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Central adenosine receptor signaling is necessary for torpor in mice

By

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Abstract

When calorically restricted at cool ambient temperatures, mice conserve energy by entering torpor, during which metabolic rate (MR), core body temperature (T_b), heart rate (HR), and locomotor activity (LMA) decrease. Treatment with exogenous adenosine produces a hypometabolic state with reductions in T_b, HR, and LMA, mimicking torpor. In this study, I used the non-specific adenosine receptor antagonists aminophylline and 8-sulfophenyltheophylline (8-SPT) to test the hypothesis that adenosine signaling is necessary for the initiation and maintenance of torpor in fasted mice. In the first experiment, male C57BL/6J mice were subcutaneously (s.c.) infused with aminophylline at a rate of 6 mg kg^{-1} hr^{-1} while T_b, HR, and LMA were continuously monitored using implanted radiotelemeters. Over the course of a 23 hour fast at an ambient temperature of 20°C, control mice were torpid for 529 ± 49 minutes, while aminophylline-treated mice were torpid for significantly less time, 108 ± 51 minutes. Aminophylline-treated fasted mice were not significantly different from saline-treated fed mice. In a second experiment, 100 mg^{-1} kg^{-1} aminophylline was infused s.c. into torpid mice to test the role of adenosine in the maintenance of torpor. Aminophylline reversed the hypometabolism, hypothermia, bradycardia, and hypoactivity of torpor, while saline did not. In the third experiment, the polar adenosine antagonist 8-SPT, which does not cross the blood-brain barrier, was infused either s.c. (50 mg/kg) or intracerebroventricularly (i.c.v.; 10 μg/mouse) to test the hypothesis that both peripheral and central adenosine receptor signaling are necessary for the maintenance of torpor. Intracerebroventricular, but not subcutaneous, infusion of 8-SPT reversed torpor. These findings support my hypothesis that adenosine is necessary for the induction and maintenance of torpor in mice, and further suggest that whereas peripheral adenosine signalling is not necessary for the maintenance of torpor, antagonism of central adenosine is sufficient to disrupt torpor.

Introduction

Characteristics of Torpor

Reduction of metabolic rate is a strategy that is widely used for the conservation of energy, particularly among small mammals and birds (Swoap 2008). When environmental conditions cue a need for high energy expenditure during a period of low energy availability, animals employing this strategy enter a hypometabolic state, allowing them to align energy use with availability. Environmental cues can include long-term cues such as a short photoperiod, indicating that winter in approaching, or short-term cues like cool temperatures, indicating an immediate need for increased thermogenesis to maintain body temperature. The length of hypometabolism can likewise vary, from the well-known long-term state of hibernation, to the much shorter term state of daily torpor.

Like hibernation, daily torpor is a hypometabolic state that allows for the conservation of energy. However, whereas hibernation typically lasts for days or weeks at a time, daily torpor bouts average only hours in length. Hibernation is also much deeper than daily torpor, with metabolic rate on average falling to 5% of basal metabolic rate (BMR) in hibernation and 30% of BMR in torpor. This difference is reflected by a similar difference in minimum body temperature (T_b), with hibernators reaching a minimum of as low as 3°C and torpid animals falling to 18°C (Geiser 2004). Despite differences in length and depth, hibernation and torpor are physiologically very similar (Wilz and Heldmaier 2000; Geiser 2004). Likewise, the biochemical mechanisms involved in each state share substantial overlap, although key differences do exist. Notably, active suppression of metabolism appears to play a greater role in the induction of hibernation

(Geiser 2004; Staples and Brown 2008). Thus, although studies of hibernation can inform the investigation of daily torpor, the states should not be assumed to be identical.

The common house mouse *Mus musculus*, though often considered a homeotherm, is more accurately described as a facultative daily heterotherm (Swoap 2008). Mice can maintain a T_b of 37°C across a wide range of ambient temperature, but their large surface area to volume ratio makes the maintenance of euthermy energetically costly in extreme ambient temperatures. When the energetic stress of low ambient temperature is accompanied by a negative energy balance due to reduced food availability, mice enter the hypometabolic state of daily torpor (referred to from this point on as torpor). When torpor is initiated, the hypothalamic temperature set point (T_{set}) is lowered, starting a chain of events that results in substantially reduced energy consumption (Heller, Colliver et al. 1977; Geiser 2004). When T_{set} is lowered below T_b, thermoregulatory heat production is reduced, quickly decreasing metabolic rate. As T_b falls, metabolic rate is further reduced due to the temperature dependence of enzymatic reactions. As T_b approaches the new T_{set}, thermoregulatory heat production resumes at a lower level, maintaining the lower T_b (Geiser 2004). The reduction in heat production and temperature-related drop in overall metabolic rate during hypothermia combine to reduce energy requirements, allowing mice to survive longer on stored energy in times of limited food availability.

In mice, torpor bouts average approximately 6 hours in length (Hudson and Scott 1979). Torpor is punctuated by inter-bout arousal periods, during which T_b returns to approximately 37°C, even in the absence of an increase in ambient temperature (Carey,

Andrews et al. 2003; Drew, Buck et al. 2007). During arousal periods, activity increases as animals forage for food (Geiser 2004).

Besides the decreases in metabolic rate and T_b, torpid mice experience a variety of cardiovascular changes. Heart rate (HR) decreases in a temperature-dependent fashion, falling from an average of around 600 beats per minute (bpm) in fed mice to approximately 150 bpm during torpor (Williams, Chambers et al. 2002; Swoap and Gutilla 2009). Heart rate at a given T_b is dramatically lower during torpor entrance than during emergence, suggesting that heart rate is actively controlled during these times (Swoap and Gutilla 2009). Indeed, fasting has long been known to decrease sympathetic nervous system signaling and studies in other species have demonstrated parasympathetic control of heart rate during the initiation of torpor (Young and Landsberg 1977; Zosky 2002; Zosky and Larcombe 2003). Systolic, diastolic, and mean arterial pressures all drop during torpor, but pulse pressure does not, suggesting that while heart rate decreases, stroke volume (SV) remains constant (Swoap and Gutilla 2009). Total peripheral resistance (TPR) is increased during torpor bouts, likely due to effects of sympathetic activation on smooth muscle, similar to previously documented sympathetic signaling to white adipose tissue (Migliorini, Garofalo et al. 1997; Swoap and Weinshenker 2008; Swoap and Gutilla 2009). The paradoxical fall in blood pressure while stroke volume is constant and total peripheral resistance is increased suggests that the drop in blood pressure is primarily driven by the decrease in heart rate, since Mean Arterial Pressure=HR x SV x TPR (Swoap and Gutilla 2009).

Neuroendocrine Control of Torpor

The arcuate nucleus of the hypothalamus (ARC) has been shown to integrate a variety of hormonal and neural signals in order to regulate feeding and energy balance (Hahn, Breininger et al. 1998; Cone, Cowley et al. 2001; Cowley, Smart et al. 2001; Kalra and Kalra 2003). Ablation of the ARC by perinatal treatment with monosodium glutamate eliminates torpor in mice and suckling rats, suggesting that the ARC plays a crucial role in the regulation of torpor (Schoelch, Hubschle et al. 2002; Gluck, Stephens et al. 2006; Pelz, Routman et al. 2008).

The two neuron populations involved in ARC regulation of energy balance are those that coexpress neuropeptide Y (NPY) and agouti-related protein (AgRP) and those that express pro-opiomelanocortin (POMC) (Schwartz and Porte 2005). Whereas NPY/AgRP neurons stimulate feeding and reduce energy expediture, POMC neurons inhibit feeding and stimulate weight loss (Clark, Kalra et al. 1985; Cone 1999; Fekete, Sarkar et al. 2002; Schwartz and Porte 2005). NPY/AgRP neurons have specifically been implicated in the control of torpor; *Npy* -/- mice, which do not express NPY protein, experience torpor bouts that are both shallower and shorter in duration than wild-type mice (Gluck, Stephens et al. 2006). In Siberian hamsters, intracerebroventricular (i.c.v.) administration of NPY or an NPY Y_1 receptor agonist induces hypothermia similar to torpor (Paul, Freeman et al. 2005; Pelz and Dark 2007). Treatment with a Y_1 antagonist blocks this NPY-induced hypothermia (Dark and Pelz 2008).

Ghrelin, a peptide hormone secreted primarily by the stomach, induces weight gain through activation of NPY/AgRP neurons (Kojima, Hosoda et al. 1999; Asakawa, Inui et al. 2001). Plasma ghrelin levels are decreased after feeding and increased during a

fast, suggesting a role as an indicator of short-term negative energy balance (Horvath, Diano et al. 2001). Vagotomy abolishes the orexigenic effect of peripheral ghrelin, suggesting that the effect is dependent on vagal nerve afferent signaling to the periphery (Asakawa, Inui et al. 2001; Date, Murakami et al. 2002). However, the ghrelin-sensitive growth hormone secretagogue receptor (GHSR) is expressed in NPY/AgRP neurons, and ghrelin producing neurons have been found in close proximity to NPY/AgRP neurons in the ARC, suggesting an additional role for central ghrelin in the regulation of energy balance (Cowley, Smith et al. 2003; van den Top, Lee et al. 2004). Peripheral ghrelin treatment increases the length and depth of hypothermia in torpor, but does not affect the body temperature of fed mice, implicating ghrelin as a modulator of the torpor response. This effect is absent in *Npy* -/- mice, suggesting that, like its orexigenic effect, ghrelin's control of torpor is mediated by NPY signaling (Gluck, Stephens et al. 2006).

Leptin, another peptide hormone that regulates energy balance, is encoded by the *Ob* gene and primarily secreted by white adipose tissue (Zhang, Proenca et al. 1994). Plasma leptin levels correlate with adiposity, with high leptin levels signaling energy sufficiency when adipose mass is large (Considine, Sinha et al. 1996; Takahashi, Funahashi et al. 1996). Leptin reduces food intake and increases energy expenditure through several pathways. Peripherally, leptin inhibits the secretion of ghrelin by the stomach, reducing its orexigenic effect (Kalra, Ueno et al. 2005). Centrally, leptin binds to long form leptin receptors (Ob-Rb) on ARC NPY/AgRP neurons, resulting in inhibition of those cells (Takahashi and Cone 2005). Leptin also acts in the ARC to activate ARC POMC neurons, both directly by binding to Ob-Rb receptors and indirectly by decreasing inhibitory signaling from NPY/AgRP neurons (Cowley, Smart et al. 2001).

Like ghrelin, leptin plays a role in fasting-induced torpor. Whereas high plasma leptin levels prevent the initiation of torpor, chronically low leptin levels facilitate torpor. *ob/ob* mice lack a functional leptin gene, resulting in hyperphagia and obesity (Ingalls, Dickie et al. 1950; Zhang, Proenca et al. 1994). Unlike wild-type mice, *ob/ob* mice experience spontaneous torpor bouts while fed, and fasted *ob/ob* mice go into deeper torpor than wild-type controls (Himmshagen 1985). During a fast, sympathetic nervous system input to white adipose tissue increases, activating the β_3-adrenergic receptor (Migliorini, Garofalo et al. 1997; Rayner 2001). This β_3-adrenergic activation suppresses leptin secretion, signaling negative energy balance and leading to the induction of torpor (Giacobino 1996; Mantzoros, Qu et al. 1996; Swoap, Gutilla et al. 2006). *Dbh* -/- mice, which are unable to synthesize epinephrine or norepinephrine and therefore cannot activate sympathetic pathways, do not reduce leptin secretion or enter torpor during a fast. Treatment with a non-specific β-adrenergic agonist or a β_3-specific agonist causes an immediate drop in plasma leptin levels and induces torpor in fasted *Dbh* -/- mice, while β_3-antagonist treatment reduces the length and depth of torpor bouts in wild-type mice (Swoap, Gutilla et al. 2006). FXR -/- mice, which lack a bile acid receptor, experience a greater drop in leptin levels during a fast compared to wild-type mice. Like *ob/ob* mice, FXR -/- mice experience deeper torpor bouts than wild-type controls (Cariou, Bouchaert et al. 2007). In both *ob/ob* and FXR -/- models of leptin depletion, as well as in calorically restricted wild-type mice, treatment with exogenous leptin blunts torpor (Doring, Schwarzer et al. 1998; Gavrilova, Leon et al. 1999; Cariou, Bouchaert et al. 2007). Thus, the reduction of plasma leptin levels is necessary, but not sufficient, for the induction of torpor, an effect that could be driven by the removal of tonic inhibition of

ARC NPY/AgRP neurons when leptin signaling decreases (Cowley, Smart et al. 2001; Takahashi and Cone 2005; Swoap 2008).

Orexin Signaling in the Hypothalamus

The orexins, also known as hypocretins, are neuropeptides expressed primarily by neurons in the lateral hypothalamus (LH; De Lecea, Kilduff et al. 1998; Sakurai, Amemiya et al. 1998; Date, Ueta et al. 1999). The orexin precursor prepro-orexin is cleaved into two orexin peptides, orexin A and orexin B. The orexins bind to two G-protein receptors, Ox1R and Ox2R. Ox1R selectively binds orexin A, while Ox2R equally binds orexin A and orexin B. Fasting and hypoglycemia increase prepro-orexin mRNA expression in orexin neurons, and i.c.v. injection of either orexin induces hyperphagy, suggesting a role for orexins in the regulation of feeding (Sakurai, Amemiya et al. 1998). Both orexins have been shown to activate NPY/AgRP neurons and inhibit POMC neurons *in vitro*, suggesting that orexin signaling may involve ARC pathways (Muroya, Funahashi et al. 2004). Axons from orexin-positive neurons have been shown to synapse on POMC neurons, suggesting that a similar relationship is found *in vivo* (Guan, Saotome et al. 2001). Furthermore, Ox1R is expressed in ARC NPY/AgRP neurons and the hyperphagia induced by orexins is blunted by i.c.v. injection of NPY Y_1 or Y_5 antagonists (Ida, Nakahara et al. 1999; Dube, Horvath et al. 2000; Jain, Horvath et al. 2000; Yamanaka, Kunii et al. 2000; Suzuki, Shimojima et al. 2002). Likewise, Ox2R is expressed in the ARC and arcuate Ox2R mRNA is increased during fasting (Marcus, Aschkenasi et al. 2001; Chen, Karteris et al. 2006).

