Activation of neuronal adenosine A₁ receptors causes hypothermia through central and peripheral mechanisms

Haley S. Province, Cuiying Xiao, Allison S. Mogul, Ankita Sahoo, Kenneth A. Jacobson, Ramón A. Piñol, Oksana Gavrilova, Marc L. Reitman

1 Diabetes, Endocrinology, and Obesity Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland, United States of America, 2 Mouse Metabolism Core, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland, United States of America, 3 Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland, United States of America

☯ These authors contributed equally to this work.
* marc.reitman@nih.gov

Abstract

Extracellular adenosine, a danger signal, can cause hypothermia. We generated mice lacking neuronal adenosine A₁ receptors (A₁AR, encoded by the Adora1 gene) to examine the contribution of these receptors to hypothermia. Intracerebroventricular injection of the selective A₁AR agonist (Cl-ENBA, 5'-chloro-5'-deoxy-N₆-endo-norbornyladenosine) produced hypothermia, which was reduced in mice with deletion of A₁AR in neurons. A non-brain penetrant A₁AR agonist [SPA, N₆-(p-sulfophenyl) adenosine] also caused hypothermia, in wild type but not mice lacking neuronal A₁AR, suggesting that peripheral neuronal A₁AR can also cause hypothermia. Mice expressing Cre recombinase from the Adora1 locus were generated to investigate the role of specific cell populations in body temperature regulation. Chemogenetic activation of Adora1-Cre-expressing cells in the preoptic area did not change body temperature. In contrast, activation of Adora1-Cre-expressing dorsomedial hypothalamus cells increased core body temperature, concordant with agonism at the endogenous inhibitory A₁AR causing hypothermia. These results suggest that A₁AR agonism causes hypothermia via two distinct mechanisms: brain neuronal A₁AR and A₁AR on neurons outside the blood-brain barrier. The variety of mechanisms that adenosine can use to induce hypothermia underscores the importance of hypothermia in the mouse response to major metabolic stress or injury.

Introduction

Extracellular adenosine, a product of excessive ATP breakdown such as from extreme metabolic demand or tissue injury, has been called a ‘retaliatory metabolite’ or danger signal [1]. Due to rapid (i.e., seconds) cellular uptake and metabolism, extracellular adenosine levels are typically low [2] and many of its effects occur locally. Adenosine elicits protective physiology to diminish metabolic demand, reduce injury, and orchestrate recovery from the instigating
‘extreme’ physiology [3]. The adenosine A₁ receptor (A₁AR) is one of the four adenosine G protein-coupled receptors and is primarily coupled to G<sub>i/o</sub> [4]. Its structure has been solved [5]. A₁AR is very widely expressed and agonism at this receptor has cardiovascular (bradycardia, protection from ischemia-reperfusion injury), nervous system (antinoception, anti-seizure, protection from ischemia injury, sleep), anti-inflammatory, antidiuretic, and tissue protective (lung, kidney) actions, among other effects [6].

Clinically, hypothermia is used to reduce injury after neonatal hypoxia or cardiopulmonary resuscitation [7]. The hypothermia is typically produced by physical cooling. However, a wide variety of pharmacologic agents can cause hypothermia (see [8]), including adenosine [9], and there is interest in using such agents clinically. This can be readily investigated using mice, as their small size makes them a sensitive, responsive model for exploring mammalian thermal physiology [10]. In the brain, the preoptic area (POA) plays a major integratory role in the control of body temperature [11]. For example, this region has neurons that can drive hypothermia/torpor [12], so this brain region is a prime candidate for pharmacologic actions.

A number of lines of evidence indicate a role for A₁AR in regulation of core body temperature (T<sub>b</sub>), particularly hypothermia. Classic pharmacologic studies identified agonism at brain A₁AR as able to cause hypothermia [13] and there is interest in using A₁AR agonists for therapeutic hypothermia [14]. Selective agonist microinjections and other localization studies suggested a role in hypothermia for A₁AR in multiple brain regions, including the POA [15–18]. However, we and others have determined that agonists selective for each of the four adenosine receptors can cause hypothermia [19–22]. Also, while a role for A₁AR in torpor has been proposed [23, 24], neither A₁AR nor any of the other ARs is required for torpor [21, 25].

Understanding the role of A₁AR in temperature regulation is complicated by the very wide distribution of the A₁AR throughout the brain [6, 26] and its presence in both neurons and glia [27]. The highest brain A₁AR concentrations are in portions of the hippocampus, thalamus, and cerebral and cerebellar cortex. Interestingly, A₁AR levels are much lower in the hypothalamus, including the POA [26]. Here we investigated where A₁AR agonists might act to cause hypothermia.

Materials and methods

Mice

Mice at 3–12 months of age were housed at 21–22°C with a 12:12-h dark:light cycle (lights on at 0600) in a clean, conventional facility with paper bedding (7099-TEK-fresh, Envigo, Indianapolis, IN) and ad libitum access to water and chow (7022, 15% kcal fat, Envigo Inc, Madison, WI). Experiments were approved by the NIDDK Institutional Animal Care and Use Committee (protocol K016-DEOB-20). Mice were studied >7 days after any operation or prior treatment, and singly housed after telemeter implantation. Reuse of mice tends to reduce physical activity levels during drug testing, presumably due to acclimatization to handling. No specific effort was made to acclimatize mice in individual experiments. Mice were euthanized by exposure to carbon dioxide followed by cervical dislocation.

