Physiology and effects of nucleosides in mice lacking all four adenosine receptors

Cuiying Xiao[1], Naili Liu[2], Kenneth A. Jacobson[3], Oksana Gavrilova[2], Marc L. Reitman[1]*

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 Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland, United States of America

* marc.reitman@nih.gov

Abstract

Adenosine is a constituent of many molecules of life; increased free extracellular adenosine indicates cell damage or metabolic stress. The importance of adenosine signaling in basal physiology, as opposed to adaptive responses to danger/damage situations, is unclear. We generated mice lacking all four adenosine receptors (ARs), $Adora1^{-/-}$;$Adora2a^{-/-}$; $Adora2b^{-/-}$;$Adora3^{-/-}$ (quad knockout [QKO]), to enable investigation of the AR dependence of physiologic processes, focusing on body temperature. The QKO mice demonstrate that ARs are not required for growth, metabolism, breeding, and body temperature regulation (diurnal variation, response to stress, and torpor). However, the mice showed decreased survival starting at about 15 weeks of age. While adenosine agonists cause profound hypothermia via each AR, adenosine did not cause hypothermia (or bradycardia or hypotension) in QKO mice, indicating that AR-independent signals do not contribute to adenosine-induced hypothermia. The hypothermia elicited by adenosine kinase inhibition (with A134974), inosine, or uridine also required ARs, as each was abolished in the QKO mice. The proposed mechanism for uridine-induced hypothermia is inhibition of adenosine transport by uridine, increasing local extracellular adenosine levels. In contrast, adenosine 5′-monophosphate (AMP)–induced hypothermia was attenuated in QKO mice, demonstrating roles for both AR-dependent and AR-independent mechanisms in this process. The physiology of the QKO mice appears to be the sum of the individual knockout mice, without clear evidence for synergy, indicating that the actions of the four ARs are generally complementary. The phenotype of the QKO mice suggests that, while extracellular adenosine is a signal of stress, damage, and/or danger, it is less important for baseline regulation of body temperature.

Author summary

Elevated extracellular adenosine generally indicates metabolic stress or cell damage and regulates many aspects of physiology. We studied "QKO" mice lacking all four adenosine

receptors. Young QKO mice do not appear obviously ill, but do show decreased survival later in life. QKO mice demonstrate that adenosine receptors are not required for growth, metabolism, breeding, and body temperature regulation. QKO mice are missing the pharmacologic effects of adenosine on body temperature, heart rate, and blood pressure. Therefore, all of these effects are mediated by the four adenosine receptors. We also determined that the hypothermic effects of a pharmacologic adenosine kinase inhibitor (A134974), uridine, or inosine each requires adenosine receptors. The uridine-induced hypothermia is likely due to its inhibition of adenosine uptake into cells. QKO mouse physiology appears to be the sum of the individual knockout mice, without evidence for synergy, indicating that the actions of the four adenosine receptors are generally complementary.

Introduction

The nucleoside adenosine is incorporated covalently into many molecules of life, including those involved in information archiving and translation, energy storage and use, regulation of gene expression and function, and intermediary metabolism. Free, extracellular adenosine levels are normally low, but are increased by cell damage and metabolic stressors, such as hypoxia and seizures. Elevated extracellular adenosine thus indicates danger or damage to tissues and elicits adaptive responses. These responses are typically adaptations to the danger state, including selective regulation of perfusion and dampening of (presumably excessive) inflammatory/immune responses. A central question in adenosine physiology is the contribution of adenosine to basal homeostasis versus its clear role in stressed states [1,2].

Adenosine signals via four G protein–coupled adenosine receptors (ARs), A_1AR, $A_{2A}AR$, $A_{2B}AR$, and A_3AR. A_1AR and A_3AR are generally coupled to Gi, reducing cAMP and considered inhibitory, while $A_{2A}AR$ and $A_{2B}AR$ are typically coupled to Gs/Gq and are stimulatory, increasing $cAMP/Ca^{++}$ [3]. AR activation causes a plethora of physiology, with some actions unique to one AR and others with contributions from multiple ARs.

Approved AR drugs include adenosine for paroxysmal supraventricular tachycardia (A_1AR agonism), adenosine and regadenoson for myocardial perfusion imaging ($A_{2A}AR$ agonism, causing coronary artery vasodilation [4]), istradefylline for Parkinson's disease ($A_{2A}AR$ antagonism [5]), and the nonselective AR antagonist theophylline for asthma. Caffeine, another nonselective AR antagonist, is found in coffee and certain medications for treatment of drowsiness, headache, or migraines. Selective AR ligands are being investigated for additional indications [6,7]. Other drugs, such as dipyridamole and low-dose methotrexate, increase local adenosine levels, thus secondarily activating ARs.

One physiologic effect of adenosine is hypothermia [8]. Adenosine-induced hypothermia was initially attributed to brain A_1AR [9,10], possibly through ARs in the nucleus of the solitary tract [11] or preoptic area [12,13]. A_1AR may also regulate torpor (a hibernation-like state with hypothermia and reduced metabolism, heart rate, and respiration) [14], but neither A_1AR nor A_3AR is required for fasting-induced torpor [15]. Additionally, activation of mouse mast cell A_3ARs also causes hypothermia [16–18], via histamine release and signaling through histamine H_1 receptors [19,20]. Hypothermia is also caused by $A_{2A}AR$ agonists, which produce vasodilation, and by $A_{2B}AR$ agonists, which may be acting centrally [21]. Thus, agonism of any of the four ARs can produce hypothermia.

It is unknown if adenosine also causes hypothermia by additional mechanisms. Could cellular uptake and phosphorylation of adenosine increase adenosine 5′-monophosphate (AMP)/

ATP ratios, activating the energy sensor adenosine monophosphate–activated protein kinase (AMPK) [22]? AMPK is proposed to have a role in signaling torpor/hypothermia [23]. We produced $Adora1^{-/-};Adora2a^{-/-};Adora2b^{-/-};Adora3^{-/-}$ mice (quad knockout [QKO] mice) to examine this idea and study the basal physiology and pathophysiology of mice lacking all four ARs.

Results

Producing QKO mice

QKO mice ($Adora1^{-/-};Adora2a^{-/-};Adora2b^{-/-};Adora3^{-/-}$) were produced by crossing the previously characterized individual AR knockout mice [18,24–26] as detailed in Mice in Materials and methods. The $Adora1^{-}$ allele was slightly underrepresented in the progeny [27,28], without pairwise interaction with genotype at the other three loci. QKO mice at 2 months of age appeared to breed normally, with litter sizes similar to controls (control, 9.8 ± 0.6 per litter, $n = 11$ litters versus QKO 9.6 ± 0.9 per litter, $n = 5$ litters; $P = 0.85$, unpaired t test). Breeding of 5-month-old QKO mice produced smaller litters (QKO litters of 1, 6, 3, and 7 pups versus control litters of 8, 9, 10, and 9 pups). In addition, the QKO mice showed decreased survival starting at about 15 weeks of age (Fig 1A). Two independent lines of $Adora1^{-/-}$ mice also show decreased survival (https://www.jax.org/strain/014161 and George Yuning Huang, National Institute of Diabetes and Digestive and Kidney Diseases, personal communication); to our knowledge, decreased survival has not been reported for $Adora2a^{-/-}$, $Adora2b^{-/-}$, or $Adora3^{-/-}$ mice. The QKO mice exhibited some eye inflammation and dermatitis, like $Adora2a^{-/-}$ mice (Dorian McGavern, National Institute of Neurological Disorders and Stroke, personal communication).

Growth, metabolism, and blood characteristics were measured in cohorts of male and female mice (summarized in Table 1). At 8–16 weeks of age, chow-fed mice had a slightly
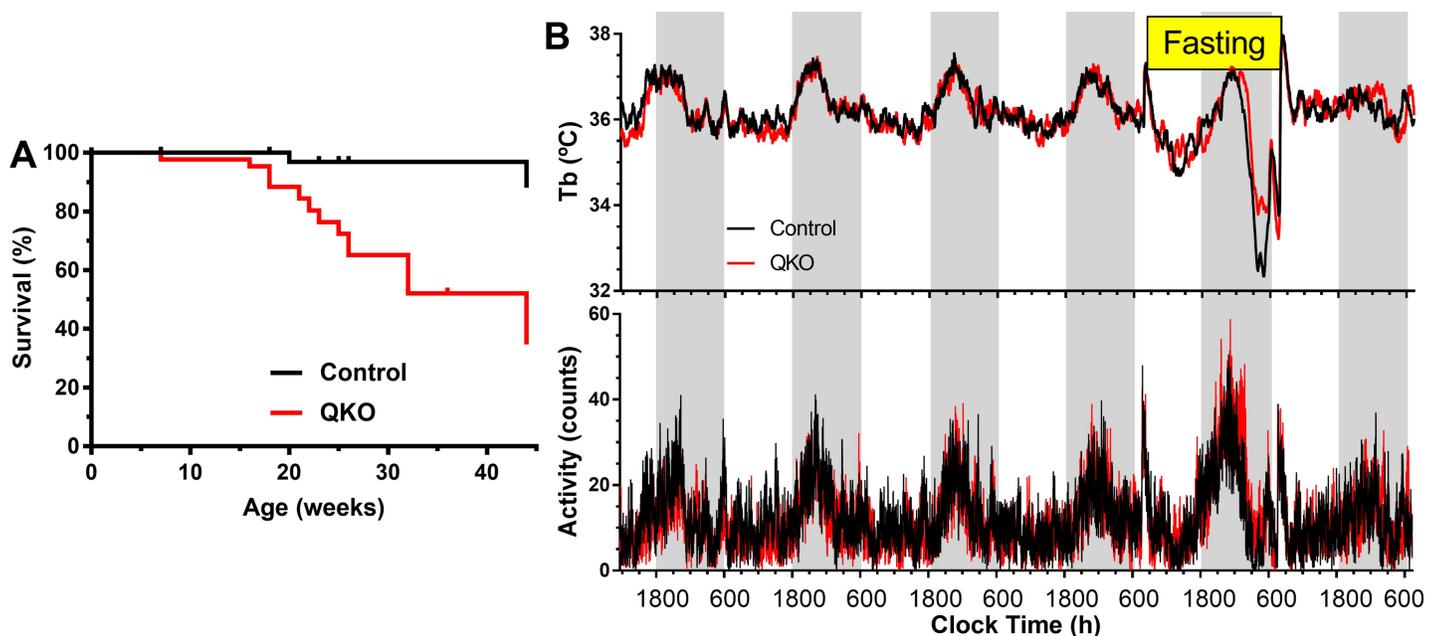
Fig 1. Decreased survival of QKO mice with normal Tb and entry into torpor. A. Kaplan–Meier survival of QKO ($N = 43$) and control ($N = 45$) mice, males and females. QKO survival is less than control, $P = 0.0005$ by Mantel–Cox test. B. QKO and control male mice show similar diurnal rhythm, and both groups enter torpor with a 24-hour fast. Data are means; SEMs omitted for visual clarity; $N = 11$–13/group. The numerical data are in Supporting information. QKO, quad knockout; Tb, core body temperature.

https://doi.org/10.1371/journal.pbio.3000161.g001

Table 1. Anatomic and metabolic characteristics of QKO mice.