Like ghrelin and leptin, orexin influences signaling in the ARC, suggesting a possible role for changes in orexin signaling in the regulation of torpor. Orexin A mRNA expression is decreased in the LH in torpid Djungarian hamsters, supporting this hypothesis (Herwig, Ivanova et al. 2007). Orexin neurons in the LH almost universally express leptin receptors, suggesting that leptin's control of torpor may be partially mediated by inhibition of orexin signaling (Hakansson, de Lecea et al. 1999). Likewise, ghrelin-containing neurons synapse on orexin neurons, i.c.v. ghrelin increases activation of orexin neurons, and i.c.v. treatment with anti-orexin antibodies blunts the hyperphagia induced by ghrelin (Toshinai, Date et al. 2003). Thus, in addition to acting on the same torpor-related ARC pathways as leptin and ghrelin, orexin signaling is regulated by the two hormones, and could be involved in mediating their effects.

Torpor, Sleep, and Orexin

It has been hypothesized that torpor is evolutionarily derived from sleep; the two processes share similarities, although their exact relationship is unclear (Heller and Ruby 2004). Hibernation is entered through a sleep state, and electroencephalogram (EEG) recordings suggest that NREM (non-rapid eye movement) sleep is common during torpor, with no REM (rapid eye movement) sleep once brain temperature falls below 25°C (Walker, Glotzbach et al. 1977; Walker, Haskell et al. 1981). As in torpor, body temperature drops during NREM sleep, suggesting possible common mechanisms for energy conservation (Heller 1988; Heller and Ruby 2004). The evidence that torpor is similar to sleep is complicated by the finding that EEG patterns after torpor and hibernation mimic sleep deprivation (Deboer and Tobler 1996; Palchykova, Deboer et al.

2002). This result can be explained if the recuperative mechanisms of sleep cease to work at low brain temperatures, a phenomenon that would also explain the function of interbout arousals; torpid animals must return to euthermy to allow such processes to work (Daan, Barnes et al. 1991; Trachsel, Edgar et al. 1991). In hibernating golden mantled ground squirrels, the EEG pattern is a function of brain temperature, with increased similarity to sleep-deprivation patterns at lower temperature. In contrast, bout length is not correlated with sleep deprivation EEGs, supporting the rejuvenation explanation for interbout arousals; as long as the brain temperature is above a threshold level, even very long torpor bouts do not mimic sleep deprivation (Larkin and Heller 1996). An alternate theory has also been proposed, that dendritic and synaptic density decreases during hypometabolic states, reducing the ability to maintain depolarization of wakefulness-promoting thalamocortical neurons. This theory suggests that after arousal, reduced signaling results in intense sleep until the repair of dendrites and synapses and the restoration of thalamocortical depolarization (Heller and Ruby 2004). Further study has shown that sleep deprivation following hibernation eliminates, rather than exacerbates, the sleep deprivation EEG patterns, suggesting that hibernation is not equivalent to sleep deprivation (Larkin and Heller 1999; Chen, Karteris et al. 2006). Additionally, like NREM sleep and unlike sleep deprivation, torpor results in improved memory retention (Nowakowski, Swoap et al. 2009). Although some questions remain unanswered, current evidence suggests that sleep and torpor are related (Heller and Ruby 2004).

The link between sleep and torpor provides additional evidence for the involvement of orexins in the control of torpor. In addition to modulating food intake,

orexin is critical for the modulation of sleep and wakefulness (Sakurai 2007). Reduced orexin signaling has been correlated with narcolepsy in humans, and mutations in the prepro-orexin or Ox2R gene result in narcolepsy-like phenotypes in animal models (Chemelli, Willie et al. 1999; Lin, Faraco et al. 1999; Peyron, Faraco et al. 2000; Thannickal, Moore et al. 2000). When orexin is administered i.c.v. to rats during the light cycle, when sleep normally occurs, wakefulness is increased and both REM and NREM sleep are decreased (Hagan, Leslie et al. 1999). While orexin neurons project to a wide variety of targets in the brain, the projections that are most likely relevant to its control of wakefulness are those to the locus coeruleus (LC), raphe nucleus (RN), and tuberomammillary nucleus (TMN). Activation of LC, RN, and TMN monoaminergic neurons is known to be correlated to wakefulness, suggesting that activation of these neurons mediates the wakefulness-promoting effects of orexin (Sakurai 2007). Orexin also activates cholinergic neurons in the basal forebrain, which have been implicated in the maintenance of arousal (Alam, Szymusiak et al. 1999; Eggermann, Serafin et al. 2001).

In addition to their well-characterized effects on wakefulness, orexins influence a variety of physiological parameters. In rats, i.c.v. injection of orexin A induces an increase in mean arterial pressure (MAP), and heart rate (HR), with simultaneous increases in plasma norepinephrine and renal sympathetic nerve activity (RSNA) (Shirasaka, Nakazato et al. 1999, Esposito, Viggiano et al. 2006). Orexin A has also been shown to have differential dose-dependent effects on white adipose tissue (WAT) sympathetic activation and plasma free fatty acid (FFA) levels in rats (Shen, Tanida et al. 2008). At high doses, i.c.v. orexin A increases WAT sympathetic activation and

increases plasma FFA consistent with increased sympathetic-induced lipolysis. This effect is eliminated by β-adrenergic or histamine H_1 antagonists, suggesting that orexin-induced WAT sympathetic activation acts through histaminergic signaling pathways.

Orexins also play a role in thermoregulation. When administered to rats i.c.v., orexin A increases brown adipose tissue (BAT) sympathetic activation, raising interscapular BAT temperature and core body temperature (Monda, Viggiano et al. 2001, Esposito, Viggiano et al. 2006; Jaszberenyi, Bujdoso et al. 2002). This effect is preceded by a transient dose-dependent decrease in BAT sympathetic activation, along with NPY-mediated hypothermia (Szekely, Petervari et al. 2002; Yasuda, Masaki et al. 2005).

The role of orexins in the regulation of feeding, body temperature, wakefulness, and the cardiovascular system suggests that they may be involved in torpor. This theory is further reinforced by the interaction between NPY, ghrelin, and leptin, which are known to be involved in the regulation of torpor, and orexinergic signaling. Puzzlingly, orexin's effects on the NPY/POMC system is opposite to its effects on arousal, temperature, and the cardiovascular system (i.e., orexin increases arousal, body temperature, and heart rate, while simultaneously signaling an energy deficit to NPY/POMC neurons), suggesting that there may be complex interaction effects. While the relationship between orexin and torpor has not been shown experimentally, orexin A expression is decreased during torpor, providing some support for a possible role of orexin signaling in torpor regulation, although this change could also be an effect of torpor. Orexinergic signaling is one way through which adenosine could affect pathways involved in sleep, energy balance, and cardiovascular control.

Adenosine and Torpor

Adenosine is a nucleoside that has effects throughout the body. Central or peripheral administration of exogenous adenosine induces a drop in T_b similar to the hypothermia observed in torpor, leading to the hypothesis that adenosine is involved in the regulation of torpor (Anderson, Sheehan et al. 1994; Swoap, Rathvon et al. 2007; Swoap 2008). In support of this hypothesis, i.c.v. injection of an A_1 adenosine receptor antagonist has been shown to cause a return to euthermia in hibernating Syrian hamsters (Tamura, Shintani et al. 2005). Adenosine acts on a number of torpor-related neural pathways, including those involved in regulation of sleep, temperature, energy balance, and the cardiovascular system. Adenosine inhibits wakefulness-promoting neurons in the basal forebrain through A_1 receptor activation and through A_{2A} receptors activates populations of neurons in the preoptic nucleus that are necessary for sleep (Alam, Szymusiak et al. 1999; Gallopin, Luppi et al. 2005). Activation of A_1 receptors in the preoptic area also results in hypothermia and increased sleep in rats (Ticho and Radulovacki 1991; Barros, Branco et al. 2006). Furthermore, treatment with an adenosine A_1 or A_2 receptor specific agonist inhibits GABA release in cultured ARC neurons, supporting a role for neuronal adenosine signaling in the regulation of energy balance (Chen and vandenPol 1997). Adenosine also inhibits activity of preganglionic sympathetic neurons in the spinal cord through A_1 receptor activation, a pathway through which adenosine could cause the cardiovascular changes of torpor (Deuchars, Brooke et al. 2001). In addition to these direct effects on torpor-related pathways, adenosine could act indirectly through inhibition of orexin signaling. Adenosine has an inhibitory effect on LH orexin neurons *in vitro*, driven by activation of adenosine A_1 receptors (Liu and

Gao 2007). A similar effect has been shown *in vivo*, as i.c.v. treatment with an adenosine A_{2A} receptor agonist decreases expression of *Fos*, a measure of neuronal activation, in orexin neurons (Satoh, Matsumura et al. 2006). Thus, adenosine could cause the changes of torpor through direct inhibition of various neuronal populations involved in regulating torpor, but also indirectly through inhibition of orexinergic neurons in the LH which project to those regions (Figure 1).

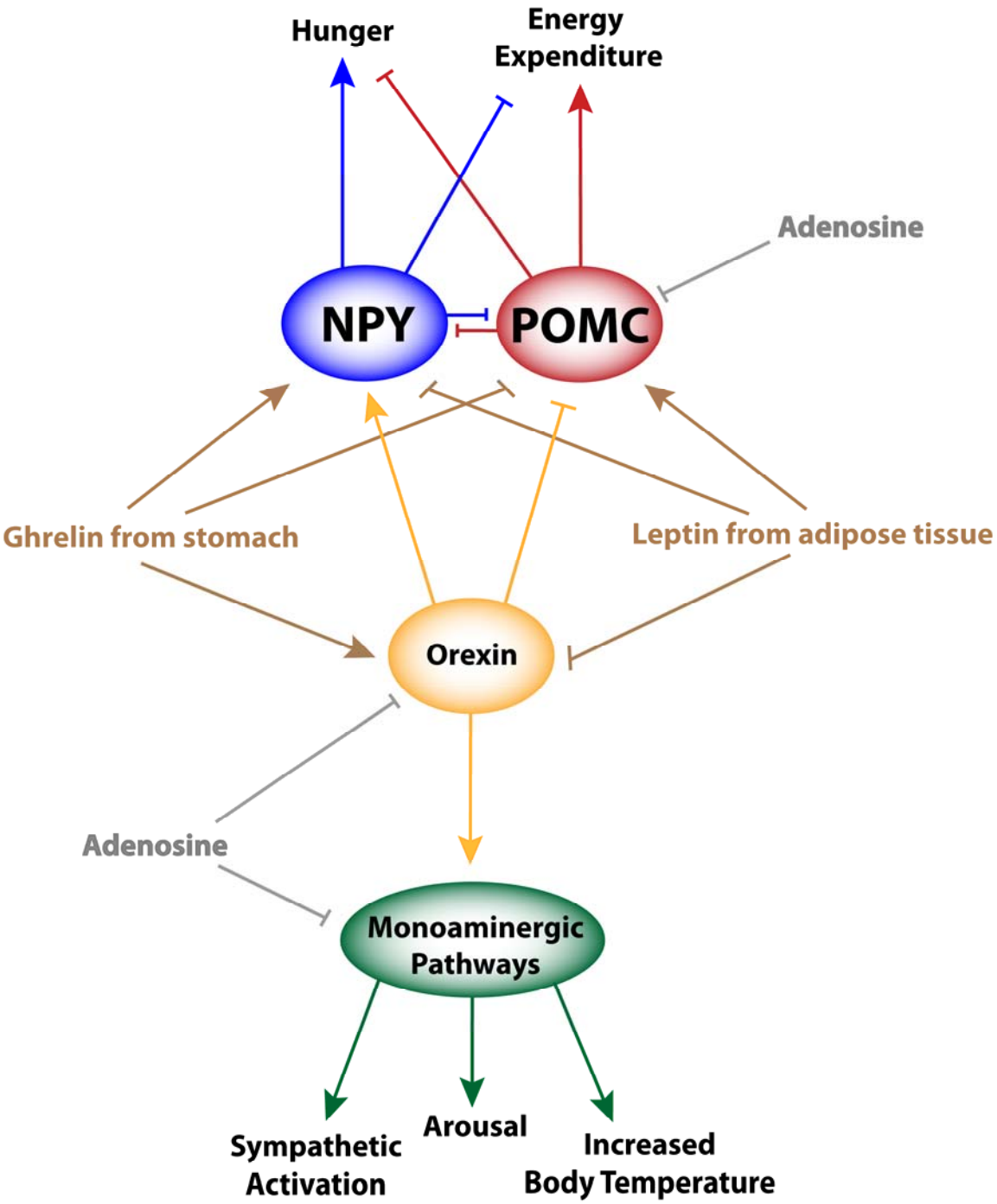

Figure 1. In the arcuate nucleus of the hypothalamus, neuropeptide Y-expressing neurons (NPY) signal energy insufficiency, resulting in hunger and reduced energy expenditure, while pro-opiomelancortin-expressing neurons (POMC) oppose this effect. The hormones ghrelin and leptin have similar opposing effects. Orexinergic neurons (Orexin) synapse on NPY and POMC neurons and may be involved in torpor, but also increase arousal through monoaminergic pathways in the locus coeruleus, raphe nucleus, and tuberomamillary nucleus. Adenosine acts through A_1 and A_{2A} to produce inhibitory effects in many of these neuronal populations, and could be involved in regulating torpor.

Adenosine Overview

Adenosine is a nucleoside composed of adenine linked to ribose by a β-N_9-glycosidic bond. *In vivo*, adenosine acts as a signal of metabolic stress after catabolism of ATP to ADP and AMP by ectonucleoside triphosphate diphosphohydrolase, followed by catabolism of AMP to adenosine by ecto-5'-nucleotidase (Hasko, Linden et al. 2008). Theoretically, drops in adenosine triphosphate (ATP) concentration could be measured to signal cellular energy status, but in practice ATP levels are so tightly controlled that they only drop under profound energetic stress (Cunha 2001). Intracellular adenosine monophosphate (AMP) concentrations are several orders of magnitude lower than ATP concentrations, so small changes in the equilibrium of ATP/ADP/AMP induce much larger relative changes in AMP concentration. This amplification of differences in ATP concentration allows AMP to function as a signal of energy insufficiency (Cunha 2001). Since AMP can be further dephosphorylated to adenosine, changes in AMP concentration can be further amplified, allowing intracellular adenosine concentration to reflect energy status in the cell. Additionally, adenosine transporters in the cell membrane allow the equilibration of intracellular and extracellular adenosine levels, letting adenosine act as a paracrine or autocrine signal during metabolic stress (Plagemann and Wohlhueter 1984; Cunha 2001). Indeed, the extracellular concentration of adenosine has been shown to increase during situations of metabolic stress (Cunha 2001). Adenosine is quickly phosphorylated to AMP by adenosine kinase, and deaminated to inosine by adenosine deaminase, thus limiting adenosine signaling in non-stressed cells (Hasko, Linden et al. 2008).

