Published in final edited form as:

Neuropharmacology. 2018 September 01; 139: 268–278. doi:10.1016/j.neuropharm.2018.02.035.

Activation of adenosine A_{2A} or A_{2B} receptors causes hypothermia in mice

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Abstract

Extracellular adenosine is a danger/injury signal that initiates protective physiology, such as hypothermia. Adenosine has been shown to trigger hypothermia via agonism at A₁ and A₃ adenosine receptors (A₁AR, A₃AR). Here, we find that adenosine continues to elicit hypothermia in mice null for A₁AR and A₃AR and investigated the effect of agonism at A_{2A}AR or A_{2B}AR. The poorly brain penetrant A_{2A}AR agonists CGS-21680 and PSB-0777 caused hypothermia, which was not seen in mice lacking A_{2A}AR. MRS7352, a likely non-brain penetrant A_{2A}AR antagonist, inhibited PSB-0777 hypothermia. While vasodilation is probably a contributory mechanism, A_{2A}AR agonism also caused hypometabolism, indicating that vasodilation is not the sole mechanism. The A_{2B}AR agonist BAY60-6583 elicited hypothermia, which was lost in mice null

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Chemical compounds

APEC, 2-[[2-[4-[2-(2-aminoethyl)-aminocarbonyl]ethyl]phenyl]ethylamino]-5'-N-ethyl-carboxamidoadenosine

BAY60-6583, 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide

CGS-21680, 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine

MRS7352, 4-((4-(3-(5-amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)methyl)benzenesulfonate triethylammonium salt

PSB-0777, 4-[2-[(6-Amino-9-b-D-ribofuranosyl-9H-purin-2-yl)thio]ethyl]benzenesulfonic acid ammonium salt

Regadenoson, 1-[6-amino-9-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]purin-2-yl]-N-methylpyrazole-4-carboxamide

SCH442416, 2-(2-furyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

for A_{2B}AR. Low intracerebroventricular doses of BAY60-6583 also caused hypothermia, indicating a brain site of action, with neuronal activation in the preoptic area and paraventricular nucleus of the hypothalamus. Thus, agonism at any one of the canonical adenosine receptors, A₁AR, A_{2A}AR, A_{2B}AR, or A₃AR, can cause hypothermia. This four-fold redundancy in adenosine-mediated initiation of hypothermia may reflect the centrality of hypothermia as a protective response.

Graphical abstract

Keywords

hypothermia; adenosine; A_{2A}AR; A_{2B}AR; preoptic area; paraventricular hypothalamus

1. Introduction

Maintenance of a warm, regulated core body temperature (T_b) is a defining feature of mammals and birds. In homeotherms, hypothermia can be caused by excessive cold exposure, severe injury or illness, and many drugs. In large mammals, such as adult humans, hypothermia is relatively difficult to achieve. In contrast, small mammals, such as mice, are at risk of hypothermia and expend relatively massive amounts of energy to maintain their T_b, a process in which brown fat has a large role. The high energetic cost of defending T_b means that small mammals have evolved strategies for using T_b reduction to conserve fuel. For example, with cold exposure or fasting, mice let their T_b fall slightly (~1°C). Mice can also enter torpor, during which T_b can fall >10°C, producing huge energetic savings (Gavrilova et al., 1999; Geiser, 2004; Hudson and Scott, 1979; Melvin and Andrews, 2009).

Hypothermia is observed with severe injury (Shafi et al., 2005) and sepsis (Fonseca et al., 2016). It is unknown if the poor prognosis associated with hypothermia is because hypothermia is a marker of clinical severity or if the hypothermia represents a physiologic response to the dire situation (Fonseca et al., 2016). Clinically, therapeutic hypothermia is used after insults such as neonatal hypoxic injury (Azzopardi et al., 2014) and cardiac arrest (Callaway et al., 2015), and prophylactically during certain types of hypoperfusion surgery (Yan et al., 2013). Therapeutic hypothermia is induced by cooling, sometimes with pharmacologic agents to block shivering. Drugs that induce hypothermia through reduction of the central T_b balance point could minimize counterregulatory mechanisms such as shivering and provide clinical benefit.

Extracellular adenosine is one of the body's danger signals, an indicator of tissue damage or metabolic stress (Borea et al., 2016). Adenosine acts at four G protein-coupled receptors: A₁AR, A_{2A}AR, A_{2B}AR, and A₃AR. A₁AR and A₃AR are typically coupled to Gi, while A_{2A}AR and A_{2B}AR are usually coupled to Gs or Gq. Adenosine has many actions that dampen immune and inflammatory responses, one of which is causing hypothermia (Bennet and Drury, 1931). The best studied mechanism of adenosine-induced hypothermia is via agonism of brain A₁AR, with proposed sites of action being the anterior hypothalamus and the nucleus of the solitary tract (Anderson et al., 1994; Carlin et al., 2017; Johansson et al., 2001; Muzzi et al., 2013; Shintani et al., 2005; Tupone et al., 2013). However, mouse genetic and pharmacologic evidence suggested that adenosine can cause hypothermia via other mechanisms (Yang et al., 2007; Yang et al., 2010). Hypothermia via A₃AR is caused by agonism of peripheral mast cell A₃AR, causing histamine release, which produces hypothermia via central histamine H₁ receptors (Carlin et al., 2016). Since A₁AR agonists must penetrate the brain to produce hypothermia, some compounds used as A₁AR agonists may be causing hypothermia via peripheral mast cell A₃AR (Carlin et al., 2017).