Characteristics	Female chow, group housed *S1 Fig*	Male chow, singly housed *S2 Fig*	Male HFD, singly housed *S2 Fig*	Male chow, singly housed *S3 Table*
Age:	*8–16 weeks*	*8–16 weeks*	*8–16 weeks*	*46 weeks*
Body weight	⇩	⇩	no Δ	⇧
Fat mass	no Δ	no Δ	no Δ	
Lean mass	⇩	⇩	no Δ	
Food intake		no Δ	no Δ	
Energy expenditure		no Δ	no Δ	
Age:	*17–19 weeks*	*17–19 weeks*	*17–19 weeks*	
GTT AUC	⇩	⇩	no Δ	
GTT insulin	no Δ	no Δ	no Δ	
ITT AUC	no Δ	no Δ	no Δ	
Glucose (non-fasting)	⇩	no Δ	no Δ	⇩
Insulin (non-fasting)	no Δ	no Δ	no Δ	⇧
Free fatty acids	⇩	⇩	no Δ	no Δ
Triglycerides	no Δ	no Δ	no Δ	no Δ
Cholesterol	⇩	⇩	no Δ	⇩
Leptin	no Δ	no Δ	no Δ	⇧
Adiponectin	no Δ	no Δ	no Δ	no Δ
Insulin-like growth factor-1	⇩	⇩	no Δ	
β-Hydroxybutyrate				no Δ
Corticosterone				no Δ
T3			no Δ	⇧
T4			no Δ	no Δ
Age:	*37 weeks*	*32 weeks*	*24 weeks*	*46 weeks*
Body length	no Δ	no Δ	no Δ	no Δ
BAT weight	no Δ	no Δ	no Δ	⇧
Inguinal WAT weight	no Δ	no Δ	no Δ	⇧
Gonadal WAT weight	no Δ	no Δ	⇩	no Δ
Liver weight	no Δ	⇩	no Δ	⇧
Spleen weight	no Δ	no Δ	⇧	no Δ

Primary data are in S1 Fig, S2 Fig, and S3 Table. Arrows indicate significant changes (without multiplicity correction) in the indicated direction of the QKO mice; no Δ indicates no significant change. Abbreviations: AUC, area under the curve; BAT, interscapular brown adipose tissue; GTT, glucose tolerance test; HFD, high-fat diet; ITT, insulin tolerance test; QKO, quad knockout; T3, triiodothyronine; T4, thyroxine; WAT, white adipose tissue.

lower body weight due to reduced lean, but not fat, mass (S1 Fig and S2 Fig). These mice also showed improved glucose tolerance and lower serum free fatty acid, cholesterol, and insulin-like growth factor-1 (IGF-1) levels (S1 Fig and S2 Fig). Corticosterone levels were similar to controls with intact diurnal rhythm (control versus QKO: 8 AM 26.0 ± 5.2 versus 19.5 ± 4.3 ng/mL and 6 PM 149.6 ± 18.1 versus 103.9 ± 14.3 ng/mL; n = 10/group). Blood monocyte and polymorphonuclear leukocyte counts were reduced (S1 Table). Serum chemistries revealed increased alkaline phosphatase and confirmed the low cholesterol levels (S2 Table). On a high-fat diet, QKO mice were remarkably like control mice. An independent cohort of chow-fed older male mice was slightly heavier (body weight, white adipose tissue [WAT], brown adipose tissue [BAT], and liver, which likely accounts for the higher leptin and insulin; S3 Table). In these mice, there was no difference in β-hydroxybutyrate or thyroxine (T4), while

Table 2. Tb and physical activity in QKO and control mice.

Characteristics	Control	QKO	P
N	11	13	
Body weight (g)	24.36 ± 0.40	23.55 ± 0.37	0.16
Tb, dark phase (°C)	36.41 ± 0.11	36.35 ± 0.06	0.60
Tb, light phase (°C)	35.98 ± 0.11	35.93 ± 0.10	0.72
Tb span (°C)	2.46 ± 0.10	2.38 ± 0.17	0.71
Activity, dark phase (beam breaks)	17.83 ± 0.67	16.15 ± 0.51	0.06
Activity, light phase (beam breaks)	12.00 ± 0.73	10.57 ± 0.51	0.13

Data were obtained using G2 E-mitters and are mean ± SEM from a 72-hour time interval. Male mice were 12 weeks old. Tb span is the difference between the 5th and 95th percentiles and is a measure of Tb variation. P is from unpaired t tests, without correction for multiple tests.

Abbreviations: Tb, core body temperature; QKO, quad knockout.

https://doi.org/10.1371/journal.pbio.3000161.t002

triiodothyronine (T3) was increased and corticosterone was reduced. Overall, QKO and control mice showed some phenotypic differences, which were generally neither striking nor present in all cohorts.

Kidney function (as assessed by serum creatinine and urea nitrogen, S2 Table) were similar in control and QKO mice. Water intake was similar in control (3.71 ± 0.11 g/day, n = 16) and QKO (4.00 ± 0.28 g/day, n = 17; P = 0.36) mice. Necropsies of male, 32-week-old QKO and control mice (three of each) did not reveal any gross differences in the heart, lungs, liver, kidneys, spleen, gastrointestinal tract, testes, epididymis, seminal vesicles, urinary bladder, preputial gland, or brain. Similarly, no histological abnormalities were apparent in kidney (with tubular lipidosis in both control and QKO mice), heart, lung, spleen, WAT, or BAT.

Normal body temperature and entry into torpor

Control and QKO male mice tolerated surgical implantation of intraperitoneal (i.p.) sensors for core body temperature (Tb) similarly (100% survival in 13 QKO and 12 controls). The light phase Tb, dark phase Tb, and Tb span, a measure of Tb variation, were the same in QKO and control mice (Table 2). Tb and activity had a similar diurnal rhythm in QKO and control mice, and all mice entered torpor when challenged with a 24-hour fast (Fig 1B). QKO and control mice increased Tb and activity similarly in response to handling for food removal (Fig 1B) or the stress of being placed in a cage previously occupied by an unfamiliar male. mRNA levels in WAT and BAT showed variable increases in Ucp1, but not Cidea or Cox8b (S4 Table). These data indicate that ARs are not required for baseline control of Tb, induction of torpor, or bidirectional Tb regulation.

Blood pressure and heart rate

We investigated heart rate and blood pressure by telemetry in unanesthetized, freely moving female mice. Implantation of radio transmitters requires unilateral carotid artery occlusion; survival from this surgery was reduced in the QKO mice (5/11 [45%] QKO versus 34/42 [81%] non-QKO mice survived; P = 0.017 by χ^2). The telemetered QKO mice had a higher light phase heart rate and lower dark phase blood pressure, but the small sample size limits the conclusions (Table 3). The usual diurnal effects (dark phase increases in systolic, diastolic, and mean arterial blood pressure (MAP), and in heart rate, Tb, and physical activity) were similar in QKO and control mice. Thus, ARs are not required for diurnal rhythmicity of blood pressure and heart rate.

Table 3. Baseline cardiovascular parameters in QKO and control mice.

Parameters	Control dark	QKO dark	P	Control light	QKO light	P
N	5	5				
Heart rate (bpm)	563 ± 17	587 ± 18	0.35	505 ± 11	558 ± 17	**0.031**
Systolic (mmHg)	128 ± 4	117 ± 3	**0.047**	111 ± 3	108 ± 2	0.46
Diastolic (mmHg)	100 ± 3	91 ± 4	0.11	84 ± 2	82 ± 3	0.65
MAP (mmHg)	114 ± 3	105 ± 3	0.057	98 ± 2	96 ± 1	0.47
Tb (°C)	37.06 ± 0.07	36.68 ± 0.14	**0.041**	35.82 ± 0.11	36.01 ± 0.08	0.20
Activity (au)	0.234 ± 0.061	0.130 ± 0.023	0.15	0.072 ± 0.018	0.061 ± 0.003	0.59

Data are mean ± SEM from a 48-hour time interval of continuous recording during a quiet period in the animal facility, measured using HD-X11 transmitters in 24-week-old female mice. Analysis of the individual 12-hour epochs produced the same conclusions. P values are from unpaired t tests, without correction for multiple tests.

Abbreviations: au, arbitrary unit; bpm, beats per minute; MAP, mean arterial blood pressure; Tb, core body temperature.

Loss of adenosine-induced hypothermia, bradycardia, and hypotension in QKO mice

Adenosine causes hypothermia in wild-type mice, via each of the four ARs. The 100 mg/kg dose of adenosine equals about 1/10th of the molar ATP content of the body, so instantaneous conversion of the adenosine (1 adenosine + 2 ATP → 3 ADP) would change a body's ATP/ADP ratio from 10 to 2 and could signal an energy-depleted state, inducing hypothermia, independent of ARs. When male QKO mice were treated with adenosine, no hypothermia occurred. Similarly, treatment of female QKO mice with adenosine had no clear effect on Tb, heart rate, blood pressure, and activity, while in control mice, adenosine reduced Tb, heart rate, MAP, and activity and increased pulse pressure (Fig 2A–2E). These results demonstrate that adenosine actions at A_1AR, $A_{2A}AR$, $A_{2B}AR$, and/or A_3AR mediate the full effects of adenosine-induced hypothermia, bradycardia, and hypotension. This result argues against the hypothesis that pharmacologic dosing of adenosine produces an AR-independent energy-depletion signal.

Response to lipopolysaccharide

Lipopolysaccharide (LPS) stimulates the innate immune system, increasing cytokine levels and causing Tb changes in a dose-dependent manner, with lower doses increasing Tb and higher doses causing hypothermia followed by fever [29,30]. $Adora2b^{-/-}$ mice are reported to have increased basal and LPS-stimulated cytokine levels [31] and, pharmacologically, $A_{2A}AR$ [32] and A_3AR [18] agonists inhibited LPS-induced cytokine production. We examined the QKO mice to see if their response to LPS was exaggerated. At a low dose (10 μg/kg, i.p.), LPS increased Tb slightly in control (Tb at 60–360 minutes versus vehicle: +0.52 ± 0.19°C, $P = 0.018$) and, similarly, in QKO (+0.58 ± 0.18°C, $P = 0.007$) mice. At a dose of 50 μg/kg, the QKO mice showed less ($P = 0.015$) increase in Tb in this interval (+0.89 ± 0.19°C, $P = 0.0009$ in control versus +0.31 ± 0.12°C, $P = 0.023$ in QKO). At a higher LPS dose (250 μg/kg, i.p.) the control mice demonstrated an increase in Tb, while the QKO mice had a mild early reduction in Tb, followed by a Tb increase (Fig 3A–3E). These data are consistent with a subtly augmented Tb response to LPS in the QKO mice.

We next studied the cytokine response to LPS (250 μg/kg, i.p.) at 2 hours after dosing. Of the 31 analytes with detectable levels, 24 increased upon treatment with LPS. Interestingly, there was no significant effect of genotype or genotype × treatment interaction on plasma levels of any of these 31 proteins (S5 Table). Thus, in the QKO mice we observed neither a change
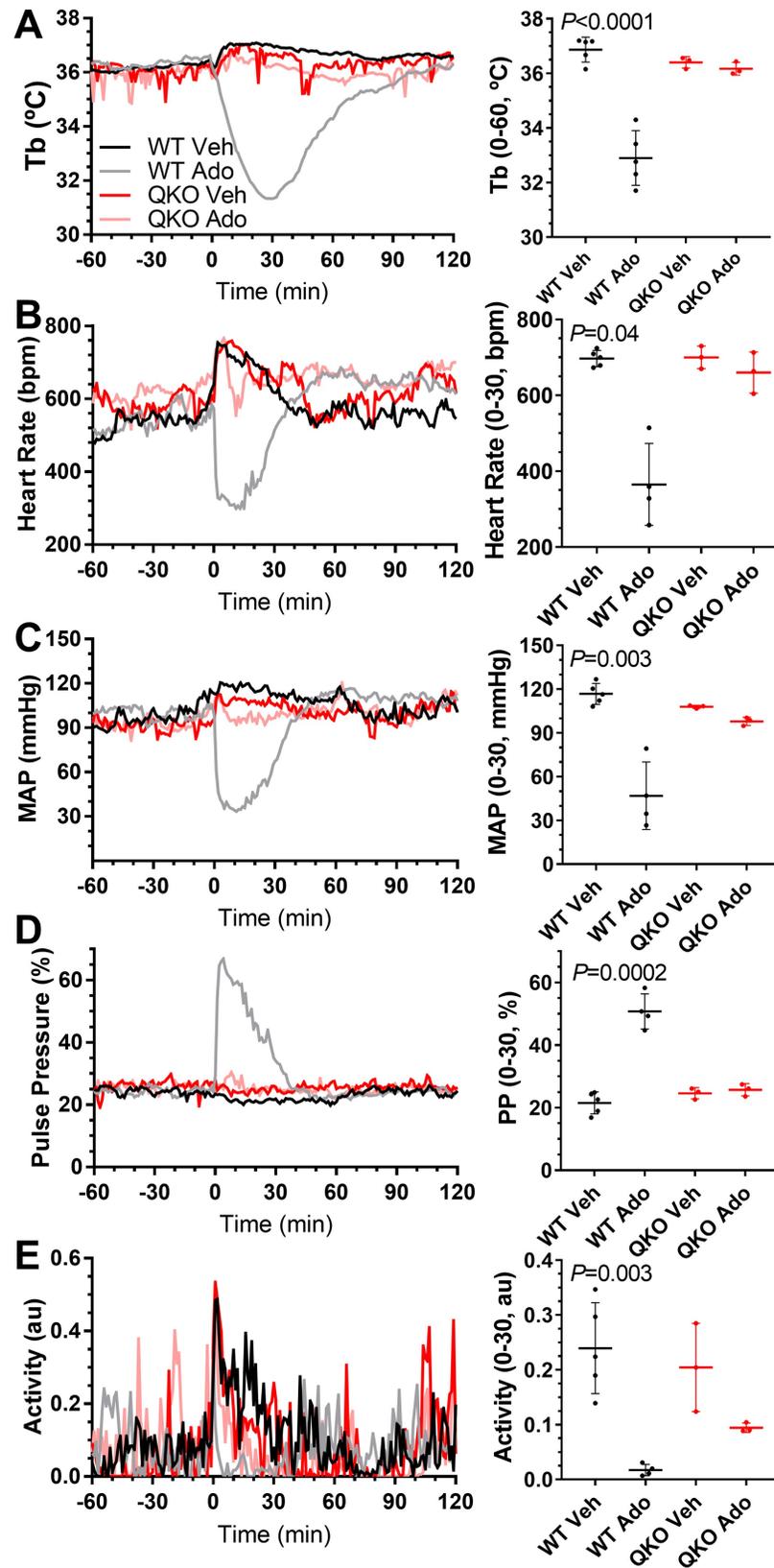
Fig 2. Loss of cardiovascular effects of adenosine in QKO mice. Female QKO and control mice were treated with adenosine (Ado, 100 mg/kg, i.p.) or vehicle (Veh). Adjusted *P* values (from two-way ANOVA with Sidak multiple comparisons test; *N* = 3–5/group) are for effect of vehicle versus adenosine in control mice; adenosine had no significant effect in QKO mice (S6 Table). Activity data were analyzed after log transformation. The numerical data are in Supporting information. Ado, adenosine; au, arbitrary unit; bpm, beats per minute; i.p., intraperitoneal; MAP, mean arterial blood pressure; QKO, quad knockout; Tb, core body temperature; Veh, vehicle; WT, wild-type.

https://doi.org/10.1371/journal.pbio.3000161.g002

in basal cytokine levels, nor an exaggerated cytokine response to LPS, suggesting that the innate immune system was not activated because of the lack of ARs.