Adenosine acts on four known G-protein-linked receptors: A_1, A_{2A}, A_{2B}, and A_3. Although adenosine binds to all four receptor types, endogenous adenosine concentrations are only sufficient to cause significant activation in A_1, A_{2A}, and A_3 receptors, suggesting that A_{2B} receptors are only activated in pathological states or after pharmacological intervention (Fredholm, Irenius et al. 2001).

Within the cell, the primary effect of A_1 receptor activation is inhibition of adenylyl cyclase by $G_{\alpha i}$, leading to decreased cyclic AMP (cAMP) concentrations and decreased activity of protein kinase A (PKA), ultimately leading to decreased cAMP response element-binding protein (CREB) activation. Through $G_{\beta\gamma}$ subunits, A_1 receptors also activate phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K), resulting in increased mitogen-activated protein kinase (MAPK) activation (Jacobson and Gao 2006). A_1 receptors also activate K^+ channels, probably through $G_{\beta\gamma}$, and inhibit Ca^{2+} channels through $G_{\alpha i}$, increasing the threshold for depolarization (Fredholm, Ijzerman et al. 2001).

A_3 receptors similarly act through $G_{\alpha i}$ to inhibit adenylyl cyclase, with the same downstream effects as A_1. A_3 $G_{\beta\gamma}$ subunits activate PLC and PI3K, leading to increased MAPK activation. A_3 activation also leads to $G_{\beta\gamma}$-mediated activation of RhoA and phospholipase D1. WNT and nuclear factor-κB pathways are also regulated by A_3 signaling. Finally, A_3 has been shown to activate ATP-dependent K^+ channels (Jacobson and Gao 2006).

In contrast to A_1 and A_3 receptors, A_{2A} receptors *activate* adenylyl cyclase through $G_{\alpha s}$, resulting in increased intracellular cAMP and increased activation of PKA and CREB. Like A_1 and A_3 receptors, A_{2A} activation also results in increased MAPK

activation (Fredholm, Ijzerman et al. 2001). A_{2A} activation can also increase intracellular inositol phosphate concentration, raising intracellular Ca^{2+} levels and increasing PKC activation (Jacobson and Gao 2006).

Like A_{2A} receptors, A_{2B} activates adenylyl cyclase through $G_{\alpha s}$. However, the most common functions of A_{2B} receptors may be primarily mediated through $G_{\alpha q}$-mediated activation of PLC. As with A_{2A} receptor activation, A_{2B} activation increases inositol phosphate production, increasing intracellular Ca^{2+} concentrations. Effects of adenosine receptor activation are shown in Figure 2.

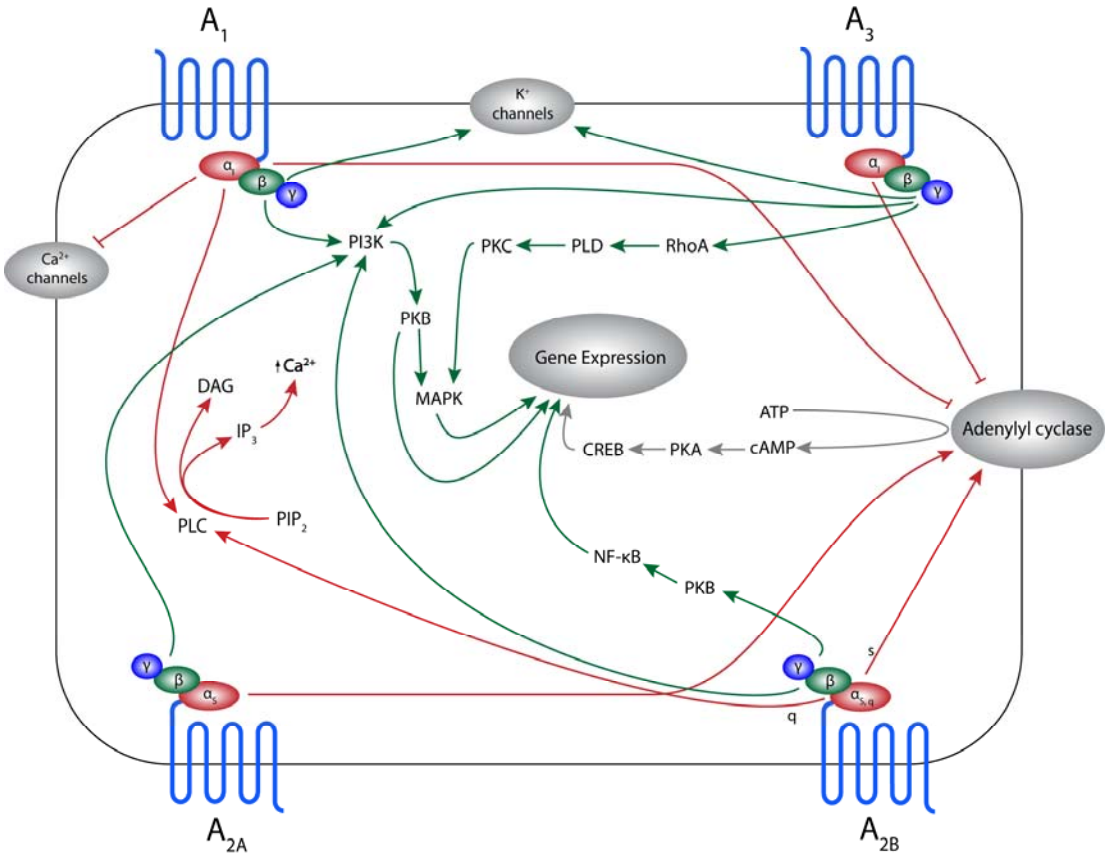

Figure 2. Downstream effects of adenosine receptor activation. The majority of short-term physiological effects are due to changes in adenylyl cyclase activity. ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate, PKA = protein kinase A, PKB = protein kinase B, PKC = protein kinase C, PLC = phospholipase C, PLD = phospholipase D1, CREB = cAMP responsive element-binding protein, NF-κB = nuclear factor κB, MAPK = mitogen-activated protein kinase, DAG = diacyl glycerol, PIP_2 = Phosphatidylinositol bisphosphate, IP_3 = inositol triphospate.

Adenosine in the Central Nervous System

The most common adenosine receptor in the CNS is the inhibitory A_1 subtype, so adenosine primarily acts as an inhibitory neurotransmitter. This inhibition is mediated by both presynaptic inhibition of neurotransmitter release and by postsynaptic hyperpolarization due to K^+ channel activation (Cunha 2001). Adenosine's A_1-mediated effects on neurotransmitter release are primarily due to inhibition of N-type calcium channels, although the release apparatus itself may also be affected; A_1 receptors have been hypothesized to decrease the affinity of the release apparatus for Ca^{2+} (Cunha 2001). A_{2A} receptors, which are generally excitatory, also play a role in neuromodulation by adenosine. Through activation of PLC and PKC, A_{2A} receptors activate P-type Ca^{2+} channels and inhibit N-type Ca^{2+} channels. The push-pull relationship of A_1 inhibition and A_{2A} excitation have been demonstrated in a number of brain regions and neurotransmitter systems, including acetylcholine (ACh) and dopamine in the striatum, ACh, serotonin (5-HT), norepinephrine (NE), and glutamate (Glu) in the hippocampus, ACh in the cortex, Glu in the superior colliculus, and glycine in the brainstem (Cunha 2001). Importantly, these effects of A_1 and A_{2A} are only reliably found in excitatory neurons. The effects in inhibitory neurons are not well defined and vary by region, with the traditional effects being reversed in some neurons (Cunha 2001). Notably, both A_1 and A_{2A} receptor activation inhibit orexinergic neurons in the LH (Satoh, Matsumura et al. 2006; Liu and Gao 2007).

While A_1 and A_{2A} are clearly important receptors for neuromodulation, A_{2B} and A_3 receptors play less of a role in neurotransmission in non-pathological states. As mentioned previously, A_{2B} receptors have insufficient affinity to bind adenosine at non-

pathological concentrations, and A_3 receptors are primarily involved in longer-term effects rather than acute neuromodulation (Gessi, Merighi et al. 2008; Hasko, Linden et al. 2008).

One major role of adenosine in the CNS is neuroprotection, mediated primarily by A_1 receptors. Administration of either adenosine or A_1-specific agonists are neuroprotective in ischemia models, while A_1 antagonists increase cell death. This neuroprotective effect is likely due to adenosine's role in maintaining homeostasis, perhaps combined with a decrease of excitotoxicity due to its inhibitory neuromodulatory effect (Cunha 2001). A_{2A} receptors may also play some role in neuroprotection, since A_{2A} activation leads to vasodilation, resulting in increased delivery of oxygen and nutrients to affected cells. A_{2A} activation also suppresses the production of hydrogen peroxide and superoxide radicals, resulting in decreased oxidative damage (Daval, Nicolas et al. 1996). However, tests of A_{2A} receptor antagonists have yielded unclear results, with different studies reporting both neuroprotective and neurotoxic effects in CNS ischemia models (Cunha 2001). A_3 receptors have also been proposed to have a role in neuroprotection, although evidence is similarly mixed. A_3 activation could provide neuroprotection by inhibition of nitric oxide synthase, suppression of glutamate-induced excitotoxicity, and suppression of the inflammatory response (Gessi, Merighi et al. 2008).

Adenosine in the Cardiovascular System

Adenosine is also a critical regulator of the cardiovascular system. Most of adenosine's effects on cardiovascular physiology during non-pathological conditions are mediated by A_1 receptors in the heart and A_{2A} receptors in the vasculature, although A_3

receptors in both the heart and blood vessels may also play a role (Shryock and Belardinelli 1997; Tabrizchi and Bedi 2001; Gessi, Merighi et al. 2008). Through activation of A_1 receptors, adenosine slows heart rate, slows atrioventricular node conduction, and reduces atrial contractility (Shryock and Belardinelli 1997). These cardiac effects are induced by A_1-mediated inhibition of adenylyl cyclase, resulting in activation of inward-rectifying K^+ channels and thus allowing increased K^+ currents that result in hyperpolarization and reduced action potential duration (Shryock and Belardinelli 1997; Fredholm, Ijzerman et al. 2001). Decreased adenylyl cyclase activity also results in reduced activation of Ca^{2+} channels, reducing contractility. Adenosine also reduces the pacemaker current in sinoatrial and atrioventricular node cells, inhibiting pacemaker activity (Shryock and Belardinelli 1997). Reduced atrial contractility decreases ventricular filling, reducing ventricular stroke volume according to Starling's Law, an effect that combines with the reduced heart rate to result in decreased cardiac output (Starling 1921).

Adenosine also works via A_1 receptors to suppress the effects of β-adrenergic receptor activation. Because the increases in contractility and heart rate caused by β-adrenergic activation are mediated by stimulation of adenylyl cyclase and increased intracellular cAMP, inhibition of adenylyl cyclase by A_1 activation opposes these effects (Belardinelli, Shryock et al. 1995). In summary, adenosine A_1 receptor activation decreases heart rate and contractility, resulting in decreased cardiac output. As an adenylyl cyclase inhibitor, the A_3 receptor subtype could have similar effects to the A_1 receptor, but because A_3 receptors are rare in the heart and blood vessels, their role is likely minor (Gessi, Merighi et al. 2008).

Adenosine A_{2A} receptors play an important role in the cardiovascular system as a modulator of blood flow. Activation of A_{2A} receptors induces vasodilation, resulting in reduced resistance and increased blood flow. This effect is mediated by receptors on endothelial and vascular smooth muscle cells (Daval, Nicolas et al. 1996). A_{2A} activation results in increased ATP-dependent K^+ channel activation, leading to hyperpolarization of smooth muscle cells, decreased intracellular Ca^{2+}, and therefore muscle relaxation and decreased resistance to blood flow (Liang 1992). Nitric oxide-mediated vasodilation may also play some role in the effects of A_{2A} activation (Vials and Burnstock 1993). As in the brain, A_1 and A_{2A} receptors in the cardiovascular system oppose each other, with A_1 receptors decreasing blood flow by reducing cardiac output, and A_{2A} receptors increasing blood flow through vasodilation.

As in the brain, adenosine may provide protection from ischemia-induced damage to the heart. Pre-treatment with adenosine reduces infarct size and severity of functional deficits after ischemia, and mice that overexpress A_1 receptors are resistant to ischemia-induced injury (Dhalla, Shryock et al. 2003). Furthermore, A_1 receptor activation has been implicated in cardioprotection during hibernation in Syrian hamsters. Whereas pentobarbital-induced hypothermia induces abnormal EKG waves related to irreversible cardiac injury, these waves do not occur during hypothermia induced by central adenosine or after administration of pentobarbital to hibernating hamsters (Miyazawa, Shimizu et al. 2008). The cardioprotective effects of adenosine may be due in part to A_1 receptor-induced inhibition of metabolic demands, or possibly due to A_1-induced changes in activation of specific pathways. Similar effects have been shown for A_3 activation, despite low A_3 receptor density in the heart (Gessi, Merighi et al. 2008). A_3 agonist

pretreatment confers resistance to ischemia-induced damage, possibly through activation of mitochondrial ATP-dependent K^+ channels and prevention of high intracellular Ca^{2+} concentrations (Gessi, Merighi et al. 2008). However, the role of A_3 receptors in cardioprotection is not entirely clear, as A_3 knockout mice are resistant to ischemia, and cardiac A_3 receptor expression is so low that the ability of physiological levels of adenosine to cause meaningful activation is questionable (Gessi, Merighi et al. 2008).

The Role of Adenosine in Torpor

The finding that exogenous adenosine causes hypothermia that mimics torpor, combined with adenosine's well-studied role as a regulator of homeostasis, suggests that adenosine may play a role in the regulation of torpor. Adenosine has been shown to act on several neural pathways that are required for torpor, and activation of adenosine receptors also reduces heart rate and blood pressure, mimicking the changes that occur in torpor. Finally, adenosine has been shown to induce neuroprotection and cardioprotection, and thereby provides a mechanism by which critical tissues could resist damage caused by low body temperature and poor tissue perfusion.