Male C57BL/6J mice were purchased from Jackson Laboratories, Bar Harbor, ME (JAX; #000664). Ai6 mice carrying Cre-dependent fluorescent protein ZsGreen [28] were purchased from JAX (#007906). Male Adora<sup>1</sup> mice on a C57BL/6J background were provided by Dr. Jurgen Schnrermann, NIDDK [29] and genotyped as described [21]. Adora<sup>1fl/fl</sup> mice [30] were generously provided by Dr. Robert Greene, UTSW, and genotyped by PCR. Primers x692 (forward, 5′-CCACCATTATCTGGCTCCCAT) and x691 (reverse, 5′-GCTGAGTCAC- CACTGTCTTGT) produce 268-bp (wild-type allele) and 302-bp (flox allele) products. Primers x692 and x708 (reverse, 5′-GCTCCCCTGTCTGACTGAAG) produce a ~230-bp product from
a deleted flox allele. Syn1-Cre mice expressing Cre in neurons [31] were purchased from JAX (#003966) and genotyped (~300 bp product) using primers x682 (forward, 5′-CTCAGCGCTGCCTCAGTCCT) and x683 (reverse, 5′-GCATCGACCGGTAATGCA). Offspring (both sexes) of Adora1fl/fl x Adora1fl/+;Syn1-Cre/+ and Adora1del/fl x Adora1fl/+;Syn1-Cre/+ matings were used for neuronal deletion experiments.

**Generation of Adora1-Cre mice**

The Adora1-Cre mouse was made in a B6D2F1/J founder at the NHLBI Transgenic Core using CRISPR/Cas9 to insert a T2A peptide sequence and Cre recombinase at the Adora1 stop codon. The T2A sequence adds EGRGSLLTCGDVEENPG to the C terminus of A1AR and a proline to the N terminus of Cre. It is expected that the two proteins are translated in a 1:1 ratio [32] and that the added T2A sequence may reduce A1AR function [33, 34]. The founder was bred to C57BL/6J once. Presence of Adora1-Cre (553 bp) vs wild type (318 bp) alleles was determined by PCR using primers x617 (common forward, 5′-AAGTTCCGGGTCACCTTTC), cre1 (Cre reverse, 5′-CCTGTTTTGCACGTTCACCG), and x630 (Adora1 reverse, 5′-AC TCAAACCTCCTCCAGGT). While homozygous Adora1-Cre mice are fertile and appear grossly normal, only heterozygous Adora1-Cre mice were used in this study. The Adora1-Cre mouse is available from the corresponding author.

**Compounds**

Compounds (source; vehicle) were obtained as follows: Cl-ENBA [(±)-5′-chloro-5′-deoxy-N6-endo-norbornyladenosine, Tocris 3576; 10% DMSO], SPA [N6-(p-sulfophenyl)adenosine, Sigma S198; saline], CNO (clozapine-N-oxide, Sigma C0832; saline).

**Quantitative PCR and RT-PCR**

DNA and RNA were extracted (Allprep DNA/RNA micro Kit, Qiagen, Germantown, MD) from whole brain, excluding cerebellum and brain stem. RNA was reverse transcribed (Transcriptor High Fidelity cDNA Synthesis Kit, Roche, Indianapolis, IN). DNA and cDNA were quantified (QuantStudio 7 Flex Real-Time PCR System, Applied Biosystems, Waltham, MA) using SYBR green. Adora1 mRNA primers were x711 (forward, 5′-CATGGGCGCAGCTACCTT) and x713 (reverse, 5′-CTGTTTTGCACGTTCACCG), and x630 (Adora1 reverse, 5′-AC TCAAACCTCCTCCAGGT). While homozygous Adora1-Cre mice are fertile and appear grossly normal, only heterozygous Adora1-Cre mice were used in this study. The Adora1-Cre mouse is available from the corresponding author.

**Telemetric monitoring of body temperature and activity**

Core body temperature (Tb) and physical activity were continuously measured by telemetry, using G2 E-Mitters implanted intraperitoneally, ER4000 energizer/receivers, and VitalView software (v 5.0 Starr Life Sciences, Oakmont, PA), with data collected each minute [35].

**Surgical procedures**

Mice were anesthetized with ketamine/xylazine (80/10 mg/kg, i.p.) and placed in a stereotaxic instrument (Digital Just for Mouse Stereotaxic Instrument, Stoelting). Ophthalmic ointment (Puralube, Dechra) was applied. Post-surgery mice received subcutaneous sterile saline injections to prevent dehydration and an analgesic (buprenorphine; 0.1 mg/kg, i.p.). G2 E-Mitters were implanted intraperitoneally as described [35].
Central infusions
Sterile guide cannulas (5.25 mm, 26 gauge; Plastics One, Roanoke, VA) were unilaterally implanted into the lateral ventricle (coordinates relative to bregma: -0.34 mm anterior, 1.0 mm lateral, 1.7 mm ventral) and fixed with dental cement (Parkell, Edgewood, NY). Compounds in 5 μl were infused (0.5 μl/min) through a 33-gauge cannula protruding 0.5 mm past the tip of the guide cannula using PE-50 tubing fitted to a 5 μl syringe (Hamilton, Reno, NV) on a dual syringe pump (KD Scientific, Holliston, MA).

Virus injections
All injections (200 nl) were done with pulled-glass pipettes (pulled 20–40 μm tip diameter; 0.275 ID, 1 mm OD, Wilmad Lab Glass) at a visually controlled rate of 50 nl per min with an air pressure system regulator (Grass Technologies, Model S48 Stimulator). The pipette was kept in place for 5 min after injection. The viruses AAV8-hSyn-DIO-hM3Dq-mCherry (gift from B. Roth; Addgene viral prep # 44361-AAV8 [36]) and AAV8-hSyn-DIO-hM4Di-mCherry (gift from B. Roth; Addgene viral prep # 44362-AAV8 [36]) were injected bilaterally into the preoptic area (POA, coordinates relative to bregma: 0.35 mm anterior, ±0.3 mm lateral, -5.25 mm ventral) or dorsomedial hypothalamus (DMH, coordinates relative to bregma: 1.85 mm posterior, ±0.25 mm lateral, -5.2 mm ventral) of Adora1-Cre mice. This batch of AAV8-hSyn-DIO-hM4Di-mCherry was functional in other experiments in our lab that were not part of this project. The hM4Di-mCherry is a fusion protein, so if mCherry is expressed, then hM4Di is also expressed.

