC GROUND SQUIRRELS

By

Tulasi Ram Jinka

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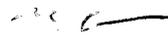
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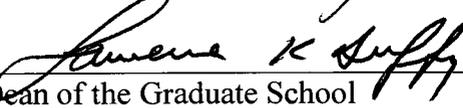
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CENTRAL NERVOUS SYSTEM REGULATION OF METABOLIC SUPPRESSION
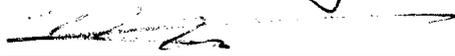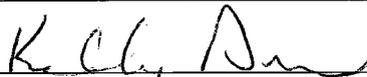
IN ARCTIC GROUND SQUIRRELS

A

THESIS

Presented to the Faculty

Of the University of Alaska Fairbanks

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

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Abstract

The main focus of this dissertation is central nervous system regulation of metabolic

suppression in hibernating mammals in general, and the Arctic ground squirrel

(*Urocitellus parryii*) as a model for seasonal hibernation. Hibernation is a unique

physiological, morphological, and behavioral adaptation to overcome the periods of

resource limitation. Metabolic suppression seen in torpor during hibernation has several

biomedical applications. A multitude of studies have revealed the role of the central

nervous system in regulating hibernation, including a role for neurotransmitters and

neuromodulators. Previous studies have shown that the neuromodulator adenosine

mediates altered thermoregulation during induction of torpor in facultative hibernators,

but it is not clear how adenosine influences torpor in seasonal hibernators. The main

focus of the current project was to test the hypothesis that a seasonal change in purinergic

signaling is necessary for the onset of spontaneous torpor in the Arctic ground squirrel.

My dissertation reports that adenosine meets all of the necessary requirements for an

endogenous mediator of torpor in the hibernating Arctic ground squirrel. A progressive

increase in sensitivity to adenosine A_1 receptors mediated signaling defines the seasonal

transition into the hibernation phenotype. I show that adenosine A_1 receptor activation is

necessary and sufficient to induce torpor in the Arctic ground squirrel. Glutamate is an

excitatory neurotransmitter which is widely studied in hibernation research. My

dissertation demonstrates that N-methyl-D-aspartate type glutamate receptors, located in

the periphery or circumventricular organs, are involved in inducing arousal from torpor in

the hibernating Arctic ground squirrel. This dissertation also presents evidence that

dietary restriction sensitizes adenosine A_1 receptors in rats through an increase in surface

expression in thermoregulatory regions of the brain (hypothalamus). This contributes to

the decline in body temperature and respiratory rate in animals subjected to a restricted

diet, which mimics a torpor-like effect.

TABLE OF CONTENTS

Chapter 4 Role of NMDA Receptors in Arousal from Torpor in Hibernating Arctic

Ground Squirrels 73

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ACKNOWLEDGEMENTS

Chapter 1 General Introduction to Hibernation and Central Nervous System Regulation of Metabolic Suppression in Mammalian Torpor

Overview of hibernation

Hibernation is an adaptation to periods of resource limitation (Carey et al. 2003a; Drew et al. 2007). Torpor in hibernation is the period of metabolic suppression and decreased core body temperature (Drew et al. 2007). True hibernation is characterized by alternating phases of torpor and euthermy that begins in the fall and continues until the hibernation season ceases in spring (Boyer and Barnes 1999; Geiser and Ruf 1995; Lyman 1958). Each torpor bout is divided into 3 phases based on the whole-body metabolic rate and core body temperature namely, entrance, steady-state, and arousal phases (Boyer and Barnes 1999; Carey et al. 2003a; Drew et al. 2007; Heldmaier et al. 2004).

Hibernators may be either facultative or obligate (seasonal) (Drew et al. 2007). Facultative hibernators, such as hamsters, undergo bouts of torpor, when conditions of food shortage and short photoperiods are mimicked (Geiser 2004; Hoffman et al. 1968). Obligate hibernators, such as Arctic ground squirrels (AGS; *Urocitellus parryii*), are strictly seasonal hibernators where torpor bouts depend on a circannual cycle of temperature and day light (Carey et al. 2003a; Drew et al. 2007; Drew et al. 2001).

During periods of hibernation obligate hibernators undergo a variety of complex morphological, behavioral, and physiological adaptive changes to overcome the seasonal

periods of resource scarcity (Carey et al. 2003a; Drew et al. 2007). They suppress their

metabolic rate (rate of oxygen consumption) to as low as 0.01 $mLg^{-1}h^{-1}$ (Buck and Barnes

2000; Geiser 1988) and body temperature to as low as -2.9°C from an euthermic body

temperature of 37°C (Barnes 1989). Periods of torpor are characterized by severe

metabolic suppression, suppressed body temperature, respiration, and cardiovascular

function at the organismal level (Carey et al. 2003a; Drew et al. 2007; Drew et al. 2001;

Heldmaier et al. 2004; Ross et al. 2006; Tamura et al. 2005; Zhou et al. 2001).

Understanding the mechanisms leading to hibernation has the potential to develop

therapies for conditions such as hemorrhagic shock and cerebral ischemia where failure

in oxygen and blood supply to vital organs leads to death and disability (Drew et al.

2007).

Phases of torpor in hibernation

The entrance phase

During the entrance phase metabolic rate and core body temperature gradually

decline until they reach the lowest limit where the core body temperature is just above the

ambient temperature (Boyer and Barnes 1999; Tamura et al. 2005). Core body

temperature can be as low as -2.9°C in arctic hibernators (Barnes 1989) and metabolic

rate falls to approximately 2% of resting metabolic rates (Buck and Barnes 2000; Geiser

1988). The mechanisms involved in regulating the entrance into torpor are still unclear.

Several explanations exists which can be broadly classified into two categories, both of

them using the concept of temperature 'set-point' in the hypothalamus of the brain. They are:

1. Temperature-dependent metabolic suppression (Passive process)

2. Temperature-independent metabolic suppression (Active process)

According to the temperature-dependent metabolic suppression hypothesis, a set-point for thermoregulation exists in the central nervous system which, when adjusted downwards, results in cooling of the body tissues followed by a slowing of the metabolism.(Heldmaier and Ruf 1992; Hosken and Withers 1997; Snapp and Heller 1981). According to the temperature-independent metabolic suppression hypothesis, active suppression of metabolism takes place first followed by a decrease in body temperature due to a decrease in heat production (Snyder and Nestler 1990). In general, daily heterotherms such as dunnart (Sminthopsis macroura) rely on passive process while small hibernators rely on active process (Geiser 1988; 2004). Temperature-independent metabolic suppression is seen in hibernators such as woodchucks (*Marmota monax*) in which the metabolism is actively suppressed followed by a drop in body temperature (Lyman 1958).

The steady-state phase

The entrance phase is followed by a steady-state phase where the animal maintains its lowest basal metabolism for about 1-3 weeks (Boyer and Barnes 1999; Buck and Barnes 2000; Carey et al. 2003a). During this phase an occasional burst of activity coupled with increased metabolism and heat production is observed and is hypothesized to be an avoidance measure to decreases in body temperature beyond a

certain point (Heldmaier et al. 2004). The steady-state phase of a hibernator represents the nadir of mammalian metabolism (Drew et al. 2007).

The arousal phase

Periodic spontaneous arousals from torpor are initiated in all true hibernators when ambient temperatures are sufficiently low (Dausmann et al. 2004) and they are energetically expensive (Karpovich et al. 2009). During arousal there is a gradual increase in metabolism, respiration and heart rate followed by a gradual increase in body temperature (Drew et al. 2007; Tahti and Soivio 1978). Rewarming from torpor during the arousal phase, without any external source of heat, suggests that hibernation is not a state of energy deficiency (Carey et al. 2003a). The arousal phase is initiated when the animal's metabolic rate and core body temperature gradually starts increasing until it reaches a core body temperature of 35-37°C. Aroused animals maintain euthermic body temperature for about one day before another torpor bout starts (Boyer and Barnes 1999; Carey et al. 2003b). The physiological mechanisms necessitating the energetically expensive periodic arousals to euthermic temperatures are unknown, but under active investigation.

Mechanisms regulating hibernation and the role of the central nervous system

The role of the central nervous system in regulation of hibernation is widely accepted (Drew et al. 2007). Some specific sites in the brain including the hypothalamus, the principal center of thermoregulation, and hippocampus are widely studied and considered to play a significant role in the regulation of hibernation (Heller and Colliver

1974; Popov et al. 1992). Central nervous system regulation of hibernation points towards the likely involvement of certain neuromodulators and neurotransmitters including the excitatory neurotransmitter glutamate (Harris and Milsom 2000), and the inhibitory neuromodulator adenosine (Shintani et al. 2005; Tamura et al. 2005)

Adenosine in mammalian hibernation and induction of torpor

Adenosine is a widely distributed inhibitory neuromodulator in the central nervous system (Shintani et al. 2005). Adenosine decreases neuronal excitability directly and modulates the actions of other neurotransmitters (Dunwiddie and Masino 2001). Adenosine functions through a family of receptors: A_1 (A_1AR), A_{2a} ($A_{2a}AR$), A_{2b} ($A_{2b}AR$), and A_3 (A_3AR) receptors (Olah and Stiles 1995). Endogenous adenosine in the central nervous system is produced from multiple sources, some of which are linked to energy levels (Dunwiddie and Masino 2001; Fredholm et al. 1994; White 1977). One of the major sources of endogenous adenosine production is by dephosphorylation of adenosine triphosphate (ATP). ATP released into the synapse is metabolized to adenosine and acts through the adenosine receptors (Dunwiddie and Masino 2001; Fredholm et al. 1994).

The adaptive process of hibernation demonstrates the use of altered thermoregulation. Miller and Hsu (1992) have shown that exogenous adenosine application to the central nervous system decreased body temperature demonstrating that adenosine can mediate altered thermoregulation state. Evidence from various studies also support the function of adenosine in hibernation (Drew et al. 2007; Shintani et al. 2005; Shiomi and Tamura 2000; Tamura et al. 2005). First, the involvement of central

adenosine in thermoregulation was evident from a study where central administration of aminophylline, a non-specific adenosine antagonist, attenuated hypoxia induced decrease in body temperature (Barros and Branco 2000). Microinjection of the A_1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine into the anteroventral preoptic area attenuated hypoxia-induced decrease in body temperature, suggesting the involvement of A_1AR (Barros et al. 2006). Second, hibernators such as ground squirrels have an increased number of A_1AR in their brains during hibernation (Lee et al. 1993) suggesting a role of adenosine during hibernation. Third, endogenous adenosine has been found to mediate a decrease in body temperature at torpor onset in hamsters (Shiomi and Tamura 2000; Tamura et al. 2005). Administration of the A_1AR agonist N^6-cyclohexyladenosine (CHA) into the lateral ventricle of hamsters induced a hypothermic like response similar to torpor (Shintani et al. 2005; Tamura et al. 2005), but, it was not clear whether CHA-induced torpor or decreased body temperature as simultaneous O_2 consumption and core body temperature were not monitored. Administration of the A_1AR antagonist 8-cyclopenthyltheophylline (CPT; 3nmol) into the lateral ventricle reversed entrance into torpor but did not induce arousal when administered during the maintenance phase of torpor, suggesting a role for adenosine in the entrance phase but not in the steady-state phase of torpor (Tamura et al. 2005).

The involvement of A_2AR in torpor was addressed by the central administration of the A_2AR antagonist 3, 7-dimethyl-1-propargylxanthine (DMPX; 3nmol) which did not induce arousal (Tamura et al. 2005). Inhibitory constant (K_i) values of DMPX for A_1AR and A_2AR populations differ by about only two-fold making DMPX a non-

selective adenosine receptor antagonist (Muller et al. 2000; Muller et al. 1997). When

CPT, with an inhibitory constant (K_i) value of 24 nM for A_1AR (Bruns et al. 1986),

reverses torpor with 3nmol dose, the same 3 nmol dose is not sufficient to induce effect

with DMPX with a K_i value of 12000 nM for A_1AR, and 5600 nM for $A_{2a}AR$ (Solinas et

al. 2005). Instead, the dose should be increased by many folds to be effective. Because

the K_i values of DMPX for A_1AR and A_2AR populations differ by about only two-fold,

the result of increasing dose might be effective but non-specific. (E)-3-(3-

hydroxypropyl)-8-[2-(3-methoxyphenyl) vinyl]-7-methyl-1-prop-2-ynyl-3,7-

dihydropurine-2,6-dione (MSX-2) with a K_i value of 9.1 $\pm$ 1.8 nM (Sauer et al. 2000) has

nearly 100 times more affinity for $A_{2a}AR$ over A_1AR and greater than 10,000 times over

A_3AR (Solinas et al. 2005), making it a selective $A_{2a}AR$ antagonist (Solinas et al. 2005).

Thus MSX-2 may be a good choice over DMPX due to high affinity. (E)-phosphoric acid

mono-(3-{8-[2-(3-methoxyphenyl) vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-

tetrahydropurin-3-yl}propyl) ester disodium salt (MSX-3) is a water soluble prodrug that

is converted to the potent $A_{2a}AR$ selective antagonist MSX-2 *in vivo* (Solinas et al. 2005).

All of these studies suggest the role of adenosine in altered thermoregulation during

entrance into torpor but it is not clear from these studies whether there is any seasonal

influence in altered thermoregulation during entrance into torpor in obligate hibernators

such as Arctic ground squirrels.

Glutamate in hibernation

Glutamate is of interest as an important signal in hibernation especially during the

arousal phase. Glutamate is an excitatory amino acid neurotransmitter (Collingridge and

Singer 1990), and has a ubiquitous presence in the central nervous system. This

transmitter acts through N-methyl-D-aspartate type glutamate receptors (NMDAR), α-

amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid receptors (AMPAR), and kainate

receptors (Monaghan and Cotman 1985; Siegel and Agranoff 1999). NMDAR are widely

distributed throughout the nervous system of all mammals, and have been demonstrated

in the peripheral nervous system and circumventricular organs of AGS or other rodent

species (Monaghan and Cotman 1985; Petralia et al. 1994; Zhao et al. 2006). NMDAR, a

subclass of ionotropic receptors which are activated by glutamate (Davies et al. 1979),

are thought to play a role in respiration in hibernators (Harris and Milsom 2001). Various

sites in the central nervous system including the hypothalamus and hippocampus, which

have a wide distribution of NMDAR are involved in arousal by regulating the

thermogenic mechanisms (Heller 1979; Lyman 1982).

The suprachiasmatic nucleus (SCN) is also considered a part of hibernation

circuitry which influences the circannual cycle of hibernation (Bitting et al. 1994; Coon

et al. 1995; Drew et al. 2007; Kilduff et al. 1982; O'Hara et al. 1999; Ruby et al. 1996;

1998; Yu et al. 2002). A decrease in glutamate immunoreactivity was observed in the

SCN of torpid animals suggesting increased transmitter release (Nürnberger et al. 2000).

Glutamate mediated NMDAR activation opens the ion channel resulting in the influx of

Na^+ and Ca^{2+} and the generation of action potentials (Mayer and Miller 1990; Purves

2008; Siegel and Agranoff 1999). Inhibition of NMDAR by intraperitoneal injections of

MK-801 (Dizocilpine hydrogen maleate, (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-

dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate, a non-competitive NMDA

glutamate receptor antagonist) in the hibernating golden-mantled ground squirrel initiated

arousal (Harris and Milsom 2000). The pharmacological non-specificity of MK-801

induced response does not exclude other hypotheses as MK-801 is known to inhibit

several non-NMDAR (Galligan and North 1990; Hung et al. 2006). Site-specific studies

are also needed to identify the exact locations where glutamate might be acting in

regulating hibernation because of wide distribution of NMDAR in central (Collingridge

and Singer 1990) peripheral (Gill and Pulido 2001; Zhao et al. 2006) and

circumventricular regions (Zhao et al. 2006) of the nervous system.

Food restriction in hibernation

Food deprivation in facultative hibernators such as hamsters encourages onset of

torpor (Geiser 2004; Hoffman et al. 1968) . In non-hibernating rodents food restriction

results in a mild decrease in body temperature (Contestabile 2009; Conti et al. 2006;

Ungvari et al. 2008). The exact mechanism of central nervous system regulation behind

either processes is unknown (Tabarean et al. 2009). Understanding the central nervous

system regulation in metabolic suppression or body temperature reduction to hibernators

will benefit non-hibernating species such as humans. Food restriction has become a key

neuroscience focus that is relevant to healthy aging and the metabolic syndromes.

Pharmacological induction of torpor in non-hibernating species

The ability to translate aspects of hibernation to a non-hibernating species will

open possibilities of applying the concept of metabolic suppression and low body

temperature to certain conditions like stroke, hemorrhagic shock, cerebral ischemia, and

multiorgan failure (Drew et al. 2007). Blackstone et al. (2005) and Scanlan et al. (2004) conducted studies to induce torpor like state in non-hibernating rodents. A torpor-like metabolic suppression, a state of suspended-animation, has been demonstrated in mice with inhalant hydrogen sulfide (H_2S); (Blackstone et al. 2005). This suspended-animation like state is characterized by a drop in metabolic rate which precedes a drop in core body temperature as ambient temperature is decreased. 3-Iodothyronamine (T_1AM) is found in the brain and is considered to be an endogenous derivative of thyroid hormone. It is an agent that, when systemically administered, causes an immediate decrease in body temperature and bradycardia in rats (Scanlan et al. 2004). However, it is unclear whether this decrease in body temperature is similar to the metabolic suppression seen in torpor of hibernators since the metabolic rate was not monitored during the experiment.

Summary of chapters and significant findings

Hibernation and daily torpor may have evolved in various species as a protective adaptation to environmental pressures and resource limitation (Carey et al. 2003a; Drew et al. 2007; Heldmaier et al. 2004). This adaptation illustrates several protective traits such as hypothermia, neurosuppression, enhanced antioxidant defense and hypometabolism (Drew et al. 2007). Unraveling the mechanisms regulating hibernation will pave the way to useful biomedical applications (Carey et al. 2003a; Drew et al. 2007). Research is being performed to understand in more detail the mechanisms of hibernation of which study about neuromodulators and neurotransmitters are also of great interest although several missing links still exist (Carey et al. 2003a; Drew et al. 2007;

Harris and Milsom 2000; Harris and Milsom 2001; Shintani et al. 2005; Tamura et al. 2005).

In chapter 2, I demonstrate that activation of the A_1 adenosine receptor (A_1AR) is necessary and sufficient to induce torpor in AGS during the hibernation season, but not during the off-season when AGS do not spontaneously hibernate. I also show that sensitization to the effects of endogenous adenosine is a seasonally regulated switch that facilitates A_1AR mediated torpor.

In chapter 3, I demonstrate that that dietary restriction lowers body temperature via sensitization of thermoregulatory or metabolic effects of endogenous adenosine by increasing surface expression of A_1AR.

In chapter 4, I demonstrate that activated peripheral or circumventricular NMDAR are necessary to maintain torpor in hibernating arctic ground squirrels.

In chapter 5, I discuss ethical issues arising with study of hibernation and develop two case studies while in chapter 6; I conclude my findings by providing future directions.

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Chapter 2 Season Primes the Brain in an Arctic Hibernator: Adenosine A$_1$ Receptor Mediates Torpor

A seasonally regulated change in the gain in purinergic signaling allows endogenous adenosine to induce torpor via adenosine A$_1$ receptor activation in hibernating arctic ground squirrels.

Hibernation, defines a nadir in metabolic demand and body temperature, termed torpor, and is fundamental to adaptation to seasonal periods of reduced resource availability. Adenosine mediates a decrease in body temperature at torpor onset. How, or if, adenosine and season interact to induce torpor has been unknown. Here we show that A_1 adenosine receptor activation is sufficient to induce torpor in arctic ground squirrels (*Urocitellus parryii*) but requires a switch in sensitivity that is mediated by a change in season. Sensitization to endogenous neuromodulators is thus a fundamental process in a complex system response to seasonal changes in environmental pressures. This fundamental mechanism found to induce a hypometabolic state and facilitate cooling may translate into pharmacotherapies for medical conditions such as stroke, cardiac arrest, hemorrhagic shock and trauma.

Hibernation is essential for survival during seasonal deficiencies in food supply in several diverse lineages of mammals(*1-3*). Survival is achieved by severe metabolic suppression, termed torpor, where rates of O_2 consumption fall to as low as 1% of resting metabolic rate and core body temperature (T_b) falls to as low as -3°C(*3-5*). Torpor in hibernating mammals thus defines the nadir of mammalian metabolism and T_b, but mechanisms regulating initiation of torpor have been poorly understood(*3, 6*). In seasonal (obligate) hibernators torpor depends on a circannual cycle(*7, 8*). Once torpor ensues animals rewarm spontaneously every 2 to 3 weeks for brief (12-24h) periods of normal body temperature (termed euthermy). This cycle continues until torpor ceases to occur in the spring. A two switch model suggests that one physiological switch initiates the onset

of the hibernation season and another switch initiates the onset of torpor(*9*). Adenosine is

involved in thermoregulation(*10, 11*) and more recently endogenous adenosine has been

found to play a role in decreasing body temperature at torpor onset in hamsters

(*Mesocricetus auratus*)(*12, 13*). A seasonal change in purinergic signaling was therefore

hypothesized to be necessary for the onset of spontaneous torpor in the arctic ground

squirrel (AGS), an obligate hibernator. Here we show that activation of the A_1 adenosine

receptor (A_1AR) is necessary and sufficient to induce torpor in AGS during the

hibernation season, but not during the off-season when AGS do not spontaneously

hibernate. Sensitization to the effects of endogenous adenosine thus serves as a

seasonally regulated switch that facilitates A_1AR mediated torpor.

To investigate if A_1AR activation by endogenous adenosine is necessary for the

onset of spontaneous torpor in AGS, the A_1AR antagonist 8-cyclopentyltheophylline

(CPT; 3nmol/10μL/min) was administered into the lateral ventricle during onset of

spontaneous torpor, via an indwelling intracerebral ventricular (icv) cannula. CPT,

delivered by an investigator unaware of treatment, reversed torpor onset in all animals

tested while the vehicle had no effect (Fig. 2.1).