This study tested the hypothesis that endogenous adenosine is involved in the regulation of torpor in mice. Adenosine signaling was be blocked in fasted mice at cool ambient temperatures, using the non-specific adenosine receptor antagonists aminophylline and 8-sulfophenyltheophylline (8-SPT). Using radiotelemetry to measure heart rate, body temperature, and activity, as well as indirect calorimetry to measure metabolic rate, the effect of adenosine receptor antagonism on the depth and length of torpor was characterized. First, the role of adenosine in initiation of torpor was tested by

continuous peripheral infusion of aminophylline. Next, peripheral aminophylline was administered during a torpor bout to test whether adenosine is necessary for maintenance of torpor. In the third and fourth experiments, the site of adenosine's action was tested by infusing 8-SPT during a torpor bout, either peripherally (Experiment 3) or centrally (Experiment 4).

Materials and Methods

Animals

Male C57BL/6J mice were either bred locally for peripheral drug infusion experiments or ordered pre-cannulated from The Jackson Laboratory (Bar Harbor, ME) for central drug infusion experiments. Animals were individually housed at approximately 25°C with a 12 hour light/12 hour dark cycle. Experiments were run between the beginning of the dark cycle and one hour before the start of the next dark cycle. The last hour of the light cycle was reserved for animal care. All experiments were conducted according to a repeated measures, crossover design to allow each animal to act as its own control and minimize the number of animals used. Between experiments, subcutaneous cannulae were capped by a sealed portion of polyethylene tubing. Intracerebroventricular guide cannulae were protected from tissue infiltration by dummy cannulae, which were removed and replaced with injection cannulae immediately before each experiment. All procedures were approved by the Williams College Animal Care and Use Committee.

Electrocardiogram Radiotelemeter Implantation

Each mouse was initially anesthetized with 5% isoflurane in oxygen through a nose cone, followed by maintenance with 2-3% isoflurane. The mouse was placed on a heating pad to maintain a body temperature of approximately 37°C. Meloxicam (5 mg kg^{-1}) was administered subcutaneously (s.c.) for perioperative and postoperative analgesia. The mouse was placed in dorsal recumbancy and the abdomen was clipped to remove

hair and prepared with povidone iodine antiseptic. A midline incision approximately 2.5 cm in length was made in the skin, and the skin was separated from the abdominal wall to facilitate later closure. A 2 cm midline incision was then made in the abdominal wall, taking care not to damage visceral tissue. An electrocardiogram/temperature/activity telemeter (hereafter referred to as an EKG telemeter) was then inserted into the abdomen (TA10ETA-F20; Data Sciences International, St. Paul, MN). Non-absorbable suture was used to simultaneously close the anterior section of the body wall incision and secure the telemeter to the abdominal wall (5-0 Ethilon; Johnson & Johnson, New Brunswick, NJ). An 18-gauge needle was used as a trocar to pass the tip of fine forceps through the abdominal wall into the abdominal cavity approximately 3mm lateral to the incision and 1cm from the anterior end. The forceps were used to grasp and pull through each EKG lead, one on each side of the incision. The incision was then completely closed using non-absorbable suture. The skin was separated from the body wall from the initial incision in two tunnels terminating at approximately the level of the heart, approximately 5mm medial to the forelimbs. The EKG leads were placed in these tunnels and secured with one non-absorbable suture, approximating a Lead I configuration. The skin was then closed with 5mm wound clips (Reflex Clips; Fine Science Tools, Foster City, CA). Bacitracin/neomycin/polymyxin B triple antibiotic ointment was applied to the incision and the mouse was returned to its cage, which was placed with one half on a heating pad to allow the mouse to behaviorally thermoregulate. Each mouse was monitored until it regained the righting reflex, then allowed to recover on the heating pad for 48 hours, with health checks every 12-24 hours. Mice were allowed to recover at least 7 days before any experimental testing. Staples were removed after 7 days.

Subcutaneous Cannula Implantation

Each mouse was anesthetized, placed on a heating pad, and treated with meloxicam as in the EKG telemeter implantation surgery. The mouse was placed in ventral recumbancy and the right dorsal flank was clipped and prepared with povidone iodine. An incision approximately 1.5cm in length was made in the skin at the posterior end of the ribcage, perpendicular to a line between the interscapular region and the site of the incision. The skin was then separated from the underlying tissue from the incision to the interscapular region. A 1.5" 21 gauge needle was used as a trocar to puncture the interscapular skin from the exterior and the tip was fed to the site of the incision. A custom cannula, constructed of coiled 25 gauge stainless steel hypodermic tubing attached to Prolene surgical mesh, was fed into the lumen of the larger needle and placed in the subcutaneous interscapular region. The incision was then stapled and triple antibiotic ointment was applied. The mouse was placed in its cage, which was half on a heating pad, and observed until it regained the righting reflex. Mice were allowed to recover on the heating pad for 24 hours and were not used in experimental testing until 7 days after surgery.

Tethering

For all torpor experiments, mice were tethered via their implanted cannula to tubing outside of the cage. For subcutaneous infusions, mice were connected to a flexible piece of 25 gauge inside diameter polyethylene tubing approximately 3cm in length (BPE-T25; Instech Solomon, Plymouth Meeting, PA), which ran through a custom polypropylene bite-guard. This tubing connected to a 10cm length of 25 gauge outside

diameter stainless steel hypodermic tubing, followed by another length of polyethylene tubing. This tubing connected to a 25 gauge swivel **(**375/25PS; Instech Solomon), which was itself connected to a gimbal to allow for pivoting along the long axis of the cage. This system was designed to minimize weight and resistance while maximizing freedom of movement and ease of attachment and detachment.

Drugs and Doses

All drugs were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Drugs were administered in sterile 0.9% NaCl solution intraperitoneally (i.p.) or subcutaneously (s.c.) or in sterile phosphate-buffered saline (PBS) intracerebroventricularly (i.c.v.). Adenosine was always used at a concentration of 3 mg ml^{-1}. Mice received a dose of 100 mg kg^{-1} i.p. or 30 µg i.c.v.

When testing the efficacy of the antagonist in the first experiment, adenosine mono-phosphate (AMP) was used instead of adenosine. AMP was dissolved at 20 mg ml^{-1} and administered at a dose of 100 mg kg^{-1}. AMP is quickly metabolized to adenosine *in vivo* and produces a similar physiological reaction (Swoap, Rathvon et al. 2007).

Two adenosine antagonists were used, aminophylline and 8-sulfophenyltheophylline (8-SPT). For continuous infusion, aminophylline was given at a s.c. dose of 6 mg kg^{-1} $hour^{-1}$ in a volume of 20 µL $hour^{-1}$. For the interrupt experiment, aminophylline was given as a 100 mg kg^{-1} s.c. dose in a volume of 100 µL (concentration was adjusted depending on body weight; approximately 20 mg ml^{-1}). 8-SPT was either prepared at a concentration of 10 mg ml^{-1} and given as a 50 mg kg^{-1} s.c. dose (volume

adjusted based on body weight; approximately 125 μL) or prepared at 2 mg ml^{-1} and given as a 10 μg i.c.v. dose.

For drug infusion through cannulae, the administration was controlled using a syringe pump (PHD-22; Harvard Apparatus, Holliston, MA), except during adenosine challenges testing acute drug infusion, which were performed by hand. The other exception was the infusion of 8-SPT and adenosine during the central 8-SPT adenosine challenge, also performed by hand. For peripheral adenosine challenges, AMP or adenosine was administered by i.p. injection using a 27G x 0.5" needle.

Adenosine Challenges

Experiment 1: Continuous Peripheral Aminophylline

Each antagonist treatment was tested for efficacy using an adenosine-induced hypothermia assay. To test the chronic aminophylline infusion treatment, mice were housed at 20°C and infused with 6 mg kg^{-1} aminophylline through a swiveling tether attached to their cannulae, starting at the beginning of the dark phase. After 16 hours, without interrupting aminophylline infusion, mice were injected with 100 mg kg^{-1} i.p. AMP.

Experiments 2, 3, 4: Torpor Interrupt Experiments

To test the treatments used in each interrupt experiment, mice were housed at 20°C starting from the beginning of the dark cycle. After one hour of acclimation, mice were injected with either antagonist or saline through the implanted cannula (Experiments 2 and 3: subcutaneous; Experiment 4: intracerebroventricular). After 20

minutes, all mice were then injected with adenosine (Experiments 2 and 3: 100 mg kg^{-1} at 3 mg ml^{-1} in saline; Experiment 4: 30 µg in 10 µL phosphate-buffered saline over 5 minutes).

Caloric Restriction

Experiment 1: Continuous Peripheral Aminophylline

In the continuous infusion experiment, mice were fed *ad libitum* at all times except during 23 hour fasts. Mice had a body weight range of 28 to 35 grams.

Experiment 2: Torpor Interrupt with Peripheral Aminophylline

Torpor depth and length have an inverse relationship with body weight, so mice were calorie restricted to increase the depth and length of torpor bouts. The torpor bouts of *ad libitum* fed mice were too short to reliably determine whether mice were exiting torpor naturally or as a result of adenosine antagonism. In the aminophylline interrupt experiment, a baseline food intake was recorded over 7 days, and mice were then fed 80% of their baseline caloric intake until they weighed less than 28 grams, at which point they were tested. After fasting, each mouse was re-fed *ad libitum* for one day before repeating the caloric restriction.

Experiment 3: Torpor Interrupt with Peripheral 8-SPT

In the peripheral 8-SPT interrupt experiment, mice were fed 80% of their 7-day baseline food intake until they weighed less than 28 grams, at which point they were fed 2.9 grams of chow per day to maintain that body weight.

Experiment 4: Torpor Interrupt with Central 8-SPT

The mice in the central 8-SPT interrupt experiment were young enough that their body weights when fed *ad libitum* were less than 28 grams, so they were not calorie restricted.

Measurement of Physiological and Behavioral Parameters

The implanted radiotelemeters were used to measure body temperature, heart rate, and locomotor activity. Telemeters were sampled once per minute and collected data over 5-30 seconds, reporting average values over that time. Data were collected by receiver pads, then routed through a hardware data matrix to a PC-compatible computer. Data were saved using the D.S.I. Acquistion software, and later loaded using the D.S.I. Analysis program. Temperature data were processed by a macro in Microsoft Excel to convert raw outputs to temperature readings using 5-point calibrations taken before implantation.

Indirect Calorimetry

In the aminophylline interrupt experiment, metabolic rate and respiratory exchange ratio (RER: CO_2 expired / O_2 consumed) were measured. Mice were housed in clear, air-tight cages. Room air was dried using calcium sulfate (Drierite; W.A. Hammond Drierite Co., Xenia, OH) and pumped in at a regulated flow of approximately 350 mL/minute. The outflow from the cage was then dried again and fed through a flow meter (Omega Engineering, Stamford, CT). The gas was then pulled through an O_2

analyzer and a CO_2 analyzer (AEI Technologies, Naperville, IL) at 140 mL/min, with excess flow vented to the atmosphere through a concentric, convoluted pathway to prevent mixing. Cages were sampled sequentially, with flow mediated by an 8-port gas flow switcher. Cages were switched no faster than once per 20 seconds to allow the gas to fully purge the previous sample from the analyzers. Ambient O_2 and CO_2 levels were measured by switching flow through an unoccupied cage. Atmospheric pressure and ambient temperature were measured once per minute to allow for conversion to standard conditions for temperature and pressure. Data passed through an custom digitizer before being fed into a Macintosh-compatible computer and saved by Labview software (National Instruments, Austin, TX).

Data Analysis

Data were collected in Microsoft Excel files. Metabolism and telemetry data were synchronized, accounting for differences in computer clocks and for delay for sampled air to flow through tubing. Times in interrupt experiments were standardized, setting the beginning of the infusion as time=0. Statistical analyses were performed in SPSS 15.0. In the continuous infusion experiment, a repeated measures ANOVA was performed, with fed/fasted condition and aminophylline/saline treatment condition as within-subject variables. This test was followed by a planned comparison to evaluate the difference between aminophylline and saline treatment in fasted mice, using a paired Student's *t*-test. For other experiments, paired *t*-tests were performed between control and experimental conditions. The α level for significance was set at 0.05. Data are reported as mean ± standard error of the mean.

Results

Experiment 1: Continuous Peripheral Aminophylline Infusion

Before testing the effect of continuous peripheral aminophylline on torpor, its ability to block the effects of adenosine was tested. Either aminophylline or saline were infused for 16 hours, followed by i.p. adenosine injection without interrupting infusion. The average body temperature, heart rate, and activity over the next 60 minutes were compared with the average values for the preceding 4 hours. Continuous aminophylline treatment blunted the drop in temperature after adenosine injection, with the body temperature of saline-treated mice dropping 1.6±0.4°C and aminophylline-treated mice dropping 0.3±0.5°C (Figure 3; $t(5)=2.64$, $p<0.05$).

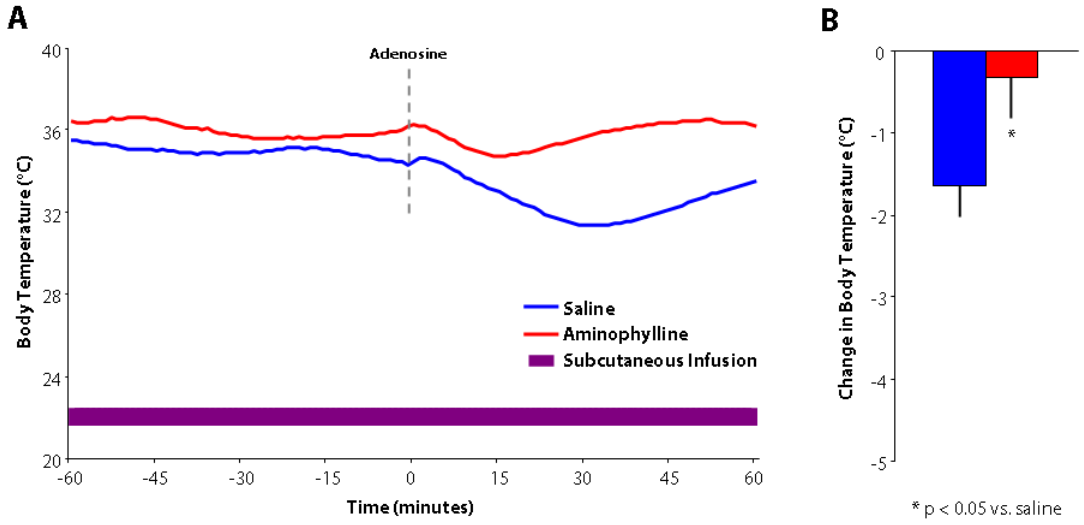

Figure 3. Continuous peripheral aminophylline infusion blunts adenosine-induced hypothermia. (A) Body temperature of a typical mouse when injected with adenosine during continuous peripheral infusion of saline or aminophylline. (B) Pooled data from 6 mice, measuring the change in average body temperature between the 4 hours prior to adenosine injection and the 60 minutes post-injeciton.