Chemogenetics experimental procedure
hM3Dq and hM4Di were activated by CNO (1 mg/kg, i.p.) or saline vehicle. After completion of all experiments, mice were anesthetized (chloral hydrate, 500 mg/kg, i.p.), perfused transcardially with 0.9% saline followed by 10% neutral buffered formalin, the brain was removed, and reporter expression was visualized by immunohistochemistry [33]. Mice without hM3Dq or hM4Di expression in the target area on at least one side were excluded from analysis.

Experimental design and statistical analysis
Drug treatments were typically administered in crossover design using randomized treatment order. Hypothermia was assessed as the mean Tb from 0 to 60 minutes after dosing. Hyperthermia was assessed from 60 to 120 minutes after dosing to exclude the handling-induced Tb increase. Physical activity was assessed as the mean from 10 to 60 (hypothermia experiments to avoid the first 10 min after handling) or 60 to 120 (hyperthermia experiments) minutes after dosing. In crossover experiments, paired t-tests were used to compare the within mouse effect of drug vs vehicle. Unpaired t-tests were used to compare the within mouse drug vs vehicle effect between genotypes. Statistical significance was defined as 2-tailed P < 0.05. Data are presented as mean ±SEM. No statistical methods were used to pre-determine sample size. Data, keyed to each figure, are available in the S1 Data.

Image capture and processing
Images were captured using an Olympus BX61 motorized microscope with Olympus BX-UCB hardware (VS120 slide scanner) and processed (including contrast adjustments) using OlyVIA software (Olympus).
Results

No effect on baseline body temperature from neuronal loss of A₁AR

To distinguish between adenosine action on glia [27] and neurons, we produced mice carrying both floxed Adora1 and Syn1-Cre to selectively ablate A₁AR function in neurons. The effect of neuronal deletion was studied in pooled Adora1<sup>fl/fl</sup>;Syn1-Cre and Adora1<sup>del/fl</sup>;Syn1-Cre mice (hereafter referred to collectively as Adora1<sup>del/fl</sup>;Syn1-Cre, where del indicates a germline-deleted allele). In whole brain, Syn1-Cre produced 45% deletion per flox allele (Fig 1A). Concomitantly, Syn1-Cre reduced Adora1 mRNA levels by 35% per flox allele (Fig 1B). Since Syn1-Cre is selectively expressed in differentiated neurons [31], these data are consistent with neuronal deletion of Adora1<sup>fl</sup>, with the remaining expression likely derived from non-Syn1-Cre-expressing (non-neuronal) cells.

At baseline, there was no difference between control and Adora1<sup>fl</sup>;Syn1-Cre littermate mice, matched for sex, in body temperature (T<sub>b</sub>) during light or dark phase, or in Tb variability (as Tb span, the difference between the 95<sup>th</sup> and 5<sup>th</sup> Tb percentiles). There was also no effect of neuronal deletion on the level of physical activity or its diurnal rhythm (Table 1).

A₁AR agonist-induced hypothermia is mediated by both peripheral and central neurons

Cl-ENBA is a selective A₁AR agonist. Peripherally-dosed Cl-ENBA (3 mg/kg, i.p.) caused robust hypothermia in Adora1<sup>fl</sup> controls (-4.93 ± 0.17°C vs vehicle at 0 to 60 minutes) and Adora1<sup>fl</sup>;Syn1-Cre (-3.50 ± 0.53°C vs vehicle) mice (Fig 2A–2C). The hypothermia in Adora1<sup>fl</sup>;Syn1-Cre mice was attenuated compared to controls (P = 0.02). Since Cl-ENBA at high doses can also cause hypothermia via activation of mast cell A₃AR [21], we tested a lower Cl-ENBA dose (1 mg/kg; -3.33 ± 0.50°C in controls vs. -1.83 ± 0.55°C in Adora1<sup>fl</sup>;Syn1-Cre mice; P = 0.06) (Fig 2D–2F). The similar level of partial loss of Cl-ENBA-induced hypothermia at
the two doses suggests that i.p. CI-ENBA is causing hypothermia partially through neuronal A₁AR.

SPA is a non-brain penetrant selective A₁AR agonist [37]. Peripherally dosed SPA (1 mg/kg, i.p.) caused hypothermia in wild-type mice that was lost in Adora₁⁻/⁻ mice (Fig 3A–3C). In Adora₁⁻;Syn1-Cre mice, SPA-induced hypothermia was greatly diminished and not statistically different from vehicle treatment (Fig 3D–3F). These results suggest that hypothermia can be elicited via A₁AR expressed by neurons that are outside the blood-brain barrier.

We next tested the ability of a low i.c.v. dose of A₁AR agonist to produce hypothermia. Central administration of CI-ENBA (3.06 μg/mouse; ~0.1 mg/kg) caused hypothermia in control but not Adora₁⁻;Syn1-Cre mice (-2.30 ± 0.43°C vs -0.23 ± 0.17°C; P = 0.001) (Fig 4A–4C). This demonstrates that A₁AR agonists can cause hypothermia by acting directly on brain neurons.