We next asked if A_1AR activation was sufficient to induce a state of torpor that

mimicked spontaneous torpor both in temporal profile and in magnitude of decline in the

rate of O_2 consumption and T_b. We also investigated if the sensitivity to torpor-inducing

effects of N^6-cyclohexyladenosine (CHA), an A_1AR agonist, would increase as the

hibernation season progressed. Six AGS instrumented with icv cannula open to the

lateral ventricle were administered CHA (0.5nmol/10μL/min) or vehicle in a blinded,

balanced, cross-over fashion at three times of the year. These 3 tests commenced during the off-season when AGS were not displaying spontaneous torpor, during the early hibernation season after all AGS had begun to display spontaneous torpor and during the middle of the hibernation season.

CHA administered during the off-season, induced a slight, temporary, reduction in O_2 consumption and T_b in all AGS tested (Fig. 2. 2a,g). Early in the hibernation season the same dose of CHA delivered to the same 6 AGS induced a torpor-like decline in O_2 consumption and T_b in 2 out of 6 animals tested (Fig. 2. 2b,h) and an off-season like response in the remaining 4 animals (Fig. 2. 2c,i). By mid-hibernation season (mid-season), the same dose of CHA induced a torpor-like response in all of these same 6 animals (Fig. 2. 2d,j). The torpor-like response to CHA resembled spontaneous entry into torpor (Fig. 2. 2e,k,). Vehicle had no effect on O_2 consumption or T_b in any of the animals at any season tested (Fig. 2.5). The two animals that displayed CHA-induced torpor when tested early in the hibernation season had exhibited slightly more bouts of spontaneous torpor than the other 4 animals. Other variables did not predict the larger response in these animals (Table 2.1).

To investigate if the seasonal change in response to CHA was specific to CHA or was due to a non-specific change in sensitivity to sedative-hypnotic drugs, pentobarbital was administered during the mid- and off-seasons. Pentobarbital induced a similar effect regardless of season and the effect was similar to that of CHA during the off-season (Table 2.2). Pentobarbital was administered by intraperitoneal (ip) injection rather than via an icv cannula because it is not well tolerated when administered icv. To insure that

ip injections did not interfere with drug-induced torpor, separate groups of AGS were treated with CHA, ip, during the mid- and off-season. During mid-season, CHA (0.5mg/kg, ip) induced torpor (Table 2.3). These results indicate that sensitivity to the torpor-inducing effects of the A_1AR agonist CHA increases as the hibernation season progresses and that the increase in sensitivity is specific to CHA. It is unlikely that the seasonal response to CHA was due to differences in cold adaptation since animals were housed at 2°C throughout the study period.

Although CHA is fairly selective for A_1AR, it has some affinity for A_3AR(*14*) leading us to ask if A_3AR activation could account for CHA-induced torpor. The A_3AR agonist, 2-CI-IB-MECA (3nmol/10μL/min, icv) delivered during mid-season, failed to induce torpor in any of the animals tested, although a subsequent injection of CHA (0.5nmol/10μL/min, icv) induced torpor as observed previously (Fig. 2. 3a) indicating that A_3AR activation is not sufficient to induce torpor. Both A_1AR and $A_{2a}AR$ play a role in sleep(*15-17*); and torpor is in part an extension of sleep(*18*). We therefore asked if $A_{2a}AR$ receptors contribute to the onset of torpor. MSX-3, a water soluble pro-drug of the selective, high affinity, $A_{2a}AR$ antagonist MSX-2(*19*), failed to reverse onset of spontaneous torpor in any of the animals tested (Fig. 2. 3b). These results indicate that $A_{2a}AR$ activation is not necessary for torpor onset.

We conclude that adenosine meets all of the necessary requirements for an endogenous mediator of torpor in hibernating AGS. Moreover, a progressive increase in the sensitivity of AGS to A_1AR mediated signaling defines the seasonal transition into the hibernation phenotype and provides an example of a seasonal switch proposed in the

two switch model for obligative hibernation(9). We show that in the context of this model increased gain in purinergic signaling serves as the first switch and stimulation of A_1AR by endogenous adenosine serves as a second switch that induces torpor (Fig. 2.4). Prolonged torpor in hibernating mammals is distinguished by at least three distinct processes that include onset of torpor, maintenance of torpor and arousal from torpor(6). In hamsters (*Mesocricetus auratus*) A_1AR activation is necessary for torpor onset, as shown here for AGS, but is not necessary to maintain prolonged torpor(13). Seasonal alterations in signaling events that may be involved in torpor maintenance or interbout arousal are as yet unclear. Moreover, while the present results demonstrate that A_1AR activation is necessary and sufficient to induce torpor in AGS, it is unlikely that adenosine is the only neuromodulator involved with torpor onset.

A_1AR signaling is sensitized in rats fed a restricted diet and these rats maintain a lower resting T_b than their fully fed cohorts(20). An increase in A_1AR sensitivity may be reflected in a lower resting T_b. Slight decreases in resting, euthermic T_b precedes onset of torpor in hamsters (*Mesocricetus auratus*)(21), Eastern chipmunk (*Tamias striatus*)(22) and AGS(6). Why a lower resting T_b was not evident in AGS that responded to CHA with a torpor-like response in the present study is unclear, but is most likely due to disturbance of animals prior to drug trials. Overall, these findings demonstrate that adenosine serves as an endogenous mediator of torpor in hibernating AGS and that sensitization to endogenous neuromodulators is a fundamental process in a complex system response to seasonal changes in environmental pressures.

The mechanism which increases gain in purinergic signaling remains unknown. In obligate hibernators such as AGS, hibernation is driven by an endogenous, circannual rhythm that persists in constant photoperiods, with food provided ad libitum and at high ambient temperatures(7, 8, 23). Seasonally regulated response to stress in birds is associated with seasonal changes in receptor expression and circulating levels of corticosteroids(24). Seasonal regulation of sensitivity to endogenous signaling molecules may therefore be fundamental to environmental adaptation.

The capacity to mimic in humans the hypometabolism observed in hibernation may have therapeutic applications. Metabolic suppression, coupled with low tissue temperatures and other adaptations make hibernation highly neuroprotective(25). Central A_1AR stimulation prevents cardiac arrhythmias during cooling in hamsters(26). The ability to induce a hibernation-like state of suspended animation with H_2S has lead to investigation of H_2S as a therapeutic agent(27). Mechanisms involved in spontaneous torpor during hibernation may translate to improved therapies for conditions where oxygen and energy supply fails to meet demand. Such conditions include stroke, cardiac arrest, hemorrhagic shock and trauma.

Acknowledgements

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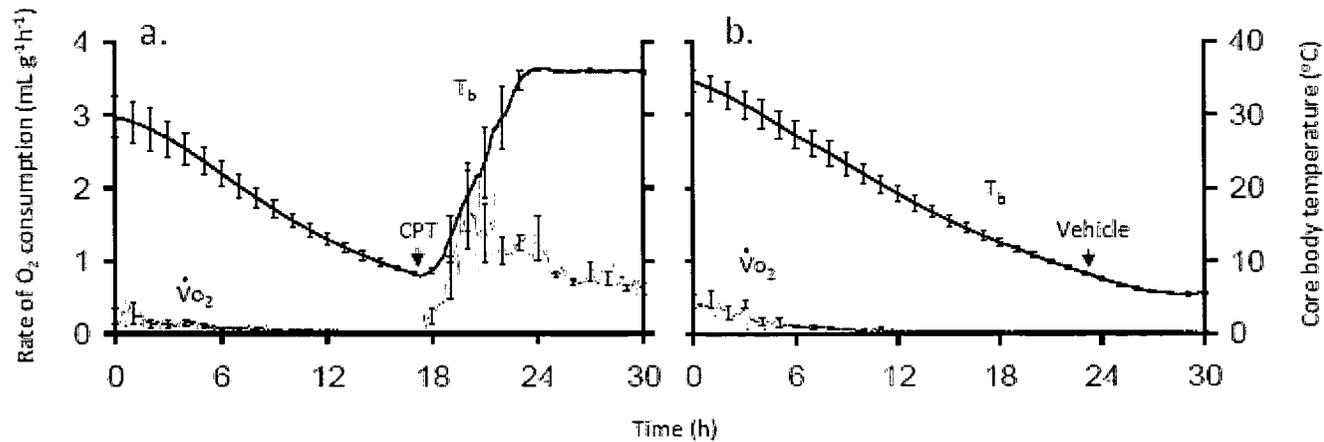
Figure 2.1 Onset of torpor requires A_1AR activation

a, An increase in the rate of O_2 consumption (Vo$_2$) and an increase in core body temperature (T_b) to euthermic levels occurred in all animals tested following administration of CPT (3nmol/10uL/min, icv) during onset of spontaneous torpor. This indicates that A_1AR activation is necessary for torpor onset. b, Vehicle had no effect in any of the animals tested. Results are shown as means and s.e.m; n=6 AGS.

a, CHA (0.5nmol, icv) administered during the off-season when animals were not displaying spontaneous torpor induced a slight, temporary, reduction in the rate of O_2 consumption (Vo_2) and T_b in all 6 AGS tested. b, Early in the hibernation season after all animals showed evidence of spontaneous torpor, CHA induced a torpor-like response in 2 of 6 animals tested. c, In the remaining 4 animals, the same dose of the drug did not induce torpor. d, By the middle of the hibernation season (mid-season), the same dose of CHA induced torpor in all 6 AGS tested. e, Spontaneous torpor in one AGS is shown to illustrate similarity to CHA-induced torpor. f, Pentobarbital (20mg/kg; ip), delivered during the off-season as well as during the mid-season induced a response similar to the response to CHA observed during the off-season (n=3). (The time scale on the x-axis in c applies to e and f and is a continuous 30h). g-l, Detail of the first 4.5 h of a-f illustrates that CHA-induced torpor resembles spontaneous torpor where a rapid drop in metabolism is followed by a slow gradual decrease in T_b. g, During the off-season CHA induces a rapid drop in T_b that begins prior to and at the same rate as the decline in O_2 consumption. By contrast, T_b declines more slowly than O_2 consumption when CHA induces torpor (h,j) and when animals spontaneously enter torpor (k). i, T_b and O_2 consumption decline at similar rates when CHA fails to induced torpor. l, T_b and O_2 consumption decline at similar rates after pentobarbital. Vehicle for CHA or pentobarbital failed to affect T_b or O_2 consumption during any of the seasons tested (Fig. 2.5). Data shown are means. Error bars are s.e.m
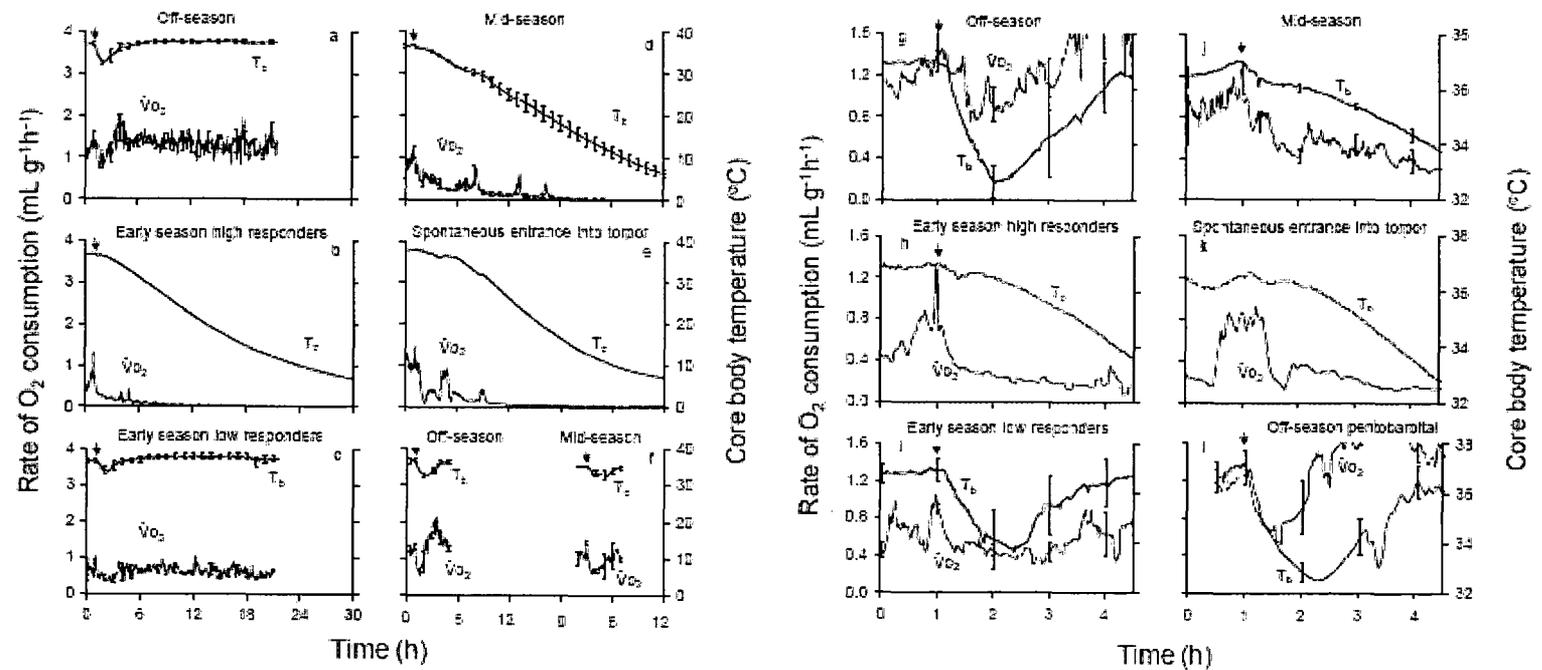
Figure 2.2 Sensitivity to the torpor-inducing effects of the A_1AR agonist CHA increases as the hibernation season progresses
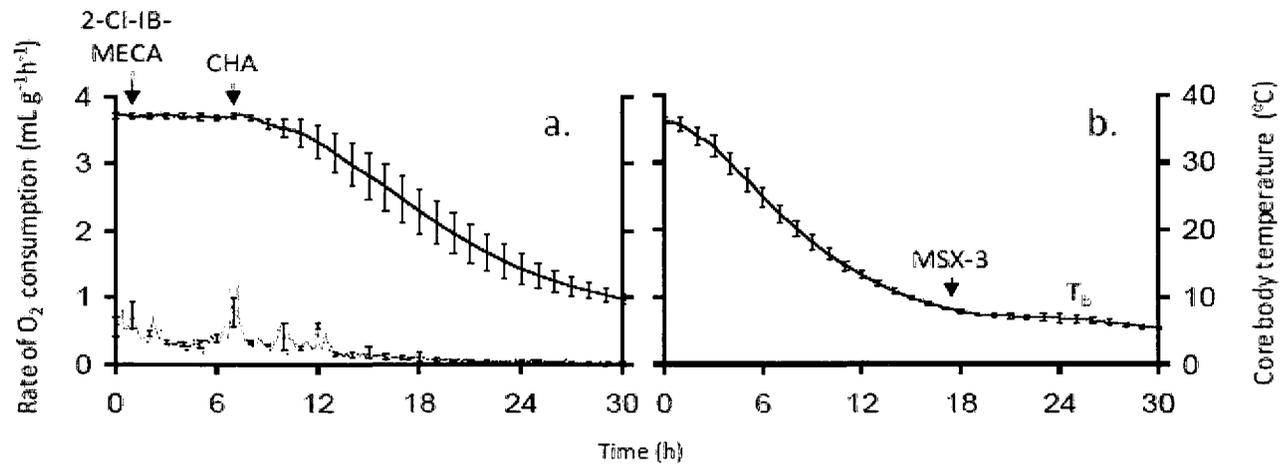
Figure 2.3 CHA-induced and spontaneous torpor is specific to A_1AR

a, The selective A_3AR agonist 2-CI-IB-MECA (3nmol, icv) failed to induce torpor in any of the animals tested, while a

subsequent injection of CHA (0.5nmol icv) induced torpor (n=3). b, MSX-3 (3nmol, icv), a water soluble pro-drug of the

A_2AR antagonist MSX-2, failed to reverse onset of spontaneous torpor (n=3). Data shown are means and s.e.m.

Supporting Material for

Season primes the brain in an arctic hibernator: Adenosine A_1 receptor mediates

torpor

Materials and Methods

Animals: Procedures were approved by UAF IACUC and DoD ACURO. Arctic ground

squirrels (AGS, *Urocitellus parryii*) were captured near 66°38′N, 149°38′W under permit

from the Alaska Department of Fish & Game. Animals were fed rodent chow and housed

at 20°C and natural lighting for their wild-trapped latitude until mid-August when they

were moved to environmental chambers set to 2°C and 4:20-h L:D. AGS remained in

these conditions until the end of the study. The hibernation season was defined by the

presence of spontaneous torpor. The off-hibernation season (off-season) was defined by

an absence of spontaneous torpor. Torpor was monitored daily by placing shavings on the

animal's backs. An animal was noted to be torpid if shavings remained undisturbed the

following day.

Surgery: Under sterile conditions, telemetry transmitters (model VM-FH, MiniMitter or

model CTA-F40, Data Sciences International) were implanted under isoflurane

anesthesia. The head was leveled in a rat stereotaxic frame (Stoelting). Copalite® (Cooley

& Cooley) was applied to the skull. A target was marked at AP_{EBZ} +8.5mm, L_{EBZ} +

3.0mm, the arm tilted 15° and the cannula tip repositioned on the target. An internal

cannula extending 1.0mm beyond the guide cannula was connected to a syringe primed

with sterile saline. The cannula assembly was lowered 5.5mm from the brain surface and

retracted until CSF was withdrawn. The guide cannula was secured to anchoring screws

(Stoelting) and plugged with a dummy cannula (Plastics One). Animals received

enrofloxacin (Bayer Health Care,) (5mg/kg, sc BID for 3 days), and ketoprofen (Fort

Dodge Animal Health) (1mg/kg, QD, sc for 3 days total). When CTA-F40 transmitters

were used animals received buprenorphine (Hospira) (0.03mg/kg, QD, im for 3 days) and

2 weeks separated transmitter surgery and icv cannula surgery. Following surgery,

animals were housed at 20°C 4:20-h L:D and wounds cleaned for at least 10 days before

returning to environmental chambers at 2°C. Surgery was performed at least 1 month

prior to drug testing.

O_2 consumption and body temperature: A cylindrical Plexiglas metabolic chamber (dia.

28cm, height 23cm) on a rat-turn (Bioanalytical Systems, Inc.) was positioned over a

telemetric receiver and T_b was acquired using DataQuest software A.R.T.2.3 (Data

Sciences International). Air was drawn from a gas tight swivel at the bottom of the

chamber, filtered, passed through a mass flow controller at 3L/min (Model, 840, 0-

5L/min, Sierra Instruments Inc.), and a subsample was passed through a multiplexing

valve system, dried by a Nafion® drier used in reflux mode (model PD-50T-24-PP, Perma

Pure LLC) before passing through the O_2 and CO_2 analyzers (Model FC-1B and CA-2A,

Sable Systems International). The automated data acquisition and analysis software

(LabGraph, developed by Tøien) interpolated between calibrations. O_2 consumption was

corrected for respiratory volume change according to the principles of the Haldane

transformation (*1, 2*). The integrity of the system was tested during and after the study

period by burning 100% ethanol. Measured O_2 consumption was within 4% of that

calculated from the weight loss of the lamp.

Drug administration: Drugs were administered using a blinded, balanced, cross-over design with at least one week between drug and vehicle administration. For icv administration of CHA, injection cannulae primed with CHA or vehicle by an observer unaware of treatment were connected to a perfusion pump (Harvard Apparatus). Animals were anesthetized as described above and fit with a harness and injection cannula in a way that allowed animals to move freely within the metabolic chamber. After recovery from anesthesia, baseline O_2 consumption and T_b were collected for 1h before delivering the drug (0.5nmol CHA,10μL/min) or vehicle (10μL/min). O_2 consumption and T_b were monitored for at least 24h or until T_b was stable. In a separate group of animals, a Y-injection cannula (Plastics One) was primed with 2-CI-IB-MECA; (3nmol/10μL) and the secondary line was primed with CHA (0.5nmol/10μL). Animals were treated as above except that the injection of 2-Cl-IB MECA (10μL/min) was followed by a second injection of CHA (3.3μL to clear the cannula of 2-Cl-IB MECA, then 10μL of CHA at 10μL/min).

Additional animals received pentobarbital (20mg/kg, ip) during the mid or off-(non-hibernating) season and T_b and O_2 consumption were monitored as described above. To insure that the stress of injections did not interfere with drug-induced torpor, a separate group of AGS was also administered CHA, ip during mid-season. As expected, CHA, (0.5mg/kg, ip) induced torpor (Table 2.3).

For antagonist studies, torpid AGS were handled daily to mimic handling necessary for the experiment until handling failed to induce arousal. At the next signs of torpor when T_b dipped to about 34°C, AGS were fit with a harness and an injection

cannula primed with antagonist (CPT, 3.0nmol/10μL) or vehicle by an experimenter

unaware of treatment. When T_b reached 10°C, 10μL was delivered over 1min and the

cannula left in place for an additional 24h. MSX-3; (3.0nmol/10μL) was administered in

the same way to another group of animals. The 3nmol dose of CPT, MSX-3 and 2-Cl-IB-

MECA was considered to be equipotent since the ligands have similar affinities for the

A_1, A_{2a} or A_3 adenosine receptors respectively(3, 4).

Drugs: N^6-Cyclohexyladenosine (CHA), 8-cyclopentyltheophylline (CPT) and

phosphoric acid mono-(3-{8-[2-(3-methoxyphenyl) vinyl]-7-methyl-2,6-dioxo-1-prop-2-

ynyl-1,2,6,7-tetrahydropurin-3-yl}propyl) ester disodium salt (MSX-3) hydrate were

purchased from Sigma-Aldrich, Inc., and 2-chloro-N^6-(3-iodobenzyl) adenosine-5'-N-

methyluronamide (2-Cl-IB MECA) was purchased from Tocris Bioscience. CHA was

dissolved in 0.01M phosphate buffer, CPT and 2-Cl-IB-MECA was dissolved in 1%

DMSO and MSX-3 hydrate was dissolved in water. All solutions were sterilized by

0.2μm filtration prior to use except for pentobarbital sodium which was obtained as an

injectable solution (50mg/mL) (Abbott Laboratories).