Adenosine-induced bradycardia trended lower in the aminophylline-treated group ($\Delta HR= 0\pm32$ beats min^{-1}) than in the saline-treated group ($\Delta HR= -61\pm25$ beats min^{-1}),

but this effect was not significant (Figure 4; $t(5)=1.69$, *ns*). Aminophylline treatment caused a trend towards increased activity after adenosine injection, but this result was also not significant (saline ΔLMA= -1±2, aminophylline ΔLMA= 7±7; $t(5)=1.126$, *ns*; data not shown).

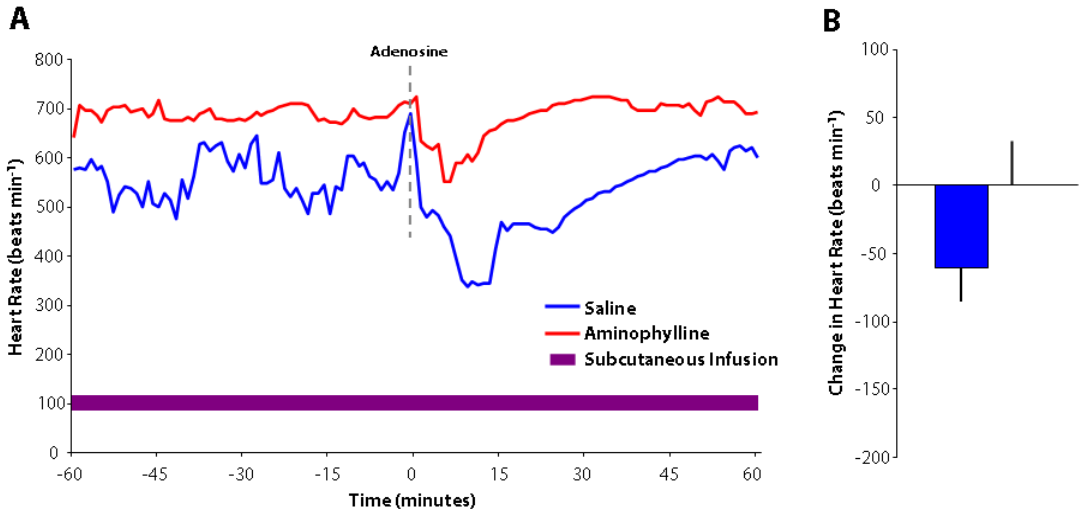

Figure 4. Continuous peripheral aminophylline infusion causes a trend toward decreased adenosine-induced bradycardia. (A) Heart rate from a typical mouse. (B) Pooled data from 6 mice.

After the hypothermia assay demonstrated that aminophylline infusion was effective at preventing adenosine-induced hypothermia, mice were continuously treated with aminophylline or saline while fasted to determine the whether adenosine blockade would prevent the induction of torpor. Mice were housed at 20°C and fasted for 23 hours, starting at the beginning of the dark cycle. To control for the effects of blocking adenosine in euthermic animals, mice were also infused while fed. Each mouse was attached to a swiveling tether and continuously infused with 6 mg kg^{-1} s.c. aminophylline at a rate of 20 µL hr^{-1}, or the same volume of saline, through a subcutaneous cannula. Cannula patency was verified by injecting a small volume of saline before the

experiment. The results were analyzed with a 2x2 (Food status * Drug Treatment) repeated measures ANOVA.

There were significant main effects of food status on minimum T_b, minimum HR, average T_b, average HR, and time in torpor (minutes T_b<34°C); each measurement was higher in fed animals except for time in torpor. There were also significant main effects of treatment on minimum T_b, average T_b, average HR, and time in torpor, with a marginally significant effect on minimum HR (p = 0.058). Every measurement was higher in the aminophylline-treated condition except for time in torpor. There were significant Food Status * Treatment interaction effects for minimum T_b, average T_b, average HR (p=0.051), and time in torpor, indicating that aminophylline had a greater effect on these measurements in the fasted condition than in the fed condition. Planned comparisons revealed that over 23 hours, aminophylline treatment significantly decreased time in torpor (Figure 5; saline time=542±43 minutes, aminophylline time=96±45 minutes; $t(6)=8.57$, $p<0.05$), minimum T_b (Figure 6A; saline $T_{b,min.}$=30.9±0.3°C, aminophylline $T_{b,min}$=33.2±0.4°C; $t(6)=5.13$, $p<0.05$), average T_b (Figure 6B; saline $T_{b,avg.}$=35.1±0.2°C, aminophylline $T_{b,avg}$=36.2±0.2°C; $t(6)=8.27$, $p<0.05$), minimum HR (Figure 7A; saline $HR_{min.}$=240±26 beats min^{-1}, aminophylline HR_{min}=311±41 beats min^{-1}; $t(6)=2.52$, $p<0.05$), and average HR (Figure 7B; saline $HR_{avg.}$=519±27 beats min^{-1}, aminophylline HR_{avg}=578±22 beats min^{-1}; $t(6)=3.87$, $p<0.05$).

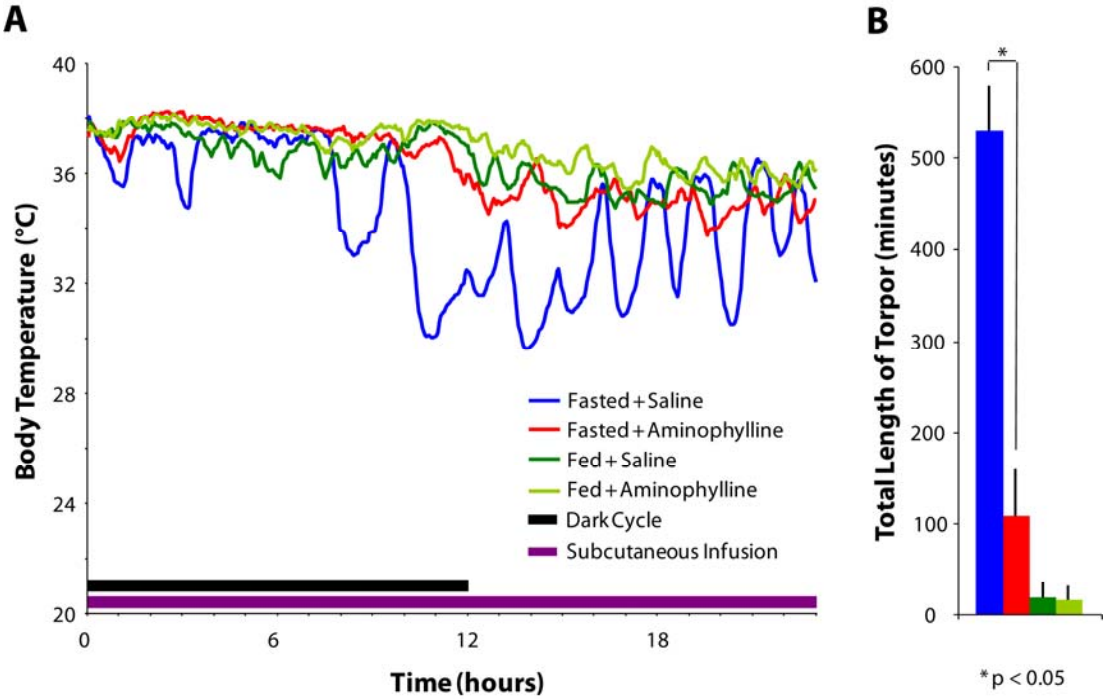

Figure 5. Continuous aminophylline treatment prevents induction of torpor. (A) Body temperature of a typical mouse when fed and fasted and treated with either aminophylline or saline. (B) Time in torpor (T_b<34°C) in each condition for 7 mice.

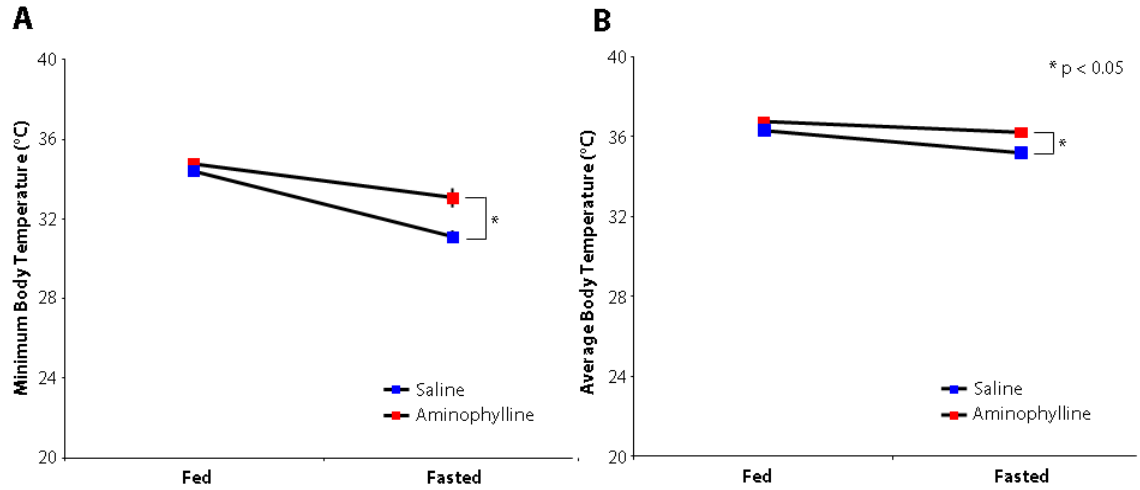

Figure 6. Aminophylline increases body temperature to a greater extent in fasted mice than in fed mice. (A) Minimum body temperature over 23 hours. (B) Average body temperature over 23 hours.

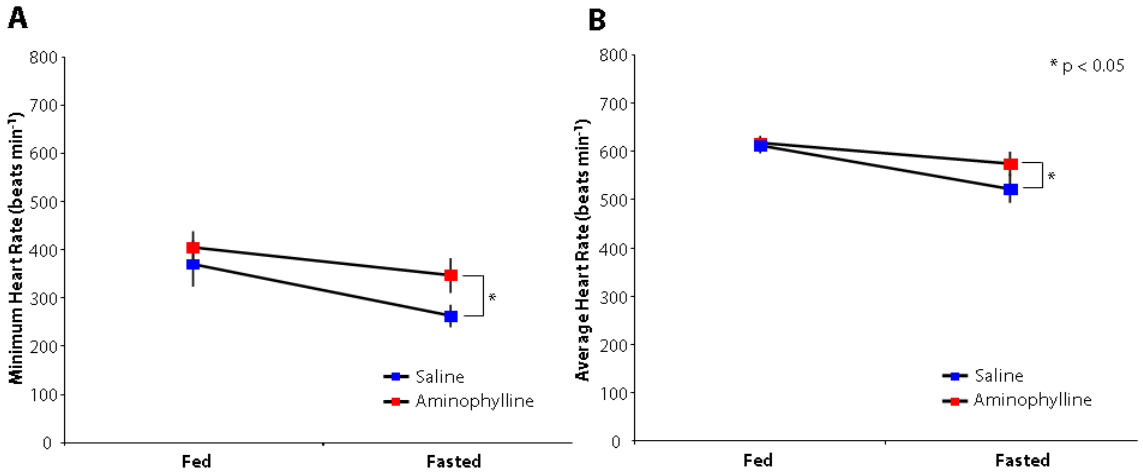

Figure 7. Aminophylline increases heart rate to a greater extent in fasted mice than in fed mice. (A) Minimum heart rate over 23 hours. (B) Average heart rate over 23 hours.

Experiment 2: Acute Peripheral Aminophylline Infusion

The role of adenosine in the maintenance of torpor was tested by interrupting a torpor bout with a short-term peripheral aminophylline infusion. First, the ability of acute peripheral aminophylline treatment to block the effects of adenosine was tested using the adenosine-induced hypothermia assay. The average body temperature, heart rate, and activity levels for the 60 minutes following adenosine injection were compared with the average values for the 30 minutes preceding the injection. Pre-treatment with 100 mg kg^{-1} peripheral aminophylline significantly reduced adenosine-induced hypothermia. The body temperature of saline-treated mice dropped 2.7±0.8°C, while the temperature of aminophylline-treated mice dropped 0.3±0.2°C (Figure 8, $t(5)=3.37$, $p<0.05$).

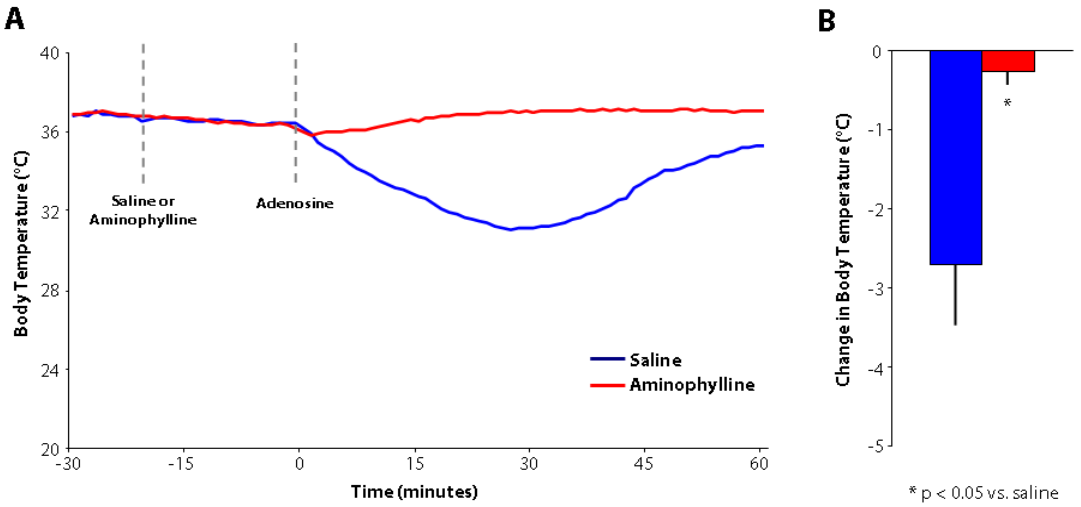

Figure 8. Acute peripheral aminophylline blunts adenosine-induced hypothermia. (A) Body temperature of a typical mouse when injected with adenosine following pretreatment with peripheral saline or adenosine. (B) Pooled data from 6 mice, measuring the change in average body temperature between the 30 minutes prior to adenosine injection and the 60 minutes post-injection.