Table 1. Deletion of neuronal A₁AR has no effect on baseline body temperature or physical activity.

| Sex             | Adora₁⁻ Female | Adora₁⁻;Syn1-Cre Female | P   | Adora₁⁻ Male | Adora₁⁻;Syn1-Cre Male | P   |
|-----------------|----------------|--------------------------|-----|--------------|------------------------|-----|
| N               | 4              | 5                        | 0.27| 7            | 8                      | 0.64|
| Tb, light phase (°C) | 36.17 ±0.07 | 36.40 ±0.16               | 0.94| 35.67 ±0.16  | 35.76 ±0.12             | 0.25|
| Tb, dark phase (°C)  | 37.37 ±0.17 | 37.39 ±0.16               | 0.50| 36.77 ±0.13  | 36.93 ±0.05             | 0.64|
| ΔTb, dark-light (°C) | 1.21 ±0.10 | 0.99 ±0.25                | 0.38| 1.10 ±0.10   | 1.17 ±0.11              | 0.64|
| Tb span (°C)      | 2.58 ±0.15 | 2.09 ±0.45                | 0.18| 2.28 ±0.08   | 2.49 ±0.14              | 0.23|
| Activity, light phase (counts) | 6.8 ±0.5 | 8.1 ±0.6                | 0.23| 5.4 ±0.3    | 6.4 ±0.4               | 0.09|
| Activity, dark phase (counts) | 19.6 ±1.1 | 25.0 ±3.5               |       | 14.5 ±1.0  | 15.5 ±1.1              | 0.51|

Data collected from 5-month old mice over a continuous 96-hour interval. Tb span is the 95th minus the 5th percentiles. Data are mean ± SEM. P values are from unpaired t-tests comparing genotype within sex.

https://doi.org/10.1371/journal.pone.0243986.t001

Fig 2. Neuronal A₁AR partially mediate CI-ENBA induced hypothermia. (A–C) Core body temperature (Tb) and physical activity response to CI-ENBA (3 mg/kg, i.p.) or vehicle in Adora₁⁻ (n = 10) and Adora₁⁻;Syn1-Cre (n = 11) mice. (D–F) Tb and physical activity response to CI-ENBA (1 mg/kg, i.p.) or vehicle in Adora₁⁻ (n = 8) and Adora₁⁻;Syn1-Cre (n = 9) mice. Data are mean ± SEM (SEM is omitted for visual clarity in A and D). P values calculated by paired t-test comparing drug vs vehicle within mouse.

https://doi.org/10.1371/journal.pone.0243986.g002
Activation of Adora1-Cre-expressing neurons in the dorsomedial hypothalamus increased body temperature

Since $A_1$AR is widely distributed in the brain, we wished to probe specific regions for their potential role in driving $A_1$AR agonist-induced hypothermia. Adora1-Cre mice were produced by targeted insertion of a Cre recombinase gene into the endogenous Adora1 locus, preserving the coding region (Methods, Fig 5A). The mice were bred with reporter mice expressing GFP in a Cre-dependent manner. The resulting GFP expression was widespread and particularly high in the cortex, hippocampus, and thalamus (Fig 5B). This GFP reporter expression pattern matches that reported for $A_1$AR ligand binding activity [26].

The preoptic area (POA) is a region of the brain with major control over Tb; other areas also contribute [38]. Local injection of $A_1$AR agonist has been shown to produce hypothermia in hypothalamic sites, including POA and dorsomedial hypothalamus (DMH) [16], so we focused on these regions. $A_1$AR is coupled to Gi/o, therefore we used chemogenetics to selectively target Adora1-Cre neurons with viral expression of a Gi-coupled designer receptor exclusively activated by designer drug (DREADD), hM4Di, to mimic the endogenous inhibitory $A_1$AR signaling. Mice with virally-delivered hM4Di in the POA were treated with DREADD agonist, clozapine-N-oxide (CNO), or vehicle. No effect of CNO on Tb was observed (Fig 6A–6C). All mice had at least unilateral virus expression in the medial preoptic area, and some mice had virus expression also in the medial septal nuclei and/or diagonal band of Broca. Thus, agonism of an inhibitory DREADD in POA Adora1 neurons did not cause hypothermia.

We next tested activation of an excitatory Gq-coupled DREADD, hM3Dq, in Adora1-Cre-expressing cells. As endogenous $A_1$AR signaling is inhibitory, an increase in Tb due to chemogenetic activation of stimulatory Gq would be a concordant observation. Activation of POA Adora1 neurons with CNO had no effect on Tb (Fig 7A–7C). All mice had unilateral or bilateral virus
expression in the medial preoptic area, with virus expression in varying extent throughout the anterior hypothalamus. In contrast, activation of DMH neurons expressing hM3Dq with CNO increased Tb (Fig 7D–7F). All mice had unilateral or bilateral virus expression restricted to the DMH/dorsal hypothalamic area. Thus, the DMH is a potential candidate region for neurons driving A1AR agonist-induced hypothermia.

Discussion

The brain controls Tb. Thus, we had anticipated identifying an A1AR-expressing neuron population, possibly in the POA, as the driver of A1AR agonist-induced hypothermia. However, our results suggest that A1AR agonism can cause hypothermia acting at neuronal A1AR both inside and outside the brain, demonstrating that adenosine can cause hypothermia via more than one A1AR neuronal mechanism.

Central mechanisms of A1AR hypothermia

While A1AR is found in microglia, oligodendrocytes, astrocytes, and neurons [39], the loss-of-function experiments demonstrate that neurons (Syn1-Cre-positive cells) are required for
hypothermia caused by central administration of an A₁AR agonist. Activation of different defined populations of POA neurons can increase or decrease Tb [12, 40–44]. In addition, local A₁AR agonist administration to this region causes hypothermia [16]. Thus, we had expected that chemogenetic inhibition or activation of POA Adora₁ neurons would affect Tb, but this was not observed. The effect on Tb from stimulation of POA Adora₁ neurons may be more variable than in controls, suggesting that there may be multiple POA Adora₁ neuron populations with differing effects on Tb.