Supplementary Figures and Tables

Schematic diagram modified from the two switch model of Serkova et al, 2007(5) (Fig.

2.4) illustrates how seasonal sensitization of purinergic signaling primes the brain for

adenosine-induced torpor during the hibernation season. The off-season, commonly

referred to as the "summer-active" season is indicated by a white background. During the

off-season, overflow of adenosine that occurs as part of normal purinergic signaling fails

to induce torpor. Here we use homeostatic sleep drive as an example of normal purinergic

signaling(6, 7). The present report shows that an increase in the gain in purinergic

signaling occurs during the hibernation season. The hibernation season is indicated by a

dark background and the shading from gray to black illustrates an increase in gain in

purinergic signaling as the season progresses. This increased gain in purinergic signaling

during the hibernation season primes the brain such that overflow of endogenous

adenosine with subsequent activation of A_1AR now induces torpor. The increase in

overflow of endogenous adenosine is inferred from the ability of an A_1AR antagonist

(CPT) to reverse onset of spontaneous torpor. The connection with sleep is hypothetical

and used here only as an example of endogenous adenosine signaling.

To control for nonspecific effects of pharmacological treatments, solutions used to

dissolve the drugs (vehicle) were administered in a balanced cross-over design by an

observer unaware of treatment. In this way, half of the animals received drug on the first

test and vehicle on the second test and the other half received vehicle on the first test and

drug on the second. Drug and vehicle tests were separated by at least 1 week. None of

the vehicles tested produced a notable effect on body temperature (T_b) or rate of oxygen

consumption (V_{O_2})

We asked if characteristics such as body weight, sex, age and timing or evidence of prior

torpor bouts predicted the magnitude or quality of the CHA-induced response during the

early hibernation season. The two animals that displayed CHA-induced torpor when

tested early in the season (Early season) had exhibited slightly more bouts of spontaneous

torpor prior to this CHA test than the other 4 animals (Table 2.1). Other variables did not

predict the larger response to CHA in these animals. Data shown in Table 2.1 also

illustrates that hibernation season was defined by the presence of spontaneous torpor. In

these animals progression of the hibernation season was evident from the number of

torpor bouts noted since the onset of spontaneous torpor. The circannual cycle of

obligate hibernators, such as AGS, will free run when animals are housed under constant

L:D conditions(8, 9). This free-running circannual cycle explains why the first day of

spontaneous torpor occurred in July in most of the animals in this study.

To determine if the seasonal difference in response to CHA was specific to CHA or if

other hypnotic drugs would induce torpor during the hibernation season, pentobarbital

was administered, ip, to 2 groups of animals. One group was tested during the off-season

when animals failed to demonstrate spontaneous torpor. Another group was tested during

the mid-hibernation season when the total number of bouts of spontaneous torpor ranged

between 12 and 16 bouts. Table 2.2 shows that the characteristics of AGS treated with

pentobarbital are similar to the off-season and mid-season groups of AGS treated with

CHA. Unlike the CHA study design, two different groups of animals were administered

pentobarbital during the 2 seasons. Injections of pentobarbital, ip, were noted to produce

a brief, but detectible increase in Vo_2 that was not noted with icv administration (Fig.

2.2f). To insure that ip injections did not interfere with drug-induced torpor, separate

groups of AGS were treated with CHA, 0.5mg/kg, ip, during the off-season and during

the mid-season. Briefly, animals were implanted with IPTT-300 transponders (Bio

Medic Data Systems, Inc. Seaford, DE), subcutaneously between the scapula. T_b was

monitored using a telemetry system (DAS-6000; BioMedic Data Systems, Seaford, DE)

in the home cage every 30-60min for at least one hour prior to drug injection and every

1h post-injection for 4h and again 30h after ip injection of CHA. Because the IPTT

transponders are not reliable below about 30°C, to confirm minimal T_b at 30h, rectal

temperature was monitored with a thermocouple (Model H H21 Microprocessor

Thermometer, Type J-K-T Thermocouple (OMEGA Engineering,INC., Stamford, CT) in

animals that were torpid after 30h. Data shown in Table 2.3 show that ip injections of

CHA induced torpor during the mid-season, but not during the off-season as seen for icv

administration. Characteristics of AGS were similar to other groups of animals tested

during these 2 seasons.

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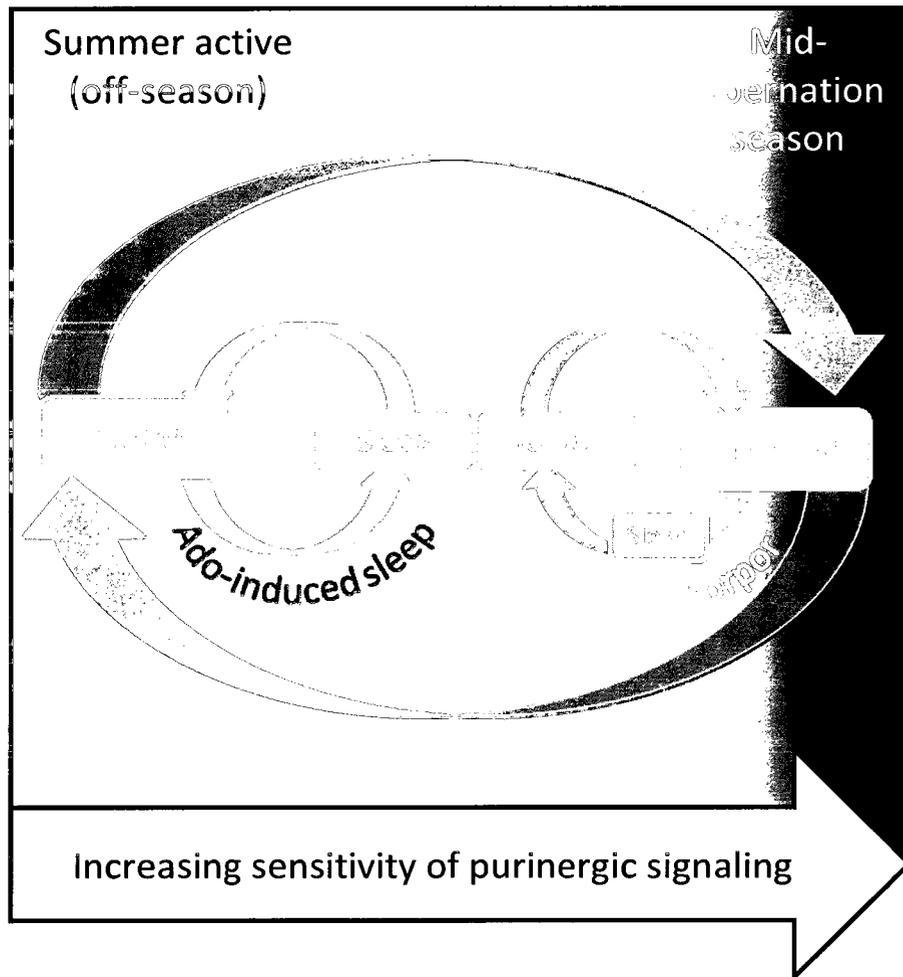
Figure 2.4 Enhanced purinergic signaling turns on the seasonal switch to hibernate in

arctic ground squirrels.
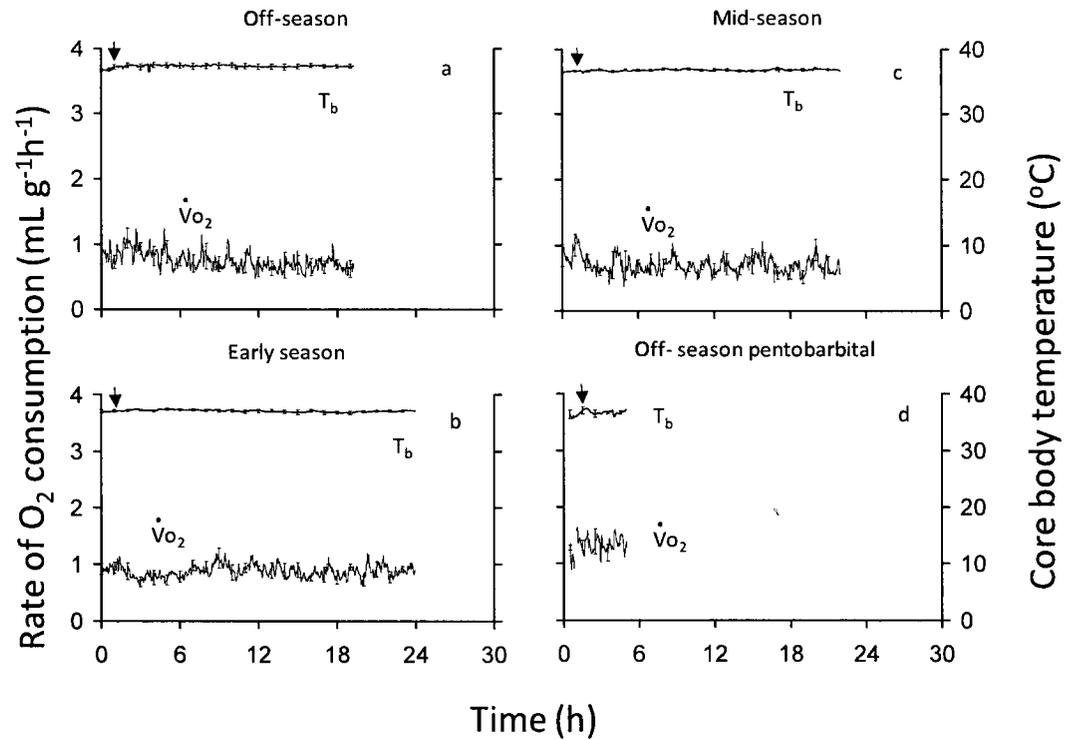
Figure 2.5 Vehicle had no effect in any of the season tested

a-c, Vehicle (0.01M phosphate buffer, icv, for CHA); and d, (saline, ip, for pentobarbital) failed to produce any notable change in T_b or rate of O_2 consumption (Vo_2). Data shown are means and s.e.m., n=6 AGS.

Table 2.1 Characteristics of AGS treated with CHA during the three test seasons

AGS ID	05-25	04-86	04-58	05-03	05-13	05-06
Age	Adult	Adult	Adult	Adult	Adult	Adult
Sex	Male	Male	Male	Male	Male	Male
Last day of torpor during previous season	18-Apr-07	4-Feb-07	19-Feb-07	3-Feb-07	17-Jan-07	15-May-07
First day of spontaneous torpor	26-Aug-07	1-Jul-07	25-Jul-07	22-Jul-07	1-Jul-07	1-Jul-07
Body weight (g)						
Off-season	601	551	820	680	630	681
Early season	860	881	801	940	700	801
Mid-season	906	800	860	808	700	751
No. of spontaneous torpor bouts prior to CHA test						
Off-season	0	0	0	0	0	0
Early season	3	5	4	4	8	7
Mid-season	9	9	8	9	13	13
Minimum T_b induced by CHA (°C)						
Early season	31.9	32.0	32.8	30.9	4.3	3.9
Mid-season	4.6	3.9	5.2	5.3	4.3	4.7
Minimum V_{O_2} induced by CHA ($mLg^{-1}h^{-1}$)						
Early season	0.22	0.17	0.26	0.3	<0.02	<0.02
Mid-season	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02

Table 2.2 Characteristics of AGS treated with pentobarbital, ip, during the off-season and during the middle of the hibernation season

Off-season			
AGS ID	04-25	04-48	04-73
Age	Adult	Adult	Adult
Sex	Male	Male	Male
Body weight (g)	714	707	689
No. of spontaneous torpor bouts prior to pentobarbital test	0	0	0
Minimum T_b induced by pentobarbital (°C)	30.0	32.6	32.0
Minimum Vo_2 induced by pentobarbital ($mLg^{-1}h^{-1}$)	0.56	0.85	0.52
Mid-season			
AGS ID	08-61	08-46	07-74
Age	Adult	Adult	Adult
Sex	Female	Female	Male
Body weight (g)	826	606	911
No. of spontaneous torpor bouts prior to pentobarbital test	12	16	13
Minimum T_b induced by pentobarbital (°C)	33.2	30.0	33.1
Minimum Vo_2 induced by pentobarbital ($mLg^{-1}h^{-1}$)	0.62	0.23	0.52

Table 2.3 Characteristics of AGS treated with CHA (0.5mg/kg, ip)					
Off-season					
AGS ID	05-27	07-106	05-21	07-54	07-98
Age	Adult	Adult	Adult	Adult	Adult
Sex	Male	Male	Male	Male	Male
Body weight (g)	929	769	722	951	684
No. of spontaneous torpor bouts prior to CHA	0	0	0	0	0
Minimum T_b induced by CHA (°C)	30.8	35.2	35.9	34.6	32.7
Mid-season					
AGS ID	08-82	08-98	08-86	08-77	
Age	Adult	Adult	Adult	Adult	
Sex	Female	Female	Female	Female	
Body weight (g)	713	711	700	722	
No. of spontaneous torpor bouts prior to CHA	9	7	7	8	
Minimum T_b induced by CHA (°C)	3.8	4.4	5.1	4.4	

Chapter 3 Altered Thermoregulation via Sensitization of A_1 Adenosine Receptors in Dietary Restricted Rats

Abstract

Rationale Evidence links longevity to dietary restriction (DR). A decrease in body temperature (T_b) is thought to contribute to enhanced longevity because lower T_b reduces oxidative metabolism and oxidative stress. It is as yet unclear how DR decreases T_b.

Objective Here we test the hypothesis that prolonged DR decreases T_b by sensitizing adenosine A_1 receptors (A_1AR) and adenosine-induced cooling.

Methods and Results Sprague-Dawley rats were dietary restricted using an every other day feeding (EODF) protocol. Rats were fed every other day for 27 days and then administered the A_1AR agonist, N^6-cyclohexyladenosine (CHA; 0.5mg/kg, ip). Respiratory rate (RR) and subcutaneous T_b measured using IPTT-300 transponders were monitored every day and after drug administration. DR

animals displayed lower RR on day 20 and lower T_b on day 22 compared to animals fed ad libitum and displayed a larger response to CHA. In all cases, RR declined before T_b. Contrary to previous reports, a higher dose of CHA (5mg/kg, ip) was lethal in both dietary groups. We next tested the hypothesis that sensitization to the effects of CHA was due to increased surface expression of A_1AR within the hypothalamus. We report that the abundance of A_1AR in the membrane fraction increases in hypothalamus, but not cortex of DR rats. *Conclusion* These results suggest that EODF lowers T_b via sensitization of thermoregulatory effects of endogenous adenosine by increasing surface expression of A_1AR.

Discussion Evidence that diet can modulate purinergic signaling has implications for the treatment of stroke, brain injury, epilepsy and aging.

Introduction

Dietary restriction (DR), defined by a decrease in food intake, lowers core body temperature, enhances longevity and attenuates progression of neurodegenerative diseases in animal models (Contestabile 2009). These effects have been suggested to be through a reduction in metabolic demand associated with a decrease in body temperature (T_b) (Ungvari et al. 2008). A recent study found that increasing temperature of the hypothalamus in mice, decreased core T_b and increased life span (Conti et al. 2006). However, a mechanistic link between

metabolism or nutrient homeostasis and temperature is lacking (Tabarean et al. 2009).

It has also been shown that stimulation of the adenosine A_1 receptor (A_1AR) within the hypothalamus decreases T_b (Shintani et al. 2005). Although there are many studies showing that A_1ARs play a role in thermoregulation (Steiner et al. 2002; Swoap et al. 2007; Tamura et al. 2005) there are no studies to our knowledge that have linked alterations in purinergic signaling to DR-induced cooling.

In this study we test the hypothesis that DR imposed by every other day feeding (EODF) lowers T_b by sensitizing the response to A_1AR stimulation. Moreover we test the hypothesis that sensitization is associated with increased A_1AR surface expression within the hypothalamus. Sensitization to cooling effects of A_1AR stimulation via increased A_1AR surface expression may account for the chronic decrease in T_b following DR and contribute to increased longevity in DR animals.

Materials and Methods

Experimental Animals: All procedures were approved by UAF's Animal Care and Use Committee. Male Sprague- Dawley rats (90 days old) (Simonson Laboratories, Gilroy, CA) were housed 2 per cage at an ambient temperature of 20°C, on 12:12 L:D and fed either ad libitum (AL) or a dietary restricted diet. IPTT-300 transponders (BioMedic, Seaford, DE) used to monitor T_b were implanted on day 1. Dietary restriction was started on day 2. Dietary restricted

animals were fed every other day (ad libitum for 24h periods) and food was

removed on alternate days. Body temperature (T_b) and respiratory rate (RR),

monitored by visual inspection, were monitored daily between 11:00 am and 1:00

pm just prior to feeding if food was added. Body weight was measured every

fourth day after collecting T_b and RR. Drug was delivered on day 29 after 27 days

of DR. On day 29, 5 animals from each dietary group were injected

intraperitoneal with either N^6-cyclohexyladenosine (CHA; 0.5 or 5.0 mg/kg)

(Sigma-Aldrich, St. Louis, MO) or vehicle (0.01M phosphate buffer pH 7.0 for

0.5 mg/kg and DMSO, 1% for 5.0 mg/kg). T_b and RR were monitored every 30

min commencing just prior to drug injection and for 4 h after drug injection. T_b

and RR were recorded again 24h after drug injection. DR was continued for 48h

after drug testing. Animals were anesthetized with isoflurane and brain tissue was

rapidly dissected and frozen in liquid nitrogen then stored at -80°C until use.

Cell Fractionation for Western blot: In order to insure that changes in receptor

levels were not influenced by the introduction of CHA only tissue from DR and

AL animals treated with vehicle were used in the western blot experiments.

Approximately 30 µg of frontal cortex or whole hypothalamus were homogenized

and fractionated as described previously ((Raval et al. 2003). Briefly, tissue was

suspended in ten times volume of freshly made ice-cold homogenization buffer [4

mM ATP, 100 mM KCl, 10 mM imidazole, 2 mM EGTA, 1 mM $MgCl_2$, 20%

Glycerol, 17 µg/mL (1mM) PMSF, 20 µg/mL soybean trypsin inhibitor, 25

μg/mL leupeptin, and 25 μg/mL aprotinin] with 0.05% Triton X-100. The

suspended tissue was homogenized in an all-glass homogenizer (10-15 strokes)

then centrifuged (1000xg, 4°C) for 10 minutes. The resulting supernatant (soluble,

cytosolic fraction) was removed and centrifuged at 16,000xg and 4°C for 15

minutes and stored at -80°C until use. The initial pellet (particulate, membrane

fraction) was re-suspended in 250μL of the same lysis buffer containing 1%

Triton X-100 and extracted on ice for 60 minutes. The extracted membrane

fraction was then centrifuged (16000xg, 4°C) for 15 minutes to remove debris and

stored at -80°C until use.

Western Blot: Protein concentration was determined using the Bio-Rad protein

assay kit (Bio-Rad, Hercules, CA). Twenty-five μg of protein was separated on

10% SDS-PAGE gels, transferred to nitrocellulose membrane and blocked with

5% milk in TBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) for 1 hour at room

temperature with gentle agitation. Membranes were then incubated overnight

with rabbit anti-A_1AR (1:1000, Cat#PC158, Calbiochem, San Diego, CA) in

TBST + 5% milk overnight at 4°C with gentle agitation. The membrane was

washed 3 times over 30 minutes in 5% milk-TBST (TBS and 0.1% Tween 20) at

room temperature with gentle agitation, then incubated in secondary antibody

(Goat anti-rabbit, 1:10,000, Santa Cruz Biotechnology) for 1 hour at room

temperature. The bands were visualized with enhanced chemiluminescense

(Supersignal West Pico Substrate, Pierce, Rockford, IL) then digitally imaged

using an AutoImager 3400 (Alpha Innotech Corporation, San Leandro, CA) . The

membrane was then stripped by incubation with TBS (pH 2.0; 10 mM Tris-HCl

and 150 mM NaCl) for 30 minutes and re-probed with rabbit anti-Na^+/K^+ATPase

β-1 (1:5000, Cat#06-170 Upstate, Lake Placid, NY) to assess the purity of the

membrane fractions. After visualization the membrane was stripped and re-probed

in the same manner with mouse Anti-β-actin (1:5,000 product# A5316, Sigma) as

a loading control using Goat anti-mouse HRP (1:10,000, BioRad) as the

secondary antibody.

Data Analysis: Digitized images of chemiluminescent bands were analyzed using

ImageQuant 5.2. Total A_1AR content was calculated as the summed densities of

the cytosolic and membrane fractions and normalized as a percent of the mean of

the control (AL) group on each gel.   Surface expression of A_1AR was assessed

from the ratio of density of the cytosolic A_1AR bands to the density of the

particulate A_1AR bands.

Statistics: To assess the effect of diet on T_b and RR, data was analyzed by

ANOVA with repeated measures over time followed by 2-way ANOVAs and

Tukey post-hoc comparisons (SAS, v. 9.1). Baseline T_b and RR prior to drug

treatment are shown and were analyzed as raw data. T_b and RR after CHA or

vehicle injection is shown as raw data, but was analyzed as percent of pre-

injection values to normalize for differences in T_b and RR between DR and AL

groups at the time of drug injection. Data are shown as mean ± SEM. Densities of

immunoreactive bands were expressed as the ratio of cytosolic to membrane

fractions normalized to the average of the AL group for each blot. Western blot

results for DR and AL groups were compared by t-tests (Excel, v. 2007).

Results

Body weight, monitored every 4 days until the day of injection, increased

in both DR and AL fed rats until the day of injection ($p<0.0001$, n=20, main

effect of time). By the day of injection, however, DR rats weighed significantly

less than the AL rats ($p<0.0001$, diet x time; $p<0.05$, n=10,10, DR vs. AL on day

29, Tukey test; Fig. 3.1). No difference was noted in weight gain between groups

of DR and AL fed animals that were subsequently tested with CHA or vehicle.