AMP-induced hypothermia occurs by AR-dependent and AR-independent mechanisms

AMP is a proposed natural regulator of torpor, and injection of large doses causes hypothermia [33]. The contribution of ARs to AMP-induced hypothermia is debated [15,34–37]. In the QKO mice, the hypothermia caused by AMP (500 mg/kg, i.p.) was attenuated, but not abolished (Fig 4A–4D). Thus, AMP-induced hypothermia has both AR-dependent and AR-independent components.

Adenosine kinase inhibition effects are via ARs

Adenosine kinase phosphorylates adenosine to AMP, keeping intracellular adenosine levels low, thereby drawing extracellular adenosine into cells down its concentration gradient. It has been suggested that inhibition of adenosine kinase causes hypothermia via AR-independent mechanisms [38]. We confirmed that treatment of wild-type mice with the adenosine kinase inhibitor A134974 [39] caused hypothermia in a dose-dependent manner (Fig 5A–5D). In QKO mice, A134974 (5 mg/kg, i.p.) had no effect on heart rate, blood pressure, or Tb, while a 10-fold lower dose caused hypothermia, bradycardia, and hypotension in control mice (Fig 5E–5I). These results suggest that the acute hypothermia and cardiovascular effects of adenosine kinase inhibition are due to increased extracellular adenosine acting on ARs, with no reason to invoke non-AR mechanisms.

Caffeine effects are lost in QKO mice

Caffeine is a brain-penetrant micromolar antagonist of all four human ARs [40] and three of the mouse ARs (not A_3AR) [41,42]. Caffeine can also act at multiple other sites. In control
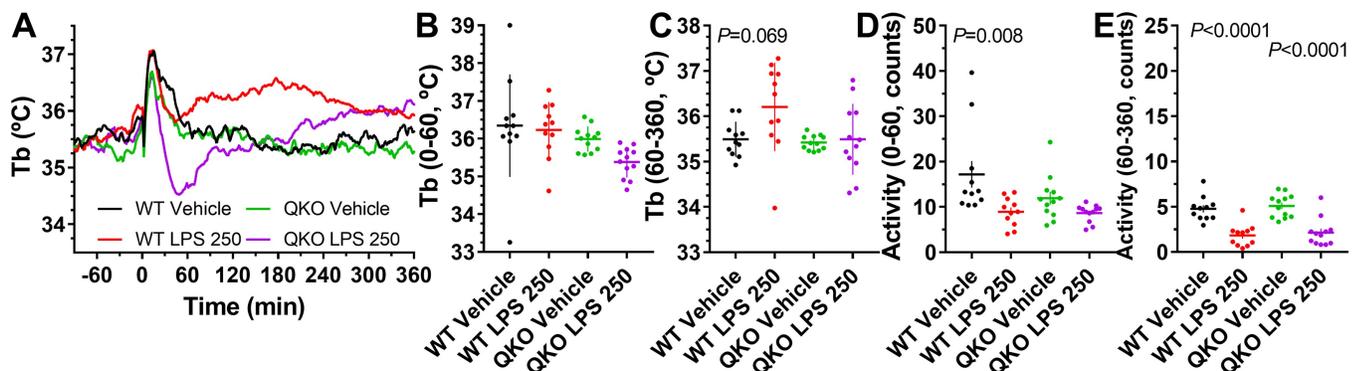

Fig 3. Altered response to LPS in QKO mice. Male QKO and control mice were treated with LPS (250 μg/kg, i.p.) or vehicle. Adjusted *P* values (from two-way ANOVA with Tukey multiple comparisons test; *N* = 11–12/group) are for effect of vehicle versus LPS within genotype (S6 Table). The numerical data are in Supporting information. i.p., intraperitoneal; LPS, lipopolysaccharide; QKO, quad knockout; Tb, core body temperature; WT, wild-type.
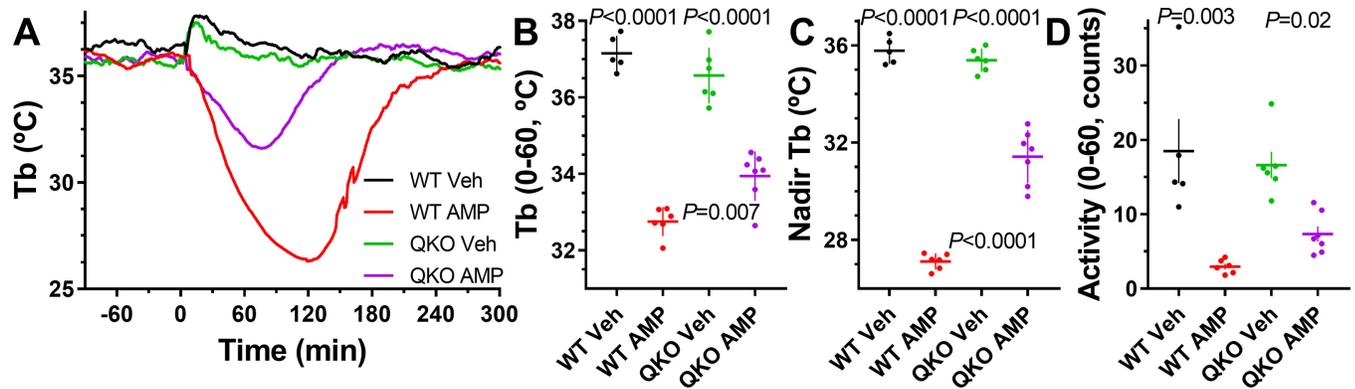
https://doi.org/10.1371/journal.pbio.3000161.g003

Fig 4. AMP causes less hypothermia in QKO mice compared with controls. Male QKO and control mice were treated with AMP (500 mg/kg, i.p.) or vehicle. Adjusted P values (from two-way ANOVA with Tukey multiple comparisons test; N = 5–7/group) are (top) for effect of vehicle versus AMP within genotype or (bottom) effect of AMP between genotype (S6 Table). The numerical data are in Supporting information. AMP, adenosine 5′-monophosphate; i.p., intraperitoneal; QKO, quad knockout; Tb, core body temperature; Veh, vehicle; WT, wild-type.

mice, caffeine (30 mg/kg, i.p.) caused a prolonged increase in Tb, activity, and blood pressure, all of which were attenuated or lost in the QKO mice (Fig 6A–6J). These data suggest that these actions of this dose of caffeine are mediated by ARs.

Inosine causes hypothermia via mast cell A₃AR

Inosine is formed by deamination of adenosine and has no known dedicated receptors, but has in vivo actions with multiple proposed mechanisms. Treatment of wild-type mice with inosine (200 mg/kg, i.p.) produced hypothermia and hypoactivity. Both effects were lost in the QKO mice (Fig 7A–7D). The hypothermia was also lost in $Kit^{W-sh/W-sh}$ mice, which lack mast cells (Fig 7E–7H), and in $Adora3^{-/-}$ but not $Adora1^{-/-}$ mice (Fig 7I and 7J). The most parsimonious explanation of the data is that inosine is acting on mast cell A₃AR, causing mast cell activation, including histamine release and hypothermia [19,43,44]. Inosine is probably not increasing extracellular adenosine by transporter inhibition [45,46], as this would cause hypothermia via other ARs, in addition to A₃AR (see below).

Uridine-induced hypothermia requires ARs

A recent study found that plasma uridine levels decreased with feeding and increased with fasting and suggested that uridine was linked to the reduction in Tb during fasting [47]. We replicated the effect of exogenous uridine (1,000 mg/kg, i.p.) to cause hypothermia in control mice. However, the uridine did not cause hypothermia in QKO mice (Fig 8A–8D). The uridine-induced hypothermia was distinct from inosine-induced hypothermia, as it was intact in $Adora1^{-/-}$ mice and attenuated but not eliminated in $Adora3^{-/-}$, $Adora1^{-/-}$;$Adora3^{-/-}$, and $Kit^{W-sh/W-sh}$ mice (Fig 8E–8J), suggesting action via A₃AR and also A₂ₐAR, and/or A₂ᵦAR.

How does uridine cause hypothermia via the ARs? Uridine and adenosine are both substrates for the equilibrative nucleoside transporter 1 (ENT1), competing with each other for transport [45,46]. An ENT1 inhibitor (6-S-[(4-Nitrophenyl)methyl]-6-thioinosine [NBMPR], 1 mg/kg, i.p.) had slight effects on Tb by itself, but greatly amplified the hypothermia caused by adenosine (100 mg/kg, i.p.) (Fig 9A–9D). We identified a uridine dose (1,000 mg/kg, i.p.) with a modest effect on Tb and tested its effect on adenosine-induced hypothermia, finding an increased effect (Fig 9E–9H). We next measured plasma adenosine in uridine-treated (2,000 mg/kg, i.p.) wild-type mice at 30 minutes after dosing, when uridine levels peak [48]. Uridine
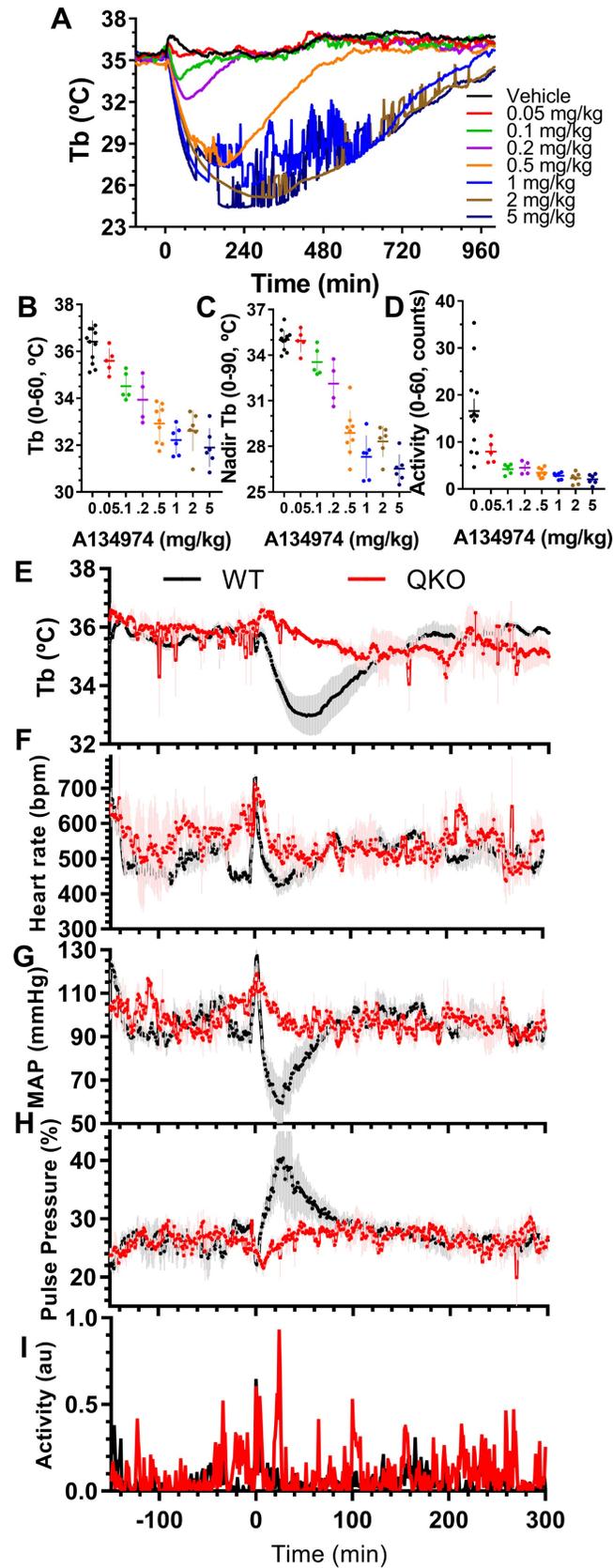
Fig 5. Tb and cardiovascular effects of adenosine kinase inhibition are due to ARs. (A–D) Dose response of A134974 in male QKO and control mice. Smallest doses significantly different (one-way ANOVA with Dunnett multiple comparisons tests; N = 4–12/group) from vehicle were 0.1 mg/kg for Tb (0–60), 0.2 mg/kg for nadir Tb, and 0.05 mg/kg for activity (0–60). (E–I) Cardiovascular effects of A134974 in female QKO (5 mg/kg, i.p.) and control (0.5 mg/kg, i.p.) mice (N = 3–5/group; statistics in S6 Table). The numerical data are in Supporting information. AR, adenosine receptor; au, arbitrary units; bpm, beats per minute; i.p., intraperitoneal; QKO, quad knockout; Tb, core body temperature; WT, wild-type.