Chemogenetic activation of Adora₁-Cre-expressing neurons of the DMH, another known thermoregulatory center, uniformly increased Tb, consistent with reports that disinhibition and activation of DMH neurons increase Tb [33, 40, 45]. One interpretation of our results is
that DMH\textsuperscript{Adora\textsubscript{1}} neurons are driving \textit{A\textsubscript{1}}AR agonist-induced hypothermia, as activation of these neurons (opposing the inhibitory signaling from endogenous \textit{A\textsubscript{1}}AR activation) increased \textit{Tb}. Another interpretation is that the chemogenetically manipulated neurons have a role in thermoregulation, but this is not directly related to pharmacologic \textit{A\textsubscript{1}}AR agonist-induced hypothermia.

Our results do not rule out the possibility of a single, specific nucleus mediating central \textit{A\textsubscript{1}}AR hypothermia. However, with widespread expression throughout the brain, we hypothesize that expression of \textit{A\textsubscript{1}}AR may not identify a discrete neuronal population that drives hypothermia. Rather, adenosine acting on \textit{A\textsubscript{1}}AR may be a general mechanism to reduce local neuronal activity, with the effect on \textit{Tb} depending on the particular neuronal subpopulation’s direct or indirect contributions to thermoregulation.

---

**Fig 6.** Chemogenetic inhibition of POA\textsuperscript{Adora\textsubscript{1}} neurons has no effect on \textit{Tb}. (A) Example of hM4Di-mCherry expression in Adora\textsubscript{1}-Cre mouse injected with AAV8-hSyn-DIO-hM4Di-mCherry in the POA. Scale bar is 500 μm. (B) \textit{Tb} response to CNO (1 mg/kg, i.p.) vs. vehicle in Adora\textsubscript{1}-Cre mice with hM4Di-mCherry in POA (\(n = 7\)). (C) Mean \textit{Tb} at 0 to 60 minutes after dosing. Data are mean ± SEM (SEM is omitted for visual clarity in B). \(P\) value was calculated by paired \textit{t}-test comparing drug vs vehicle within mouse. Aca, anterior commissure; MnPO, median preoptic area; MPA, medial preoptic area; VMPO, ventromedial preoptic area.

https://doi.org/10.1371/journal.pone.0243986.g006

**Fig 7.** Chemogenetic activation of DMH\textsuperscript{Adora\textsubscript{1}}, but not POA\textsuperscript{Adora\textsubscript{1}} neurons increases \textit{Tb}. A-C tests activation of the POA. (A) Example of hM3Dq-mCherry expression in Adora\textsubscript{1}-Cre mouse injected with AAV8-hSyn-DIO-hM3Dq-mCherry in the POA. (B) \textit{Tb} response to CNO (1 mg/kg, i.p.) vs. vehicle in Adora\textsubscript{1}-Cre mice with hM3Dq-mCherry in POA (\(n = 11\)). (C) Mean \textit{Tb} at 60 to 120 minutes after dosing with CNO (1 mg/kg, i.p.) vs. vehicle. D-F tests activation of the DMH. (D) Example of hM3Dq-mCherry expression in Adora\textsubscript{1}-Cre mouse injected with AAV8-hSyn-DIO-hM3Dq-mCherry in the DMH. (E) \textit{Tb} response to CNO (1 mg/kg, i.p.) vs. vehicle in Adora\textsubscript{1}-Cre mice with hM3Dq-mCherry in DMH (\(n = 8\)). (F) Mean \textit{Tb} at 60 to 120 minutes after dosing with CNO (1 mg/kg, i.p.) vs. vehicle (\(n = 8\)). Data are mean ± SEM (SEM is omitted for visual clarity in B and E). \(P\) values were calculated by paired \textit{t}-test comparing drug vs vehicle within mouse. Scale bar is 500 μm. Aca, anterior commissure; dDMH, dorsomedial hypothalamus, dorsal part; DHA, dorsal hypothalamic area; MnPO, median preoptic area; MPA, medial preoptic area; vDMH, dorsomedial hypothalamus, ventral part; VMPO, ventromedial preoptic area; VLPO, ventrolateral preoptic area.

https://doi.org/10.1371/journal.pone.0243986.g007
Peripheral mechanisms of A<sub>1</sub>AR hypothermia

Our results indicate A<sub>1</sub>AR agonist-induced hypothermia can be caused by neuronal A<sub>1</sub>AR outside the central nervous system. The hypothermic effect of the non-brain penetrant, peripherally-restricted A<sub>1</sub>AR agonist SPA was lost in Adora<sup>fl</sup>β-Syn1-Cre mice, indicating that peripheral A<sub>1</sub>AR agonist-induced hypothermia is mediated by cells expressing synapsin-1 (i.e. peripheral nerves). A<sub>1</sub>AR is found throughout the peripheral nervous system (PNS): in motor and sensory nerve terminals of rats, as well as sympathetic and parasympathetic nerves [46–49]. Like central A<sub>1</sub>AR, PNS A<sub>1</sub>AR play a role in regulating numerous physiological processes (nociception, vascular tone, etc.) by providing an inhibitory tone on neurotransmission. Therefore, the effect of PNS A<sub>1</sub>AR agonism on Tb depends on the function of the peripheral neuron being inhibited.