Acute aminophylline treatment also prevented the bradycardia caused by adenosine. The heart rate of aminophylline-treated mice *increased* by 45±19 beats min^{-1} after adenosine treatment, compared to a decrease of 50±44 beats min^{-1} in saline-treated mice (Figure 9; $t(5)=2.61$, $p<0.05$). The change in activity after adenosine injection was similar between saline- and aminophylline-treated mice (saline ΔLMA= -7±1, aminophylline ΔLMA= -8±3; $t(5)=0.57$, *ns*; data not shown).

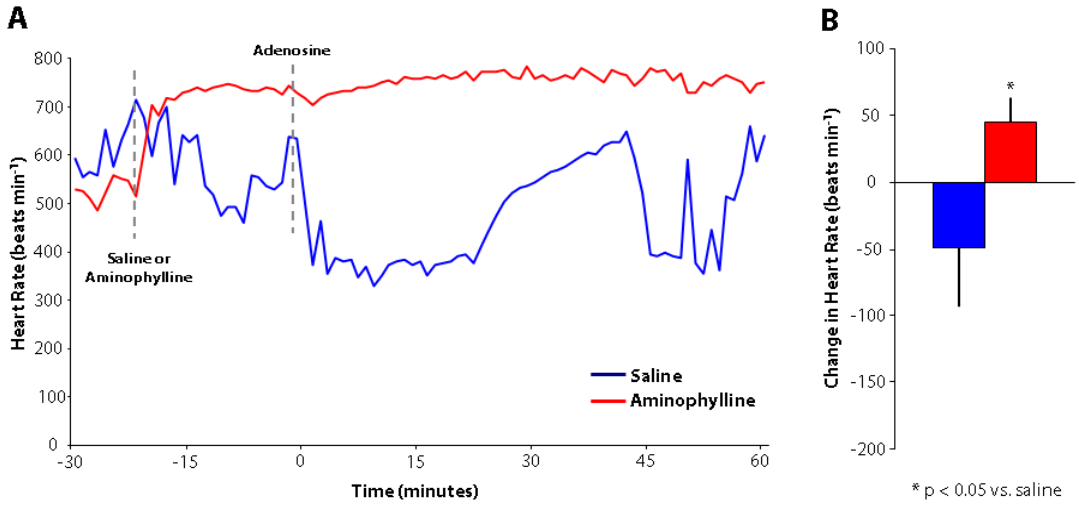

Figure 9. Acute peripheral aminophylline treatment suppresses adenosine-induced bradycardia. (A) Heart rate of a typical mouse. (B) Pooled data from 6 mice.

The role of adenosine receptor activation in the maintenance of torpor was then investigated by infusion of aminophylline during a torpor bout. Mice were housed at 20°C and fasted for 23 hours, starting at the beginning of the dark cycle. Each mouse was attached to a swiveling tether previously primed with aminophylline solution or saline. During the first torpor bout of the light cycle, as determined by the presence of steep, persistent drops in T_b and heart rate, mice were infused with either 100 mg kg^{-1} s.c. aminophylline in a volume of 100 µL, or the equivalent volume of saline. The infusion was conducted over the course of 20 minutes so that the mice were not disturbed. This experiment was conducted in sealed metabolic cages to allow measurement of metabolic parameters.

Aminophylline caused significant increases in metabolic rate (Figure 10A), body temperature (Figure 10B), heart rate (Figure 10C), and locomotor activity (Figure 10D) compared to saline treatment. These measures first diverged after approximately 10 minutes of infusion, and all measures were significantly different within 45 minutes of the start of infusion.

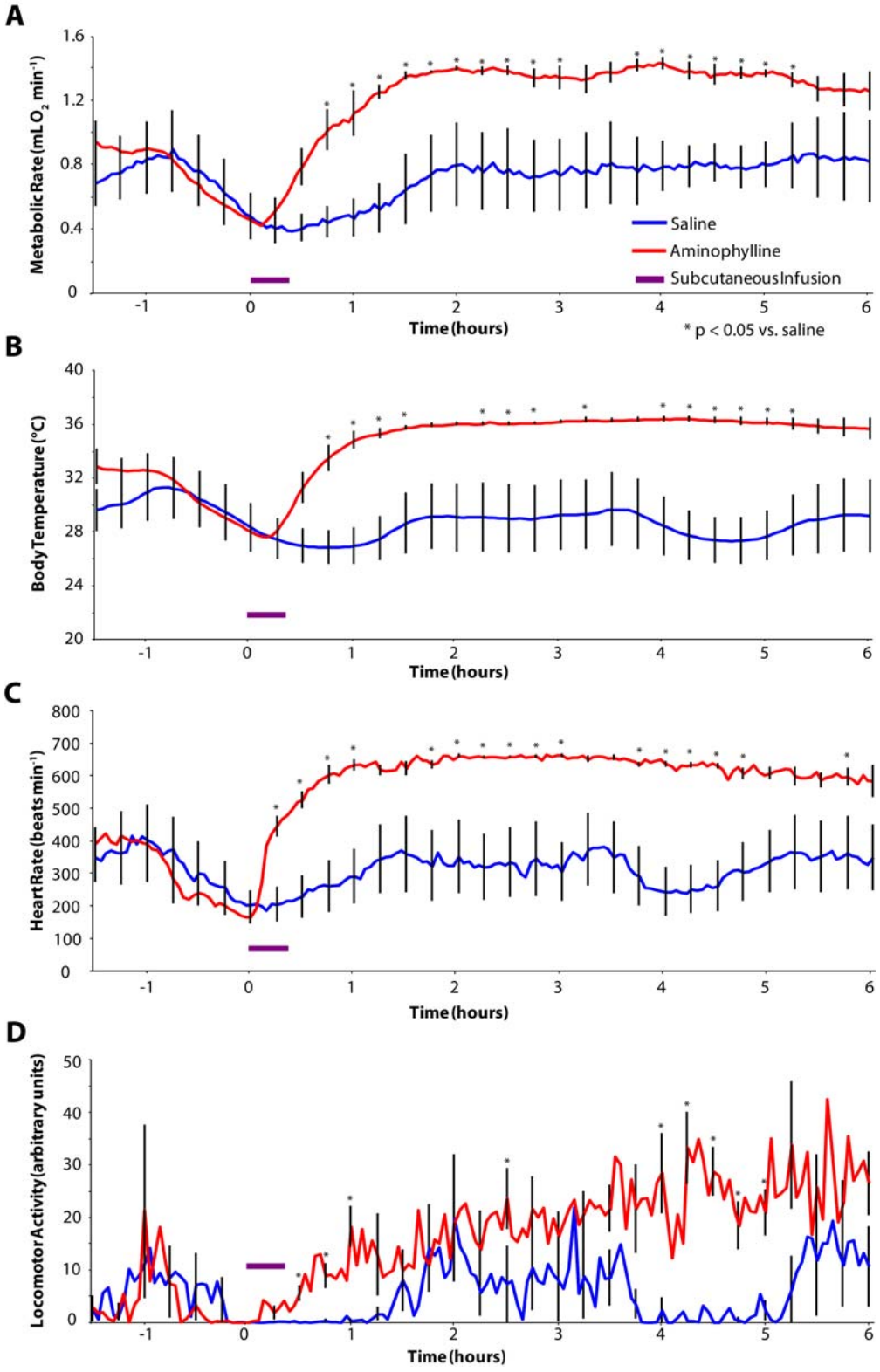

Figure 10. Peripheral aminophylline infusion reverses torpor. (A) Metabolic rate. (B) Body temperature. (C) Heart rate. (D) Locomotor activity.

Experiment 3: Acute Peripheral 8-SPT Infusion

Experiments 1 and 2 demonstrated that treatment with the adenosine antagonist aminophylline was sufficient to block the induction of torpor, as well as torpor maintenance. Further experiments were conducted with the polar non-selective adenosine receptor antagonist 8-sulfophenyltheophylline, which does not cross cell membranes or the blood-brain barrier. Additionally, while aminophylline has the side-effect of inhibiting phosphodiesterases (PDE), 8-SPT does not. Because of these characteristics, 8-SPT was used to clarify whether adenosine acts centrally or peripherally to regulate torpor, and to determine whether aminophylline's effects on torpor were solely a result of PDE inhibition.

In Experiment 3, Experiment 2 was repeated using 8-SPT to determine whether the reversal of torpor was caused by peripheral adenosine receptor blockade. Prior to the experiment, the effectiveness of acute peripheral 8-SPT treatment was assessed using the adenosine hypothermia assay. Average body temperature, heart rate, and locomotor activity for the 60 minutes following adenosine injection were again compared with the average values for the 30 minutes preceding the injection. Mice pretreated with 8-SPT exhibited greatly reduced hypothermia, with a reduction in body temperature of 0.8±0.2°C, versus hypothermia of 3.1±0.9°C in controls (Figure 11; $t(5)=2.95$, $p<0.05$).

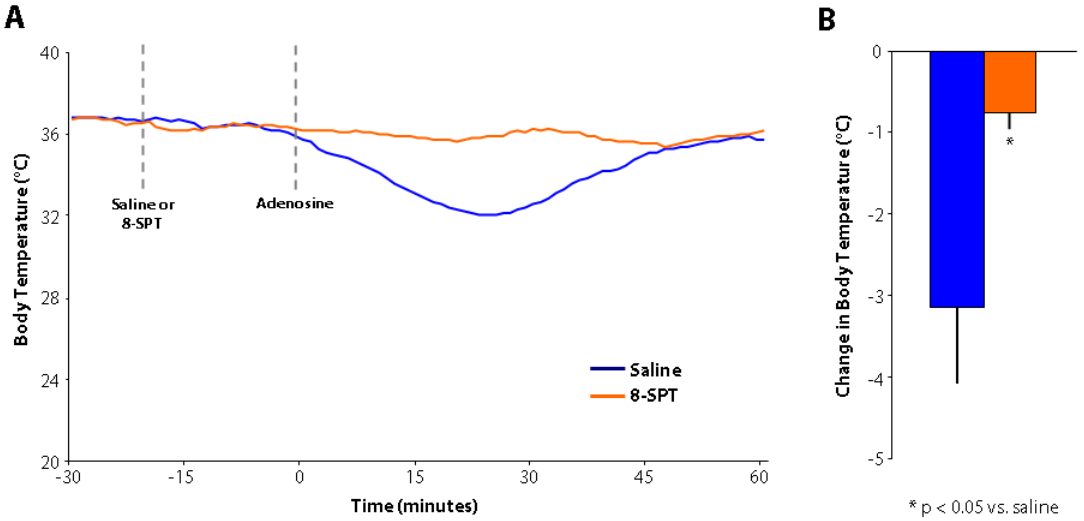

Figure 11. Acute peripheral 8-SPT blunts adenosine-induced hypothermia. (A) Body temperature of a typical mouse pre-treated peripherally with either saline or 8-SPT before an injection of adenosine. (B) Pooled data from 6 mice.

8-SPT treatment caused a trend toward decreased adenosine-induced bradycardia. The heart rate of saline-treated mice dropped 118±66 beats min^{-1}, while 8-SPT-treated mice experienced an increase of 10±25 beats min^{-1} after adenosine injection (Figure 12; $t(5)=2.21$, $p=0.078$). There was also a marginally significant difference in the effect of adenosine on activity, with saline-treated mice (ΔLMA= -14±4) exhibiting a larger decrease in activity than 8-SPT treated mice (ΔLMA= -8±3; $t(5)=2.43$, $p=0.06$; data not shown).

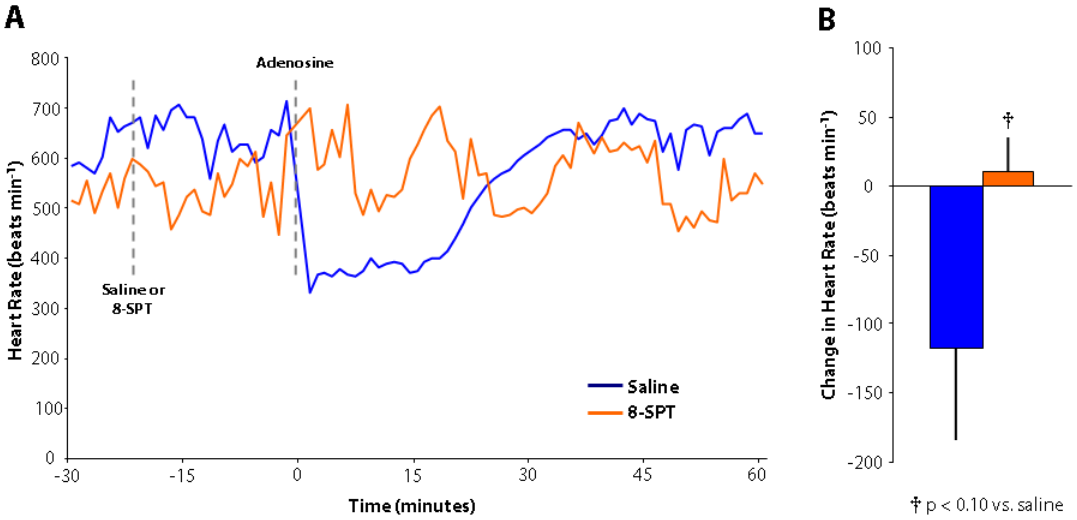

Figure 12. Acute peripheral 8-SPT blunts adenosine-induced bradycardia. (A) Heart rate of a typical mouse after peripheral 8-SPT or saline pretreatment. (B) Pooled data from 6 mice.

As in the previous experiments, mice were then fasted at an ambient temperature of 20°C while connected to a swiveling tether. The tether was primed with 8-SPT solution or saline, and during the first torpor bout of the light cycle, mice were infused with either 50 mg kg^{-1} 8-SPT in a volume of about 125 µL, or an equivalent volume of saline. In this experiment, infusions were conducted over approximately 25 minutes, varying slightly depending on the dose required.