levels were low ($\leq$2 μM) in vehicle-treated controls and 3,360 ± 190 μM in uridine-treated mice (Fig 9I); uridine treatment tended to increase plasma adenosine levels (0.842 ± 0.086 μM in vehicle-treated versus 1.33 ± 0.06 μM in uridine-treated mice; P = 0.08) (Fig 9J). Taken together, these results suggest that uridine inhibits adenosine transporters such as ENT1, thereby increasing extracellular adenosine levels and causing hypothermia via mast cell A_3AR and other ARs.

Discussion

We generated QKO mice to examine adenosine physiology and test whether exogenous adenosine-induced hypothermia occurs independent of ARs, such as by energy depletion. For example, brain glucoprivation produces hypothermia [49] and glucose and fatty acids levels are sensed in the hypothalamus [50,51]. Specifically, AMPK is a cellular energy sensor and we hypothesized that uptake and phosphorylation of adenosine might trigger hypothermia such as via AMPK. While we cannot formally rule out complex multihit mechanisms requiring both ARs and non-AR mediators, the most parsimonious interpretation of the lack of adenosine-induced hypothermia in the QKO mice is that adenosine causes hypothermia via ARs and not by cellular energy depletion.

QKO and adenosine physiology

We were surprised by the baseline phenotype of mice lacking all ARs. We had hypothesized that QKO mice might have a basally activated immune system and not survive or be healthy. Young QKO mice turned out to be viable and breed readily. We did observe reduced blood monocyte and neutrophil levels, but we do not know the mechanism. Like $Adora1^{-/-}$ mice (https://www.jax.org/strain/014161), they showed decreased survival starting at about 4 months of age, with no identified cause of death. While the QKO mice showed no mortality from the surgery for i.p. implantation of Tb telemeters, they did exhibit increased mortality from surgery for cardiovascular telemeter implantation, which requires ligation of one carotid artery. A crucial difference is that carotid ligation is an ischemic event, increasing brain adenosine, which is neuroprotective via multiple ARs [52]. These protective effects cannot occur in the QKO mice, confirming adenosine's beneficial effects in certain "danger" situations.

While LPS treatment showed a subtly modified effect on Tb, the QKO mice had cytokine levels (basal and LPS-stimulated) that were the same as those in control mice. This differs from the reported elevated basal and LPS-stimulated TNF-α and IL-6 levels in $Adora2b^{-/-}$ mice [31]. Possible explanations for this difference include LPS dose, genetic background, and effect of the other AR genotypes. However, most literature on the role of ARs in modulating the LPS response examines the effect of exogenous drugs [18,32,44,53], not of AR loss. Basal cytokine levels were reported unchanged with loss of $A_{2A}AR$ [32] or A_3AR [18]. This evidence again suggests that adenosine is more important for "emergency physiology" in danger/damage situations, and not under basal conditions.

Adenosine physiology can be difficult to study. Adenosine, the natural ligand, is locally cleared in a few seconds [54], with a plasma half-life of about 1 minute in humans [55]. The
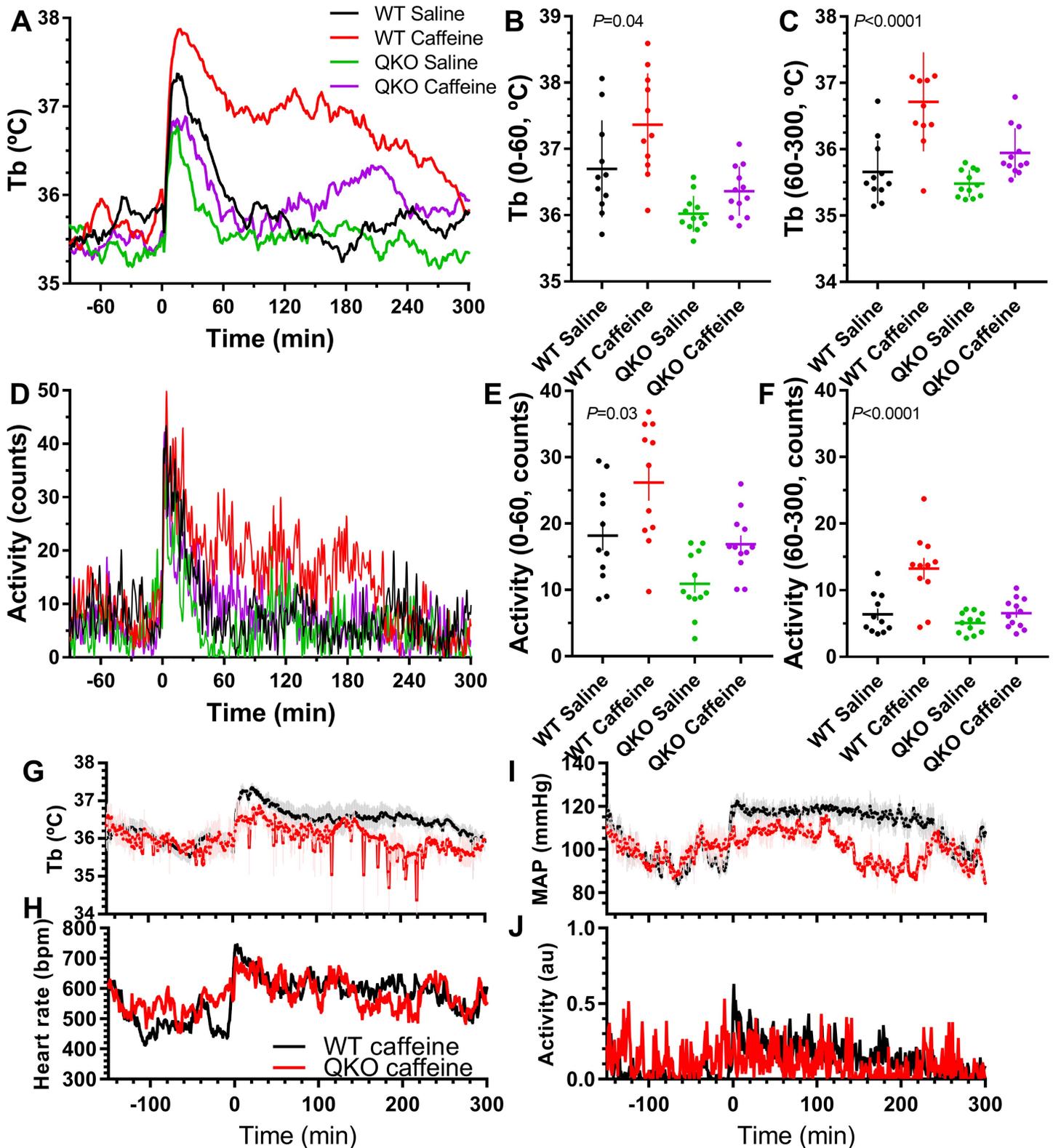
Fig 6. Tb and activity effects of caffeine are lost in QKO mice. (A-F) Male QKO and control mice were treated with caffeine (30 mg/kg, i.p.) or saline. Adjusted *P* values (from two-way ANOVA with Sidak multiple comparisons test; *N* = 11–12/group) are for effect of vehicle versus caffeine in control mice; caffeine effect was not significant in QKO mice. (G-J) Cardiovascular effects of caffeine (30 mg/kg, i.p.) or saline in female QKO mice (*N* = 3–5/group; statistics in S6 Table). The numerical data are in

rapid clearance means that experimentally, massive adenosine doses are used, with a complicated relationship to endogenous physiology. One solution is use of synthetic ligands, which increase the effect duration and likely provide supraphysiologic activation. It is not known if AR physiology from transient adenosine binding differs from the prolonged activation from agonist ligands. Some synthetic ligands are selective for one AR, but ligand selectivity can vary by >1,000-fold between species [41]. The use of knockout mice is a powerful tool for ensuring ligand selectivity, with loss of drug effect in the null being strong evidence for an "on-target" action, due to the identified AR. However, QKO mice develop from conception without any ARs, which may elicit compensatory mechanisms, underestimating the role of the missing AR. Therefore, a more prominent phenotype of the QKOs might appear in different experimental stress models. Finally, ARs can heterodimerize with other G protein-coupled receptors and use multiple signaling routes [56], so it is possible that probing adenosine effect may require understanding of dimerization partners and differential effects on signaling pathways.

Role of adenosine in hypothermia induced by various nucleosides and their modulators

The clear result that adenosine-induced hypothermia is absent in QKO mice makes these mice extremely valuable for investigating the possible role of adenosine in physiology thought to be caused by other agents. For example, the AR contribution to AMP-induced hypothermia is controversial [15,34–37]. The attenuation of AMP-induced hypothermia in the QKO mice demonstrates that both AR-independent and AR-dependent mechanisms contribute. Additionally, the QKO mice enable study of the non-AR mechanism.

In contrast, the QKO mice demonstrate that the acute hypothermia caused by an adenosine kinase inhibitor is completely attributable to ARs. Some of the hypothermia is due to the increased extracellular adenosine activating A_1AR [38]. While possible explanations for the remaining A134974-induced hypothermia have been proposed, the QKO results dictate that the non–A_1AR-mediated hypothermia must occur via $A_{2A}AR$, $A_{2B}AR$, and/or A_3AR.

Inosine does not have identified dedicated receptors. Mechanisms that might explain inosine's neuroprotective and immunomodulatory actions include all four ARs and non-AR routes (reviewed in [57]). Inosine can activate A_3AR on mouse mast cells [43,58]. Inosine has also been reported to be an agonist at A_1AR [59], $A_{2A}AR$ [60,61], and $A_{2B}AR$ [62]. Our QKO and follow-up experiments demonstrate that inosine-induced hypothermia is due to A_3AR agonism.

Caffeine is an AR antagonist [40,41]. At higher concentrations, it inhibits phosphodiesterases [63], γ-aminobutyric acid A ($GABA_A$) receptors [63], and glycine receptors [64] and activates ryanodine receptors [65]. We examined a 30 mg/kg dose, which approximates the human plasma exposure of 2–3 cups of coffee [66]. Our data confirm that the effects of caffeine (30 mg/kg) to increase Tb and activity are mediated by ARs, likely $A_{2A}AR$ [42,67,68].