There is limited information on the role of A_{2A}AR and A_{2B}AR in hypothermia. The A_{2A}AR agonist CGS-21680 produced mild hypothermia with a high i.c.v. dose (Anderson et al., 1994) or with peripheral dosing (Eisner et al., 2017). These effects were smaller than those seen with agonism at other adenosine receptors and were not investigated mechanistically. Intraarterial CGS-21680 caused hypotension in wild type but not *Adora2a*^{-/-} mice, which may have been accompanied by hypothermia, but Tb was not measured (Ledent et al., 1997). In contrast, the A_{2A}AR agonist PSB-0777 increased energy expenditure by stimulation of brown adipose tissue; effects on Tb were not reported (Gnad et al., 2014). No role for A_{2B}AR in hypothermia has been identified (Fredholm et al., 2011).

Here we explored whether agonism at A₁AR and A₃AR completely accounts for the hypothermia potential of adenosine, or if A_{2A}AR and A_{2B}AR can also contribute. We find that agonism of either A_{2A}AR or A_{2B}AR does cause hypothermia, and investigate the pharmacology and mechanisms.

2. Materials and methods

2.1. Mice

Male C57BL/6J and *Kit*^{W^{-sh}/W^{-sh} (JAX #012861) (Grimbaldeston et al., 2005; Nigrovic et al., 2008) mice were obtained from the Jackson Laboratory. *Adora1*^{-/-} (Sun et al., 2001), *Adora3*^{-/-} (Salvatore et al., 2000), and *Adora1*^{-/-};*Adora3*^{-/-} mice were obtained and genotyped as reported (Carlin et al., 2017). *Adora2a*^{-/-} mice (Chen et al., 1999) on a mixed background were obtained from Dr. Dorian McGavern and genotyped as described (JAX #010685). *Adora2b*^{-/-} mice (Hua et al., 2007) on a C57BL/6J background were obtained from Dr. Stephen Tilley and genotyped as described (JAX #022499). Mice were singly housed at ~22°C with a 12:12-h light-dark cycle (lights on at 6:00 am). Chow (NIH-07, Envigo Inc, Madison, WI) and water were available ad libitum. Drugs were typically dosed at 10:00 am. Mice were studied 7 days after any operation or prior treatment. Reuse of mice tends to reduce physical activity levels, presumably due to acclimatization. No specific effort was made to acclimatize mice to handling in individual experiments. Studies were}

approved by the Animal Care and Use Committee of National Institute of Diabetes and Digestive and Kidney Diseases.

2.2. Drugs

MRS7352 (4-((4-(3-(5-amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)methyl)benzenesulfonate triethylammonium salt) was synthesized and purified by HPLC as described (Duroux et al., 2017). The following compounds (vehicle) were purchased from Tocris (Minneapolis, MN): CGS-21680 (15% DMSO/15% KolliphorEL/70% saline for i.p.; 5% DMSO/95% PBS neutralized with one equivalent of NaOH for i.c.v.); PSB-0777 (saline); BAY60-6583 (25% PEG); SCH442416 (15% DMSO/15% KolliphorEL/70% saline). Adenosine (10% DMSO in water) was obtained from Sigma. APEC (2-[[2-[4-[2-(2-aminoethyl)-aminocarbonyl]ethyl]phenyl]ethylamino]-5'-N-ethyl-carboxamidoadenosine; water) was obtained through NIMH Chemical Synthesis and Drug Supply Program, <http://nimh-repository.rti.org/>. Regadenoson was purchased from Santa Cruz Biotechnology, Inc. Screening of PSB-0777, SCH442416, BAY60-6583, and CGS-21680 for off target activities at 45 human receptors, channels, and transporters with radioligand binding assays was performed by the National Institute of Mental Health's Psychoactive Drug Screening Program (PDSP) directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill.

2.3. Adenosine receptor binding affinities

Binding affinity for mouse A₁AR, A_{2A}AR, and A₃ARs was measured as described (Kreckler et al., 2006) using membranes from human embryonic kidney (HEK)-293 cells stably expressing individual recombinant mouse adenosine receptors and using the agonists [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)adenosine-5'-methyluronamide ([¹²⁵I]AB-MECA; A₁AR and A₃AR) and [³H]CGS-21680 (A_{2A}AR) as radioligands. Nonspecific binding was defined using 100 μM adenosine-5'-N-ethylcarboxamide (NECA). K_i values were obtained using the Cheng-Prusoff equation from IC₅₀ values calculated by non-linear regression analysis of specific binding data using GraphPad Prism software (San Diego, CA).