Dietary restriction decreases resting body temperature and respiratory rate

As expected, DR decreased resting T_b and RR.Three way ANOVAs

showed main effects of diet on T_b ($p<0.0001$) and RR ($p<0.001$). Interestingly,

the diet-induced decrease in resting RR occurred 2 days prior to the diet-induced

decrease in resting T_b. RR was consistently different between groups beginning

on day 20 ($p<0.05$ Tukey tests; Fig. 3.2c). By contrast, resting T_b was consistently

different between dietary groups beginning on day 22 through to the day of drug

testing ($p<0.05$ Tukey tests; Fig. 3.2a).

Dietary restriction sensitizes rats to the effects of CHA on body temperature and respiratory rate

The A_1AR agonist CHA produced a significant decrease in T_b in both AL and DR groups (Fig. 3.2b). [$p < 0.0001$, n=5,5, diet x drug x time for 3-way ANOVA; $p < 0.0001$ drug x time in AL group and $p < 0.0001$ drug x time in DR group in subsequent 2-way ANOVAs]. Post-hoc analysis showed that in animals fed ad libitum, CHA induced a decrease in T_b that was different from vehicle treated animals between 0.5 to 2.5h after drug injection ($p < 0.05$ CHA vs veh at 0.5 to 2.5h, Tukey test). In animals fed a restricted diet CHA induced a decrease in T_b that was different from vehicle treated animals between 0.5 and 3.5h after drug injection ($p < 0.05$ CHA vs veh at 0.5 to 3.5h Tukey test).

The effect of CHA on T_b was greater in DR rats than in AL rats ($p < 0.0001$, n=5,5 diet x time where DR animals treated with CHA were compared to AL animals treated with CHA and vehicle treated animals were not included in the 2-way ANOVA). Post-hoc comparisons showed a difference between AL and DR groups treated with CHA at 0.5 to 3.5h after drug injection ; $p < 0.05$ Tukey tests, Figure 3.2b). These results demonstrate that prolonged DR sensitizes rats to the cooling effects of CHA.

In addition to decreasing T_b, CHA also induced a significant decrease in RR in both dietary groups (Figure 3.2d). [$p < 0.05$, n=5,5, diet x drug x time for 3-way ANOVA; $p < 0.01$ drug x time in AL group, and $p < 0.0001$ drug x time in DR

group in subsequent 2-way ANOVAs]. Post-hoc analysis showed that in animals

fed ad libitum, CHA induced a decrease in RR that was different from vehicle

treated animals between 1.0 to 1.5h after drug injection ($p<0.05$ CHA vs veh at

1.0 to 1.5h, Tukey test). Likewise, in animals fed a restricted diet CHA induced a

decrease in RR that was different from vehicle treated animals between 0.5 and

3.5h after drug injection ($p<0.05$ CHA vs veh at 0.5 to 3.5h, Tukey test). These

data suggested that the effects of CHA on RR were enhanced in DR animals.

DR-induced decrease in T_b is thought to enhance longevity, in part, by

decreasing metabolism. RR declines with metabolic rate in studies of metabolic

suppression during onset of torpor (Elvert and Heldmaier 2005) and is thus an

indirect indicator of metabolic suppression. We therefore asked if DR sensitized

animals to the effects of CHA on RR. The CHA-induced decrease in RR was

indeed greater in the DR group than in the AL group out to 3.5h after drug

administration (Fig. 3.2d). This was evident from a 2-way ANOVA comparing

the effects of CHA in DR and AL fed animals that excluded vehicle treated

animals ($p<0.0001$, n=5,5 diet x time). Post-hoc comparisons showed a

difference between AL and DR groups treated with CHA between 0.5 and 3.5h

after drug injection ($p<0.05$ Tukey tests). These results demonstrate that

prolonged DR sensitizes rats to the effects of CHA on RR.

Contrary to expectations that metabolic suppression results from a

reduction in T_b we found that a decrease in RR preceded the decline in T_b. This

was true for resting RR during 27 days of DR and for CHA-induced decreases in

RR. In support of this interpretation, a 1-way ANOVA with repeated measures over time showed that in DR animals RR reached a minimum within 0.5h after CHA while T_b continued to decline for 2h after CHA ($p<0.05$, Tukey test). Similarly, the small decrease in T_b in the AL fed rats was preceded by a decrease in RR. In the AL group a one-way ANOVA showed that RR reached a minimum at 1 and 1.5h after CHA. By contrast, T_b did not reach a minimum until 2h after CHA.

Effects of the higher 5mg/kg dose of CHA were compromised by side effects and subsequent death. All 10 animals were administered this dose in one test session and subsequent use of this high dose was abandoned.

Dietary restriction increases surface expression of A_1AR in hypothalamus

We next asked if surface expression of A_1AR increased in DR animals as a potential mechanism of sensitization. The relative concentrations of A_1AR were determined in the cytosolic and membrane fractions of vehicle treated AL or DR rat cortex and hypothalamus by western blot. In cortex, the relative density of total A_1AR protein, calculated from the sum of band densities in the cytosolic and membrane fractions was not different between the DR and AL animals ($p>0.05$, n=9,10, t-test). Similarly, the relative proportion of A_1AR in the cytosolic and membrane fractions was not different between the AL and DR groups ($p>0.05$, n= 9,10, t-test). When the total expression of A_1AR was determined in

hypothalamus, again by summing density of bands in the cytosolic and membrane

fractions, there was no significant difference between the AL and DR animals

($p>0.05$, $n=8,10$, t-test). However, the ratio of densities of A_1AR in cytosolic and

membrane fractions of the AL animals was significantly different than the ratio in

DR animals ($p<0.05$, $n=8,10$, t-test, Fig. 3.3). This shift in the relative densities of

the A_1AR from the cytosol to the membrane suggests that A_1AR are mobilized

from a nonfunctional cytosolic pool to a functional surface pool.

We next asked if the effect of DR on A_1AR surface expression or CHA-induced

lowering of body temperature depended on a change in food intake or body weight. In

the DR group, all 5 animals tested gained less weight than the AL fed animals. The body

weight gain in the DR group ranged from 7 to 14g and the drug induced decrease in body

temperature in this group ranged from 6.6 to 7.1°C. The body weight gain in the AL

group ranged from 15 to 44g and the drug-induced decrease in body temperature ranged

from 2.4 to 2.8°C, ($n=5$ per group). These results show that despite a variable weight

gain in the AL fed animals, weight gain had little influence on drug-induced lowering of

body temperature. The small range of body weight gain and drug-induced change in

body temperature in the DR animals made it difficult to assess how weight gain

influenced other dependent variables.

Discussion

Here we show for the first time that DR-induced modification of

thermoregulation is associated with changes in components of the purinergic

neuromodulatory system. DR imposed by EODF sensitizes rats to the cooling and

metabolic depressant effects of CHA, an A_1AR agonist. We also report that DR is

associated with altered surface expression of A_1AR in hypothalamus, but not

cortex. Taken together, these data suggest that DR sensitizes A_1AR through an

increase in surface expression in thermoregulatory regions of the hypothalamus

and in this way contributes to the decline in T_b and RR in animals subjected to

DR.

Here we used a well characterized paradigm of DR, described in the literature as

every other day feeding (EODF) or intermittent fasting (IF) to test the hypothesis that a

decrease in T_b is associated with sensitization to the cooling effects of an A_1AR agonist.

The EODF paradigm, like other DR paradigms decreases T_b (Fraifeld and Kaplanski

1997) typically within 4 weeks of onset (Wan et al. 2004) and increases longevity

(Goodrick et al. 1983). Effects of EODF on T_b (Fraifeld and Kaplanski 1997) are similar

to the effects observed with several other paradigms of DR that are associated with

increased longevity (Goodrick et al. 1983), and decreased metabolism (McCarter and

McGee 1989; McCarter and Palmer 1992; Valle et al. 2005).

The amount of food consumed by rodents fed every other day varies with strain.

Food intake has been reported to be 30% less than rats fed ad libitum (Wan et al. 2004).

However, in C57BL/6 mice increased food intake on feeding days offsets absence of food

intake on fasting days such that overall intake is not decreased (Anson et al. 2003).

Individual food intake was not monitored in the present study to avoid the potential

effects of stress associated with individual housing. Body weight gain in rats fed every

other day in the present study differed only slightly from rats fed ad libitum suggesting that food intake was decreased but was not dramatically different from AL fed rats.

When compared with 40% caloric restriction EODF produces slightly different decreases in heart rate, blood pressure and blood glucose (Mager et al. 2006). It remains to be determined if all DR paradigms enhance the cooling effects of CHA and if sensitization of A_1AR is a common mechanism for altering thermoregulation in all DR paradigms or if a decrease in food intake is necessary for the observed effect. Stress and other aspects of EODF that are not related to decreased food intake may contribute to lowered resting T_b and sensitization to CHA.

A negative energy balance could compromise thermogenic capacity and contribute to lower resting T_b. Others (Fraifeld and Kaplanski 1997) show that lipopolysaccharide (LPS) induces similar maximal T_b in rats fed every other day suggesting that in their study, thermogenic capacity was not compromised. Compromised thermogenic capacity due to a negative energy balance is unlikely since body weight gain in DR rats differed so slightly from AL fed animals.

Restricted feeding schedules which limit food availability to a single meal each day induce and entrain circadian rhythms in food-anticipatory activities in rodents (Verwey and Amir 2009). Animals in the DR group of rats may have entrained to feeding which occurred at the same time every other day. Anticipation of food on feeding days would have been expected to increase activity and T_b for 2 to 3h prior to feeding in the DR group (Fuller et al. 2008). Since T_b and RR were measured just before feeding, food entrainment would have

increased T_b and RR in the DR group, but not in the AL fed animals. While a food entrainment-induced increase on T_b and RR in the DR group can not be ruled out, it was not sufficient to override a significant decrease in resting T_b and RR in this group of animals. Similarly, since RR and T_b were monitored during the middle of the light period, activity and resting T_b should have been at a minimum in AL animals. This assumed minimum in RR and T_b in the AL fed animals was still higher than resting RR and T_b in the DR animals. Thus, while food entrainment in the DR animals and circadian rhythms in the AL animals may have influenced RR and T_b, it is unlikely that the observed differences between these two groups in resting values or CHA-induced decreases in T_b or RR are artifacts of these influences.

In the present study DR induced a decrease in resting RR, an indicator of oxidative metabolism prior to a decrease in resting T_b. Moreover, the CHA-induced decrease in RR occurred prior to the CHA-induced decrease in T_b. A decrease in respiration that precedes a decrease in T_b mimics what is observed in hibernating animals during entrance into torpor (Drew et al. 2007; Elvert and Heldmaier 2005; Karpovich et al. 2009) and suggests that inhibition of metabolism precedes the decline in T_b rather than vice-versa as is often suggested for DR (Conti 2008).

During onset of torpor the decline of metabolism, heart rate and respiratory rate occur in parallel and before a decline in T_b (Elvert and Heldmaier 2005). Thus, in studies of metabolic suppression RR and heart rate serve as

indirect indicators of metabolic suppression. In the present study a decrease in RR is interpreted as a decrease in metabolism. This interpretation is made with the caveat that direct effects of adenosine or CHA on respiratory control centers or stress-induced sympathetic responses could influence RR independent of effects on metabolism. Stress-induced effects on resting RR are unlikely since rats were observed daily and would have been expected to habituate to the observer. It is more difficult, however, to discount direct effects on respiratory control centers.

Adenosine plays a neuromodulatory role in thermoregulation (Barros et al. 2006). Recent evidence supports an emerging role for A_1AR signaling in thermoregulatory alterations during onset of torpor in hibernating animals (Tamura et al. 2005) as well as in daily torpor in mice (Swoap et al. 2007). Because CHA-induced cooling in DR rats qualitatively resembled torpor in hibernating animals where metabolic suppression precedes a gradual decline in T_b, we asked if a maximal dose of CHA would induce torpor and drive T_b closer to ambient temperature. The high (5.0mg/kg) dose of CHA proved to be lethal in AL and DR rats. This was unexpected because previous studies showed that 3mg/kg was well tolerated in rats (Tuovinen and Tarhanen 2004) and 5mg/kg produced sedation and cooling in mice (Assi 2001). Further deaths were avoided by immediately abandoning use of this dose as soon as side-effects and death were noted in the first group of animals tested.

Because sensitization is defined by an altered dose-response curve we asked if a maximally effective dose of CHA in AL fed rats would produce an effect similar to a lower dose of CHA in DR rats. Indeed, we tested a higher dose of CHA expecting to see results consistent with a shift in the dose response curve, however, the higher dose proved lethal in both dietary groups. Inverted U-shaped dose-response curves can cause decreased responsiveness to appear as sensitization when a single dose is tested. Because drug-induced hypothermic effects can follow inverted dose response curves (Jaszberenyi et al. 2002) the possibility exists that enhanced responsiveness to CHA is due to a rightward shift in the dose response relationship and not sensitization. Nonetheless we interpret the current results as evidence for increased responsiveness to CHA because; 1) our hypothesis predicted that DR animals would show an increased response to CHA; 2) a decrease in resting T_b supports increased sensitivity to endogenous adenosine; and, 3) increased surface expression of A_1AR is consistent with sensitization.

A_1AR agonists inhibit lipolysis via direct effects on adipocytes in white and brown adipose tissue (WAT and BAT) (Viswanadha and Londos 2006). Inhibition of lipolysis in BAT inhibits nonshivering thermogensis (Asakura 2004) and therefore the oxidative metabolism associated with this energy demanding process. Although we suggest that CHA suppresses RR and T_b via effects on central thermoregulatory pathways, we can not rule out a peripheral site of action. Sensitization of A_1AR in BAT would increase endogenous adenosine- or CHA-induced inhibition of nonshivering thermogenesis and oxygen consumption and

be expected to produce an immediate decline in RR and a subsequent more gradual decrease in T_b.

If glycogen reserves are depleted in DR animals direct inhibition of lipolysis in WAT could limit fuel availability and impair thermogenesis. Data reported here argues against this interpretation, however, because RR and T_b in the DR group of animals did not vary between days following 24h fasts and days following 24h food availability.

Receptor sensitization suggested by the present results may be a common means to modulate purinergic signaling. Sleep deprivation increases adenosine receptor surface expression which may contribute to sleep drive in humans (Elmenhorst et al. 2007). Although we did not detect a change in total A_1AR expression, studies involving transgenic mice indicate that receptor levels limit response to endogenous adenosine as most effects studied were amplified upon increases in receptor level (Yaar et al. 2005). Results suggest that sensitization may result from a change in the distribution of A_1AR from the cytosol to the membrane. A_1AR interactions with other membrane receptors, enzymes, adaptor and scaffolding proteins as well as dimerization of A_1AR within the membrane play a role in receptor trafficking, internalization and desensitization (Franco et al. 2005). Knowledge of these mechanisms opens the possibility for further investigation into mechanisms of A_1AR sensitization following DR. Moreover, an effect of DR on extracellular levels of adenosine may also play a role in the observed effect. A change in surface expression does not rule out an influence of

presynaptic mechanisms where adenosine kinase plays a primary role in regulating extracellular levels of adenosine (Li et al. 2007).

Adenosine is neuroprotective in a variety of brain injury models via multiple mechanisms including a decrease in T_b (Bischofberger et al. 1997; Xu et al. 2006). The present results suggest that diet may be a means to sensitized A_1AR responsiveness and in this way facilitate therapeutic effects of endogenous or exogenously applied adenosine. Ketogenic diets, like adenosine, protect the brain from several types of injuries (Prins 2008) and decrease incidence of seizure in intractable epilepsy potentially via purinergic mechanisms (Etherington et al. 2009; Masino and Geiger 2009). EODF leads to intermittent periods of ketosis (Anson et al. 2003), but unlike EODF, ketogenic diets have not been shown to decrease T_b or increase life span.

In summary, we show that DR sensitizes rats to the respiratory depressant and cooling effects of an A_1AR agonist. Sensitization of thermoregulatory effects of endogenous adenosine through increased surface expression of A_1AR may play a role in lowered body temperature and enhanced longevity associated with DR. Evidence that diet can modulate purinergic signaling has implications for the treatment of brain injury, stroke, epilepsy and aging.

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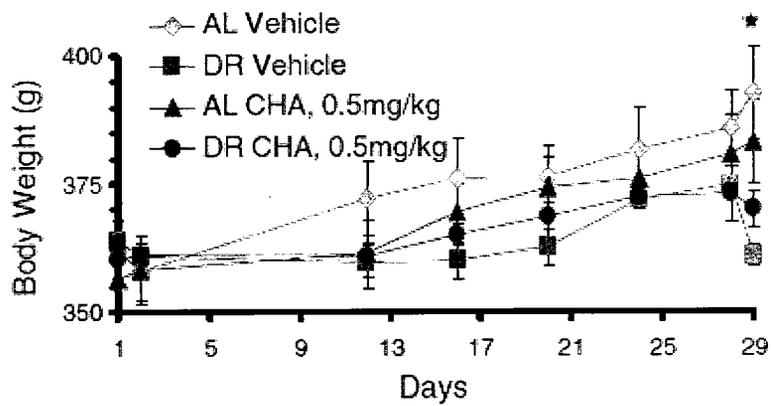
Figure 3.1 Body weight (g) increases over days.

*p<0.05, n=10,10, DR vs AL, Tukey test.
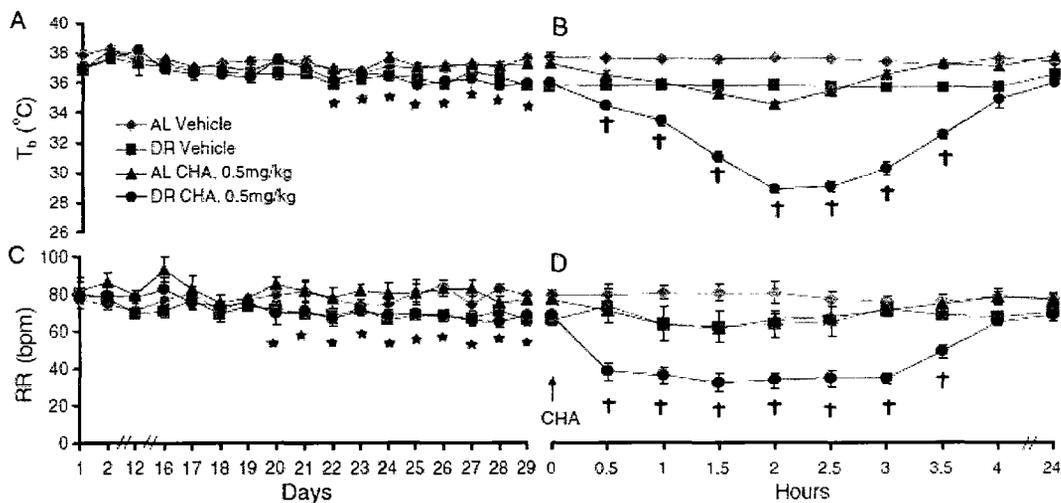
Figure 3.2 Dietary restriction decreases resting body temperature and respiratory rate

Resting T_b is shown over days (A), and every 30 min for 4h and at 24h after CHA

administration (B). Similarly, resting RR is shown over days (C) and every 30 min for 4h

and at 24h after CHA (D). *p<0.05, n=10,10; DR vs AL, Tukey test; †p<0.05, n=5,5;

CHA DR vs CHA AL, Tukey test.
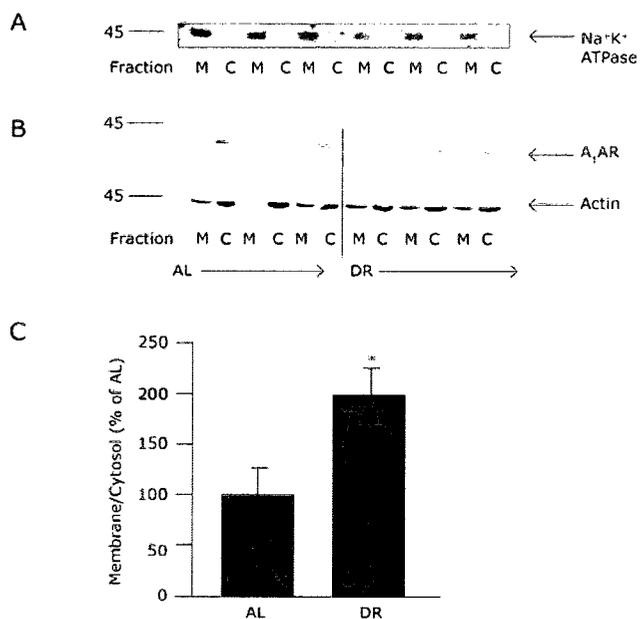
Figure 3.3 Dietary restriction increases surface expression of A_1AR in hypothalamus

Blot (A) demonstrates fraction purity using Na+/K+ ATPase as a marker for the

membrane fraction. Blot (B) shows one representative blot of hypothalamus where

density of A1AR bands in the cytosolic fractions are decreased and density of A1AR

bands in the membrane fractions are increased in rats fed every other day. Graph in (C)

shows that normalized ratios of cytosolic to membrane band densities in hypothalamus

from rats fed a restricted diet are significantly lower than for rats fed ad libitum (*p<0.05,

n=5 per group).

Chapter 4 Role of NMDA Receptors in Arousal from Torpor in Hibernating Arctic Ground Squirrels

Abstract

Rationale: Arctic ground squirrels (AGS, *Urocitellus parryii*) undergo hibernation characterized by profound metabolic suppression and decreased body temperature. Hibernation is broadly divided into 3 distinct phases known as the entrance phase, maintenance phase, and arousal phase. Physiological mechanisms regulating the spontaneous arousals are poorly understood. N-methyl-D-aspartate (NMDA) type glutamate receptors are thought to play a regulatory role in maintenance of torpor in hibernation, and controlling respiration.

Objective: The present study aims at testing the hypothesis that activation of NMDA receptors (NMDAR) are involved in maintenance of torpor in AGS.