The protective effects of guanosine in the central nervous system (CNS) have been studied, revealing apparent adenosine-dependent and -independent effects, but a guanosine receptor has not been identified [69–71]. While more mechanistic studies of guanosine's effects are warranted, we were unable to explore this with QKO mice, because a high, solubility-limited dose of guanosine (60 mg/kg, i.p.) [70] failed to lower Tb in wild-type mice.
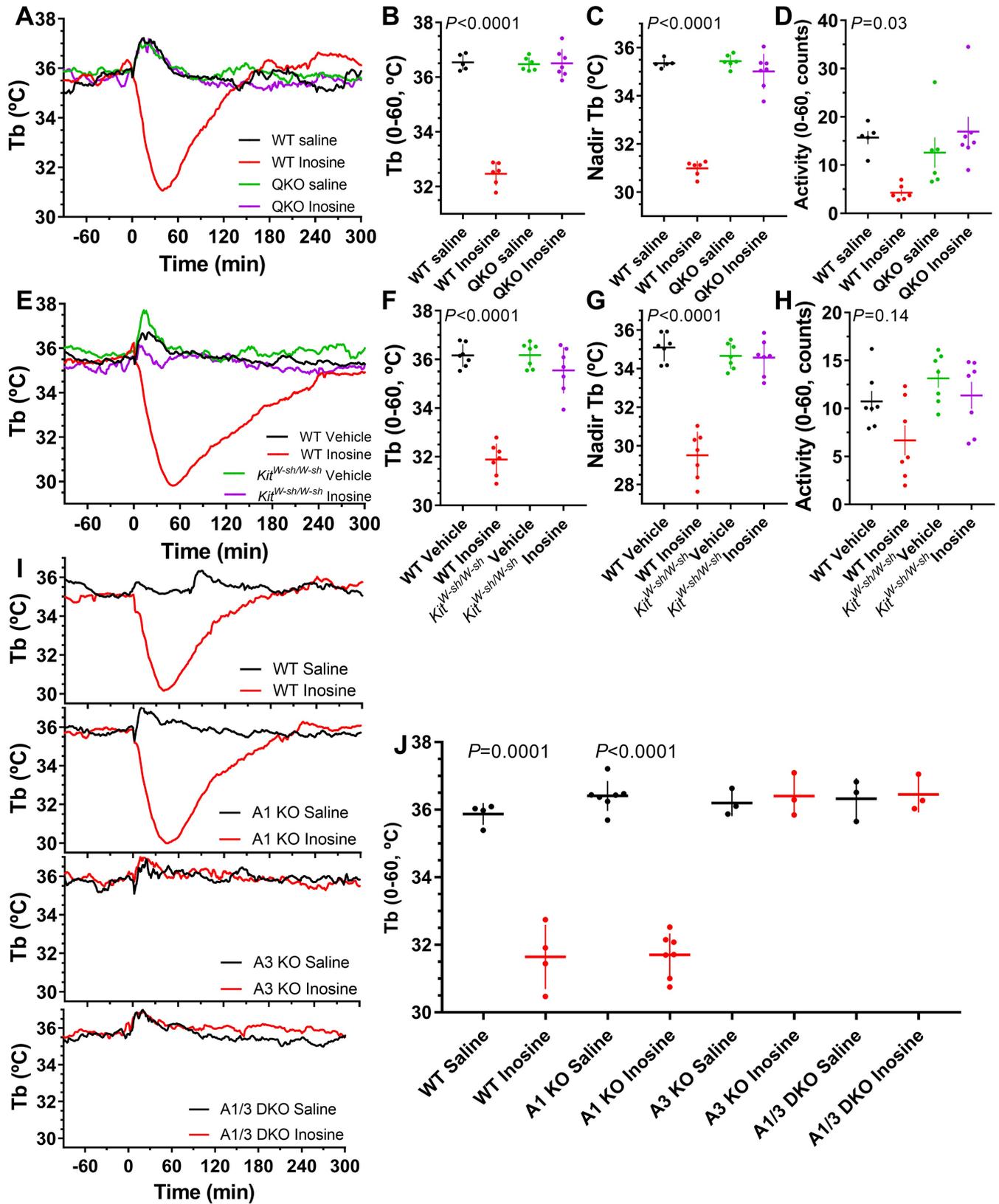
Fig 7. Inosine causes hypothermia via adenosine receptor A₃AR. (A-D) Effect of inosine (200 mg/kg, i.p.) or saline in male QKO and control mice; N = 5–7/group. (E-H) Effect of inosine (200 mg/kg, i.p.) or saline in $Kit^{W-sh/W-sh}$ and control mice. Adjusted P values (from two-way ANOVA with Tukey multiple comparisons test; N = 7/group) are for effect of vehicle versus inosine within genotype. (I-J) Effect of inosine (200 mg/kg, i.p.) or saline in control (WT), $Adora1^{-/-}$ (A1 KO), $Adora3^{-/-}$ (A3 KO), and $Adora1^{-/-}$ $Adora3^{-/-}$ (A1/3 DKO) mice. P values (paired t test) are for effect of vehicle versus inosine (N = 3–7/group; S6 Table). The numerical data are in Supporting information. DKO, double knockout; i.p., intraperitoneal; KO, knockout; QKO, quad knockout; Tb, core body temperature; WT, wild-type.

https://doi.org/10.1371/journal.pbio.3000161.g007

Mechanism of uridine-induced hypothermia

We were surprised by the QKO results demonstrating that uridine-induced hypothermia requires ARs. Uridine (at doses reaching ≥10 mM in plasma) causes hypothermia in mice and rats [48]. A recent study found that physiological plasma uridine levels decreased with feeding and increased with fasting (albeit in a much lower range, 4–10 μM) and suggested that uridine was linked to the reduction in Tb that occurs during fasting [47]. The result that uridine-induced hypothermia is absent in QKO mice caused us to investigate further. Uridine itself does not bind to ARs [72], is not a substrate for adenosine kinase [73], and does not block adenosine deaminase [74]. However, uridine is a substrate for transport by ENT1 and a competitive inhibitor of adenosine transport by ENT1 [45, 46], and we showed that uridine mimicked the effects of the selective ENT1 inhibitor, NBMPR. Thus, we propose that the pharmacologic effects of uridine are due at least in part to inhibition of ENT1, preventing adenosine uptake into cells. The increased extracellular adenosine activates multiple ARs (mast cell A₃AR and other ARs). This may explain the sleep-promoting and anticonvulsant effects of uridine (reviewed in [75]). It seems likely that this AR-dependent mechanism for Tb regulation with large exogenous uridine doses does not explain Tb effects of changes of uridine levels in the physiologic range.

AR-independent effects of adenosine

The QKO mice described here are a valuable research tool to study AR-independent effects of adenosine. Adenosine is an obligatory product of S-adenosylmethionine (SAM)–dependent transmethylation reactions, which include DNA methylation and histone methylation. Through mass action, adenosine provides negative feedback control for transmethylation—low levels of adenosine drive DNA methylation, whereas high levels of adenosine block DNA methylation [76]. The QKO mice would be an ideal investigative tool to study the epigenetic mechanisms of adenosine in the absence of potentially confounding ARs.

Limitations

We monitored growth and metabolic parameters, Tb, activity, and cardiovascular end points. Other assays might expose additional differences in the QKO mice. While the mice have a mixed genetic background, both QKO and control mice were bred using a scheme to minimize founder/inbreeding effects. Lastly, mice with germline AR deletion may have undergone adaptive changes during development, masking deficits due to loss of ARs [77,78]. Use of conditional AR alleles with deletion in adulthood would avoid this potential confound.

Implications

We have demonstrated that the physiology elicited by adenosine, uridine, inosine, caffeine, and (partially) AMP requires ARs. It is notable that the QKO mouse phenotype replicates that of individual AR knockout mice, without evidence for synergy, suggesting that the actions of the four ARs are generally complementary. The phenotype of the QKO mice supports the
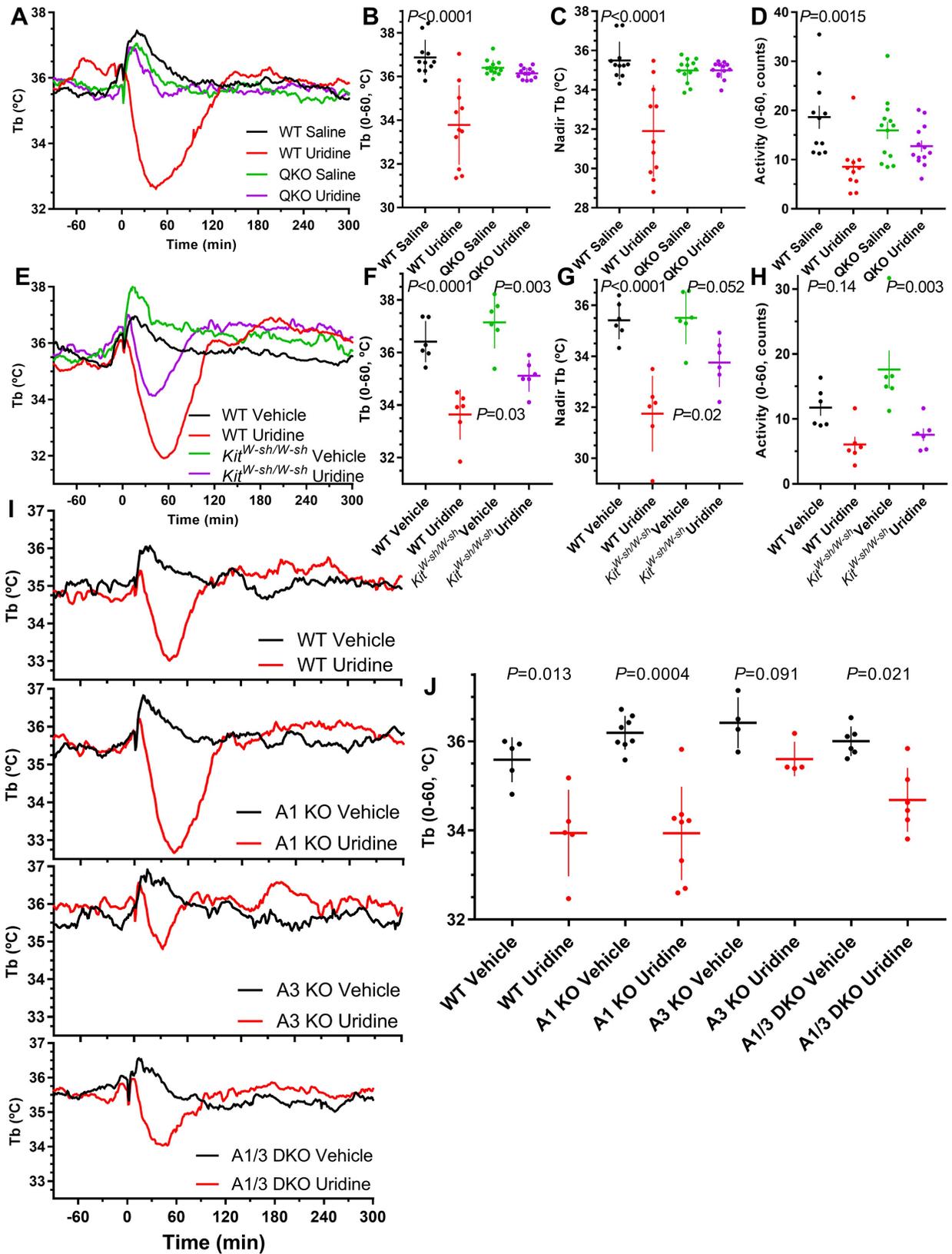
Fig 8. Uridine causes hypothermia via multiple ARs. (A-D) Effect of uridine (1,000 mg/kg, i.p.) or saline in male QKO and control mice; N = 11–13/group. (E-H) Effect of uridine (1,000 mg/kg, i.p.) or saline in $Kit^{W-sh/W-sh}$ and control mice. Adjusted P values (from two-way ANOVA with Tukey multiple comparisons test; N = 6/group) are for effect of vehicle versus uridine within genotype (S6 Table). (I-J) Effect of uridine (1,000 mg/kg, i.p.) or saline in control (WT), $Adora1^{-/-}$ (A1 KO), $Adora3^{-/-}$ (A3 KO), and $Adora1^{-/-};Adora3^{-/-}$ (A1/3 DKO) mice. P values (unpaired t test; N = 4–8/group) are for effect of vehicle versus uridine. The numerical data are in Supporting information. AR, adenosine receptor; DKO, double knockout; i.p., intraperitoneal; KO, knockout; QKO, quad knockout; Tb, core body temperature; WT, wild-type.
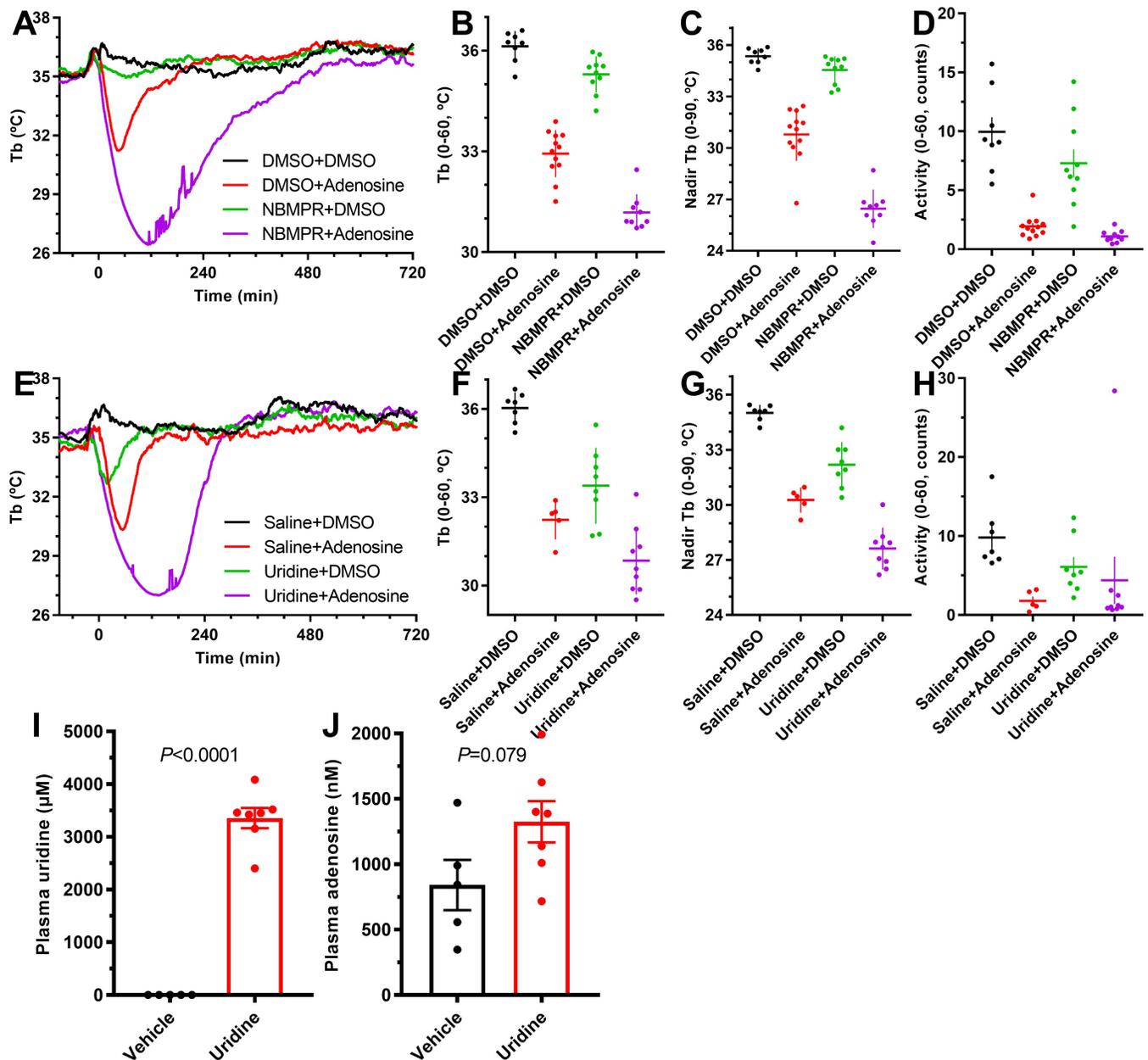
https://doi.org/10.1371/journal.pbio.3000161.g008