2.4. Thermal physiology phenotyping

Implantation (coordinates relative to bregma: -0.34 mm anterior, 1.0 mm lateral, and +1.7 mm ventral) and use of chronic intracerebroventricular cannulas are described in (Carlin et al., 2017), as are the following procedures. Continuous telemetric monitoring of Tb and activity were performed using G2 E-mitters (Starr Life Sciences, Oakmont, PA) implanted intraperitoneally. Average Tb response was calculated using 0 to 60 min from agonist dosing and activity from 10 to 60 min. Inhibitors were dosed 20–25 min before agonists. Indirect calorimetry was performed using a 12-chamber CLAMS (Columbus Instruments, Columbus, OH).

2.5. Drug-induced neuronal activation

Sixty min after i.c.v. infusion, mice were euthanized with 7% chloral hydrate and perfused with saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4).

Brains were post fixed in 4% paraformaldehyde/30% sucrose in PBS at 4°C overnight. Coronal brain sections (40 µm) were cut, stored at 4°C for up to one week in PBS containing 0.02% sodium azide, washed in 0.1 M PBS, preincubated in blocking buffer (PBS + 0.2 % triton X-100 + 10% normal horse serum (NHS)) for 2 h at room temperature, and incubated with antibody to Fos (1:2000, Cell Signaling, #5348) in 2% NHS blocking buffer for 48 h at 4°C. After washing in PBS, tissue was incubated for 4 h with donkey anti-rabbit Alexa Fluor 488 antibody (1:500 in PBS + 2% NHS blocking buffer, ThermoFisher Scientific #A31556). Fos was visualized by fluorescence microscopy with Olyvia software (Olympus Life Sciences). A blinded individual counted the Fos-positive neurons using Image J software (NIH). Display images were adjusted for brightness and contrast. Brain anatomy was defined using (Franklin and Paxinos, 2007).

Data are reported as mean ± SEM. Significance (two-tailed $p < 0.05$) was determined by t -test or ANOVA followed by post hoc Holm-Sidak multiple comparison tests.

3. Results

3.1 Adenosine Causes Hypothermia via Mechanisms Independent of A₁AR and A₃AR Receptors

To determine if there are mechanisms in addition to A₁AR and A₃AR by which adenosine causes hypothermia, we studied *Adora1*^{-/-}; *Adora3*^{-/-} mice, which lack both of these receptors. Handling for dosing with vehicle increased Tb and activity. Treatment with a relatively modest adenosine dose (100 mg/kg, i.p. (Eisner et al., 2017)) produced robust hypothermia in *Adora1*^{-/-}; *Adora3*^{-/-} mice, approaching that seen in C57BL/6J controls (Fig. 1). Thus, adenosine can cause hypothermia independent of A₁AR and A₃AR.

3.2 Agonism at A_{2A}AR causes hypothermia

We used CGS-21680, a modestly selective A_{2A}AR agonist (Table 1), to test if A_{2A}AR activation could produce hypothermia. CGS-21680 (0.01–1 mg/kg, i.p.) caused dose-dependent hypothermia and hypoactivity (Fig. 2A–C). CGS-21680 is only ~5-fold selective for A_{2A}AR over A₃AR (Table 1); therefore, we tested its specificity using mice lacking A_{2A}AR. Treatment with CGS-21680 did not cause hypothermia in *Adora2a*^{-/-} mice (Fig. 3A–C). In contrast, CGS-21680 hypothermia was intact in *Adora1*^{-/-}; *Adora3*^{-/-} mice (Fig. 3D–F). *Adora2a* is expressed in mast cells, but *Kit*^{W^{-sh}/W^{-sh} mice, which lack mast cells, showed a hypothermic response to CGS-21680 (Fig. 3G–I). Screening of CGS-21680 for off-target activities detected none at <10 µM (see 2.2). Taken together, these data suggest that the hypothermic effects of CGS-21680 (at 1 mg/kg, i.p.) are via agonism at A_{2A}AR, with little or no contribution from A₁AR, A₃AR, or mast cells.}

Given the modest adenosine receptor selectivity of CGS-21680, we examined the binding affinities of other commonly used A_{2A}AR agonists on mouse receptors. In humans, regadenoson is used as a selective A_{2A}AR agonist (Gao et al., 2001). However, in mouse it is not selective, binding to A₁AR 10-fold more tightly than to A_{2A}AR (Table 1), and we did not study it further. PSB-0777 is a selective A_{2A}AR agonist in rat (El-Tayeb et al., 2011) and had few off-target activities, binding only human β1 (K_i 4.4 µM) and β3 (K_i 3.3 µM)

adrenergic receptors. However, PSB-0777 bound mouse A₁AR 1000-fold better than rat A₁AR (Table 1), so in mouse PSB-0777 is a better ligand for A₁AR than for A_{2A}AR. Despite this lack of binding selectivity, in vivo, PSB-0777 caused dose-dependent hypothermia and hypoactivity (Fig. S1A-C) that was abolished in *Adora2a*^{-/-} mice (Fig. S1D-F). This effective selectivity of PSB-0777 for A_{2A}AR in causing hypothermia may be due to its poor CNS penetration (El-Tayeb et al., 2011), which precludes PSB-0777 from reaching the brain A₁AR that trigger hypothermia. These data demonstrate that the hypothermic effects of PSB-0777 (at 3 mg/kg, i.p.) in mice occur via agonism at peripheral A_{2A}AR.