A<sub>1</sub>AR is present in many non-neuronal cells (smooth muscle, epithelial, immune cells) and tissues (heart, adipose tissue, pancreas, kidney) that have a very wide variety of functions (reviewed in [1]). Agonism of non-neural A<sub>1</sub>AR can produce physiologic effects such as hypotension that could secondarily decrease Tb. The lack of significant hypothermia produced by the peripheral A<sub>1</sub>AR agonist SPA in Adora<sup>fl</sup>β-Syn1-Cre mice suggests that while non-neuronal cells may contribute, neuronal A<sub>1</sub>AR are required for hypothermia by this mechanism. Further research will be needed to identify the specific neuronal populations involved.

Adenosine as a physiologic and general danger signal

Adenosine can act at each of the four adenosine receptors to cause hypothermia. Activation of A<sub>3</sub>AR on peripheral mast cells causes hypothermia via histamine release, followed by activation of H<sub>1</sub> receptors [22, 50, 51]. Agonism at peripheral A<sub>2A</sub>AR or central A<sub>2B</sub>AR both cause hypothermia [20, 52]. However, the specific cells that elicit hypothermia when directly activated by A<sub>2A</sub>AR and A<sub>2B</sub>AR agonists have not been identified. Including A<sub>1</sub>AR on central and on peripheral neurons, there are five identified paths by which adenosine agonism elicits hypothermia. When hypothermia is studied in sufficient detail, it is accompanied by hypometabolism, decreased physical activity, and hypotension. While hypometabolism is likely required to achieve hypothermia, different driving physiology (eg., hypotension vs torpor) can produce hypothermia.

Adenosine is a ubiquitous molecule that acts locally to signal metabolic stress or injury. The distinct mechanism(s) by which activation of each AR causes hypothermia suggests that adenosine signaling is harnessed to minimize damage, regardless if the signaling occurs centrally or peripherally. Hypothermia can be due to direct adenosine agonism on thermoregulatory neurons, or a secondary effect of other adenosine-driven physiology. Taken with previous studies, these results demonstrate that at least five distinct mechanisms of hypothermia mediated by adenosine receptors exist. The redundancy in AR signaling highlights the importance of hypothermia as a component of protective response mechanisms.

Supporting information

S1 Data.
(XLSX)

Acknowledgments

We thank Alice Franks, Naili Liu, Yuning Huang, and Zhenzhong Cui for experimental support, and Chengyu Liu of the NHLBI Transgenic Core for collaborating in generating the Adora1-Cre mouse.
Author Contributions

Conceptualization: Marc L. Reitman.

Investigation: Haley S. Province, Cuiying Xiao, Allison S. Mogul, Ankita Sahoo, Ramón A. Piñol, Oksana Gavrilova.

Visualization: Marc L. Reitman.

Writing – original draft: Haley S. Province, Cuiying Xiao, Marc L. Reitman.

Writing – review & editing: Haley S. Province, Cuiying Xiao, Allison S. Mogul, Ankita Sahoo, Kenneth A. Jacobson, Ramón A. Piñol, Oksana Gavrilova, Marc L. Reitman.

References

1. Borea PA, Gessi S, Merighi S, Vincenzi F, Varani K. Pharmacology of Adenosine Receptors: The State of the Art. Physiol Rev. 2018; 98(3):1591–625. Epub 2018/06/01. https://doi.org/10.1152/physrev.00049.2017 PMID: 29848236.

2. Moser GH, Schrader J, Deussen A. Turnover of adenosine in plasma of human and dog blood. Am J Physiol. 1989; 256(4 Pt 1):C799–806. https://doi.org/10.1152/ajpcell.1989.256.4.C799 PMID: 2539728.

3. Fredholm BB. Adenosine—a physiological or pathophysiological agent? J Mol Med (Berl). 2014; 92(3):201–6. https://doi.org/10.1007/s00109-013-1101-s PMID: 24362516.

4. Fredholm BB, Uzerman AP, Jacobson KA, Linden J, Müller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors—an update. Pharmacol Rev. 2011; 63(1):1–34. Epub 2011/02/10. https://doi.org/10.1124/pr.110.003285 pr.110.003285 [pii]. PMID: 21303899; PubMed Central PMCID: PMC3061413.

5. Glukhova A, Thal DM, Nguyen AT, Vecchio EA, Jorg M, Scammells PJ, et al. Structure of the Adenosine A1 Receptor Reveals the Basis for Subtype Selectivity. Cell. 2017; 168(5):867–77 e13. Epub 2017/02/25. https://doi.org/10.1016/j.cell.2017.01.042 PMID: 28235198.

6. Varani K, Vincenzi F, Merighi S, Gessi S, Borea PA. Biochemical and Pharmacological Role of A1 Adenosine Receptors and Their Modulation as Novel Therapeutic Strategy. Adv Exp Med Biol. 2017; 1051:193–232. Epub 2017/06/06. https://doi.org/10.1007/978-3-319-58211-3_11 PMID: 28767923.

7. Arrich J, Holzer M, Havel C, Mullner M, Herkner H. Hypothermia for neuroprotection in adults after cardiopulmonary resuscitation. Cochrane Database Syst Rev. 2016; 2:CD004128. Epub 2016/02/16. https://doi.org/10.1002/14651858.CD004128.pub4 PMID: 26878327; PubMed Central PMCID: PMC6516972.

8. Clark WG. Changes in body temperature after administration of antipyretics, LSD, delta 9-THC and related agents: II. Neurosci Biobehav Rev. 1987; 11(1):35–96. Epub 1987/01/01. S0149-7634(87)80003-9 [pii]. https://doi.org/10.1016/s0149-7634(87)80003-9 PMID: 3033566.

9. Bennet DW, Drury AN. Further observations relating to the physiological activity of adenine compounds. J Physiol. 1931; 72(3):288–320. https://doi.org/10.1113/jphysiol.1931.sp002775 PMID: 16994210; PubMed Central PMCID: PMC1403115.