Fig 9. Effect of treatment with ENT1 inhibitor or uridine on hypothermia. (A-D) Effect of the ENT1 inhibitor, NBMPR (1 mg/kg, i.p.), or vehicle on adenosine (100 mg/kg, i.p.) hypothermia in C57BL/6J mice; N = 9–12/group. (E-H) Effect of uridine (1,000 mg/kg, i.p.) or saline on adenosine (100 mg/kg, i.p.) hypothermia in C57BL/6J mice. Adjusted P values (N = 5–9/group) are in S6 Table. (I-J) Plasma uridine and adenosine levels 30 minutes after uridine (1,000 mg/kg, i.p.) or saline in C57BL/6J mice. P values (unpaired t test; N = 5–7/group) are for effect of uridine versus vehicle. The numerical data are in Supporting information. ENT1, equilibrative nucleoside transporter 1; i.p., intraperitoneal; NBMPR, 6-S-[(4-Nitrophenyl)methyl]-6-thioinosine; Tb, core body temperature.

https://doi.org/10.1371/journal.pbio.3000161.g009

hypothesis that extracellular adenosine has less of a contribution to baseline physiology and is likely more important for its roles as a signal of stress, damage, and/or danger.

Materials and methods

Ethics statement

Studies were approved by the Animal Care and Use Committee of National Institute of Diabetes and Digestive and Kidney Diseases, animal protocol K016-DEOB-17. Mice were anesthetized with isoflurane or ketamine and xylazine. Euthanasia of anesthetized mice was performed by exsanguination via retro-orbital bleeding followed by cervical dislocation.

Mice

Male C57BL/6J and $Kit^{W-sh/W-sh}$ (JAX 012861) mice were obtained from the Jackson Laboratory. $Adora1^{-/-}$ [24], $Adora2b^{-/-}$ [26] (JAX 022499), and $Adora3^{-/-}$ [18] mice were on a C57BL/6J genetic background. The $Adora2a^{-/-}$ mice [25] (JAX 010685) were on a mixed background. Mice were genotyped as described (S3 Fig) [15,21]. Mice were singly housed at about 22˚C with a 12:12-hour light–dark cycle. Chow (NIH-07, Envigo, Madison, WI) or high-fat diet (D12492, 60% kcal fat, 5.24 metabolizable kcal/g; Research Diets) and water were available ad libitum. Mice were studied ≥7 days after any operation or prior treatment. Reuse of mice tends to reduce physical activity levels, presumably due to acclimation. No specific effort was made to acclimate mice to handling in individual experiments.

To generate QKO mice, mice heterozygous at all four loci ($Adora1^{+/-}$;$Adora2a^{+/-}$; $Adora2b^{+/-}$;$Adora3^{+/-}$) were crossed. Of 334 progeny (310 successfully genotyped at all four loci), the $Adora2a$, $Adora2b$, and $Adora3$ alleles were transmitted in the expected mendelian ratios. The $Adora1^-$ allele was slightly underrepresented (+/+:+/−:−/−, observed 109:146:65 versus expected 80:160:80, χ^2 P = 0.0007), as observed previously [27,28]. There was no pairwise interaction of the $Adora1^{-/-}$ genotype with genotype at the other three loci (the mice with $Adora1^{-/-}$ genotype had the expected 1:2:1 +/+:+/−:−/− ratios for each of the $Adora2a$, $Adora2b$, and $Adora3$ loci). The QKO mice studied were the second- or third-generation progeny from the quad heterozygote intercross. Controls were +/+ or +/− at the four AR loci and are also second- or third-generation progeny of quad heterozygote intercross.

Compounds

Compounds (source; vehicle) were obtained as follows: 5′-AMP (Sigma A1752; saline), adenosine (Sigma; 10% DMSO in saline), uridine (Sigma 1707114; saline), A134974 (Sigma A2846; saline), caffeine (Sigma; saline), dipyridamole (Sigma; 10% DMSO in saline), EHNA (erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride, Tocris 1261; 10% DMSO; 10 mg/kg dose chosen to avoid phosphodiesterase inhibition [79]), inosine (Sigma I4125; saline; dose based on [80]), NBMPR (6-S-[(4-Nitrophenyl)methyl]-6-thioinosine, Tocris 2924; warm 10% DMSO in saline), and LPS (Sigma L6511; saline).

In vivo physiology

Tb and activity were continuously measured by telemetry, using i.p. G2 E-mitters, ER4000 energizer/receivers, and VitalView software (Starr Life Sciences, Oakmont, PA), with data collected each minute [81]. Average Tb over 0–60 minutes and nadir Tb during 0–90 minutes after drug injection were used to quantitate hypothermic effects. Average activity over 0–60 minutes captures the reduced activity that accompanies hypothermia. Total minutes <34˚C, a

better measure of prolonged hypothermia, did not add useful information. Inhibitors were dosed 20–25 minutes before agonists.

Continuous ambulatory intra-arterial blood pressure, heart rate, physical activity, and Tb were measured with radio transmitters (HD-X11, Data Sciences International, St Paul, MN) implanted under ketamine and xylazine anesthesia in the carotid artery as described [82]. Data were sampled at 1,000 Hz using a PhysioTel RPC-1 receiver and Ponemah v6.30 (Data Sciences International) software, with 1-minute averages used for analysis. The pulse pressure is the difference between the systolic and diastolic blood pressure, expressed as a percentage of the systolic pressure.

The following assays were used: β-hydroxybutyrate (colorimetric, #K632, BioVision), corticosterone (RIA, #07120102, MP Biomedicals), T3 (ELISA, #T3043T-100, Calbiotech) and T4 (ELISA, #T4044T-100, Calbiotech). Cytokines were measured in plasma obtained 2 hours after dosing [83] LPS (250 µg/kg, i.p.) or vehicle using the Mouse Magnetic Luminex Assay Kit (LXSAMSM, R&D Systems), performed by the NHLBI Flow Cytometry Core.

Plasma adenosine and uridine measurement: 30 minutes after dosing with uridine or vehicle, C57BL/6J mice were anesthetized with isoflurane, and blood was drawn retro-orbitally into an equal volume of ice-cold stop solution (5 µM EHNA, 200 µM dipyridamole, 4 mM EDTA in saline) to prevent generation or loss of adenosine [84,85]. Samples were centrifuged (4˚C, 13,000 rpm, 3 minutes), 50 µL of plasma was pipetted into 200 µL of methanol containing internal standards ($^{13}C_9,^{15}N_2$-uridine and $^{13}C_5$-adenosine, Cambridge Isotopes), vortexed (5 minutes), centrifuged (4˚C, 14,000 rpm, 10 minutes), and the supernatant was dried at room temperature under nitrogen and resuspended in 125 µL H_2O. Samples were analyzed by UPLC-MS/MS using a Vanquish UPLC (Thermo Scientific) and Altis LC-Triple quadrupole mass spectrometer (Thermo Scientific) with heated electrospray ionization (HESI-II, Thermo Scientific) in positive ion mode (3,500 V). Injection volume was 1 µL (autosampler at 5˚C), using a Waters Cortecs C18 + 2.7 µm, 2.1 × 100 mm UPLC column (35˚C) at 350 µL/minute with solvent A (0.1% formic acid in H_2O) and solvent B (0.1% formic acid in methanol). Initially, 99% solvent A for 0.25 minutes, linearly changing to 10% A at 2.15 minutes, remaining at 10% A until 3 minutes, linearly changing to 99% A by 3.5 minutes, and stabilizing for 3 minutes. Transitions quantitated were (m/z): adenosine, 268→119 and 268→136; $^{13}C_5$-adenosine 273→119 and 273→136; uridine, 245→96 and 245→113; and $^{13}C_9,^{15}N_2$-uridine, 256→101 and 256→119. Adenosine was calibrated ($R^2 \geq 0.999$) from 1.0 to 2,500 ng/mL and uridine from 0.25 to 750 µg/mL in the original samples. Literature plasma adenosine levels are 100 to 1,000 nM in human [54] and 145 to 6,100 nM in mouse [38,86,87].

Metabolic phenotyping, including glucose and insulin tolerance tests and hormone and metabolite profiles, were performed as previously described [88].

Complete blood count with differential and serum chemistry tests were performed by the Department of Laboratory Medicine, NIH Clinical Center.

Statistical analyses were all two tailed, performed using Prism, and are detailed in S6 Table.