APEC is readily brain penetrant, unlike CGS-21680 (Nikodijevic et al., 1990) and PSB-0777. APEC binds mouse A_{2A}AR at 3.6 nM, but also binds mouse A₁AR with high affinity (4.2 nM in mouse vs 400 nM in rat) (Table 1). Low peripheral doses of APEC caused hypothermia, blunting the handling hyperthermia at 1.7 µg/kg i.p. and producing robust hypothermia and hypoactivity at 50 µg/kg (Fig. S1G-I). APEC-induced hypothermia was attenuated in both *Adora2a*^{-/-} and *Adora1*^{-/-}; *Adora3*^{-/-} mice but fully intact in *Adora2b*^{-/-} mice (Fig. S1J-L). To specifically test if central A_{2A}AR activation can induce hypothermia, we dosed CGS-21680 i.c.v. No hypothermia was observed with 0.01 mg/kg, while 0.1 mg/kg i.c.v. produced similar hypothermia to 0.25 mg/kg i.p. (Fig. 4). These data are consistent with CGS-21680, PSB-0777, and APEC each causing hypothermia via peripheral but not central A_{2A}AR, with APEC but not CGS-21680 or PSB-0777 also causing hypothermia via central A₁AR.

3.3 Effect of A_{2A}AR antagonist on hypothermia

Treatment with the selective, brain-penetrant A_{2A}AR antagonists SCH442416 (3 mg/kg, i.p.) or preladenant (1 mg/kg, i.p.) caused increased locomotor activity (data not shown), probably from activation of the A_{2A}AR on striatal neurons. The increased activity likely explains (Lateef et al., 2014) the observed increase in Tb (data not shown). Screening SCH442416 for off-target activities by the PDSP detected binding to the human 5HT_{2B} receptor (K_i 0.91 µM), M₃ acetylcholine receptor (K_i 6.8 µM), translocator protein (TSPO, K_i 4.1 µM), and serotonin transporter (SERT, K_i 6.1 µM). A lower dose of SCH442416 (0.6 mg/kg, i.p.) trended to increase activity (p=0.078) and Tb (p=0.10) by itself and largely blocked the hypothermia caused by PSB-0777 (1 mg/kg i.p.) (Fig. 5A-E). To distinguish a central vs peripheral site of action for A_{2A}AR antagonism, we tested MRS7352 (Duroux et al., 2017), a polar derivative of SCH442416 that is unlikely to reach brain parenchymal A_{2A}AR. Although less potent than SCH442416, MRS7352 retains selectivity for A_{2A}AR (Table 1). MRS7352 (3 mg/kg, i.p.) completely blocked PSB-0777-induced hypothermia, without affecting physical activity (Fig 5F-J). These data further support a peripheral site of action of A_{2A}AR agonists to cause hypothermia.

3.4 A_{2A}AR agonist-induced hypothermia is preceded by hypometabolism

A_{2A}AR agonist-induced hypothermia (CGS-21680, 0.25 mg/kg, i.p.) was accompanied by a marked reduction in metabolic rate, with the hypometabolism preceding the hypothermia (energy expenditure nadir at ~26 vs ~65 minutes for Tb) (Fig. 6A-B). The hypothermia and

hypoactivity were accompanied by reduced food intake, which likely contributes to the lower respiratory exchange ratio, indicating relatively more fatty acid oxidation (Fig. 6C-E).

3.5 Agonism at A_{2B}AR causes hypothermia

We next examined if A_{2B}AR agonism can also cause hypothermia. The non-nucleoside A_{2B}AR agonist BAY60-6583 (0.5–2 mg/kg, i.p.) caused dose-dependent hypothermia and hypoactivity (Fig. 7A–C). BAY60-6583 shows modest adenosine receptor binding selectivity in mouse (Table 1) but appears to activate only mouse A_{2B}AR and not mouse A₁AR, A_{2A}AR, or A₃AR (van der Hoeven et al., 2011). BAY60-6583 also has a 5.6 μM K_i for SERT, from the PDSP screen. The BAY60-6583-induced hypothermia was abolished in *Adora2b*^{−/−} mice (Fig. 7D–F), intact in *Adora1*^{−/−}; *Adora3*^{−/−} mice (not shown), and also intact in *Kit*^{W^{−sh}/W^{−sh} mice (Fig. 7G–I). These results indicate that the hypothermia caused by BAY60-6583 is via A_{2B}AR with little or no contribution from A₁AR, A₃AR, or mast cells.}