Peripheral 8-SPT did not significantly affect body temperature (Figure 13A), heart rate (Figure 13B), or locomotor activity (Figure 13C) compared to saline. Whereas aminophylline-treated mice in Experiment 2 diverged from saline-treated controls within 10 minutes and had significantly different body temperature, heart rate, and activity after 45 minutes, 8-SPT treatment had no such effect.

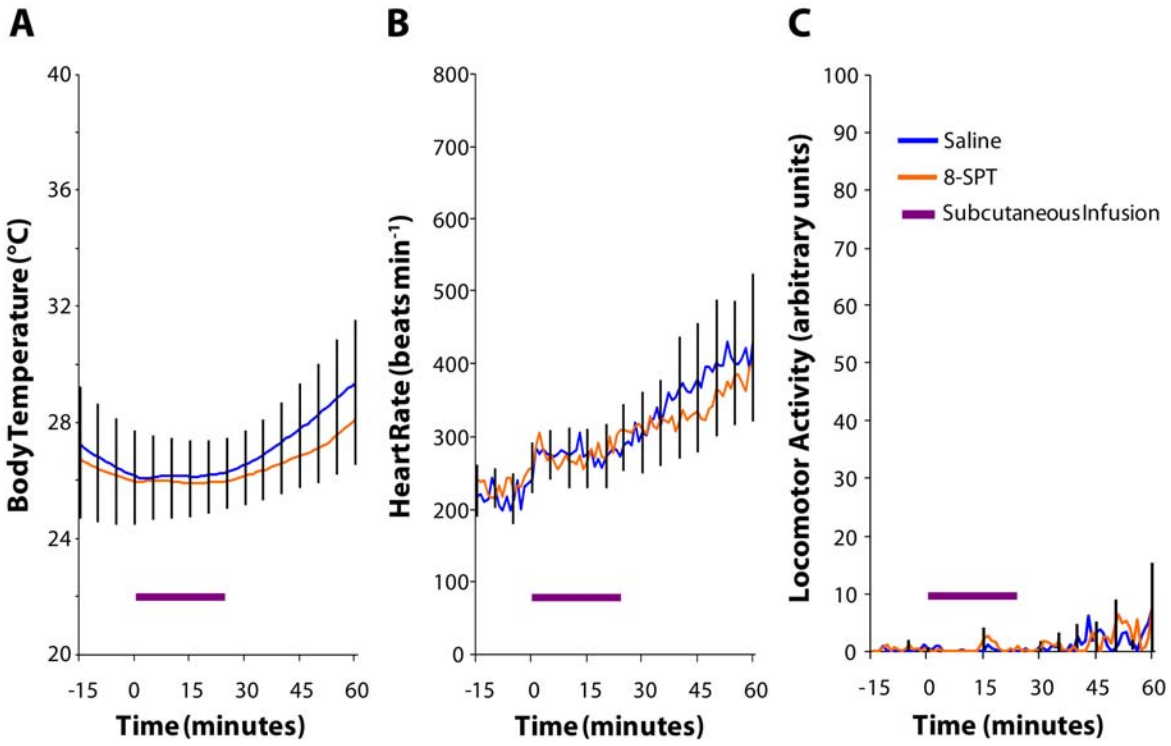

Figure 13. Peripheral 8-SPT infusion does not reverse torpor. (A) Body temperature. (B) Heart Rate. (C) Locomotor activity.

Experiment 4: Acute Central 8-SPT Infusion

The effects of central 8-SPT infusion on torpor were then evaluated. As in previous experiments, the effectiveness of adenosine antagonism by acute central 8-SPT infusion was tested by evaluating its effects on adenosine-induced hypothermia. The average body temperature, heart rate, and locomotor activity for the 60 minutes following adenosine infusion were compared with the average values for the 30 minutes preceding the injection. Central 8-SPT reduced the hypothermia induced by central adenosine infusion, with body temperature decreasing 0.3±0.4°C in the 8-SPT condition, compared to 1.8±0.5°C in the saline condition (Figure 14; $t(4)=3.61$, $p<0.05$). This effect, however, was complicated by the fact that 8-SPT itself induced hypothermia, with a reduction of

1.4±0.7°C between the average temperature for the 30 minutes prior to the 8-SPT infusion and 20 minutes following 8-SPT treatment (saline ΔT_b = 0±0.4°C). This effect was not, however, significant at an α of 0.05 ($t(4)=1.84$, $p=0.14$).

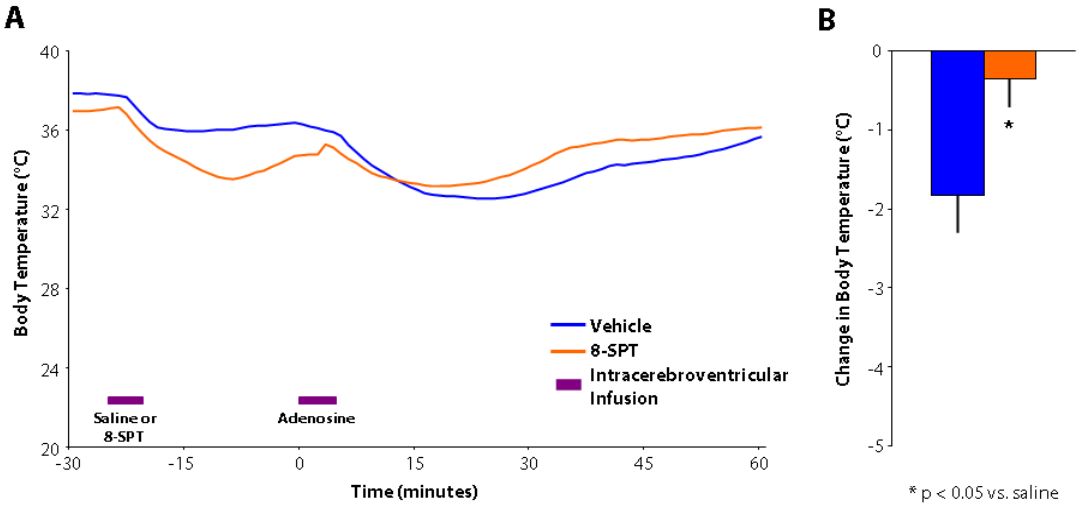

Figure 14. Acute central 8-SPT blunts adenosine-induced hypothermia (A) Body temperature of a typical mouse when pre-treated with central infusions of 8-SPT or vehicle, followed by central adenosine infusion. (B) Pooled data from 5 mice.

Central 8-SPT also prevented adenosine-induced bradycardia. The heart rate of 8-SPT-treated mice *increased* 44±18 beats min^{-1} compared to the average before adenosine infusion, while the heart rates of saline-treated mice fell 37±18 beats min^{-1} (Figure 15; $t(4)=12.36$, $p<0.05$). As with body temperature, 8-SPT produced a decrease in heart rate prior to adenosine infusion (8-SPT ΔHR= -106±28 beats min^{-1}, saline ΔHR= -37±40 beats min^{-1}), but this effect was not significant ($t(4)=1.96$, $p=0.12$). The change in locomotor activity produced by adenosine was not significantly different between saline and 8-SPT groups (saline ΔLMA= -11±5, 8-SPT ΔLMA= -9±10; $t(4)=0.29$, *ns*; data not shown).

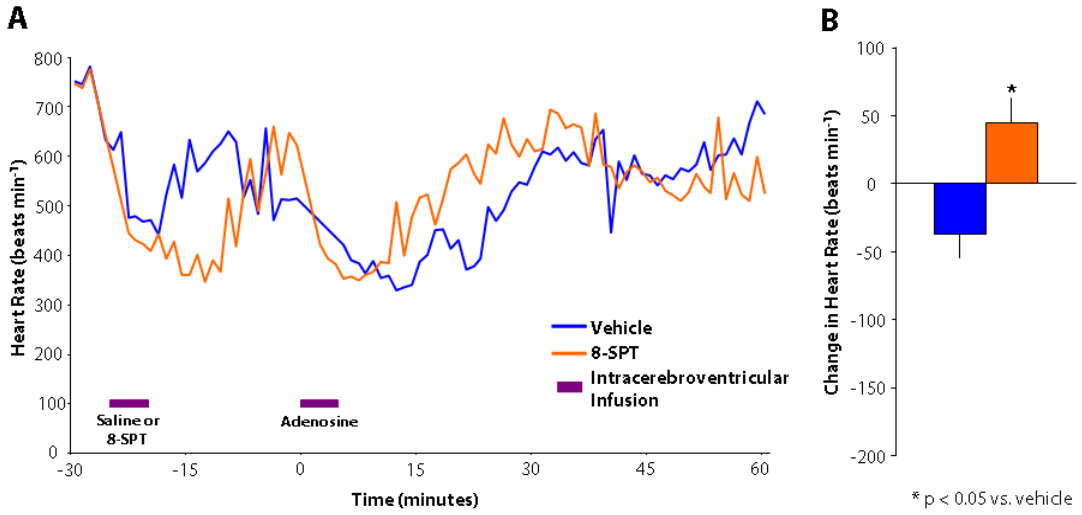

Figure 15. Acute central 8-SPT blunts adenosine-induced bradycardia. (A) Heart rate of a typical mouse when pre-treated with central 8-SPT or vehicle before central infusion of adenosine. (B) Pooled data from 5 mice.

Torpid mice were infused with i.c.v. 8-SPT to determine whether central adenosine antagonism could reverse torpor. Mice were fasted at 20°C while connected to a freely swiveling section of tubing that was clamped shut outside the cage. This setup reduced torque exerted on the animal during movement as compared to the swivel using in the peripheral experiments. The tubing was primed with 8-SPT solution or phosphate-buffered saline. During the first torpor bout of the light cycle, a primed infusion tube was connected to the clamped tubing via a short length of 25 gauge stainless steel hypodermic tubing and the clamp was removed. Mice were then infused with either 10 µg 8-SPT in 5 µL or an equivalent volume of PBS. Infusion was conducted over 5 minutes to minimize discomfort; this period of time was limited by the inability of the tubing to swivel during infusion.

Central 8-SPT infusion caused increased body temperature (Figure 16A), heart rate (Figure 16B), and locomotor activity (Figure 16C) relative to infusion with

phosphate-buffered saline vehicle. As in the interrupt experiment with using peripheral aminophylline, physiological and behavioral parameters began diverging approximately 10 minutes after the start of infusion, with all parameters significantly different after 45 minutes.

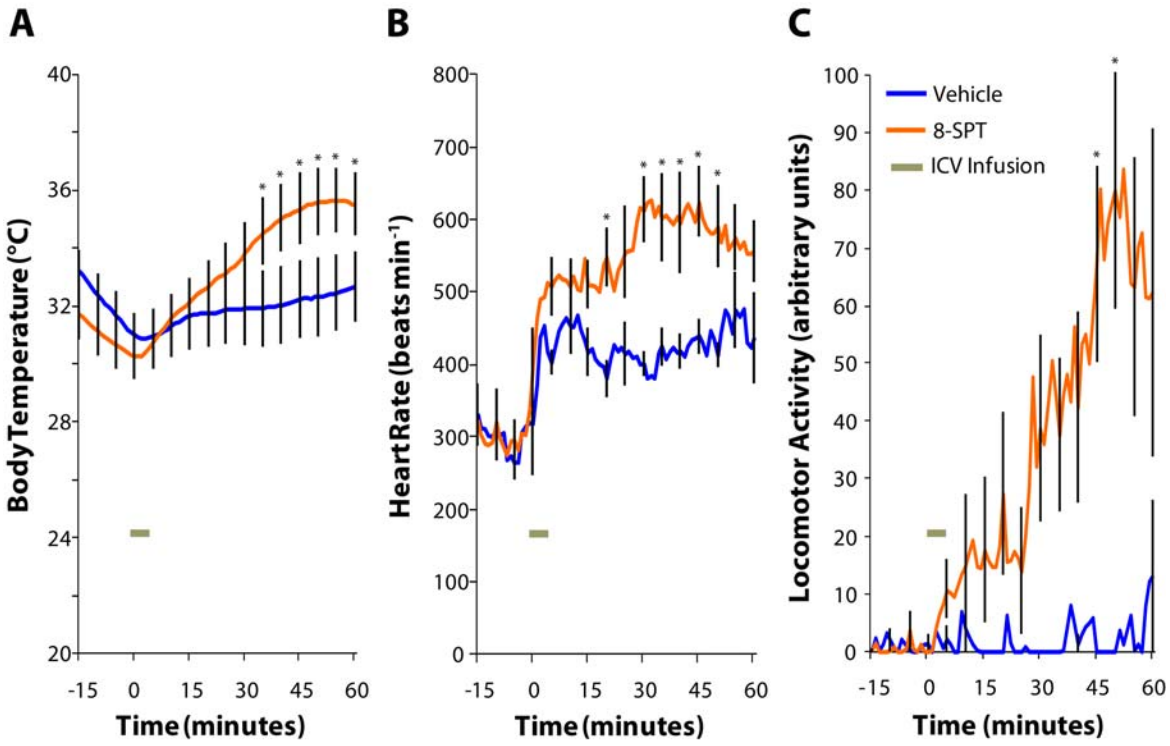

Figure 16. Central 8-SPT infusion reverses torpor. (A) Body Temperature. (B) Heart Rate. (C) Locomotor activity.

Discussion

These data support the hypothesis that adenosine receptor activation is required for the induction and maintenance of torpor in mice. In the first experiment, continuous peripheral infusion of a nonselective adenosine receptor antagonist, which blocks A_1, A_{2A}, A_{2B}, and A_3 receptors, prevented torpor. Importantly, aminophylline treatment greatly reduced the time spent in a hypothermic state in fasted mice. A variety of physiological measures, including minimum and average body temperature and heart rate, were higher in fasted aminophylline-treated mice than in fasted saline-treated controls. While many of these indicators were also elevated when fed mice were treated with aminophylline, the interaction effect was significant, indicating that adenosine receptor blockade had greater effects on fasted mice. This is consistent with the idea that mice have some basal adenosine tone even when fed, but that adenosinergic signaling is increased during times of metabolic stress. Aminophylline could also increase heart rate through its activity as a phosphodiesterase inhibitor, causing increased intracellular cyclic AMP and therefore increased intracellular Ca^{2+} through PKA-mediated pathways. However, one might expect aminophylline's effect on phosphodiesterase to be similar in fed and fasted states, in which case blockade of adenosine receptors would be the most plausible explanation for the interaction effect.