Supporting information

S1 Fig. Phenotype of chow-fed female QKO and control mice, group housed. QKO mice showed reduced body weight due to decreased lean mass at younger age (8–19 weeks, A-C), reduced fed glucose, improved glucose tolerance without changes in insulin tolerance test and insulin levels (D-J). QKO mice also had lower serum FFA, TG, and cholesterol, as well as IGF-1 levels (K-P). At euthanasia (37 weeks), there was no difference in body weight, body length, and organ weights (Q-T). FFA, free fatty acid; IGF-1, insulin-like growth factor 1; QKO, quad knockout; TG, triglyceride.
(PDF)

S2 Fig. Phenotype of male QKO and control mice. Mice were singly housed, fed either on chow or a HFD starting at 8 weeks of age. QKO mice had lower body weight when young (8–16 weeks) due to reduced lean mass on both chow and HFD (A-C). Chow-fed QKO mice had improved glucose tolerance without changes in fed glucose and insulin levels (D-H), reduced levels of FFA, cholesterol, and IGF-1 (P-U). Spleen weight was increased in HFD-fed mice (O, rightmost panel) and trending increase in chow-fed mice (N, rightmost panel). HFD-fed QKO mice showed no clear changes compared with controls (I-M, O first three panels, V-AC). Numerical data are in Supporting information. FFA, free fatty acid; HFD, high-fat diet; QKO, quad knockout.
(PDF)

S3 Fig. Genotyping of Adora1, Adora2a, Adora2b, and Adora3 alleles. Protocols are in [15,17] and references therein. The spurious band in *Adora2a* genotyping can be eliminated by using a hot start protocol.
(PDF)

S1 Data. Underlying data for Fig 1 in GraphPad Prism v7.
(PZFX)

S2 Data. Underlying data for Fig 2 in GraphPad Prism v7.
(PZFX)

S3 Data. Underlying data for Fig 3 in GraphPad Prism v7.
(PZFX)

S4 Data. Underlying data for Fig 4 in GraphPad Prism v7.
(PZFX)

S5 Data. Underlying data for Fig 5 in GraphPad Prism v7.
(PZFX)

S6 Data. Underlying data for Fig 6 in GraphPad Prism v7.
(PZFX)

S7 Data. Underlying data for Fig 7 in GraphPad Prism v7.
(PZFX)

S8 Data. Underlying data for Fig 8 in GraphPad Prism v7.
(PZFX)

S9 Data. Underlying data for Fig 9 in GraphPad Prism v7.
(PZFX)

S10 Data. Underlying data for S1 Fig in GraphPad Prism v7.
(PZFX)

S11 Data. Underlying data for S2 Fig in GraphPad Prism v7.
(PZFX)

S1 Table. Hematology in control and QKO mice. QKO, quad knockout.
(PDF)

S2 Table. Serum chemistries in control and QKO mice. QKO, quad knockout.
(PDF)

S3 Table. Related to Table 1. Phenotype of older male mice.
(PDF)

S4 Table. Adipose tissue mRNA levels.
(PDF)

S5 Table. Cytokine response to LPS in QKO and control (WT) mice. LPS, lipopolysaccharide; QKO, quad knockout; WT, wild-type.
(PDF)

S6 Table. Statistical results in excel.
(XLSX)

Acknowledgments

Author Contributions

Conceptualization: Cuiying Xiao, Kenneth A. Jacobson, Oksana Gavrilova, Marc L. Reitman.

Data curation: Cuiying Xiao, Naili Liu, Oksana Gavrilova.

Formal analysis: Cuiying Xiao, Naili Liu, Oksana Gavrilova, Marc L. Reitman.

Funding acquisition: Marc L. Reitman.

Investigation: Cuiying Xiao, Naili Liu, Oksana Gavrilova, Marc L. Reitman.

Methodology: Naili Liu, Oksana Gavrilova.

Project administration: Kenneth A. Jacobson, Oksana Gavrilova, Marc L. Reitman.

Resources: Marc L. Reitman.

Supervision: Kenneth A. Jacobson, Oksana Gavrilova, Marc L. Reitman.

Visualization: Oksana Gavrilova, Marc L. Reitman.

Writing – original draft: Cuiying Xiao, Oksana Gavrilova, Marc L. Reitman.

Writing – review & editing: Cuiying Xiao, Naili Liu, Kenneth A. Jacobson, Oksana Gavrilova, Marc L. Reitman.

References

1. Fredholm BB, Chen JF, Masino SA, Vaugeois JM. Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. Annu Rev Pharmacol Toxicol. 2005; 45:385–412. https://doi.org/10.1146/annurev.pharmtox.45.120403.095731 PMID: 15822182.

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

3. Antonioli L, Blandizzi C, Csoka B, Pacher P, Hasko G. Adenosine signalling in diabetes mellitus-pathophysiology and therapeutic considerations. Nat Rev Endocrinol. 2015; 11(4):228–41. https://doi.org/10.1038/nrendo.2015.10 PMID: 25687993.

4. Al Jaroudi W, Iskandrian AE. Regadenoson: a new myocardial stress agent. J Am Coll Cardiol. 2009; 54(13):1123–30. https://doi.org/10.1016/j.jacc.2009.04.089 PMID: 19761931.

5. Nazario LR, da Silva RS, Bonan CD. Targeting Adenosine Signaling in Parkinson's Disease: From Pharmacological to Non-pharmacological Approaches. Front Neurosci. 2017; 11:658. Epub 2017/12/

09. https://doi.org/10.3389/fnins.2017.00658 PMID: 29217998; PubMed Central PMCID: PMCPMC5703841.

6. Chen JF, Eltzschig HK, Fredholm BB. Adenosine receptors as drug targets—what are the challenges? Nat Rev Drug Discov. 2013; 12(4):265–86. https://doi.org/10.1038/nrd3955 PMID: 23535933; PubMed Central PMCID: PMC3930074.

7. 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.

8. Bennet DW, Drury AN. Further observations relating to the physiological activity of adenine compounds. J Physiol. 1931; 72(3):288–320. PMID: 16994210; PubMed Central PMCID: PMC1403115.

9. 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. PMID: 7889296; PubMed Central PMCID: PMC1510495.

10. Johansson B, Halldner L, Dunwiddie TV, Masino SA, Poelchen W, Gimenez-Llort 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.

11. Tupone D, Madden CJ, Morrison SF. Central activation of the A1 adenosine receptor (A1AR) induces a hypothermic, torpor-like state in the rat. J Neurosci. 2013; 33(36):14512–25. https://doi.org/10.1523/JNEUROSCI.1980-13.2013 PMID: 24005302; PubMed Central PMCID: PMCPMC3761054.

12. 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. PMID: 1780343.

13. 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. PMID: 15764835.

14. Silvani A, Cerri M, Zoccoli G, Swoap SJ. Is Adenosine Action Common Ground for NREM Sleep, Torpor, and Other Hypometabolic States? Physiology (Bethesda). 2018; 33(3):182–96. Epub 2018/04/05. https://doi.org/10.1152/physiol.00007.2018 PMID: 29616880; PubMed Central PMCID: PMCPMC5966658.

15. Carlin JL, Jain S, Gizewski E, Wan TC, Tosh DK, Xiao C, et al. Hypothermia in mouse is caused by adenosine A1 and A3 receptor agonists and AMP via three distinct mechanisms. Neuropharmacology. 2017; 114:101–13. https://doi.org/10.1016/j.neuropharm.2016.11.026 PMID: 27914963; PubMed Central PMCID: PMCPMC5183552.

16. Auchampach JA, Rizvi A, Qiu Y, Tang XL, Maldonado C, Teschner S, et al. Selective activation of A3 adenosine receptors with N6-(3-iodobenzyl)adenosine-5'-N-methyluronamide protects against myocardial stunning and infarction without hemodynamic changes in conscious rabbits. Circ Res. 1997; 80 (6):800–9. PMID: 9168782.

17. Fozard JR, Pfannkuche HJ, Schuurman HJ. Mast cell degranulation following adenosine A3 receptor activation in rats. Eur J Pharmacol. 1996; 298(3):293–7. PMID: 8846829.

18. 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. PMID: 10660615.

19. Carlin JL, Tosh DK, Xiao C, Pinol RA, Chen Z, Salvemini D, et al. Peripheral Adenosine A3 Receptor Activation Causes Regulated Hypothermia in Mice That Is Dependent on Central Histamine H1 Receptors. J Pharmacol Exp Ther. 2016; 356(2):474–82. https://doi.org/10.1124/jpet.115.229872 PMID: 26606937; PubMed Central PMCID: PMCPMC4746492.

20. 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.

21. Carlin JL, Jain S, Duroux R, Suresh RR, Xiao C, Auchampach JA, et al. Activation of adenosine A2A or A2B receptors causes hypothermia in mice. Neuropharmacology. 2018; 139:268–78. Epub 2018/03/20. https://doi.org/10.1016/j.neuropharm.2018.02.035 PMID: 29548686; PubMed Central PMCID: PMCPMC6067974.

22. Lin SC, Hardie DG. AMPK: Sensing Glucose as well as Cellular Energy Status. Cell Metab. 2018; 27 (2):299–313. Epub 2017/11/21. https://doi.org/10.1016/j.cmet.2017.10.009 PMID: 29153408.

23. Melvin RG, Andrews MT. Torpor induction in mammals: recent discoveries fueling new ideas. Trends Endocrinol Metab. 2009; 20(10):490–8. https://doi.org/10.1016/j.tem.2009.09.005 PMID: 19864159.

24. 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.

25. Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, et al. A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J Neurosci. 1999; 19(21):9192–200. PMID: 10531422.

26. Hua X, Kovarova M, Chason KD, Nguyen M, Koller BH, Tilley SL. Enhanced mast cell activation in mice deficient in the A2b adenosine receptor. J Exp Med. 2007; 204(1):117–28. https://doi.org/10.1084/jem.20061372 PMID: 17200408; PubMed Central PMCID: PMCPMC2118413.

27. Minelli A, Liguori L, Bellazza I, Mannucci R, Johansson B, Fredholm BB. Involvement of A1 adenosine receptors in the acquisition of fertilizing capacity. J Androl. 2004; 25(2):286–92. PMID: 14760015.

28. Gimenez-Llort L, Fernandez-Teruel A, Escorihuela RM, Fredholm BB, Tobena A, Pekny M, et al. Mice lacking the adenosine A1 receptor are anxious and aggressive, but are normal learners with reduced muscle strength and survival rate. Eur J Neurosci. 2002; 16(3):547–50. PMID: 12193199.

29. Rudaya AY, Steiner AA, Robbins JR, Dragic AS, Romanovsky AA. Thermoregulatory responses to lipopolysaccharide in the mouse: dependence on the dose and ambient temperature. Am J Physiol Regul Integr Comp Physiol. 2005; 289(5):R1244–52. Epub 2005/08/06. https://doi.org/10.1152/ajpregu.00370.2005 PMID: 16081879.

30. Everhardt Queen A, Moerdyk-Schauwecker M, McKee LM, Leamy LJ, Huet YM. Differential Expression of Inflammatory Cytokines and Stress Genes in Male and Female Mice in Response to a Lipopolysaccharide Challenge. PLoS ONE. 2016; 11(4):e0152289. Epub 2016/04/28. https://doi.org/10.1371/journal.pone.0152289 PMID: 27120355; PubMed Central PMCID: PMCPMC4847773.

31. Yang D, Zhang Y, Nguyen HG, Koupenova M, Chauhan AK, Makitalo M, et al. The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. J Clin Invest. 2006; 116 (7):1913–23. https://doi.org/10.1172/JCI27933 PMID: 16823489; PubMed Central PMCID: PMCPMC1483170.

32. Hasko G, Kuhel DG, Chen JF, Schwarzschild MA, Deitch EA, Mabley JG, et al. Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A2a receptor-dependent and independent mechanisms. FASEB J. 2000; 14(13):2065–74. Epub 2000/10/12. https://doi.org/10.1096/fj.99-0508com PMID: 11023991.

33. Zhang J, Kaasik K, Blackburn MR, Lee CC. Constant darkness is a circadian metabolic signal in mammals. Nature. 2006; 439(7074):340–3. https://doi.org/10.1038/nature04368 PMID: 16421573.

34. Swoap SJ, Rathvon M, Gutilla M. AMP does not induce torpor. Am J Physiol Regul Integr Comp Physiol. 2007; 293(1):R468–73. https://doi.org/10.1152/ajpregu.00888.2006 PMID: 17409259.

35. Rittiner JE, Korboukh I, Hull-Ryde EA, Jin J, Janzen WP, Frye SV, et al. AMP is an adenosine A1 receptor agonist. J Biol Chem. 2012; 287(8):5301–9. Epub 2012/01/05. https://doi.org/10.1074/jbc.M111.291666 M111.291666 [pii]. PMID: 22215671; PubMed Central PMCID: PMC3285310.

36. Daniels IS, Zhang J, O'Brien WG 3rd, Tao Z, Miki T, Zhao Z, et al. A role of erythrocytes in adenosine monophosphate initiation of hypometabolism in mammals. The Journal of biological chemistry. 2010; 285(27):20716–23. Epub 2010/05/01. https://doi.org/10.1074/jbc.M109.090845 PMID: 20430891; PubMed Central PMCID: PMC2898302.

37. O'Brien WG 3rd, Ling HS, Zhao Z, Lee CC. New insights on the regulation of the adenine nucleotide pool of erythrocytes in mouse models. PLoS ONE. 2017; 12(7):e0180948. Epub 2017/07/27. https://doi.org/10.1371/journal.pone.0180948 PMID: 28746349; PubMed Central PMCID: PMCPMC5528878.

38. 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.