Methods and Results: AGS were habituated with intraperitoneal (ip) saline injections prior to drug injections. Post-habituation, ip administration of MK-801(5mg/kg/mL; n=3) induced arousal while intracerebroventricular (icv) administration had no effect. When icv MK-801 (0.2, 2, 20 or 200 μg/10μL, delivered over 1 min; n=5) of habituated AGS failed to induce arousal a peripheral or circumventricular site of action was confirmed by administering intraperitoneal AP5 (5mg/kg/mL; n=5) which does not cross the blood-brain barrier unlike MK-801. Because MK-801 is also known to inhibit 5-HT3 receptors we finally asked whether 5-HT3 receptor activation is necessary for maintenance of

torpor through intraperitoneal administration of MDL-72222 (5mg/kg/mL; n=3), which did not induce arousal in any of the animals tested.

Conclusion: Results suggest that ability of MK-801 and AP5 to induce arousal in hibernating Arctic ground squirrels following intraperitoneal injection is due to a peripheral or circumventricular site of action.

Introduction

Arctic ground squirrels (AGS; *Urocitellus parryii*) hibernate to endure long periods of scarce resource availability. Based on changes in core body temperature (T_b) and metabolic rate (MR), hibernation can be sub-divided into 3 separate stages-stage of entry where T_b and MR decreases; steady-state torpor where T_b and MR remain minimal; and interbout arousal where T_b and MR increase followed by short periods of interbout euthermy (Lyman, 1982, Geiser and Ruf, 1995, Boyer and Barnes, 1999, Carey et al., 2003, Drew et al., 2007). Different mechanisms are thought to regulate entry, steady-state torpor, and arousal from torpor (Lyman and O'Brien, 1963, Twente and Twente, 1978, Lyman, 1982, Carey et al., 2003, Drew et al., 2007). Energetically expensive periodic arousals are observed in hibernators (Geiser, 1988, Buck and Barnes, 2000, Karpovich et al., 2009) and the exact mechanisms are not yet clearly understood (Harris and Milsom, 2000, Drew et al., 2007)

N-methyl-D-aspartate type glutamate receptors (NMDAR) are thought to play a regulatory role during hibernation (Drew et al. 2007; Harris and Milsom 2000; Harris and Milsom 2001). Glutamate synthesized from glucose is converted to glutamine in glia which is then converted to glutamate in neurons (Hamberger et al. 1979; Henry et al.

2007; Siegel and Agranoff 1999). It is hypothesized that glutamatergic

neurotransmission maintains torpor and decreased levels of glucose upon prolonged

torpor leads to depletion of glutamate and glutamatergic neurotransmissions, leading to

arousal. It is hypothesized that activated NMDAR inhibit arousal and facilitate the

maintenance of torpor (Harris and Milsom, 2000). Further research is needed to study the

pharmacological specificity of MK-801 induced response as MK-801 is known to inhibit

several non-NMDAR (Galligan and North, 1990, Hung et al., 2006). Site-specific studies

are also needed to identify the locations where glutamate might be acting in regulating

hibernation because of wide distribution of NMDAR in central (Collingridge and Singer,

1990) peripheral (Gill and Pulido 2001) and circumventricular regions (Zhao et al., 2006)

and the ability of MK-801 to cross blood-brain barrier (Simon et al., 1984, Ozyurt et al.,

1988, Park et al., 1988). We hypothesize that activated NMDA receptors (NMDAR)

maintain torpor in Arctic ground squirrels. We also test the regional specificity of

NMDAR in maintenance of torpor and pharmacological specificity of MK-801 in

inducing arousal from torpor in Arctic ground squirrels.

Materials and Methods

Animals

All procedures were in accordance with the UAF Institutional Animal Care and Use

Committee. Arctic ground squirrels (*Urocitellus parryii*) were captured in the northern

foothills of the Brooks Range in Alaska (66°38′N, 149°38′W) and transported to the

animal facility at the Institute of Arctic Biology, University of Alaska Fairbanks. A

trapping permit was obtained from the State of Alaska Department of Fish and Game.

Animals were maintained on a diet of rodent chow, with daily supplements of carrots and

apples, and water ad lib at an ambient temperature (T_a) of 20°C and natural lighting for

their wild-trapped latitude. Diet was supplemented with sunflower seeds from August 1

until August 15 when AGS were moved to environmental chambers set to an ambient

temperature (T_a) of 2°C and a 4:20-h light-dark cycle. After moving to environmental

chambers carrots and apples were discontinued.

Drugs: MK-801(Dizocilpine hydrogen maleate, (5R,10S)-(+)-5-Methyl-10,11-dihydro-

5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate, a non-competitive NMDA

glutamate receptor antagonist) and AP5 (2-Amino-5-phosphonopentanoic acid, a

competitive NMDA receptor antagonist) were purchased from Sigma-Aldrich, Inc., St.

Lois, MO, USA. MDL-72222 (Tropanyl 3,5-dichlorobenzoate, a 5-HT3 antagonist) was

purchased from Tocris Bioscience, Ellisville, MO, USA. MDL-72222 was dissolved in

1% dimethyl sulfoxide (DMSO). MK-801 and AP5 was dissolved in saline.

Experiments

Intraperitoneal MK-801 was administered initially to test if NMDAR activation is

necessary for maintenance of torpor. We next asked whether central NMDAR are

involved in maintenance of torpor by giving the drug into the lateral ventricle of the brain

through chronically implanted guide cannula. When icv MK-801 failed to induce arousal

we confirmed a peripheral or circumventricular site of action by administering

intraperitoneal AP5. AP5 does not cross the blood-brain barrier (Tonkiss and Rawlins,

1991) unlike MK-801 (Simon et al., 1984, Ozyurt et al., 1988, Park et al., 1988). Because

MK-801 is also known to inhibit 5-HT3 receptors (Galligan and North, 1990, Hung et al.,

2006), we finally asked whether 5-HT3 receptor activation is necessary for maintenance

of torpor through intraperitoneal administration of MDL-72222.

Before administering MK-801 (ip), AP5 (ip) and MDL-72222 (ip), AGS were

habituated to ip injections of saline until they were no longer responsive to saline

injections. Once, habituated AGS were administered vehicle (1mL/kg) or drug [MK-801,

AP5 or MDL-72222 (5mg/kg, i/p)] Arousal was quantified from an arousal index based

on a nominal scale of 0 to 6, where 0 was deep torpor indicated by a respiratory rate of

less than 5 breaths per minute (bpm) and 6 was a fully active animal (Table 1). Later, in

an another experiment using a separate group of animals, the rate of oxygen consumption

and core body temperature were measured using open-flow respirometry and telemetry.

To determine the effect of icv (intracerebroventricular) MK-801, AGS with

chronically implanted guide cannula into lateral ventricle and ip temperature transmitters

were habituated for handling necessary to introduce an injection cannula into the

chronically implanted guide cannula. On the 3rd day of a torpor bout, a cannula primed

with MK-801 was introduced through the guide cannula. On the 3rd day of another torpor

bout, a cannula primed with saline was introduced through the guide cannula.

Approximately 12 hours after inserting the injection cannula into the guide cannula,

saline or MK-801, was injected into the lateral ventricle using a syringe pump. MK-801

was delivered in doses of 0.2, 2, 20 or 200 $\mu g/10\mu l$, delivered over 1 min. Increasing concentrations were delivered in series at 2h intervals. After the last injection, the cannula was left in place for 24 hours while oxygen consumption and core body temperature were monitored. For ip AP5 and MDL-72222 the response to the drug or vehicle injections were monitored through oxygen consumption. The drugs were delivered by a blind observer unaware of the treatment. Handling induced arousal was monitored for comparison with drug-induced arousals. After acquiring at least 2h of baseline O_2 consumption and core body temperature records, AGS were subjected to handling sufficient to induce arousal from torpor (n=3) and O_2 consumption and core body temperature were collected.

Surgery

Surgeries were done before AGS began to hibernate. Animals were anesthetized with isoflurane (induced at 5%; maintained at 2-3% in 100% medical grade oxygen at 1.5 L/min). During surgery, body temperature was maintained at 37°C with a fluid-filled heating pad (Gaymar Industries Inc., Orchard Park, NY). Under sterile conditions, pre-calibrated, temperature and activity telemetry transmitters (model VM-FH, Minimitter Company, Inc., Bend, OR and model CTA-F40, Data Sciences International, Inc., St.Paul, MN) were implanted intraperitoneally. For lateral ventricular cannulation, the squirrels were placed in a rat stereotaxic frame (Stoelting, Wooddale, IL) and the head was leveled. An incision was made from the frontal nasal suture to the area in between the ears and the underlying muscle and fascia was separated by blunt dissection. Copalite® (Cooley & Cooley, LTD. Houston, TX) varnish was applied on the exposed

skull to protect the skull from contamination. Four holes were drilled to accommodate the anchoring screws (Bioanalytical Systems, Inc. W.Lafayette, IN). An icv guide cannula (Plastics One Inc, Roanoke, VA) was stereotaxically implanted into the right ventricle at the coordinates AP_{EBZ} + 8.5 mm, L_{EBZ} + 3.5 mm, $D_{Brain\ surface}$ − 4.0 mm. An internal cannula (Plastics One Inc, Roanoke, VA) fit into the guide cannula extended 1.0 mm beyond the guide cannula was connected to a syringe to withdraw cerebrospinal fluid (CSF). Cannula depth was adjusted until cannula placement in the lateral ventricle was verified by withdrawal of CSF. The guide cannula was secured with dental cement (Stoelting Co., Wood Dale, IL) to stainless steel screws. A metal hook was secured in the cement. The guide cannula was sealed with a dummy cannula (Plastics One Inc, Roanoke, VA)

O_2 consumption and body temperature

A cylindrical Plexiglas metabolic chamber (dia. 28cm, height 23cm) on a rat-turn (Bioanalytical Systems, Inc.) was positioned over a telemetric receiver and T_b was acquired using DataQuest software A.R.T.2.3 (Data Sciences International). Air was drawn from a gas tight swivel at the bottom of the chamber, filtered, passed through a mass flow controller at 3L/min (Model, 840, 0-5L/min, Sierra Instruments Inc.), and a subsample was passed through a multiplexing valve system, dried by a Nafion® drier used in reflux mode (model PD-50T-24-PP, Perma Pure LLC) before passing through the O_2 and CO_2 analyzers (Model FC-1B and CA-2A, Sable Systems International). The automated data acquisition and analysis software (LabGraph, developed by Tøien)

interpolated between calibrations. O_2 consumption was corrected for respiratory volume

change according to the principles of the Haldane transformation(Wagner et al., 1973,

Karpovich et al., 2009). The integrity of the system was tested during and after the study

period by burning 100% ethanol. The system was regarded to be satisfactory if measured

O_2 consumption was within 4% of that calculated from the weight loss of the lamp.

Results

Intraperitoneal MK-801 induces arousal from hibernation

On the 4th day of a torpor bout MK-801 (5mg/kg/, ip) or saline was administered to

hibernating AGS habituated to ip injections of saline. Arousal from hibernation was

quantified using the nominal scale defined in Table-1. MK-801 induced arousal in all

AGS tested while saline injections did not induce arousal in any of the AGS tested (n=3

AGS) (Fig. 4.1).

Intraperitoneal MK-801 induces arousal from hibernation, as monitored through

changes in oxygen consumption and core body temperature over time

In subsequent experiments the rate of O_2 consumption and core body temperature was

simultaneously monitored upon ip injections of MK-801 (5mg/kg) or saline on the 4th day

of torpor bout. Administration of MK-801 (5mg/kg; ip) induced arousal in all the animals

tested (Fig. 4.2a) while animals which received saline injections does not arouse from

hibernation (n=3) (Fig. 4.2b). The rationale for monitoring simultaneous O_2 consumption

and core body temperature is to compare drug-induced arousal to handling-induced

arousal from torpor. Handling-induced arousal is faster than MK-801 induced arousal

(Fig. 4.2c vs 4.2a).

Intracerebroventricular (icv) administration of MK-801 does not induce arousal

We next asked whether central NMDAR are involved in the regulation of hibernation and

if inhibition of central NMDAR by MK-801 results in arousal from hibernation. Centrally

administered MK-801 into the lateral ventricle (0.2, 2, 20 or 200 μg/10μl, delivered over

1 min at 2h intervals) of habituated AGS did not induce arousal in any of the animals

(Fig. 4.3). A two-way ANOVA with repeated measures between higher dose of MK-801

(200μg) and HIA for a period of 2.5h followed by either drug injection or handling the

animals for induced arousal shows a significant difference (*p<0.05) between the drug-

induced effect and HIA starting from 1h post-injection or handling the animal for

disturbance (Fig. 4.3 insert).

Intraperitoneal AP5 induces arousal from hibernation

To confirm that central administration of an NMDAR antagonist is not necessary for

antagonist-induced arousal, AP5, which does not cross blood-brain barrier (Tonkiss and

Rawlins, 1991), was administered intraperitoneally. Administration of AP5 (5mg/kg; ip)

induces arousal in hibernating AGS (n=5) (Fig. 4.4a) while vehicle had no effect in all the animals tested (n=4) (Fig. 4.4b)

Intraperitoneal MDL-72222 does not induce arousal from hibernation

MK-801 is known to inhibit 5-HT3 receptors (Galligan and North, 1990, Hung et al., 2006). To test the specificity of the NMDAR in the maintenance of torpor, MDL-7222 was administered intraperitoneally. Neither MDL-7222 (5mg/kg; ip) nor vehicle induced arousal (n=3) (Fig. 4.5) from hibernation.

Discussion

MK-801 is an anticonvulsant which antagonizes NMDAR as a non competitive antagonist (Collingridge and Singer, 1990). Our results demonstrate that intraperitoneal injections of MK-801 induce arousal from torpor in hibernating Arctic ground squirrels. This suggests that NMDAR activation maintains torpor in hibernating AGS. This result is consistent with a study in Golden mantled ground squirrels (Harris and Milsom, 2000) and confirms the involvement of NMDAR in maintenance of torpor in Arctic ground squirrels.

Involvement of central NMDAR in maintenance of torpor was investigated by administering various doses of MK-801 into lateral ventricles, which did not induce arousal in hibernating Arctic ground squirrels suggesting that central NMDAR are not involved in maintenance of torpor in Arctic ground squirrels. This interpretation was

further supported by results of ip administration of AP5, a selective competitive NMDA-type glutamate receptor antagonist (Lodge et al., 1988, Watkins et al., 1990), which does not cross blood brain barrier (Tonkiss and Rawlins, 1991). These results suggest that peripheral or circumventricular NMDAR may be involved in maintenance of torpor. Although the exact site of action is not clear, peripheral NMDAR are widely distributed in gastrointestinal tract, heart, ovary, uterus, kidney and circumventrucular organs in rodents (Gill and Pulido 2001; Zhao et al. 2006). The possible involvement of circumventricular NMDAR, especially at area postrema and median eminence is a possibility due to a high density of NMDAR at these areas in this species (Zhao et al., 2006). Median eminence is just below the third ventricle and area postrema is at the base of the fourth ventricle. Cerebrospinal fluid from the lateral ventricle passes through third and fourth ventricle before reaching the blood stream (Weed 1938). Drug injected into the lateral ventricle might have been diluted by the time it reached to third ventricle and this might be why MK-801 injected into the lateral ventricle did not show any effect.

The rationale for monitoring simultaneous O_2 consumption and core body temperature is to compare drug-induced arousal to handling-induced arousal from torpor. A previous study has shown that induced-arousal is faster than spontaneous arousal in hibernating hedgehogs (Tahti and Soivio, 1978). Handling-induced arousal tended to be faster than MK-801 induced arousal. MK-801 induced arousal is similar to spontaneous arousal in Arctic ground squirrel at the same ambient temperature of 2°C (Karpovich et al., 2009), suggesting that mechanisms regulating the arousal in both MK-801 induced

and spontaneous arousal may be the same. A further investigation is needed to understand this more clearly.

MK-801 is known to inhibit 5-HT3 receptors (Halliwell et al., 1989, Yamakura et al., 2000) which leads to the interpretation that MK-801 induced arousal may be due to the inhibition of 5-HT3 receptors. This possibility is ruled out by antagonizing 5-HT3 receptors with MDL-72222, which did not induce arousal in hibernating Arctic ground squirrels. In conclusion, our results suggest that ability of MK-801 and AP5 to induce arousal in hibernating Arctic ground squirrels following intraperitoneal injection is due to a peripheral or circumventricular site of action. Future studies may be needed to test the involvement of circumventricular NMDAR in hibernation.

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Table 4.1 Arousal Index Scale

AOUSAL INDEX SCALE	OBSERVATIONS
0	1-5 rpm
1	6-10 rpm
2	Greater than 10 rpm
3	Observable shivering
4	Sporadic body movements
5	Frequent large body movements
6	Full alertness (arousal)

Following intraperitoneal injections of MK-801, arousal was quantified from an arousal index scale based on a nominal scale of 0 to 6 where 0 was deep torpor indicated by a respiratory rate of less than 5 breaths per minute (bpm) and 6 was fully active animal which is completely aroused from hibernation.
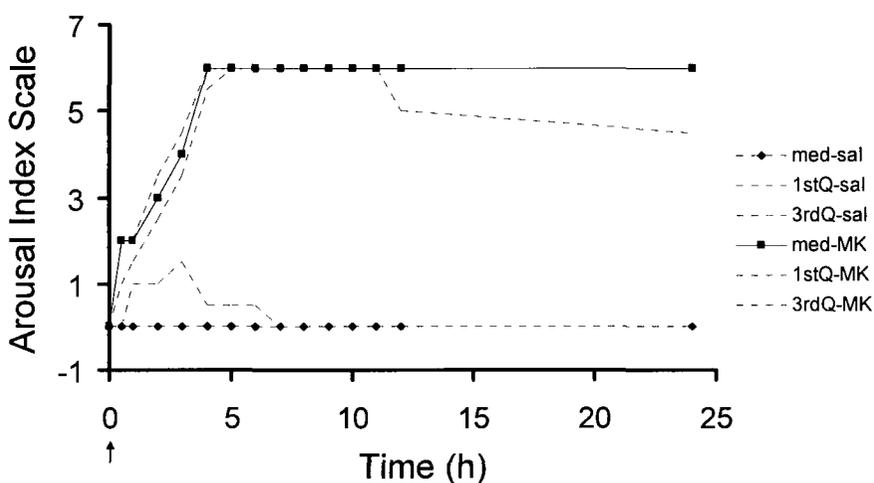
Figure 4.1 Intraperitoneal injections of MK-801 induce arousal in hibernating Arctic

ground squirrels.

On the 4th day of a torpor bout MK-801 (5mg/kg, ip) or saline was administered to

hibernating AGS habituated to ip injections of saline. Arousal from hibernation was

quantified using the nominal scale defined in Table-1. MK-801 induced arousal in all

AGS tested (n=3 AGS) while saline injections did not induce arousal in any of the AGS

tested (n=3 AGS). Arrow represents the time point of injections. Data expressed as

median with first quartile (dotted line below the median) and third quartile (dotted line

above the median).
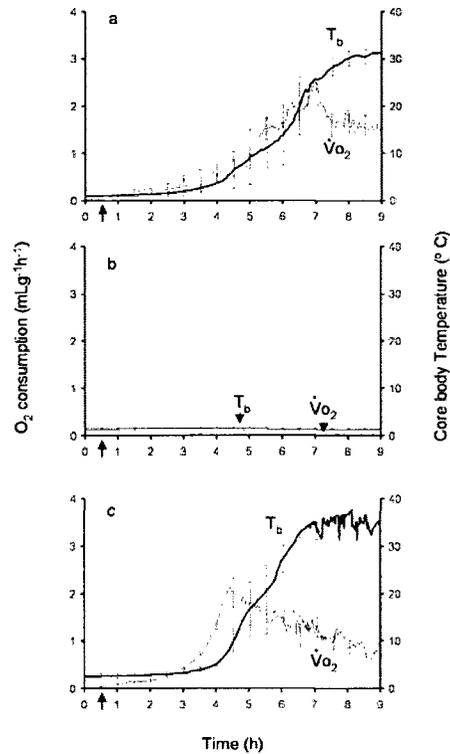
Figure 4.2 MK-801 induced arousal is slower than handling-induced arousal in hibernating Arctic ground squirrels

MK-801(5mg/kg; ip) induced arousal in all the animals tested (n=3) (a) resembles handling-induced arousal (n=3); except that handling-induced arousal is faster than MK-801 induced arousal. Intraperitoneal injections of saline did not induce arousal in any of the animals tested (n=3) (b). Note that the O_2 consumption in Fig. 4.2b is almost merged with x-axis due to a very low metabolism during hibernation. Arrow represents the time point where either drug (a) or saline (b) has been delivered or arousal has been initiated in torpid animals (c). Data are expressed as mean ± s.e.m. Rate of O_2 consumption and core body temperature are abbreviated as V_{O2} and T_b respectively.
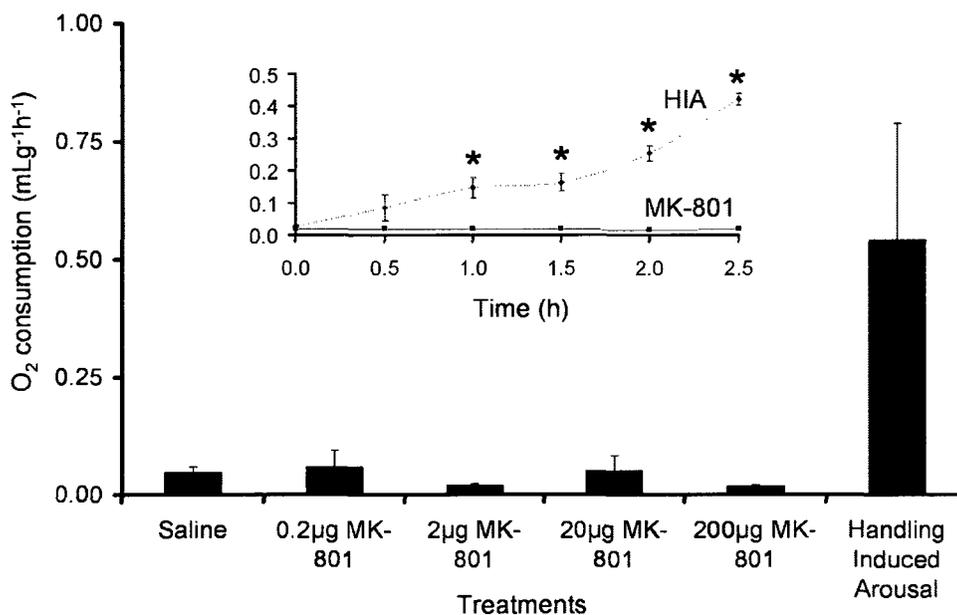
Figure 4.3 Intracerebroventricular (icv) administration of MK-801 does not induce

arousal from torpor in hibernating Arctic ground squirrels.