If it is aminophylline's adenosine receptor antagonism that is responsible for the lack of torpor in treated mice, there are several possible explanations. Adenosine is closely involved with the regulation of sleep, acting as a somnogen, while torpor and other hypometabolic states are closely related to sleep (Gallopin and Luppi 2005; Tamura, Shintani et al. 2005). By continually blocking adenosine, we may have

prevented the mice from entering sleep and thereby prevented torpor. It is also possible that there is a body temperature threshold that must be crossed for entry into torpor, and that aminophylline treatment prevented mice from falling below this threshold, either through action on adenosine receptors or by inhibition of phosphodiesterase. Similarly, aminophylline could have disinhibited sympathetic pathways, leading to increased heart rate and thermogenesis, thus counteracting the changes normally seen in torpor.

It is plausible that adenosine could be involved only in initiating torpor, with other mechanisms maintaining torpor. In Syrian hamsters, an A_1 antagonist injected i.c.v. reversed hibernation if injected during induction, but not if injected during the maintenance phase (Tamura, Shintani et al. 2005). We tested whether peripheral aminophylline infusion could interrupt torpor. Within approximately 10 minutes of the start of peripheral aminophylline infusion in fasted mice, body temperature, metabolic rate, heart rate, and locomotor activity rapidly increased (see Figure 10). Furthermore, these measures stayed elevated for approximately 6 hours after infusion, while saline-treated mice remained torpid, supporting our findings from the first experiment. These results suggest that adenosine receptor signaling is necessary for both induction and maintenance of torpor in mice. The difference between mice and hamsters can be explained by the fact that bouts of daily torpor in mice are much shorter than hibernation bouts. It is possible that the entire 1-6 hour course of a daily torpor bout is analogous to only the induction phase of hibernation, which lasts for approximately 20 hours (Tamura, Shintani et al. 2005).

By answering the question of when adenosine signaling is necessary for torpor, this experiment also gave some clue about where adenosine may be acting to promote

torpor. While torpor is entered through NREM sleep, slow-waves during hypothermia shift outside normal frequencies, and REM sleep is abolished below 24°C, suggesting that the neurological state of torpor is likely distinct from sleep (Deboer and Tobler 1995). The fact that adenosine blockade interrupts torpor maintenance, combined with the fact that maintenance of torpor is neurologically distinct from sleep, suggests that it is not adenosine's effects on sleep centers like the basal forebrain that mediate torpor.

In addition, the finding that blockade of adenosine receptors is capable of reversing the hypothermia of torpor provides evidence against the temperature threshold hypothesis. The fact that hypothermia could be reversed, and not just prevented, by aminophylline treatment suggests that the inhibition of torpor is because of blockade of normal signaling pathways, and not for example because mice with a body temperature of 37.5°C cannot enter torpor while mice at 37.0°C can. While aminophylline could prevent torpor and disrupt torpor maintenance through distinct mechanisms it is more likely that both effects are manifestations of the same changes.

The interruption of torpor by aminophylline did not address the question of whether adenosine could be disinhibiting neural pathways such as sympathetic signaling to the heart and brown adipose tissue, increasing heart rate, thermogenesis, and metabolic rate. Adenosine could also be acting directly on receptors on the heart or BAT to produce these effects, a hypothesis that is supported by the well-documented cardiovascular effects of adenosine and the fact that adenosine mediates BAT cold-acclimation in hamsters (Unelius, Mohell et al. 1990; Hori and Kitakaze 1991).

I hypothesized that adenosine acts both centrally and peripherally to promote torpor. In order to test this hypothesis, and to rule out phosphodiesterase inhibition as the

mechanism for the prevention and reversal of torpor by aminophylline, we used the polar adenosine antagonist 8-sulfophenyltheophylline. Because 8-SPT is polar, it does not cross cell membranes, preventing it from inhibiting phosphodiesterase or crossing the blood-brain barrier.

Contrary to my hypothesis, peripheral 8-SPT infusion did not interrupt torpor. Body temperature, heart rate, and locomotor activity were not different between mice in the 8-SPT and saline conditions. The dose used, 50 mg kg^{-1}, was effective in blocking the hypothermia induced by peripheral adenosine injection, suggesting that our treatment was indeed capable of blocking adenosine signaling in the periphery. Adenosine blockade was insufficient to reverse torpor, conflicting with our earlier findings using aminophylline. This result had two possible interpretations: that adenosine signaling in the periphery is not necessary for torpor, or that the effects of aminophylline on torpor induction and maintenance are primarily due to its effects on phosphodiesterase. The idea that adenosine in the periphery is not necessary for torpor, or possibly that it is not involved at all in the physiological changes of torpor, is surprising given adenosine's cardiovascular effects and role in signaling metabolic stress.

To determine whether the negative result produced by peripheral 8-SPT was due to its lack of central adenosine antagonism or its lack of PDE activity, we tested whether 8-SPT infused into the central nervous system could reverse torpor. Central 8-SPT treatment caused mice to come out of torpor, increasing body temperature, heart rate, and locomotor activity compared to the vehicle-treated control condition. This result suggests that central adenosine signaling has at least some role in maintaining torpor, although we

cannot rule out that the effects of aminophylline treatment were partially due to phosphodiesterase inhibition.

One major question about our central 8-SPT protocol is whether the treatment actually blocked adenosine signaling in the brain. In the adenosine hypothermia assay, 8-SPT, caused paradoxical hypothermia and bradycardia following injection, an effect that was absent in the vehicle-treatment condition. As a result, it is difficult to compare the hypothermia induced by adenosine, since the 8-SPT-treated mice were already hypothermic to some degree prior to injection. It is difficult to ascertain the exact cause of the hypothermia, particularly since as an adenosine antagonist, 8-SPT would be expected to cause hyperthermia. Mice did not appear to have any adverse reaction to 8-SPT injection, and were not impaired when later retrieved for adenosine injection. High doses of peripheral aminophylline also cause paradoxical hypothermia, suggesting the possibility that an overdose of 8-SPT may have caused the drop in body temperature. However, the dose used, 10μg, has been used previously with no reported ill effects (Dar 1990).

If the hypothermia was due to adenosine-independent mechanisms, then mice should have exhibited a further drop in body temperature after adenosine injection, resulting in similar measurements of adenosine-induced hypothermia in treated and control conditions. Adenosine-induced hypothermia is dose-dependent, so even if 8-SPT treatment somehow caused adenosine receptor activation, subsequently adding adenosine should have resulted in a deeper hypothermia. However, the depth of hypothermia following adenosine treatment was similar, as compared to a euthermic body temperature of 37°C, between 8-SPT and saline groups. Because mice in the 8-SPT-treated condition

did not experience a further drop in body temperature after adenosine infusion, this result suggests that 8-SPT was effective at blocking adenosine receptors. Notably, if 8-SPT had any hypothermic effect when we injected it into torpid mice, it would have increased the depth of torpor. Instead, the hypothermia of torpor was reversed.

The torpor-reversing effect of 8-SPT was modest when compared to peripheral aminophylline treatment. Whereas aminophylline caused a long-lasting increase to maximal metabolic rate, body temperature, and heart rate, the effects of 8-SPT had a smaller magnitude and duration. There are a few different ways to explain this discrepancy. First and perhaps most obviously, the dose of 8-SPT was limited by solubility and the maximum injection volume, while the aminophylline dose was limited only by potential toxicity. It is possible that the 8-SPT dose was insufficient to fully antagonize central adenosine receptors, while the aminophylline dose was more effective. The difference in duration could also be attributed to differences in dose, as 10μg of 8-SPT may be metabolized in the brain more quickly than 100 mg kg^{-1} (approximately 2.5 mg per mouse) aminophylline is metabolized in the liver. Another possible explanation is that aminophylline also blocked peripheral adenosine receptors, increasing its torpor-reversing effects. While we have shown that inhibition of peripheral adenosine receptor signaling is not necessary for torpor, we do not know that it is not involved in producing the physiological changes of torpor. It is very likely that adenosine is present in the periphery during torpor, and its effects would be consistent with the changes seen in torpor. The third possible explanation is that aminophylline's effects on central adenosine receptor activation were augmented by its effects on phosphodiesterase, increasing its ability to reverse torpor. The effects of phosphodiesterase inhibition would be expected to

have similar consequences as inhibition of adenosine receptors; in fact, both converge on a common pathway, resulting in increased intracellular cAMP. One step to attempt to resolve this question would be to treat mice with a PDE inhibitor like luteolin and see if torpor can be blocked or interrupted, but because of the common pathways involved, that approach would not necessarily yield useful information (Yu, Chen et al. 2010). Because PDE hydrolyzes cAMP to AMP, which is then dephosphorylated to adenosine, blocking PDE could suppress endogenous adenosine signaling. A better approach would be to continue to evaluate the effects of adenosine using adenosine antagonists which are polar, like 8-SPT, or that lack intrinsic PDE inhibition activity.

These experiments allow us to create a model for adenosine's role in mediating torpor in mice. When temperatures become cool, mice compensate by increasing thermogenesis to maintain euthermia. However, when food is scarce, attempting to maintain a stable body temperature causes an energy deficit, resulting in slightly reduced levels of ATP. The equilibrium between ATP, ADP, AMP, and adenosine shifts, greatly increasing adenosine levels. Adenosine may act primarily as a paracrine or autocrine signal, being secreted by brain cells, or it could be produced by cells all over the body and transported to the brain across the blood-brain barrier. While all cells produce adenosine, it is difficult to say which source is more physiologically relevant in torpor. Once adenosine reaches the brain, it could act on a number of targets to induce and maintain torpor. A_1 receptors are known to exist in the orexinergic neurons of the lateral hypothalamus and cholinergic neurons of the basal forebrain, both integrally involved in the regulation of sleep (Arrigoni, Chamberlin et al. 2006; Liu and Gao 2007). By acting on central neurons in the sympathetic pathway, adenosine could modulate blood pressure,

heart rate, and BAT thermogenesis. A third mechanism through which adenosine could promote torpor is through action on the preoptic area of the hypothalamus, which regulates body temperature. These are only some of the possible targets of adenosine; adenosine receptors, particularly A_1 receptors, are widely distributed in the brain, so it is likely that adenosine acts on many different neural pathways.

Importantly, body temperature, metabolic rate, heart rate, and activity level interact, so it is not necessary for adenosine to directly affect pathways regulating any given parameter in order to produce a change in that parameter. For example, a decrease in T_b causes a decrease in HR through temperature-dependent reduction of enzyme activity, and a decrease in HR causes a decrease in metabolic rate. Because of these interactions, adenosine could produce many effects through action on a small number of pathways, though it is likely that adenosine acts on many different targets.

Although our data support a role for adenosine in the mediation of torpor, this study had significant limitations. First and foremost, we were unable to use completely free-moving animals. Neither aminophylline nor 8-SPT is sufficiently soluble to deliver using an alternative to tethering, like an implanted osmotic pump, at the doses needed for peripheral administration. Aminophylline has too short a half-life and too high toxicity to deliver as a bolus dose to block torpor for long periods of time, a significant limitation when studying torpor initiation (Neese and Soyka 1977; Derkenne, Curran et al. 2005). Furthermore, the disturbance of an injection could prevent re-entry into torpor over the time-scale of aminophylline's half-life. While tethering is not ideal, it has the advantage of allowing very precise dosing compared with alternatives like osmotic pumps or extended-release drug emulsions. Tethering decreased the depth of torpor bouts, but

otherwise, the torpor bouts of tethered mice appeared normal in every way. Between torpor bouts, tethered mice were also more active than non-tethered mice. While tethering had minor effects on torpor, it is unlikely that these effects altered the outcome of the experiment.

The other main problem with drug delivery is that it cannot be ruled out that the effects of our antagonists were due to discomfort at the injection site, causing arousal from torpor. Subjectively, mice that were injected with aminophylline or saline during hypothermia assays did not seem to experience greater discomfort than saline-injected mice. Furthermore, since peripheral 8-SPT did not reverse torpor, discomfort from drug injection is an unlikely explanation for the observed effects of aminophylline. However, this explanation cannot be ruled out, since the pH of aminophylline solution would be expected to be higher than that of 8-SPT, as aminophylline is a complex of the drug theophylline and the base ethylenediamine. In the case of the central infusion, there is a similar problem. As we observed in the hypothermia assay, injection into the brain can cause unexpected effects. It is likely that our injection changed the temperature of the brain to some extent, which could have had an effect. The volume of the injection could have caused discomfort, resulting in arousal from torpor. In addition, although drugs were delivered via tether to cannulae, preventing the disturbance of injection, starting the pump required entry into the room, and central infusions required connecting a tube to the tether. These disturbances undoubtedly contributed to arousal, but those effects were controlled for by concurrently testing mice in the saline-treated condition.

This study has provided evidence that adenosine receptor signaling is involved in the regulation of torpor, but there are several areas that merit further research. While we

showed that central adenosine is necessary for the maintenance of torpor, the role of central adenosine in torpor induction was not evaluated. While aminophylline blocked torpor induction, that effect could have been cause by PDE inhibition or peripheral adenosine blockade. Repeating the first experiment of this study using central and peripheral 8-SPT infusion would address this question, since 8-SPT does not inhibit PDE.

Another interesting avenue for further research is the pathways through which central adenosine modulates torpor. Possibly the easiest to test is the sympathetic nervous system. Central 8-SPT infusion could be combined with an acute or continuous peripheral infusion of a beta-adrenergic antagonist like propranolol. If 8-SPT fails to reverse torpor when the sympathetic nervous system is blocked, then adenosine is likely acting either directly or indirectly to inhibit sympathetic premotor or preganglionic neurons. Although this experiment could not distinguish where adenosine might act in the sympathetic pathway, it would provide some insight into how adenosine causes the physiological changes of torpor.

Different adenosine receptor subtypes could also be evaluated to determine their role in adenosine's control of torpor. The inhibitory A_1 receptor is the most likely candidate, but receptor-specific antagonists could be used to conclusively determine which receptor types are involved. Adenosine receptor knockout mice also exist, and could be evaluated to determine whether they exhibit torpor deficits.

In this study, we have shown that treatment with the nonspecific adenosine receptor antagonist aminophylline can prevent the induction of torpor in mice and interrupt torpor maintenance. Furthermore, we have shown that central, but not peripheral infusion of the polar antagonist 8-sulfophenyltheophylline is sufficient to reverse torpor.

These results suggest that central adenosine signaling is necessary for the maintenance of torpor, and that adenosine may be involved in torpor induction. Further research will clarify the role of adenosine in the regulation of torpor.

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