To further probe the sites of A_{2B}AR agonist action, BAY60-6583 was given intracerebroventricularly, initially in *Adora1*^{−/−}; *Adora3*^{−/−} mice to avoid possible actions at A₁AR or A₃AR. A dose of 0.04 mg/kg produced profound, prolonged hypothermia, while 0.004 mg/kg had more modest effects (Fig. 8A–C). The lower dose (0.004 mg/kg, i.c.v.) caused hypothermia in C57BL/6J (wild type, WT) but not *Adora2b*^{−/−} mice (Fig. 8D–F). These data suggest that the hypothermic effects of BAY60-6583 are via agonism at central (or central and peripheral) A_{2B}AR.

3.6 A_{2B}AR agonist-induced hypothermia is driven by hypometabolism

The effect of BAY60-6583 was studied using indirect calorimetry, where it lowered metabolic rate, respiratory exchange ratio, and food intake, in addition to Tb and physical activity (Fig. 9A–E). The kinetics indicate that the reduction in metabolic rate is helping drive, not simply responding to the hypothermia.

3.7 A_{2B}AR agonist induced hypothermia is accompanied by Fos induction in the preoptic area and the paraventricular hypothalamus

To identify candidate sites of BAY60-6583 action, we assessed neuronal activation after i.c.v. infusion (0.004 mg/kg, using *Adora1*^{−/−}; *Adora3*^{−/−} mice to preclude any effects via A₁AR or A₃AR). BAY60-6583, but not vehicle, increased Fos immunostaining in the preoptic area (POA) and paraventricular hypothalamus (PVH) (Fig. 10). Fos immunoreactivity was not significantly different in the ventromedial hypothalamus (VMH) or the dorsomedial hypothalamus (DMH), including subregions of the DMH (Wanner et al., 2017). These results indicate that A_{2B}AR agonist causes neuronal activation in the PVH and POA, suggesting that these nuclei are involved in hypothermia.

4. Discussion

The results demonstrate that agonism of either peripheral A_{2A}AR or central A_{2B}AR causes hypothermia (Fig. S2). Thus, agonism at any one of the four canonical adenosine receptors, A₁AR, A_{2A}AR, A_{2B}AR, or A₃AR can cause hypothermia. The clear evidence demonstrating

four mechanisms notwithstanding, our experiments do not rule out additional mechanisms of adenosine-induced hypothermia, such as intracellular depletion of ATP causing AMPK activation (Eisner et al., 2017; Melvin and Andrews, 2009).

4.1 Mechanism of A_{2A}AR hypothermia

A_{2A}AR are widely expressed in the cardiovascular, immune, and nervous systems. Brain-penetrant A_{2A}AR antagonists acting on striatal neurons increase physical activity and are sometimes used clinically to treat Parkinson's disease (Dungo and Deeks, 2013; Kulisevsky and Poyurovsky, 2012). However, our results suggest that brain A_{2A}ARs are not where A_{2A}AR agonists initiate hypothermia. Supporting data include the observations that two poorly brain penetrant agonists, CGS-21680 and PSB-0777, cause hypothermia, that infusion of CGS-21680 into the brain did not produce hypothermia, and that MRS7352, a presumably non-brain penetrant A_{2A}AR antagonist, inhibited the hypothermia. These data are consistent with the observation that the hypotensive effects of CGS-21680 in rats are mediated by peripheral A_{2A}ARs (Schindler et al., 2005).

The hypoactivity caused by treatment with an A_{2A}AR agonist will reduce heat generation, but this probably makes a limited contribution to the reduced metabolic rate and hypothermia (Abreu-Vieira et al., 2015; Virtue et al., 2012). A peripheral mechanism that could contribute to A_{2A}AR agonist-induced hypothermia is vasodilation, increasing heat loss. A_{2A}AR agonists cause vasodilation in cerebral, mesenteric, renal, and coronary arteries (Al Jaroudi and Iskandrian, 2009; Hansen et al., 2005; Kleppisch and Nelson, 1995; Phillis, 2004), but the evidence for cutaneous vasodilation is modest (Fieger and Wong, 2010). In addition, heat loss from isolated vasodilation should elicit compensatory hypermetabolism for heat generation (Warner et al., 2013). In contrast, A_{2A}AR agonist-induced hypothermia is accompanied by the opposite, severe hypometabolism. Thus, while A_{2A}AR agonists cause vasodilation, they also trigger hypotension, hypoactivity, and hypometabolism. This coordinated response is likely a larger contributor to the hypothermia than heat loss from vasodilation.