Mean O_2 consumption for a period of 2.5h following icv administration of MK-801 at

doses of 0.2μg (n=5), 2.0μg (n=4), 20μg (n=3), 200μg (n=3) or saline (n=5) suggest no

significant arousal in hibernating Arctic ground squirrels when compared with handling-

induced arousal (HIA) (n=3). A two-way ANOVA with repeated measures between

higher dose of MK-801 (200μg) and HIA for a period of 2.5h followed by either drug

injection or handling the animals for induced arousal shows a significant difference

(*p<0.05) between the drug-induced effect and HIA starting from 1h post-injection or

handling the animal for disturbance (Insert).
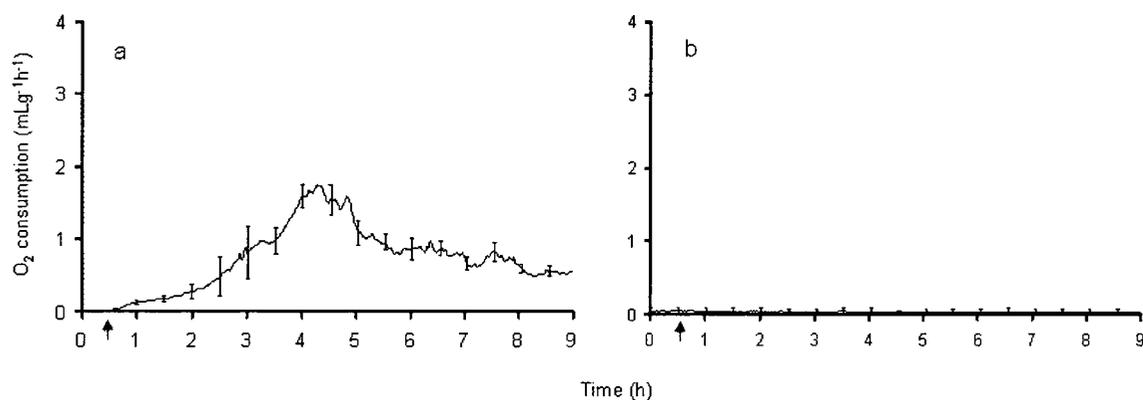
Figure 4.4 NMDA antagonist, AP5, induces arousal in hibernating Arctic ground

squirrels.

Intraperitoneal injections of AP5 (5mg/kg) induced arousal from torpor in all the animals

tested (n=5) (a) while vehicle had no effect in any of the animals tested (n=5) (b). Arrow

represents the time point where drug (a) or vehicle (b) is delivered. Data expressed as

mean ± s.e.m.
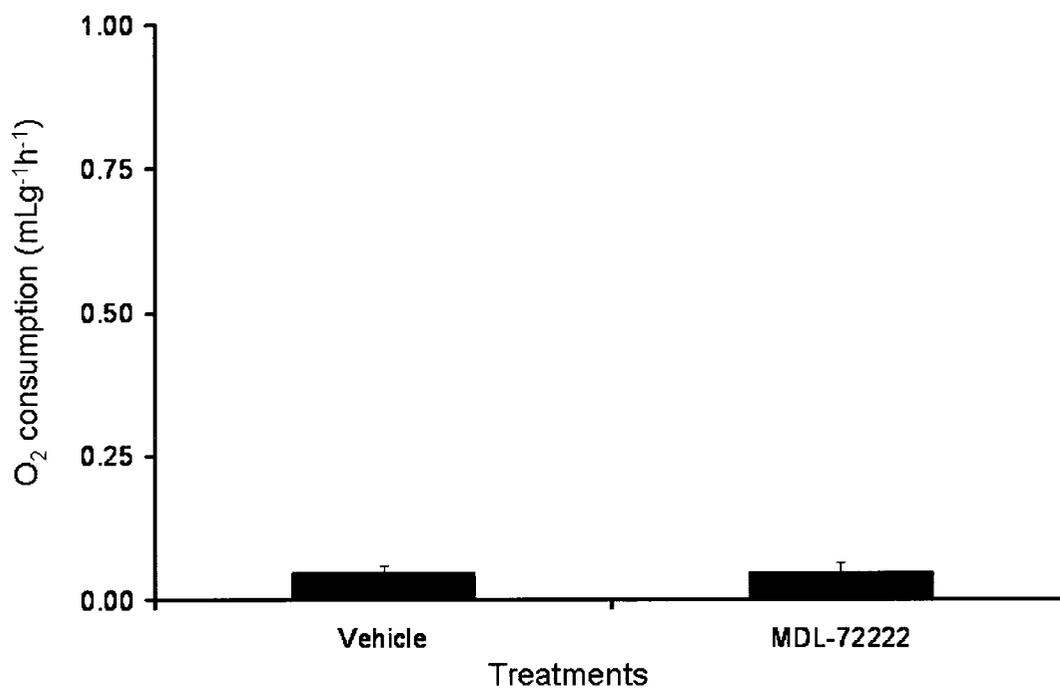
Figure 4.5 5-HT3 antagonist, MDL-72222, does not induce arousal in hibernating Arctic

ground squirrels.

Mean O_2 consumption for 2.5h following intraperitoneal injections MDL-72222

(5mg/kg) or vehicle (n=3) suggests that MDL-72222 or vehicle does not induce arousal

from torpor in Arctic ground squirrels. Data expressed as mean ± s.e.m.

Chapter 5 Hibernation research: a note on animal welfare issues

Abstract

Hibernation research has specific ethical issues in regard to animal welfare. Identifying and addressing important issues the researchers are facing will lead to improved quality in both research outcomes and welfare of hibernators. In this review paper significant ethical issues in hibernation research are identified, critically analyzed, and evaluated in light of past research and future directions. Two case studies and proposed solutions to the issues are discussed, providing best practice recommendations where appropriate to hibernation researchers. Ethical research practices are a key component of scientific integrity and improve public support of research. The animal welfare issue in hibernation research is unique, and an interdisciplinary approach towards identifying specific issues is necessary and will result in more humane outcomes.

Introduction to hibernation

Hibernation is a phenomenon where the animal enters into a stage of suspended

animation [1]. The whole body metabolism of the hibernator drops down drastically to as

low as 1% of the resting metabolism and the core body temperature drops to as low as -

3°C [2, 3, 4, 5, 6] as is defined by the term torpor [5]. All vital activities like respiration,

heart rate, and blood circulation drop down to bare minimum [7], but the animal still

possesses an inherent capacity to revive and survive [6, 5]. The exact mechanisms

underlying this phenomenon of hibernation are under active investigation [8, 9, 10, 1, 11,

12, 13, 14, 15, 16] and there is a progressive increase in the number of hibernation studies

over the past two to three decades (Fig. 5.1). Understanding the physiological

mechanisms regulating hibernation could potentially benefit society by helping to treat

life threatening conditions such as cardiac arrest, stroke, cerebral ischemia, and

hemorrhagic shock [5].

Responsible conduct in hibernation research

The main objective of the animal experiments should be knowledge advancement leading

to a public good. Proper consideration of appropriateness of the experimental procedures,

species selection, research design, and number of animals utilized should be the priority

for ethical research [17, 18]. Professional societies encourage researchers to evaluate

non-animal model alternatives in their respective fields that could answer their scientific

questions before considering animal models [19, 20]. To our best knowledge there is no

alternative that mimics the complexities of the integrated metabolism and seasonal

behavior of hibernators. Scientists in animal research should be well aware that usage of animals is a privilege, not a right [21]. Researchers should avoid unnecessary suffering of the research animals if it can be avoided [22], but replacement is not usually possible in hibernation research. Animal experimentation is justified if the pain to the animals is more than outweighed by an outcome resulting in alleviation of suffering to humans and other animals [23, 24, 25, 26]. Thus, use of hibernation animals in research may be ethically and morally justified only if the benefits of the research to human society can outweigh the suffering.

Ethical aspects in hibernation research

Hibernation research has certain ethical issues to be considered. Animal experimentation for hibernation and any comparative control such as rats has unique considerations. In an attempt to understand the mechanisms of hibernation, various pharmacological studies need to be conducted where drug-induced hibernation experiments push the animal into a "suspended-animation" like state [1], an apparent near-to-death phase. Such situations are ethically challenging to hibernation researchers in the context of animal welfare where the life of animals are at risk.

Ethically challenging situations illustrated by case-studies

Pharmacological induction of torpor is an area of ethically challenging biomedical research which needs to be addressed to find solutions. Two commonly encountered situations are discussed below with a possible solution to each case.

Case 1: X is a graduate student studying the behavior of Arctic ground squirrel (AGS; *Urocitellus parryii*), a seasonal hibernator (Figs. 5.2, 5.3 & 5.4), in response to a drug that is expected to induce torpor. One group was housed in a cold room at an ambient temperature of 2°C. This group was induced to arouse followed by drug injections resulting in torpor induction. Drug injections to cohorts that had also displayed spontaneous torpor in a warm room at an ambient temperature of 20°C resulted in mortality without any signs of distress. Because no obvious signs of distress were exhibited by the animals, intervention was not indicated and would have compromised the outcome of the study. Although the graduate student was unsure whether the drug treated animals were becoming torpid or dying, the circumstances did not warrant intervention. The findings suggest that ambient temperature may be a crucial factor in thermoregulation of AGS. These results led to a modification of the protocol (Fig. 5.5) so that AGS showing signs of spontaneous torpor in the warm room are not treated with this dose of torpor-inducing drug. This protected animals in future studies. The key concept is that in certain types of animal research like hibernation, it is not always possible to predict the outcomes with clarity until the experiment is done. This observation may have implications for pre-operation preparation for subjects undergoing hypothermic surgery.

Case 2: Y is a graduate student working with rats. In an effort to induce a torpor-like state in rats, Y injected a higher dose noted in the literature to be tolerated in mice. The higher dose expected to induce a deeper torpid-like effect, in comparison to a lower dose,

actually resulted in an unexpected respiratory distress and mortality in all the animals tested. Results lead to a modification of protocol (Fig. 5.5) that limited the dose range of the drug. This illustrates that dose-response relationships may differ between species. When testing drugs for the first time in a novel species, a pilot study with small sample size and dose is advised. Animals should be closely monitored post drug administration. Signs of distress, distinct from signs typical of onset of torpor may require intervention. If signs of distress are noted the researcher should request assistance from vet services and administer euthanasia or supporting care as instructed.

Development of tools for efficient utilization of hibernating animals and better animal welfare

Pharmacological induction of torpor: Drug dose information applied to hibernation studies are derived from studies on other rodent species usually mice or rats. Predicting their outcome in hibernators is difficult. A carefully designed pilot study is needed in these cases where the doses cannot be anticipated precisely (Fig. 5.6). Behavioral variations occur within hibernators; a facultative hibernator (eg: hamster) undergoes torpor when suitable conditions are mimicked while torpor in a seasonal hibernator (eg: AGS) is driven by a circannual cycle. Pharmacological agents used on facultative hibernators may or may not have an influence on seasonal hibernators and vice versa. Conducting a direct study involving 8-10 animals per group without a prior pilot study on a smaller group of animals is not in line with precautionary principle due to the possibility of unexpected mortality in the study group of animals.

During pharmacological induction of torpor, it is almost impossible to determine whether the animal is becoming torpid. Ethically, an animal should be relieved of the distress, pain and suffering whenever needed by human interference [17]. But, it is not possible in pharmacologically-induced hibernation studies. An investigator may not be able to decide whether to interfere or not with the experiment. A careful discretionary approach is needed in such situations (Fig. 5.7).

Stereotaxic atlas: Infusion of drugs into specific areas of brain in seasonal hibernators requires chronic implantation of guide cannula (Surgery section of Chapter 2), which in turn requires a brain atlas which is lacking for many species. Researchers rely on an established rodent atlas [27, 28, 29, 30, 31, 32, 33, 34] or sacrifice some animals to establish the brain coordinates on a trial and error basis, a deviation from the 3R principle (Refinement, Reduction, and Replacement) [35]. Variations in the cannula placement may be reduced by developing an atlas through careful post experimental anatomical exploration of the brain and skull. When a group of animals are implanted with intracranial probes aimed at drug delivery to a specific target site, it is not possible to figure out the exact placement until the brain tissue is collected and analyzed for probe placement after completion of the study. The best possible approach would be to develop an atlas based on stereotaxic placement of intracerebral probes, careful histological evaluation [36] of their placement and determining their coordinates. Animals that receive drug with probes placed outside the target site can be used as controls thus improving the efficient utilization of hibernation animals.

Diet and hibernation: Use of AGS in seasonal hibernation studies is justified due to their ability of robust seasonal hibernation [37]. AGS are wild-caught and maintained in the research facilities. Diet influences hibernation pattern [38, 39, 40, 41, 42]. Hibernation animals fed with a diet comprising of high poly unsaturated fatty acids (PUFA) have lower body temperatures during torpor and longer torpor bouts than animals fed with low PUFA diet [39]. Captive AGS are fed with rodent chow. A thorough understanding of diet in their natural habitat is required to insure similar diet in captivity. An initial study, then, needs to be conducted in two groups of captive AGS where one group receives the same diet they would have in the wild and another group receives rodent chow. The researcher should evaluate and compare all the parameters in question in the two groups and find out if there is any significant difference between groups.

Habitat: AGS are colonial [43, 44] though they hibernate in individual burrows in their natural habitat (Øivind Tøien, personal communication). In captivity they are individually housed. Enrichment of housing by providing plastic tubes which mimics the burrows in their natural habitat should be considered. Enough housing space should be provided that allows the animals for free adjustments of their postures. Type of habitat influences stress levels in AGS [43]. Behavioral changes in reproduction and stress physiology owing to their individual housing cannot be ignored as seasonality-induced stress results in physiological changes [45]. Fecal monitoring of corticosteroids can be used to monitor the stress of capturing and handling [46, 47, 48]. A study can be developed conducted

throughout the year in two groups of animals in which monitoring their feces for change in corticosteroid levels; one being maintained in captivity and the other in wild. Any significant difference in the change of corticosteroid levels would lead to improvements in quality housing conditions for AGS.

Latitude and hibernation: Latitudes influence hibernation [49]. Hibernators in northern latitude hibernate longer than their peers in southern latitudes [49]. Collaboration of hibernation researchers at different places with varying latitudes and with different hibernation species is not uncommon. Care should be taken in such cases to set the housing light-dark conditions suitable for each individual species instead of using a blanket light-dark cycle protocol.

Conclusions

As the hibernation research progresses, the understanding of the animal behavior of hibernators in captivity also advances, throwing light on certain new ethical issues. Researchers should develop an interdisciplinary approach in finding solutions to observed problems. Identifying ethical issues, animal welfare concerns, and addressing them promptly establishes scientific integrity and improves quality of research.

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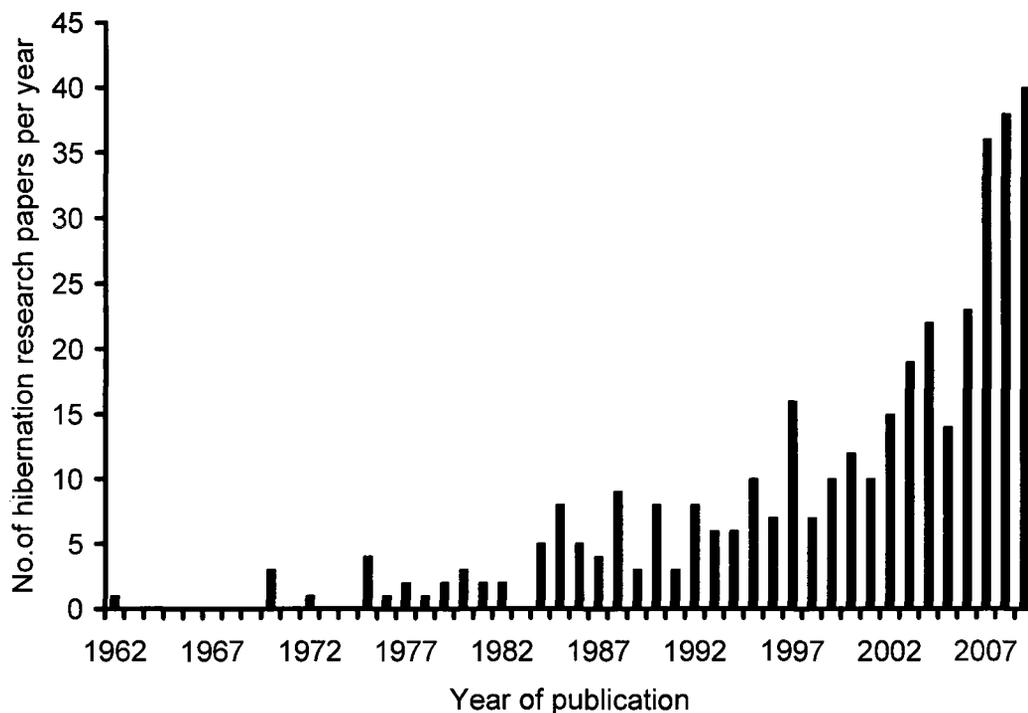
Figure 5.1 Increasing frequency of publications in hibernation research in the last 47

years

Frequency of publications relating to hibernation research over the past 47 years. A

Pubmed search for the key words "hibernation" and "torpor" obtained the data that

demonstrates a gradual increase in the hibernation research publications in the past 47

years. The last column represents the data as of 4 November 2010.
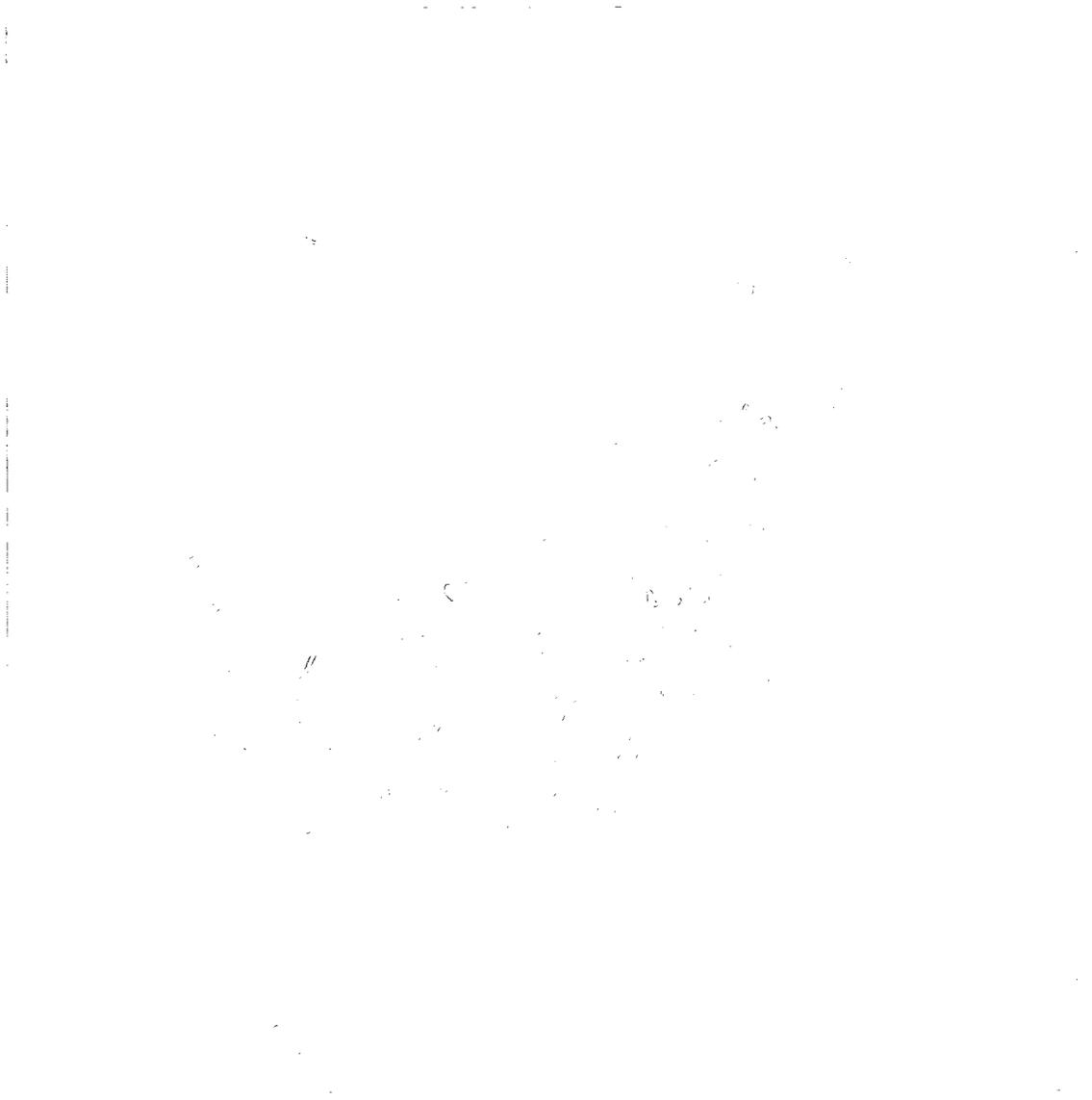
Figure 5.2 Hibernating Arctic ground squirrel (Dorsal view)