10. Skop V, Guo J, Liu N, Xiao C, Hall KD, Gavrilova O, et al. Mouse Thermoregulation: Introducing the Concept of the Thermoneutral Point. Cell Rep. 2020; 31(2):107501. Epub 2020/04/16. https://doi.org/10.1016/j.celrep.2020.03.065 PMID: 32294435; PubMed Central PMCID: PMC7243168.

11. Morrison SF, Nakamura K. Central Mechanisms for Thermoregulation. Annu Rev Physiol. 2019. Epub 2018/09/27. https://doi.org/10.1146/annurev-physiol-020518-114546 PMID: 30256726.

12. Takahashi TM, Sunagawa GA, Soya S, Abe M, Sakurai K, Ishikawa K, et al. A discrete neuronal circuit induces a hibernation-like state in rodents. Nature. 2020. Epub 2020/06/13. https://doi.org/10.1038/s41586-020-2163-6 PMID: 32528181.

13. Anderson R, Sheehan MJ, Strong P. Characterization of the adenosine receptors mediating hypothermia in the conscious mouse. Br J Pharmacol. 1994; 113(4):1386–90. Epub 1994/12/01. https://doi.org/10.1111/j.1476-5381.1994.tb17151.x PMID: 7889296; PubMed Central PMCID: PMC1510495.

14. Drew KL, Romanovsky AA, Stephen TK, Tupone D, Williams RH. Future approaches to therapeutic hypothermia: a symposium report. Temperature (Austin). 2015; 2(2):168–71. https://doi.org/10.4161/23328940.2014.976512 PMID: 27227020; PubMed Central PMCID: PMC4843898.
26. Fastbom J, Pazos A, Palacios JM. The distribution of adenosine A1 receptors and 5’-nucleotidase in
25. Xiao C, Liu N, Jacobsen KA, Gavrilova O, Reitman ML. Physiology and effects of nucleosides in mice
Silvani A, Cerri M, Zoccoli G, Swoap SJ. Is Adenosine Action Common Ground for NREM Sleep, Tor-
24. Boison D, Chen JF, Fredholm BB. Adenosine signaling and function in glial cells. Cell Death Differ.
23. Frare C, Jenkins ME, McClure KM, Drew KL. Seasonal decrease in thermogenesis and increase in
22. Carlin JL, Jain S, Gizewski E, Wan TC, Tosh DK, Xiao C, Auchampaeh JA, et al. Hypothermia in mouse is caused by
21. Carlin JL, Jain S, Duroux R, Suresh RR, Xiao C, Auchampaeh JA, et al. Activation of adenosine A2A or
20. https://doi.org/10.1016/j.neuropharmacology.2018.02.035 PMID: 29548666; PubMed Central PMCID: PMC55434.
19. Johansson B, Halldner L, Dunwiddie TV, Masino SA, Pochi W, Gimenez-Lloret L, et al. Hyperalgesia,
anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. Proc Natl
Acad Sci U S A. 2001; 98(16):9407–12. Epub 2001/07/27. https://doi.org/10.1073/pnas.161292398
161292398 [pii]; PMID: 11470917; PubMed Central PMCID: PMC55434.
18. Frare C, Jenkins ME, McClure KM, Drew KL. Seasonal decrease in thermogenesis and increase in
vasoconstriction explain seasonal response to N(6)-cyclohexyladenosine-induced hibernation in the
Arctic ground squirrel (Urocitellus parryi). J Neurochem. 2019; 151(3):316–35. Epub 2019/07/06.
https://doi.org/10.1111/jnc.14814 PMID: 31273786; PubMed Central PMCID: PMC6819227.
17. Shintani M, Tamura Y, Monden M, Shiomi H. Characterization of N(6)-cyclohexyladenosine-induced
hypothermia in Syrian hamsters. J Pharmacol Sci. 2005; 97(3):451–4. https://doi.org/10.1254/jphs.sc0040178 PMID: 15764835.
16. Shintani M, Tamura Y, Monden M, Shiomi H. Characterization of N(6)-cyclohexyladenosine-induced
hypothermia in Syrian hamsters. J Pharmacol Sci. 2005; 97(3):451–4. https://doi.org/10.1254/jphs.sc0040178 PMID: 15764835.
15. Ticho SR, Radulovacki M. Role of adenosine in sleep and temperature regulation in the preoptic area of
rats. Pharmacol Biochem Behav. 1991; 40(1):33–40. Epub 1991/09/01. https://doi.org/10.1016/0091-
3057(91)90317-u PMID: 1708343.
14. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, et a. A robust and high-throughput
Cre reporting and characterization system for the whole mouse brain. Nat Neurosci. 2010; 13(1):133–40. Epub 2009/12/22. https://doi.org/10.1038/nn.2467 PMID: 2023653; PubMed Central PMCID: PMC2885470.
13. Sun D, Samuelson LC, Yang T, Huang Y, Paliege A, Saunders T, et al. Mediation of tubuloglomerular
feedback by adenosine: evidence from mice lacking adenosine 1 receptors. Proc Natl Acad Sci U S A.
2001; 98(17):9983–8. Epub 2001/08/16. https://doi.org/10.1073/pnas.171317998 98/17/9983 [pii].
PMID: 11504952; PubMed Central PMCID: PMC55564.
12. Scammell TE, Arrigoni E, Thompson MA, Ronan PJ, Saper CB, Greene RW. Focal deletion of the
adenosine A1 receptor in adult mice using an adeno-associated viral vector. J Neurosci. 2003; 23(13):5762–70. Epub 2003/07/05. https://doi.org/10.1523/JNEUROSCI.23-13-05762.2003 [pii]. PMID: 12843280.
11. Zhu Y, Romero MI, Ghosh P, Ye Z, Charnay P, Rushing EJ, et al. Ablation of NFl function in neurons
induces abnormal development of cerebral cortex and reactive gliosis in the brain. Genes Dev. 2001; 15
70. Epub 2003/07/05. https://doi.org/10.1523/JNEUROSCI.23-13-05762.2003 [pii]. PMID: 12843280.
10. https://doi.org/10.1016/j.neuropharmacology.2018.02.035 PMID: 29548666; PubMed Central PMCID: PMC55434.
9. Silvani A, Cerri M, Zoccoli G, Swoap SJ. Is Adenosine Action Common Ground for NREM Sleep, Tor-
por, and Other Hypometabolic States? Physiology (Bethesda). 2018; 33(3):182–96. Epub 2018/04/17.
https://doi.org/10.1152/physiology.00007.2018 PMID: 29616880; PubMed Central PMCID: PMC5966658.
8. Ticho SR, Radulovacki M. Role of adenosine in sleep and temperature regulation in the preoptic area of
rats. Pharmacol Biochem Behav. 1991; 40(1):33–40. Epub 1991/09/01. https://doi.org/10.1016/0091-
3057(91)90317-u PMID: 1708343.
32. Szymczak-Workman AL, Vignali KM, Vignali DA. Design and construction of 2A peptide-linked multisegronic vectors. Cold Spring Harb Protoc. 2012; 2012(2):199–204. Epub 2012/02/04. https://doi.org/10.1101/pdb.ip067876 PMID: 22301656.