The CGS-21680 doses causing hypothermia (e.g., 0.25 mg/kg, i.p.) are in the typical range of in vivo dosages used. Thus, the possible role of hypothermia and hypometabolism must be considered, as they could contribute to observed phenotypes. For example, the hypoactivity caused by peripheral CGS-21680 (El Yacoubi et al., 2000; Ledent et al., 1997) is likely peripherally initiated not due to agonism at brain A_{2A}AR.

A_{2A}AR are also present in multiple immune system cell types, which could contribute to driving the hypothermia. Our data rule out a role for mast cells. Further research is needed to define the cells (presumably in addition to vascular smooth muscle cells) on which A_{2A}AR agonists act to cause hypothermia and how the panoply of physiology needed to achieve hypothermia is choreographed.

4.2 Mechanism of A_{2B}AR hypothermia

A_{2B}AR has a lower affinity for adenosine and more divergent synthetic ligand selectivity compared to the other three adenosine receptors. A_{2B}AR are widely distributed, including in various hematopoietic cells, blood vessels, lung, astrocytes, and neurons (Fredholm et al.,

2011; Goncalves et al., 2015; Lemos et al., 2015; Ohta and Sitkovsky, 2014). Hypothermia was produced by BAY60-6583 with an i.c.v. dose only 1/150th the amount of a similarly hypothermic i.p. dose. This suggests that A_{2B}ARs causing hypothermia are in the brain (or in both the brain and periphery). To identify where A_{2B}AR agonists might act in the brain, we mapped the neurons activated during BAY60-6583-induced hypothermia, with drug given i.c.v. to preclude effects from peripheral A_{2B}AR activation. A_{2B}AR is coupled to G_{s/q}, so neurons are activated by BAY60-6583 action. The preoptic area of the hypothalamus (POA), a known thermoregulatory site (Morrison, 2016; Tan et al., 2016), was activated, as was the paraventricular nucleus of the hypothalamus (PVH), which can inhibit thermogenesis (Madden and Morrison, 2009; Nakamura et al., 2017). The experiment was underpowered to detect lower levels of activation in the other regions studied. While neuronal activation could occur with rewarming the body to reverse the hypothermia, the 1-hour time point might minimize later contributions. Our data do not address whether the relevant A_{2B}ARs are on neurons or if the neuronal activation is indirect, due to agonist activation of astrocytes, vascular, immune system, or other cells (Sun and Huang, 2016).

4.3 A_{2A}AR ligand selectivity

To identify adenosine receptor ligands as suitable tools for our in vivo mouse studies, we selected candidates based on rat or human affinity data and measured their binding affinity at mouse receptors. There can be profound species differences in adenosine receptor ligand affinities (Alnouri et al., 2015), and our results expand this literature. Examples of ligands binding more tightly to mouse A₁AR than other species include: APEC, which is ~100-fold better than for rat (Kim et al., 1994), PSB-0777 which is >500-fold better than for rat (El-Tayeb et al., 2011), and regadenoson, which is >1000-fold better than for human (Gao et al., 2001). Thus, both PSB-0777 and regadenoson, which are A_{2A}AR-selective in other species, are not selective in mouse, having both A₁AR and A_{2A}AR activity. Some of these differences might be due to the molecular environment of the binding measurement. For example, a 57-fold difference in ligand affinity for human A_{2A}AR was observed, depending on which G-protein β subunit was present (Murphree et al., 2002). These examples underscore the risks of extrapolating ligand binding affinities across species, even between rat and mouse.

A drug's utility strongly depends on pharmacokinetics in addition to binding affinity. Adenosine is useful for coronary vasodilation since intravenous dosing gives the high exposure to vascular tissue and the very short half-life limits adverse effects from wider exposure (Al Jaroudi and Iskandrian, 2009). Although not tested, intravenous regadenoson might appear selective in mouse vascular assays, since it should not reach brain A₁AR. Similarly, pharmacokinetic considerations (lack of brain penetration) nullified N⁶-cyclopentyladenosine's 2000-fold advantage in binding affinity (A₁AR vs A₃AR), so that this "A₁AR" agonist initiated hypothermia via peripheral A₃ARs (Carlin et al., 2017).

4.4 A_{2B}AR ligands

BAY60-6583 is the most commonly used A_{2B}AR agonist. It is a partial A_{2B}AR agonist with a modest affinity (K_i at A_{2B}AR = 136 nM). BAY60-6583 also binds A₁AR and, less well, A₃AR, and is an antagonist at these receptors (Alnouri et al., 2015). Interestingly, in a cell-

based assay BAY60-6583 had a 2.8 nM EC₅₀ at mouse A_{2B}AR, 50-fold better than the binding affinity (van der Hoeven et al., 2011). Notably, the hypothermia induced by BAY60-6583 was due to A_{2B}AR, being completely abolished in the *Adora2b*^{-/-} mouse. Thus, BAY60-6583 is a valid tool to study induction of hypothermia in mice by A_{2B}AR.