Figure 5.3 Hibernating Arctic ground squirrel (Lateral view)
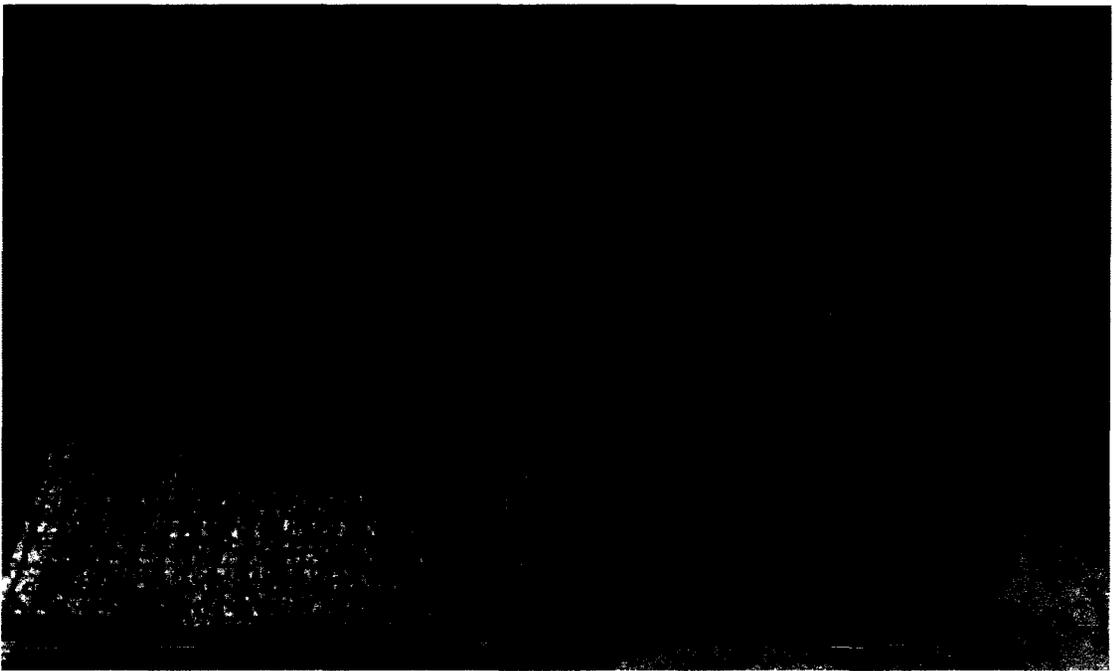
Figure 5.4 Arctic ground squirrel during interbout euthermy

Institutional PI performing animal research

↓

Submit protocol to institute's IACUC by completing
Research Protocol form

↓

Research protocol sent to IACUC members for
review

↓

Discussion at IACUC monthly meeting and voting

Research
protocol
approval

IACUC informs PI if revision or
more information is needed

Modification of
protocol

Research Protocol will be
sent back to PI for
suggested revisions and for
extra information, if any

Research protocol sent to
designated review

Submit revised protocol
to institute's IACUC

Research protocol sent to
IACUC members for review

Figure 5.5 A brief summary of protocol approval and modification by a typical

Institutional Animal Care and Use Committee (IACUC)

PI=Principal Investigator

Pharmacological study
in hibernation

Previously tested
agent with proven
safety in the study
species

Previously not tested

Pilot study

No adverse
effects

Adverse
effects

Stop
the
study

Re-design with reduced
dose or a different drug

Proceed with
main study

No adverse
effects
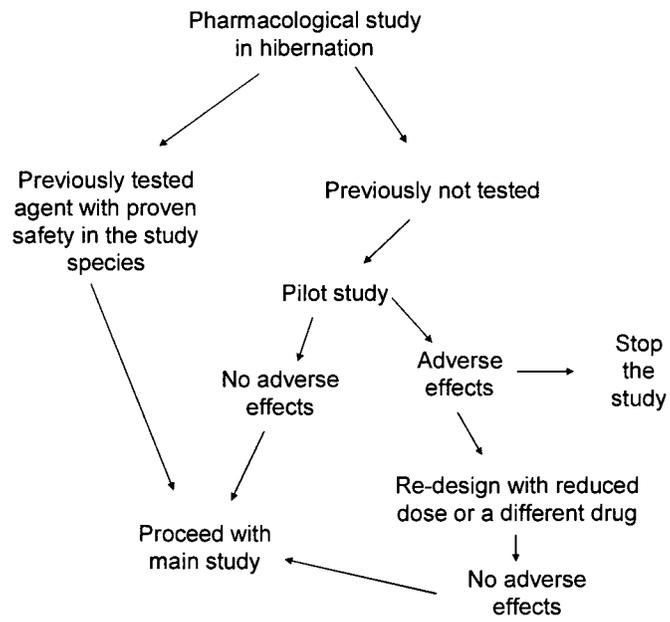
Figure 5.6 A recommended sequence for conducting a pilot study during pharmacological

induction of torpor in hibernators.
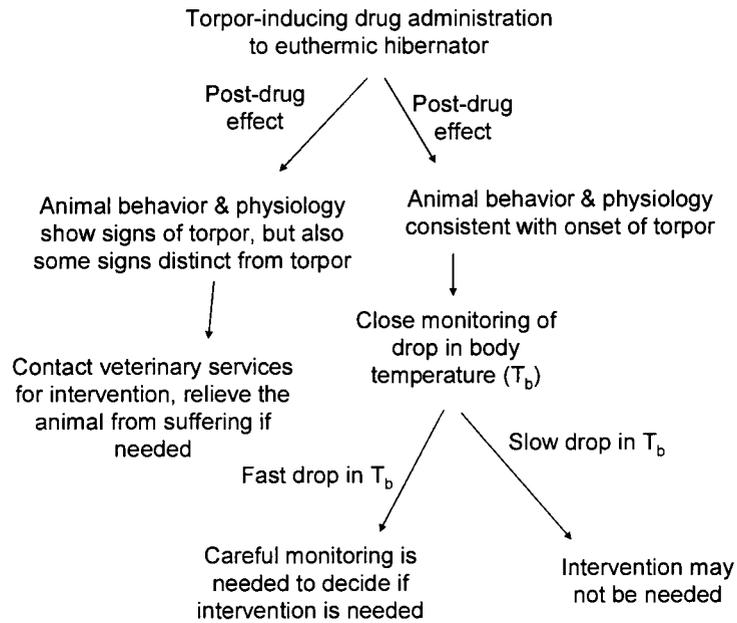
Figure 5.7 Discretionary approach for intervention during drug induced-torpor.

Chapter 6 Conclusions

To summarize, this dissertation demonstrates the role of adenosine in torpor induction with a seasonal variation, involvement of NMDAR in inducing arousal from torpor in hibernating Arctic ground squirrels, and the role of dietary restriction in sensitizing adenosine system to induce a torpor-like effect in rats and finally discusses ethical aspects in hibernation research which is of significance to Alaskan biomedical research.

Here, I demonstrate that adenosine meets all of the necessary requirements for an endogenous mediator of torpor in hibernating AGS. A progressive increase in the sensitivity of AGS to A_1AR mediated signaling defines the seasonal transition into the hibernation phenotype. I show that A_1AR activation is necessary and sufficient to induce torpor in AGS. This part of my overall research fills the missing link of unknown switches which facilitates induction of torpor with a seasonal variation. It is anticipated that future studies may be aimed at looking at other neuromodulators as well because it is unlikely that adenosine is the only neuromodulator involved with torpor onset. It is also possible that the seasonal effect of adenosine may be studied in other seasonal hibernators.

The role of glutamatergic-type NMDAR in regulating arousal from torpor is confirmed in my studies, as demonstrated in this dissertation. My study also demonstrates that peripheral or circumventricular NMDAR are involved in inducing arousal from torpor in hibernating Arctic ground squirrels. Future studies may be aimed at studying the

location of peripheral or circumventricular NMDAR in induction of arousal in Arctic ground squirrels.

Dietary restriction-induced modification of thermoregulation in rats is associated with changes in components of the purinergic neuromodulatory system, as demonstrated in this dissertation. My results confirm that dietary restriction sensitizes A_1AR through an increase in surface expression in thermoregulatory regions of the brain (hypothalamus) and in this way contributes to the decline in body temperature and respiratory rate in animals subjected to restricted diet, which mimics a torpor-like effect. Future studies may be aimed at successful translation of diet modulated purinergic signaling which has implications for the treatment of brain injury, stroke, epilepsy, multiorgan failure, and aging.

Hibernation research is one of the important areas of biomedical importance. My dissertation identifies some ethical areas to be considered in hibernation research and suggests possible solutions to those concerns, which are of significance in terms of animal welfare and biomedical research program.

APPENDICES

Appendix A

Surgical procedure for intracerebroventricular (icv) cannulation

GENERAL PREPARATION:

Devices or equipment to be implanted or affixed to the animal:

Plastics One C315G (26ga; 0.46mmOD; pedestal height is 8.0mm) (with C315DC and

C315I, dummy and injection cannula) guide cannula are affixed to 3-4 screws attached

to the skull using dental cement. Injection cannula or microdialysis probes are inserted

through guide cannulae at the time of sampling.

Sterilization Methods for Instruments, Supplies and Sterile Packs:

Steam Autoclave: All the instruments used for surgery should be wrapped in a drape or

cloth, which is secured with a thermosensitive tape. The temperature, pressure, and time

combination for general wrapped items are 250 F, 20 psi, and 30 min respectively (same

for liquids too).

Gas sterilization: Steam is unsuitable for plastic with a low melting point (microdialysis

probes, sleeve of trocar etc.,), powders or anhydrous solutions. In such cases we use gas

sterilization (ethylene oxide).

Autoclave packs will be stored in closed cabinets and the packs can be considered sterile

for six weeks, if they are double layered. Single layer wrapped surgical packs can be

considered sterile for three weeks. Expiry date will be noted on each pack.

The surgery room floor will be mopped with diluted Vindicator, all the surgery lamps,

microscope and counter tops will be disinfected with dilute chlorhexidine.

Sterilization of Solutions:

All solutions are either purchased in sterile containers or sterilized by filtering through

0.2um filters into sterile red top tubes. Solution is withdrawn using sterile technique.

Opened solutions are discarded within 30 days of opening.

Sterilization & Aseptic Technique:

The surgery table will be wiped with Nolvosan. The surgeon will be wearing a mask,

head cover and clean lab coat or scrubs. Immediately before starting the surgery, the

surgeon must wash the hands and put on a pair of sterile gloves. Once this is done a

sterile drape is placed over the animal with the hole over the incision site. Sterile packs

will be set next to the surgeon, the outer layer opened by an assistant using sterile

technique and the inner layer opened by the surgeon wearing sterile gloves. Surgeon may

change gloves when necessary and will orient tips of surgical instruments towards the

center of the sterile field indicated by a cirlce drawn on the drape. Assistant helps to

maintain sterility by opening packages into the center of the sterile field.

PRE-SURGICAL PROCEDURES/CARE:

Screening Procedure: Rodents are in good health based on daily inspection of animal

quarter's personnel

Procedures & Care:

With-holding of Food and/or Water: 2-4h or overnight fast.

Pre-Operative Medication, Fluids, or other agents: Baytril 5mg/kg, sc in the back of the neck, begin 24 hours before surgery and continue BID for 3 days minimum.

ANESTHETIC PROCEDURE:

Agent Information

Name: Isoflurane

Dose (i.e. mg/kg or % if by inhalation): Induced at 5%, maintained at 2.5-3%

Route of Administration: Inhalation

Induced in anesthetic chamber at 5% (mixed with medical grade O_2, delivered via stereotax face mask (Stoelting) at 1.5L/min; maintained at 3% (for AGS) 2% (for rats) or lower depending on respiratory frequency.F/Air Disposable Anesthesia Gas Scavenger Canister will be used for scavenging the waste gases. Because the face mask is not gas tight, surgery will be performed on a down-draft table.

Expected Duration of Anesthesia: 4-6h

Monitoring Procedure to Evaluate Depth of Anesthesia: Sharp pinch to the toe should not produce a response. Respiratory rate, HR and sO_2 (pulseoximeter) are monitored to prevent overdose.

Procedures to Ensure Maintenance of Normal Body Temperature: circulating water blanket set to 37oC

Emergency Procedures to be Employed in Case of Anesthetic Over-dose:

100% O_2 (mask is not airtight and does not allow for mechanical ventilation. Over-dose

has not been a problem with isoflurane anesthesia).

Monitoring Protocol to Ensure Animal's Complete Recovery from Anesthesia:

Observe until animal is ambulatory (~45 min after anesthesia)

SURGICAL PROCEDURE

List all physical location(s) where the surgical procedure may be performed:

Irving-1 AQ or BIRD

Preparation of Surgical Site: Head is shaved, scrubbed 3 times each with alternating

applications of 70% isopropyl alcohol and concentrated betadine allowing the 3rd

betadine scrub to dry. Scrub is started in the center and spiraled out to the edges, never in

reverse. Animal is draped with clear, sterile plastic drape beginning at the nose. Rear

end of animal is draped with sterile drape so as not to obscure observation of respiratory

movements. Eye lubricant is placed on eyes

Expected Duration of Surgery: 4-6h

Incision Site: An incision will be made from the frontal nasal suture to the area between

the ears (~2in). The underlying muscle and fascia will be separated by blunt dissection

Specific Surgical Procedure to be Performed:

Overview: Holes will be drilled to accommodate the anchoring bone screws

(Bioanalytical Systems, Inc. W.Lafayette, IN-or equivalent). Additional holes will be

drilled to accommodate guide cannula (up to 2 per rat or 3 per AGS). Guide cannula will

be lowered to desired depth and cemented in place. For icv placement, an internal

cannula will be inserted through the guide cannula to a depth that extends 1.0 mm beyond the tip of the guide cannula. The injection cannula will be connected to a 1cc syringe and slight negative pressure applied to withdraw cerebrospinal fluid (CSF). Cannula depth will be adjusted until cannula placement in the ventricle is verified by withdrawal of CSF. The guide cannula will be secured with dental cement (Stoelting Co., Wood Dale, IL or equivalent). A metal hook may be secured in the cement to assist with in vivo sampling. The guide cannula will be sealed with a plug. See attached sample surgery sheet and sample check lists for surgery packs. If transmitter is to be implanted at the same time the abdominal incision is covered with sterile gauze before turning animal onto stomach. Ear bar zero (EBZ) is recorded before putting animal into the stereotax. The skull is exposed and painted with Copalite, while keeping it from touching the tissues. The nose bar is adjusted until the skull is flat (dorsal coordinates are within 0.1 mm when measured at from APEBZ +10mm and at APFNS). Screw holes are drilled and 4 stainless steel bone screws (BAS Inc, Lafayette, IN, CMA, Acton, MA or equivalent) are secured in the skull. The nose bar is lowered 20 mm. Anterior/posterior (AP) and lateral (L) position of guide cannulae are determined according to established sterotaxic coordinates for that area. Cannulae positions are marked on the skull with a #2 pencil. Skull is removed at cannula sites using a 1mm dia. trephine drill bit (CMA). Dorsal coordinate at the surface of the brain is recorded. Dura is punctured with a bent 26 ga needle. Guide cannulae are slowly lowered to the final depth.

Note: For icv placement, an internal cannula will be inserted through the guide cannula to a depth that extends 1.0 mm beyond the tip of the guide cannula. The injection cannula

will be connected to a 1cc syringe and slight negative pressure applied to withdraw cerebrospinal fluid (CSF). Cannula depth will be adjusted until cannula placement in the ventricle is verified by withdrawal of CSF.

Gel foam is placed around cannulae to completely cover exposed brain tissue. Cannulae are cemented in place with dental cement (Stoelting). Before the last layer of cement is applied a small ring made from a paper clip is secured in the cement

Wound Closure:

The skin is sutured with 000-prolene using an FS-2 cutting needle with 2 full knots (4 throws), interrupted stitch leaving space for tissue swelling. Two stitches in front of head stage and 2-3 stitches behind head stage are usually sufficient. Toe nails are clipped to avoid interference with stitches.

POST-SURGICAL PROCEDURES/CARE

Care and Monitoring:

Post-Operative Care Procedures:

Animal will be observed until it regains a righting reflex and becomes ambulatory. The animal will be placed in a clean, stainless steel cage with a small amount of cotton and/or drape but without wood shavings. Animals will be fed ad libitum. Personnel approved for post-operative monitoring will participate in daily cleaning and inspection of head stage for 10 days post-op or until wound is healed and will be responsible for weekly monitoring after wound is healed and AGS is returned to the cold chamber. Animal Quarter's staff will be responsible for daily monitoring for presence of dummy cannula

and will contact laboratory personnel immediately if dummy is missing or damaged. Vet services will be contacted in case of complications encountered during regular work hours or during after-hour, weekend or holiday care.

Post-operative Antibiotic or Other Therapeutic Treatments (not analgesia): Baytril 5mg/kg, sc in the back of the neck, begin 24 hours before surgery and continue BID for 3 days minimum

Responsible Individual(s): Approved laboratory personnel

Analgesia

Assessment of Pain/Distress:

Analgesics are administered for 1-3 days. After 1 day pain is assessed by observing animal for lethargy, lack of appetite or abnormal postures. Analgesics will be given on an as needed basis if animal shows signs of distress. If pain and other complications are absent animal will be returned to hibernacula when experimental design requies minimal disruption to hibernation patterns. If disruption of hibernation patterns are not a concern then animal will be administered analgesics for 3 days and pain assessed after 3 days.

Analgesic Agent(s):

Ketofen 1mg/kg (if not combined with tunneling surgery) or buprenorphine 0.03mg/kg (if combined with tunneling surgery)

Analgesic Treatment Schedule:

Ketofen: 1mg/kg im on day of surgery; sc back of neck thereafter. buprenorphine: 0.03mg/kg im (thigh). Once per day for ketofen or buprenorphine

Wound Care

Incision Care: Using sterile cotton swabs, wounds will be scrubbed with 1-3% betadine (diluted to tea colour) for 10 days post-op or until wounds are healed. After initial healing, head stage is inspected once per week for signs of infection including scabbing around edge of headstage. Scabs are removed by cleaning with 3% betadine solution followed by daily cleaning with 3% betadine until wound is healed. If wound clips, non-absorbable sutures, or other temporary items are used specify when they will be removed (i.e. number of days post-surgery): 2 wks post-operative care is required.

Care of Implants or Catheters:

Care: Guide cannula will be inspected daily for presence and integrity of dummy cannula. Missing or damaged dummy cannula will be replaced with a sterile dummy cannula.

Duration and Removal: Up to 2y in AGS or until complications require euthanasia or at end of experiment if indicated in experimental design.

Post-Surgical Changes

Anticipated Outcomes: Slight change in posture may be noted for a couple of days until animal adapts to weight of headstage.

Humane Endpoints:

Loss of head stage

Loss of dummy cannula. (A daily regular observation would help to discover missing dummy cannula within 24h and replace it with a sterile replacement cannula. If implanted site is infected it poses a risk of abscess)

Unrelated health concerns giving cause for euthanasia as noted by attending vet.

Appendix B

Setting up AGS for intracerebroventicular (icv) drug delivery

Setting up of non-hibernating AGS for intracerebroventricular (icv) drug delivery

Before the procedure (At least day before)

1. Reserve procedure room for the day and time

2. Figure out the AGS for the procedure (Decide to leave it in cold room or move to warm room, based on the type of experiment)

3. Check the respirometry, Dataquest® and the pump and make sure they are working.

4. An autoclaved surgical pack should be ready with the following contents in it:

   - Micropipette with lid (Two in number)

   - Small forceps

5. Gas sterilized internal cannula and connector.

6. Sterile surgical gloves

7. Adhesive tape

8. Timer

9. Check oxygen tank and isoflurane and make sure enough quantity is available.

10. Make sure that the anesthetic jar, dessicator, and weighing scale are in place and ready to use.

11. Clean and disinfect the metabolic chamber

12.

On the day of the procedure:

Phase-1:

1. Prepare the drug/vehicle (at least 2mL; blinded) and get it ready in a red top tube

Phase-2 (Working with rat-turn):

1. Take the cart to the rat-turn (Cold chamber, etc)
2. Remove the arm of the rat-turn
3. Remove the electric chord connecting the rat-turn
4. Put the entire rat-turn set up on the cart
5. Take the set-up to procedure room

Phase-3 (Working with syringe pump):

1. Place the syringe pump in the cold chamber
2. Come back to procedure room

Phase-4 (Working with internal cannula and connector in the procedure room):

1. Put the drug/vehicle on the table
2. Keep a 21G needle ready on the table
3. Put a 1 cc syringe on the table
4. Wear regular gloves
5. Take the 1 cc syringe, fix the needle, and fill the drug/vehicle. Make sure there are no bubbles. Place the needle cap back to the needle and keep it on the table.
6. Place the autoclaved surgical pack on the surgery table
7. Open the first layer of the pack carefully following the tabs
8. Open the gas sterilized internal cannula and gently allow them to drop on the exposed sterile field in the surgical pack
9. Remove the regular gloves

10. Don the sterile surgical gloves

11. Open the sterile field of the surgical pack

12. Open the internal cannula and connector

13. Attach internal cannula to the connector

14. To avoid any contamination to the internal cannula which is at one end of the connector, insert the internal cannula into sterile micropipette. With the help of adhesive tape attach the outer portion of the micropipette to the spring portion of the connector. This will help the internal cannula to stay sterile even if touched accidentally.

15. There will be one more pipette left along with forceps

16. Leave the entire area untouched and undisturbed

17. Move the internal cannula tubing away from the sterile area and prime it with drug/vehicle. This will avoid any fluid falling into the sterile area.

18. Fix the tubing to the rat-turn

19. Move the entire set-up to the cold chamber and keep it in place.

20. Fix the syringe to the pump

21. Prime the internal cannula tubing with the drug/vehicle

Phase-5 (Working with AGS):

1. Wear thick gloves and place the AGS in the anesthetic jar. Thick gloves help preventing animal bites.

2. Weigh the AGS

3. Anesthetize with the standard and IACUC approved procedure

4. Take out the dummy out of the guide cannula located on the head stage of AGS

5. Drop it into the sterile field of the surgical pack.

6. Take the jar with AGS to the cold room

7. Place the AGS in the metabolic chamber

8. Fix the internal cannula to the guide cannula

9. Secure the AGS with zip ties around the abdomen and fix the hooks to the abdomen strap and head stage ring

10. Close the lid of the metabolic chamber

Phase-6 (Working with sterile field in procedure room):

1. Come back to procedure room
2. Don sterile surgical gloves
3. Pick up the dummy cap with forceps
4. Put it in the micropipette and close the lid. This will help the dummy cannula from damage and loosing.