33. Pinol RA, Zahler SH, Li C, Saha A, Tan BK, Skop V, et al. BRS3 neurons in the mouse dorsomedial hypothalamus regulate body temperature, energy expenditure, and heart rate, but not food intake. Nat Neurosci. 2018; 21(11):1530–40. Epub 2018/10/24. https://doi.org/10.1038/s41593-018-0249-3 PMID: 30349101.

34. Xiao C, Liu N, Province H, Pinol RA, Gavrilova O, Reitman ML. BRS3 in both MC4R- and SIM1-expressing neurons regulates energy homeostasis in mice. Mol Metab. 2020; 36:100969. Epub 2020/04/02. https://doi.org/10.1016/j.molmet.2020.02.012 PMID: 32229422; PubMed Central PMCID: PMC7113433.

35. Lute B, Jou W, Lateef DM, Goldgof M, Xiao C, Pinol RA, et al. Biphasic effect of melanocortin agonists on metabolic rate and body temperature. Cell Metab. 2014; 20(2):333–45. Epub 2014/07/02. https://doi.org/10.1016/j.cmet.2014.05.021 PMID: 24981835; PubMed Central PMCID: PMC4126889.

36. Xiao C, Liu N, Province H, Pinol RA, Gavrilova O, Reitman ML. BRS3 in both MC4R- and SIM1-expressing neurons regulates energy homeostasis in mice. Mol Metab. 2020; 36:100969. Epub 2020/04/02. https://doi.org/10.1016/j.molmet.2020.02.012 PMID: 32229422; PubMed Central PMCID: PMC7113433.

37. Jacobson KA, Nikodijevic O, Ji XD, Berkich DA, Eveleth D, Dean RL, et al. Synthesis and biological activity of p- (p-sulfophenyl)alkyl and p-sulfoalkyl derivatives of adenosine: water-soluble and peripherally selective adenosine agonists. J Med Chem. 1992; 35(22):4143–9. https://doi.org/10.1021/jm00100a020 PMID: 1433217; PubMed Central PMCID: PMC3420980.

38. Hackett TA. Adenosine A1 Receptor mRNA Expression by Neurons and Glia in the Auditory Forebrain. Anat Rec (Hoboken). 2018; 301(11):1882–905. Epub 2018/10/14. https://doi.org/10.1002/ar.23907 PMID: 30315630; PubMed Central PMCID: PMC6282551.

39. Machado NLS, Bandaru SS, Abbott SBG, Saper CB. EP3R-Expressing Glutamatergic Preoptic Neurons Mediate Inflammatory Fever. J Neurosci. 2020; 40(12):2573–88. Epub 2020/02/23. https://doi.org/10.1523/JNEUROSCI.2887-19.2020 PMID: 32079648; PubMed Central PMCID: PMC7083539.

40. Searl TJ, Dynda DI, Alanez SR, El-Zawahry AM, McVary KT, Silinsky EM. A1 Adenosine Receptor-Mediated Inhibition of Parasympathetic Neuromuscular Transmission in Human and Murine Urinary Thalamus. J Pharmacol Exp Ther. 2013; 345(1):32–40. Epub 2013/02/12. https://doi.org/10.1124/jpet.112.199612 PMID: 23397055; PubMed Central PMCID: PMC3608448.
50. Salvatore CA, Tilley SL, Latour AM, Fletcher DS, Koller BH, Jacobson MA. Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. J Biol Chem. 2000; 275(6):4429–34. Epub 2000/02/08. https://doi.org/10.1074/jbc.275.6.4429 PMID: 10660615.

51. Jain S, Panyutin A, Liu N, Xiao C, Pinol RA, Pundir P, et al. Melanotan II causes hypothermia in mice by activation of mast cells and stimulation of histamine 1 receptors. Am J Physiol Endocrinol Metab. 2018; 315(3):E357–E66. Epub 2018/05/31. https://doi.org/10.1152/ajpendo.00024.2018 PMID: 29812984.

52. Eisner C, Kim S, Grill A, Qin Y, Hoerl M, Briggs J, et al. Profound hypothermia after adenosine kinase inhibition in A1AR-deficient mice suggests a receptor-independent effect of intracellular adenosine. Pflugers Arch. 2017; 469(2):339–47. https://doi.org/10.1007/s00424-016-1925-3 PMID: 27975140.