4.5 Significance of A_{2A}AR- and A_{2B}AR-agonist induced hypothermia

Extracellular adenosine is a danger/injury signal, a general inducer of protective and anti-inflammatory mechanisms. Combined with previous data, our results demonstrate that agonism at any adenosine receptor, A₁AR, A_{2A}AR, A_{2B}AR, or A₃AR, can cause hypothermia. Thus, the body has redundant ways to sense adenosine and triggers multiple mechanisms to achieve a common response, hypothermia. Of the four receptors, A₁AR is the best studied and most directly implicated in the regulation of Tb, possibly having direct actions on brain thermoregulatory centers (Shintani et al., 2005; Tupone et al., 2013). The adenosine receptors probably do not have a major role in regulation of Tb in the non-challenged state: knockout of any one of the four receptors does not affect baseline Tb, its diurnal rhythm, or the ability to enter fasting-induced torpor ((Carlin et al., 2017; Carlin et al., 2016) and unpublished observations in *Adora2a*^{-/-} and *Adora2b*^{-/-} mice; but see (Yang et al., 2007; Yang et al., 2010)). The multiplicity of mechanisms that can sense adenosine and evoke hypothermia likely reflects the importance and centrality of the protective/anti-inflammatory effects of hypothermia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Naili Liu, Anna Panyutin, Ramón Piñol, Atreyi Saha, Lex Kravitz, and Alice Franks for experimental contributions and advice, and the National Institute of Mental Health's Psychoactive Drug Screening Program and its Chemical Synthesis and Drug Supply Program. This research was supported in part by the Intramural Research Program of the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases [ZIA DK075063; ZIA DK031117]

Abbreviations

i.p.	intraperitoneal
i.c.v.	intracerebroventricular
A×AR	adenosine × receptor

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Highlights

1. Adenosine A_{2A} agonists cause hypothermia via peripheral receptors
2. Adenosine A_{2B} agonists cause hypothermia via central action
3. Adenosine A_{2B} agonism activates preoptic area and paraventricular hypothalamic neurons

Fig. 1. Adenosine-mediated hypothermia remains intact in *Adora1*^{-/-};*Adora3*^{-/-} mice (A,B) Tb and activity response to adenosine (ADO, 100 mg/kg, i.p.) or vehicle (Veh) in C57BL/6J (WT) and *Adora1*^{-/-};*Adora3*^{-/-} mice. (C,D) Average Tb (0–60 min) and total physical activity (10–60 min). Data are mean ± SEM, n = 5–6/group in a crossover design; ***p < 0.001 vs vehicle within genotype.

Fig. 2. Systemic CGS-21680 administration causes hypothermia and decreases physical activity (A) Tb response to the indicated i.p. CGS-21680 doses in C57BL/6J mice. (B,C) Effect of CGS-21680 on average Tb (0–60 min) and physical activity (10–60 min). Data are mean $\pm$ SEM, n = 3-7/group; **p<0.01, ***p < 0.001 vs vehicle.

Fig. 3. Systemic CGS-21680 causes hypothermia through A_{2A}AR, not involving A₁AR, A₃AR, or mast cells

(A-C) Tb and activity response to CGS-21680 (0.5 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora2a*^{-/-} mice. (D-F) Tb and activity response to CGS-21680 (0.5 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora1*^{-/-}; *Adora3*^{-/-} mice. (G-I) Tb and activity response to CGS-21680 (0.5 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Kit*^{W-sh/W-sh} mice. Data are mean ± SEM, n = 3–7/group; *p < 0.05, **p < 0.01, ***p < 0.001 vs vehicle within genotype, *p < 0.05 or ***p = 0.001 vs vehicle.

Figure 4. No hypothermic response to intracerebroventricular CGS-21680
(A–C) Tb and activity response to i.c.v. CGS-21680 (0.01 or 0.1 mg/kg) or vehicle in C57BL/6J mice. Data are mean $\pm$ SEM, $n = 4$ –6/group in a crossover design.

Fig. 5. Effect of A_{2A}AR antagonists on PSB-0777-induced hypothermia (A–D) Tb and activity response to SCH442416 (0.6 mg/kg, i.p.) or vehicle at -15 min and then PSB-0777 (1 mg/kg, i.p.) or saline at 0 min. (E) Structure of SCH442416. (F–I) Tb and activity response to MRS7352 (3 mg/kg, i.p.) or vehicle at -15 min and then PSB-0777 (1 mg/kg, i.p.) or saline at 0 min. (J) Structure of MRS7352. Data are mean ± SEM, n = 5–12/group, *p < 0.05 or ***p < 0.001 indicates significance at this level or better vs the other three groups by 1-way ANOVA with Sidak multiple comparison testing.

Fig. 6. CGS-21680 reduces metabolic rate prior to Tb

The effect of CGS-21680 (0.25 mg/kg, i.p.) or vehicle in C57BL/6J (WT) on (A) Tb, (B) total energy expenditure (TEE), (C) respiratory exchange ratio (RER), (D) physical activity, and (E) food intake. Data are mean $\pm$ SEM, $n = 11$ /group, * $p < 0.05$.