Phase-7 (In B5):

1. Start collecting the data on respirometry and Dataquest®
2. Allow the real-time graphs window to run on the Dataquest® (This will tell you that parameters viz, temperature, are getting recorded and saved)
3. Keep an eye on the rat-turn at least for the first few hours and make sure that the AGS is not tangled up.

Phase-8 (Drug/vehicle delivery; blind-coded):

1. When sufficient baseline data is obtained walk into the cold chamber with the timer
2. Deliver the drug/vehicle for the fixed period of time (1 minute) with the help of timer.
3. Make sure that everything is going on fine with the data collection, by frequent visits to the system.

Setting up of non-hibernating AGS for intracerebroventricular (icv) drug delivery

Follow all the above procedures with few exceptions

- Do not move the AGS outside cold room

- Arrange a working table for maintaining sterile conditions.

- No need of anesthesia as the animal remains torpid

- Leave some gloves inside the cold chamber. Using this gloves which are already acclimatized to cold chamber will not cause temperature variation when compared to using gloves from the warm room.

4. Gently insert the internal cannula. Use a head lamp for clear vision.

Appendix C

Protocol for intraperitoneal (ip) injections of MK-801

- Select the number of AGS required for the study based on their hibernation history (Number of bouts, present bout)

- Record the weights.

- Habituate for ip saline injections (1mL/kg B.wt)).

- Prepare drug, label with date and blind code the drug and vehicle.

- Prepare a data sheet with all details going to be recorded.

- On 4th day of a bout inject the AGS with normal saline (ip @ 1mL/kg B.Wt) or MK-801(ip, 5 mg/kg B.Wt @1 mL/kg volume).

- Observe the animal before the injection of saline in its cage, undisturbed.

- Use the red/blue light for observing the animals (supposed to be of low intensity and will not disturb the animal)

- Record the breaths per minute (bpm)

- Inject the animal

- Record bpm post-injection as per the timings on the data sheet.

- Quantify arousal using arousal index scale

- Observe continuously for the first 12 hours at the regular intervals.

- Add shavings on the AGS after 12 hours if there are no signs of arousal

- Observe again at 24th hour of post-injection (next day after injection)

- Record and analyze data

Appendix D
Protocol for intraperitoneal (ip) injection of CHA for bioassay

Protocol for cold room AGS

1. Select the AGS and take down their hibernation history.

2. Record whether the AGS is torpid or not, RR is greater than or less than 5, AGS is responsive to touch or not.

3. Weigh and move them to warm room the day prior to CHA injection.

4. Next day record their body temperature. If they did not reach 37°C allow some more time till they reach that level (If needed, disturb by gentle handling to make them come up soon)

5. After reaching 37°C move them back to their home cages in the cold chamber (Ambient temp of 2°C)

6. Collect baseline data before injections.

7. Inject 0.5mg/kg CHA (N^6-Cyclohexyladenosine; ip) and place them back to their home cages.

8. Record the data at regular intervals.

9. Record whether the AGS is torpid or not, RR is greater than or less than 5.

10. Observe the AGS for the next 30 hours (with time intervals spaced apart based on the number of observations needed for the study)

11. Take the temporal and rectal temperatures and respiratory rate (breaths per 2/3 minutes) at 30 hrs post-injection, if they are torpid. If not record their sub-cutaneous temperature from IPTT tags.

12. Move the AGS to warm room and allow them to warm up until they reached 37°C (rectal and temporal temperatures) if they are torpid.

13. After 24 hours, anesthetize with isoflurane and euthanize the AGS

14. Collect the tissue.

15. Fill out the datasheets from time to time.

Data sheet

Name of experiment	CHA bioassay-ip injection					
ID			Temp (°C)	Date	Time of day	
Sex						
Age		transponder implantation				
Date		2h pre-inj (optional)				
No. of torpor bouts		1h pre-inj				
Day of torpor bout		To (before injection)				
Day into warm room(L:D;T_a)		T1h				
Day into cold room(L:D;T_a)		T2h				
Date of CHA ip injection		T3h				
Dose		T4h				
Body Wt		T20h (rectal T_b if torpid)				
Amb temp						
Experiment	CHA bioassay-tissue collection					
animal #		Date				
date of tissue collection		Time of day				
	Brain				Body	
	tissue	Peripheral Tissues collected in Liquid N_2			blood (cardiac puncture;500uL))	
sample #	region	aprox weight (mg)	tissue (label)	~ weight or whole organ	Plasma	Serum
Liq N2			heart			
cerebellum						

brainstem			liver			
hypothalamus						
pituitary						
forebrain-1						
forebrain-2						
hippocampus-1						
hippocampus-2						
choroid plexus-1						
choroid plexus-2						
forebrain excludes hippocampus and striatum						

Appendix E

Operation basics of open-flow respirometry

Equipment used in the system and suppliers

- Air Filters (Balston DFU®, Haverhill, MA)

- Mass flow meter (Sierra Instruments, L Monterey, CA)

- Mass flow controller -2 channel v1.0 (Sable Systems International, Las Vegas, NV)

- Pump, type UNMP830 KNDCB (KNF Neuberger, Trenton, New Jersey)

- UI2 Data Acquisition Interface (Sable Systems International, Las Vegas, NV)

- TC-1000 thermometer Sable Systems International, Las Vegas, NV)

- Multiplexer TR-RM4 (Sable Systems International, Las Vegas, NV)

- Cole-parmer

- Nafion® dryer

- FC-1B O_2 analyzer (Sable Systems International, Las Vegas, NV)

- CA-2A CO_2 analyzer (Sable Systems International, Las Vegas, NV)

- Gas analyzer-subsampler-version1 (Sable Systems International, Las Vegas, NV)

- Molecular sieves 3A, 8 to 12 mesh (Acros Organics-Product of Switzeland; New Jersey, USA: 1-800-ACROS-01)

- Ascarite II (Thomas Scientific, Sweedesboro, NJ)

Software:

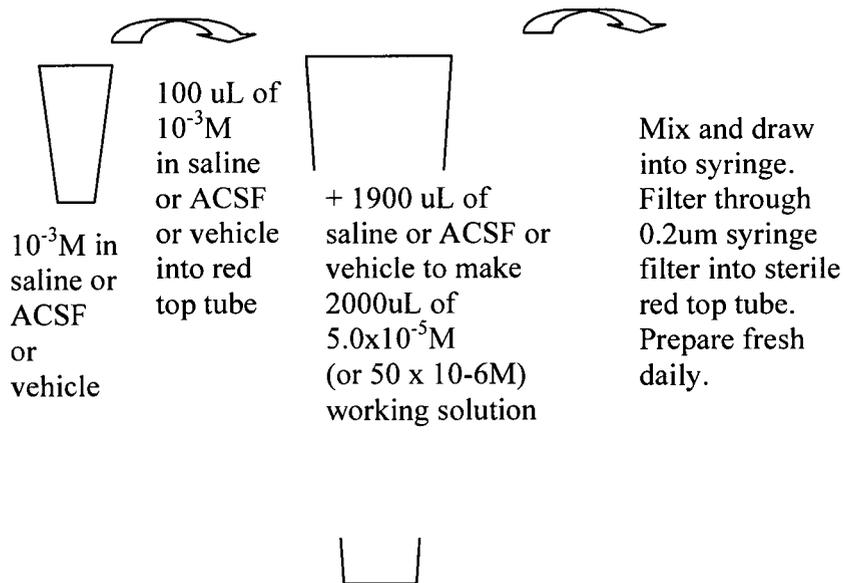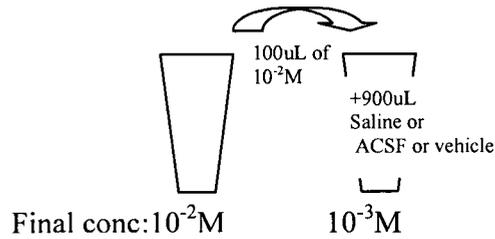- Labgraph (developed by Dr. Øivind Tøien, University of Alaska Fairbanks)

1. Turn on the computer, click on the LABGRAPH icon. This opens the software.
2. Go the CALIBRATION and click on the NEW SAMPLE.
3. Fill out the File Name. Usually the File Name would be like 55060606.The first two digits will be the AGS ID and the rest six digits will represent the date.
4. Press ENTER
5. A screen with constants appears. Make sure that the constants are correct.
6. Press ESCAPE and the screen goes to recording phase automatically.
7. Turn ON the red switch on the top right corner of the respirometer. It starts the pump.
8. Start calibration. First press CTRL+Z for zero gas. The red light on the respirometer comes up. This usually lasts for 4 minutes.
9. Then press CTRL+R. The yellow light on the respirometer comes up. This lasts for 4 minutes.
10. Then turn on the span gas and press CTRL+S. The green light comes up. This also lasts for 4 minutes.
11. After the calibration place the AGS in the metabolic chamber and the system starts recording.

- Adjust the flow rate prior to calibration and work on it. Do not try to change or adjust the flow rate while the study is going on. It may lead to errors in the data.

- Try to hang the reference air tube just above the chamber so that it can mimic the metabolic chamber atmosphere.

- Take care that there won't be any blunt ends in the tubing. This helps in avoiding the condensed water accumulation in the tubing on the way to metabolic chamber inside the cold chamber.

- Be gentle while releasing the span gas. More pressure will blow up the circuits inside the analyzer.

Appendix F
Preparing drugs for icv injections

Question or Procedure	Answer or record of what you did
Inj vol?	10uL
Dose (or amount to be injected)?	0.5nmol
Final conc of (0.5nmol/10uL)?	50uM
Total vol of working solution needed?	1.5-2.0 mL
Solubility of drug in water?	0.01M in PB at pH 7.0
Concentration of stock solution?	10^{-3}M or 10^{-2}M in H_2O
MW of drug (example: CHA)	349.38 g/mol
How many mg/mL to = 10^{-3}M Final conc in (mM) x MW (g/mol) x final vol (mL)	Xmg = 10^{-3}mol/L x 349.38g/mol x 1 mL = 0.349 mg
Is the amount of drug between 2-10mg? Is final dilution from water to saline or ACSF or vehicle at least 100 fold? if yes, proceed if no, increase concentration or volume of stock solution so amount weighed is between 2-10mg	Yes No
Increase volume of water to add to make final concentration 10^{-3}M For example if mass of drug is 3mg calculate total volume as: X mL = 3mg x (1mol/349g) x (1L/10^{-3}mol) X = 8.60 mL	Final volume of stock solution (X mL) = mass of drug in mg x (1mol/349g) x (1L/10^{-3}mol)
OR,	
Increase concentration to 10^{-2}M For example if mass of drug is 3mg calculate total volume as: X mL = 3mg x (1mol/349g) x (1L/10^{-2}mol) X = 0.860 mL = 860uL	
Because final dilution to 5×10^{-5}M is less than 100 fold, it will be better to make stock solution 10^{-2}M in water and then dilute to 5×10^{-5}M in saline.	X mL = mass of drug in mg x (1mol/349g) x (1L/10^{-2}mol) = 0.XXX mL = XXXuL
Tare scale and small piece of weigh paper	
Pour a few grains of drug onto paper until mass is between 2-10mg (try to get it around 3 mg)	
Mass of drug?	
Volume added?	

Concentration of Stock solution?	
Draw figure like below to illustrate dilutions	

100uL of
10^{-2}M

+900uL
Saline or
ACSF or vehicle

Final conc:10^{-2}M 10^{-3}M

10^{-3}M in saline or ACSF or vehicle

100 uL of 10^{-3}M in saline or ACSF or vehicle into red top tube

+ 1900 uL of saline or ACSF or vehicle to make 2000uL of 5.0×10^{-5}M (or 50 x 10-6M) working solution

Mix and draw into syringe. Filter through 0.2um syringe filter into sterile red top tube. Prepare fresh daily.

Drugs and vehicles will be blind-coded.

Appendix G
Abdominal transmitter implantation

GENERAL INFORMATION

Title of Surgery/Procedure: Abdominal transmitter

Species Name: Arctic ground squirrels (AGS)

Devices or equipment to be implanted or affixed to the animal:

model VM-FH, (Minimitter, Bend, OR), 2 cm disc, 5g

model TA-F40 (Data Sciences, St. Paul, MN), 3x1x1.5cm (3.5cc, 7-8g)

Sterilization Methods for Instruments, Supplies and Sterile Packs:

Steam Autoclave: All the instruments used for surgery should be wrapped in a drape or cloth, which is secured with a thermosensitive tape. The temperature, pressure, and time combination for general wrapped items are 250 F, 20 psi, and 30 min respectively (same for liquids too). Autoclave packs will be stored in closed cabinets and the packs will be considered sterile for six weeks, if they are double layered. Single layer wrapped surgical packs can be considered sterile for three weeks. Expiry date will be noted on each pack. Once sterile surgical packs are openned, instruments are positioned with tips pointed towards the inside of a circle and handled only by the handles.

Gas sterilization: Transmitters or e-mitters will be gas sterilized (ethylene oxide) or purchased in sterile packs and used prior to expiration date.

Sterilization of Stationary Equipment (i.e. surgical scope, drill or bone saw, etc.):

Room is swept, mopped and wiped down with dilute chlohexidine solution

Sterilization of Solutions:

All solutions are either purchased in sterile containers or sterilized by filtering through 0.2um filters into sterile red top tubes. Solution is withdrawn using sterile technique. Opened solutions are discarded wihtin 30 days of opening.

Sterilization & Aseptic Technique:

The surgery table will be wiped with Nolvosan. The surgeon will wear a mask, headcover and clean lab coat or scrubs. Immediately before starting the surgery, the surgeon will scrub his/her hands and put on a pair of sterile gloves. Once this is done a sterile drape with a hole over the incision site is placed over the animal. Assistant helps to maintain sterility by opening packages into sterile field.

PRE-SURGICAL PROCEDURES/CARE:

Screening Procedure: Rodents are in good health based on daily inspection of animal quarters personnel.

Procedures & Care:

With-holding of Food and/or Water: 2-4h or overnight fast.

ANESTHETIC PROCEDURE

Agent Information

Name: Isoflurane

Dose (i.e. mg/kg or % if by inhalation): Induced in anesthetic chamber at 5% (mixed with medical grade O_2, delivered via face mask at 1.5L/min; maintained at 3% or lower depending on respiratory frequency.

Route of Administration: Isoflurane vaporizor scavanged with Omnicon f/air (Bickford, Inc; Wales Center, NY)

Expected Duration of Anesthesia: 0.5-1h unless combined with other procedures

Monitoring procedure to evaluate depth of anesthesia: Sharp pinch to the toe should not produce a response. Respiratory rate is monitored to prevent overdose.

Procedures to Ensure Maintenance of Normal Body Temperature: Circulating temperature controlled water blanket set to 36-37°C

Emergency procedures to be employed in case of anesthetic over-dose: 100% O_2

Monitoring Protocol to Ensure Animal's Complete Recovery from Anesthesia: Observe until animal is ambulatory.

SURGICAL PROCEDURE

List all physical location(s) where the surgical procedure may be performed: Irving I or BIRD

Preparation of Surgical Site: Abdomen area will be shaved and washed 3 times with betadine (or generic equivalent). First 2 washed have a water rinse and the final wash has a 70& isopropyl alcohol rinse. Once the alcohol is dry the final prep is to apply full strength betadine solution by painting it on with a cotton tip swab. This is applied using a circling motion starting at the center of the shaved site and end at the shaved margin Eye lubricant is placed on eyes.

Expected Duration of Surgery: 0.5-1h unless combined with other procedures

Incision Site: 2cm incision on the abdominal midline through linea alba

Specific Surgical Procedure to be Performed:

Wound Closure: Make about a 2cm incision on the abdominal midline using a #10 or #15 scalpel blade or blunt scissors. The skin is retracted and blunt end scissors are used to separate muscle from sub-cutaneous tissues. An incision is made along the linea alba taking care to not cut muscle and the transmitter, moistened with sterile saline, is inserted into the peritoneal cavity. Note the transmitter #_____. The linea alba is sutured closed using a simple interrupted pattern with 3-0 chromic gut (absorbable) taper, RB-1, ~17mm needle. Sub-cutaneous tissue is sutured with 3-0 dexon or 5-0 PDS (absorbable), taper, RB-1, ~17mm needle, using a simple continous pattern. The skin is then sutured with 3-0 Prolene/Surgilene (non absorbable), FS-2 or FS-1 cutting needle using a simple interrupted pattern. Typically 4 or 5 skin sutures are required to close a 2cm incision.

POST-SURGICAL PROCEDURES/CARE

Care and Monitoring:

Post-Operative Care Procedures: After surgery animals are housed in stainless steel cages with small amount of cotton BUT NO wood shavings. Wounds are inspected and cleaned with dilute betadine daily for the first 3 days. Thereafter wounds are inspected daily and cleaned if necessary. Animals are monitored for normal behavior and activity. Sutures are removed 10-14 days post-op.

Post-operative Antibiotic or Other Therapeutic Treatments (not analgesia):

Analgesia

Assessment of Pain/Distress: Lack of voluntary feeding and drinking or lethargy will be interpreted as signs of distress. Vet services will be contacted to advise on treatment.

Analgesic Agent(s): Analgesics are administered for 3 days as specified here.

Or, if abdominal transmitter surgery is combined with another surgery, the analgesic specified in the accompanying surgery will be used. After 3 days, pain is assessed by observing animal for lethargy, lack of appetite or abnormal postures. After 3 days, analgesics will be given on an as needed basis if animal shows signs of distress.

Analgesic Treatment Schedule: Buprenorphine (0.03mg/kg, im in thigh) once per day for 3 days beginning at start of surgery. Buprenorphine is the analgesic of choice for this surgery unless it is expected to interfere with hibernation. If justified in research design we will use ketoprofen as an alternative analgesic that is less likely to interfere with hibernation.

Wound Care

Incision Care: Personnel approved for post-operative monitoring will participate in daily cleaning and inspection of wound. Vet services will be contacted in case of complications encountered during regular work hours or during after-hour, weekend or holiday care. Prolene sutures are removed 10-12 days post-op or when wound is sufficiently healed.

Humane Endpoints: Supportive care will be provided or animal will be euthanized based on recommendation of attending veterinarian if complications arise.

Appendix H

Dietary Restriction (DR) Protocol

Pre-experimental set-up:

Material:

1. Scale

2. IPTT transponders

3. Individual cages

4. A clean working table for transponder implantations

5. Isopropyl alcohol in spray bottle

6. Gloves

7. Lab coats

IPTT transponders implantation:

1. Wear lab coat and gloves

2. Disinfect the working table it with isopropyl alcohol and let it dry

3. Weigh the animal

4. Test the transponders with the reader (before and after implantation)

5. Hold animal securely on table and implant IPTT transponders

6. Return animal to the cage

7. House two rats per cage

8. Re-disinfect table with isopropyl alcohol

9. Repeat the steps 2-7 as many times as the sample size.

10. Record the particulars on data sheet

Experimental set-up:

Divide rats into 4 groups as shown

	AL	DR
Drug	10	10
Vehicle	10	10

AL = ad libitum

DR = Dietary Restriction

Feeding schedules:

- DR group will be fed on alternate days. Animals will be moved to clean cages on alternate days to avoid food caches.

- AL group will be fed everyday ad lib

Body temperature (T_b) & respiratory rate (RR):

- Monitor RR and T_b daily in both AL and DR groups prior to adding/removing food or handling them to weigh and move to clean cages.

Body weights:

- Body weights will be taken once every 4 days (when the animals are moved to clean cages)

- Animals which drop by 20% of their initial body weight will be eliminated from the study.

Injections:

- Administer drug/vehicle by a blind-observer when the DR group shows a consistent decrease in T_b.

- Collect data at regular intervals as planned on the datasheet

Continue DR for 48h after drug testing.

Tissue collection (48h post-injection):

- Anesthetize with isoflurane

- Collect brain tissue by rapid dissection

- Freeze in liquid nitrogen

- Store at $-80°C$ until analyzed

Appendix I

Setting up of telemetry device for collecting body temperature

Equipment used in the system and suppliers

- Receiver (Datasciences International, DSI™; PhysioTel™ Receiver; Model RPC-1)

- Transmitter (model VM-FH, Minimitter Company, Inc., Bend, OR and model CTA-F40, Data Sciences International, Inc., St.Paul, MN)

- Matrix (Data Exchange Matrix, Data Sciences International, Inc., St.Paul, MN)

Software:

- Dataquest® A.R.T. 2.3, Data Sciences International, Inc., St.Paul, MN)

Connecting receiver to matrix

- Arrange receiver under the animal chamber

- Plug-in J1-output of the receiver to one end of the cable and the other end to a jack on the back panel of the matrix

Connecting matrix to data acquisition system

- Plug one end the communication cable to jack J1 on the data acquisition system

- Plug the other end to matrix with jack labeled COM

- Plug-in the matrix to power supply

Configuration process of the system

- Select PROGRAMS-DATAQUEST A.R.T-ACQUISITION-ACQUISITION-NO CONFIGURATION-CONFIGURATION-NEW

- Enter the details and click OK

- Select CONFIGURATION-HARDWARE

- Configure matrix, receiver, and transmitter

Configuring a matrix

- Go to acquisition window; select CONFIGURATION-HARDWARE-

  HARDWARE-VERIFY

- A window appears with Hardware Verification Errors

- Click ACCEPT

Configuring a receiver

- Connection RPC-1 and matrix

- From the configuration window, select HARDWARE-VERIFY

- A window appears with Differences that Configuration can resolve automatically

- Click ACCEPT

Configuring a transmitter

- Select HARDWARE-NEW TRANSMITTER

- Enter the details of the transmitter

- Finish configuration of transmitter

Finishing configuration

- Go to ACQUISITION PROGRAM-HARDWARE-VERIFY

- Click OK

- A window appears with Differences that Configuration can resolve automatically

- Click ACCEPT

- Go to HARDWARE-VERIFY

- Click OK

- Click YES to save changes to exit Configuration or click NO to exit without saving any changes

- Create a folder

- Go to Acquisition

- Select the animal ID and start sampling