Fig. 7. BAY60-6583 causes hypothermia and decreased physical activity via A₂BAR
 (A-C) Tb and physical activity response to the indicated i.p. BAY60-6583 doses in C57BL/6J mice. (D-F) Tb and activity response to BAY60-6583 (1 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora2b*^{-/-} mice. (G-I) Tb and activity response to BAY60-6583 (1 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Kit*^{W-sh/W-sh} mice. Data are mean ± SEM, n = 3–8/group; *p<0.05, **p<0.01, ***p<0.001 vs vehicle within genotype.

Fig. 8. Intracerebroventricular BAY60-6583 causes hypothermia

(A–C) Tb and physical activity response to BAY60-6583 (0.04 or 0.004 mg/kg, i.c.v.) in *Adora1^{-/-}; Adora3^{-/-}* mice. Data are mean ± SEM, n = 7/group (vehicle, 0.004 mg/kg) or n = 2 for 0.04 mg/kg. (D–F) Tb and physical activity response to BAY60-6583 (0.004 mg/kg, i.c.v.) or vehicle in *Adora2b^{-/-}* or C57BL/6J (WT) mice. Data are mean ± SEM, n = 5–8/group in a crossover design; *p < 0.05, **p < 0.01 vs vehicle within genotype.

Fig. 9. BAY60-6583 reduces metabolic rate before Tb

The effect of BAY60-6583 (1 mg/kg, i.p.) or vehicle in C57BL/6J (WT) mice on (A) Tb, (B) total energy expenditure (TEE), (C) respiratory exchange ratio (RER), (D) physical activity, (E) food intake. Data are mean $\pm$ SEM, $n = 11$ /group, * $p < 0.05$.

Fig. 10. Neuronal activation after intracerebroventricular infusion of BAY60-6583

(A) BAY60-6583 (0.004 mg/kg, i.c.v.) or vehicle was infused into *Adora1^{-/-};Adora3^{-/-}* mice, with brains removed 1 h later and stained for Fos. (A-D) Representative coronal Fos immunofluorescence images following vehicle (left) or BAY60-6583 (right) treatment. Nuclei are: POA, preoptic area; PVH, paraventricular hypothalamus; Arc, arcuate; VMH, ventromedial hypothalamus; and DMH, dorsomedial hypothalamus. (E) Quantitation of Fos-positive neurons. Means and p values are indicated.

Table 1

Binding affinity of adenosine receptor agonists and antagonists used in this study.

<u>Agonists</u>	mouse K _i , nM (or % inhibition at 10 µM)*				rat K _i , nM		LogPS** or brain penetrant
	A1AR	A2AAR	A2BAR	A3AR	A1AR	A1AR rat: mouse ratio	
CGS-21680	193 ^a	10 ^a	>10,000 ^b	48 ^a	16 ^c	0.083	no ^d
regadenoson	7.75 ± 0.05 ^e	77.2 ± 6.3 ^e	>100,000 (h) ^f	7 ± 2% ^e	>16,000 (h) ^f		-4.37**
PSB-0777	18.4 ± 1.1 ^e	193 ± 28 ^e	>10,000 (h) ^g	26 ± 1% ^e	10,000 ^g	>500	no ^g
APEC	4.17 ± 0.15 ^e	3.57 ± 0.22 ^e	inactive (r) ^h	55.1 ± 4.3 ^e	400 ⁱ	95	yes ^d
BAY60-6583	351*** ^b	>10,000 ^b	136*** ^b	3920*** ^b	514 ^b	1.5	-2.77**
<u>Antagonists</u>							
SCH442416	765 ^a	1.3 ^a	>10,000 (h) ^j	>10,000 ^a	1815 ^j	2.4	yes ^j
MRS7352	21 ± 4% ^k	64.1 ± 5.3 ^k		4 ± 1% ^k			-3.89**

* Data are for mouse, except if denoted (r) rat or (h) human.

** The logPS is the blood-brain permeability-surface area product, calculated using <http://bleoberis.bioc.cam.ac.uk/pkcsim/prediction>. A logPS >2 is considered penetrating, while logPS < -3 is non-penetrating (Pires et al., 2015).

*** BAY60-6583 may be an antagonist at A1 and A3AR and a partial agonist at A2BAR.

Superscripts indicate data source. SEM s are included for experimental results.

^a (Carlin et al., 2017)

^b (Alnouri et al., 2015)

^c (Jarvis et al., 1989)

^d (Nikodijevic et al., 1990)

^e Current results

^f (Gao et al., 2001)

^g (El-Tayeb et al., 2011)

^h (Hilde et al., 1992)

ⁱ (Kim et al., 1994)

$$\begin{matrix} j \\ k \end{matrix} \begin{matrix} \text{(Todde et al., 2000)} \\ \text{(Duroux et al., 2017)} \end{matrix}$$

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