39. McGaraughty S, Chu KL, Wismer CT, Mikusa J, Zhu CZ, Cowart M, et al. Effects of A-134974, a novel adenosine kinase inhibitor, on carrageenan-induced inflammatory hyperalgesia and locomotor activity in rats: evaluation of the sites of action. J Pharmacol Exp Ther. 2001; 296(2):501–9. Epub 2001/02/13. PMID: 11160637.

40. Fredholm BB, AP IJ, Jacobson KA, Linden J, Muller 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.

41. Alnouri MW, Jepards S, Casari A, Schiedel AC, Hinz S, Muller CE. Selectivity is species-dependent: Characterization of standard agonists and antagonists at human, rat, and mouse adenosine receptors. Purinergic Signal. 2015; 11(3):389–407. https://doi.org/10.1007/s11302-015-9460-9 PMID: 26126429; PubMed Central PMCID: PMCPMC4529847.

42. Yang JN, Chen JF, Fredholm BB. Physiological roles of A1 and A2A adenosine receptors in regulating heart rate, body temperature, and locomotion as revealed using knockout mice and caffeine. Am J

Physiol Heart Circ Physiol. 2009; 296(4):H1141–9. Epub 2009/02/17. https://doi.org/10.1152/ajpheart. 00754.2008 00754.2008 [pii]. PMID: 19218506; PubMed Central PMCID: PMC2670688.

43. Jin X, Shepherd RK, Duling BR, Linden J. Inosine binds to A3 adenosine receptors and stimulates mast cell degranulation. J Clin Invest. 1997; 100(11):2849–57. Epub 1998/02/12. https://doi.org/10.1172/ JCI119833 PMID: 9389751; PubMed Central PMCID: PMCPMC508491.

44. Gomez G, Sitkovsky MV. Differential requirement for A2a and A3 adenosine receptors for the protective effect of inosine in vivo. Blood. 2003; 102(13):4472–8. Epub 2003/08/30. https://doi.org/10.1182/blood-2002-11-3624 PMID: 12947007.

45. Ward JL, Sherali A, Mo ZP, Tse CM. Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine. J Biol Chem. 2000; 275(12):8375–81. Epub 2000/03/18. PMID: 10722669.

46. Nagai K, Nagasawa K, Fujimoto S. Transport mechanisms for adenosine and uridine in primary-cultured rat cortical neurons and astrocytes. Biochem Biophys Res Commun. 2005; 334(4):1343–50. Epub 2005/07/27. https://doi.org/10.1016/j.bbrc.2005.07.032 PMID: 16043124.

47. Deng Y, Wang ZV, Gordillo R, An Y, Zhang C, Liang Q, et al. An adipo-biliary-uridine axis that regulates energy homeostasis. Science. 2017; 355(6330). https://doi.org/10.1126/science.aaf5375 PMID: 28302796.

48. Peters GJ, van Groeningen CJ, Laurensse EJ, Lankelma J, Leyva A, Pinedo HM. Uridine-induced hypothermia in mice and rats in relation to plasma and tissue levels of uridine and its metabolites. Cancer Chemother Pharmacol. 1987; 20(2):101–8. PMID: 3664929.

49. Freinkel N, Metzger BE, Harris E, Robinson S, Mager M. The hypothermia of hypoglycemia. Studies with 2-deoxy-D-glucose in normal human subjects and mice. N Engl J Med. 1972; 287(17):841–5. Epub 1972/10/26. https://doi.org/10.1056/NEJM197210262871702 PMID: 5071962.

50. Lam TK, Pocai A, Gutierrez-Juarez R, Obici S, Bryan J, Aguilar-Bryan L, et al. Hypothalamic sensing of circulating fatty acids is required for glucose homeostasis. Nat Med. 2005; 11(3):320–7. Epub 2005/03/01. https://doi.org/10.1038/nm1201 PMID: 15735652.

51. Lam TK, Gutierrez-Juarez R, Pocai A, Rossetti L. Regulation of blood glucose by hypothalamic pyruvate metabolism. Science. 2005; 309(5736):943–7. Epub 2005/08/06. https://doi.org/10.1126/science. 1112085 PMID: 16081739.

52. Pedata F, Dettori I, Coppi E, Melani A, Fusco I, Corradetti R, et al. Purinergic signalling in brain ischemia. Neuropharmacology. 2016; 104:105–30. Epub 2015/11/20. https://doi.org/10.1016/j.neuropharm. 2015.11.007 PMID: 26581499.

53. Mayne M, Shepel PN, Jiang Y, Geiger JD, Power C. Dysregulation of adenosine A1 receptor-mediated cytokine expression in peripheral blood mononuclear cells from multiple sclerosis patients. Ann Neurol. 1999; 45(5):633–9. Epub 1999/05/13. PMID: 10319886.

54. 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.

55. Blardi P, Laghi Pasini F, Urso R, Frigerio C, Volpi L, De Giorgi L, et al. Pharmacokinetics of exogenous adenosine in man after infusion. Eur J Clin Pharmacol. 1993; 44(5):505–7. Epub 1993/01/01. PMID: 8359193.

56. Vecchio EA, Baltos JA, Nguyen ATN, Christopoulos A, White PJ, May LT. New paradigms in adenosine receptor pharmacology: allostery, oligomerization and biased agonism. Br J Pharmacol. 2018; 175 (21):4036–46. Epub 2018/04/22. https://doi.org/10.1111/bph.14337 PMID: 29679502; PubMed Central PMCID: PMCPMC6177620.

57. Hasko G, Sitkovsky MV, Szabo C. Immunomodulatory and neuroprotective effects of inosine. Trends Pharmacol Sci. 2004; 25(3):152–7. Epub 2004/03/17. https://doi.org/10.1016/j.tips.2004.01.006 PMID: 15019271.

58. Tilley SL, Wagoner VA, Salvatore CA, Jacobson MA, Koller BH. Adenosine and inosine increase cutaneous vasopermeability by activating A(3) receptors on mast cells. J Clin Invest. 2000; 105(3):361–7. Epub 2000/02/17. https://doi.org/10.1172/JCI8253 PMID: 10675362; PubMed Central PMCID: PMCPMC377446.

59. Fredholm BB, Irenius E, Kull B, Schulte G. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. Biochem Pharmacol. 2001; 61 (4):443–8. Epub 2001/02/28. PMID: 11226378.

60. Welihinda AA, Kaur M, Raveendran KS, Amento EP. Enhancement of inosine-mediated A2AR signaling through positive allosteric modulation. Cell Signal. 2018; 42:227–35. Epub 2017/11/12. https://doi. org/10.1016/j.cellsig.2017.11.002 PMID: 29126977; PubMed Central PMCID: PMCPMC5732040.

61. Welihinda AA, Kaur M, Greene K, Zhai Y, Amento EP. The adenosine metabolite inosine is a functional agonist of the adenosine A2A receptor with a unique signaling bias. Cell Signal. 2016; 28(6):552–60. Epub 2016/02/24. https://doi.org/10.1016/j.cellsig.2016.02.010 PMID: 26903141; PubMed Central PMCID: PMCPMC4826793.

62. Doyle C, Cristofaro V, Sack BS, Lukianov SN, Schafer M, Chung YG, et al. Inosine attenuates spontaneous activity in the rat neurogenic bladder through an A2B pathway. Sci Rep. 2017; 7:44416. Epub 2017/03/16. https://doi.org/10.1038/srep44416 PMID: 28294142; PubMed Central PMCID: PMCPMC5353659.

63. Daly JW. Caffeine analogs: biomedical impact. Cell Mol Life Sci. 2007; 64(16):2153–69. Epub 2007/05/22. https://doi.org/10.1007/s00018-007-7051-9 PMID: 17514358.

64. Duan L, Yang J, Slaughter MM. Caffeine inhibition of ionotropic glycine receptors. J Physiol. 2009; 587 (Pt 16):4063–75. Epub 2009/07/01. https://doi.org/10.1113/jphysiol.2009.174797 PMID: 19564396; PubMed Central PMCID: PMCPMC2756438.

65. Zucchi R, Ronca-Testoni S. The sarcoplasmic reticulum Ca2+ channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. Pharmacol Rev. 1997; 49(1):1–51. Epub 1997/03/01. PMID: 9085308.

66. Bonati M, Latini R, Tognoni G, Young JF, Garattini S. Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. Drug Metab Rev. 1984; 15(7):1355–83. Epub 1984/01/01. https://doi.org/10.3109/03602538409029964 PMID: 6543526.

67. Huang ZL, Qu WM, Eguchi N, Chen JF, Schwarzschild MA, Fredholm BB, et al. Adenosine A2A, but not A1, receptors mediate the arousal effect of caffeine. Nat Neurosci. 2005; 8(7):858–9. Epub 2005/06/21. https://doi.org/10.1038/nn1491 PMID: 15965471.

68. El Yacoubi M, Ledent C, Menard JF, Parmentier M, Costentin J, Vaugeois JM. The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A(2A) receptors. British journal of pharmacology. 2000; 129(7):1465–73. Epub 2000/04/01. https://doi.org/10.1038/sj.bjp.0703170 PMID: 10742303; PubMed Central PMCID: PMC1571962.

69. Bettio LE, Gil-Mohapel J, Rodrigues AL. Guanosine and its role in neuropathologies. Purinergic Signal. 2016; 12(3):411–26. Epub 2016/03/24. https://doi.org/10.1007/s11302-016-9509-4 PMID: 27002712; PubMed Central PMCID: PMCPMC5023624.

70. Soares FA, Schmidt AP, Farina M, Frizzo ME, Tavares RG, Portela LV, et al. Anticonvulsant effect of GMP depends on its conversion to guanosine. Brain Res. 2004; 1005(1–2):182–6. Epub 2004/03/27. https://doi.org/10.1016/j.brainres.2004.01.053 PMID: 15044076.

71. Lakatos RK, Dobolyi A, Todorov MI, Kekesi KA, Juhasz G, Aleksza M, et al. Guanosine may increase absence epileptic activity by means of A2A adenosine receptors in Wistar Albino Glaxo Rijswijk rats. Brain Res Bull. 2016; 124:172–81. Epub 2016/05/08. https://doi.org/10.1016/j.brainresbull.2016.05.001 PMID: 27154620.

72. van Galen PJ, van Bergen AH, Gallo-Rodriguez C, Melman N, Olah ME, AP IJ, et al. A binding site model and structure-activity relationships for the rat A3 adenosine receptor. Mol Pharmacol. 1994; 45 (6):1101–11. Epub 1994/06/01. PMID: 8022403; PubMed Central PMCID: PMCPMC3479652.

73. Miller RL, Adamczyk DL, Miller WH, Koszalka GW, Rideout JL, Beacham LM 3rd, et al. Adenosine kinase from rabbit liver. II. Substrate and inhibitor specificity. J Biol Chem. 1979; 254(7):2346–52. Epub 1979/04/10. PMID: 218934.

74. Wiginton DA, Coleman MS, Hutton JJ. Purification, characterization and radioimmunoassay of adenosine deaminase from human leukaemic granulocytes. Biochem J. 1981; 195(2):389–97. Epub 1981/05/01. PMID: 6947796; PubMed Central PMCID: PMCPMC1162902.

75. Dobolyi A, Juhasz G, Kovacs Z, Kardos J. Uridine function in the central nervous system. Curr Top Med Chem. 2011; 11(8):1058–67. Epub 2011/03/16. PMID: 21401495.

76. Williams-Karnesky RL, Sandau US, Lusardi TA, Lytle NK, Farrell JM, Pritchard EM, et al. Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis. J Clin Invest. 2013; 123 (8):3552–63. Epub 2013/07/19. https://doi.org/10.1172/JCI65636 PMID: 23863710; PubMed Central PMCID: PMCPMC3726154.

77. Rodrigues RJ, Marques JM, Cunha RA. Purinergic signalling and brain development. Semin Cell Dev Biol. 2019:https://doi.org/10.1016/j.semcdb.2018.12.001. Epub 2018/12/12. PMID: 30529149.

78. Rivkees SA, Wendler CC. Long-term consequences of disrupting adenosine signaling during embryonic development. Mol Aspects Med. 2017; 55:110–7. https://doi.org/10.1016/j.mam.2017.02.001 PMID: 28202385; PubMed Central PMCID: PMCPMC5487283.

79. Podzuweit T, Nennstiel P, Muller A. Isozyme selective inhibition of cGMP-stimulated cyclic nucleotide phosphodiesterases by erythro-9-(2-hydroxy-3-nonyl) adenine. Cell Signal. 1995; 7(7):733–8. Epub 1995/09/01. PMID: 8519602.

80. Liaudet L, Mabley JG, Pacher P, Virag L, Soriano FG, Marton A, et al. Inosine exerts a broad range of antiinflammatory effects in a murine model of acute lung injury. Ann Surg. 2002; 235(4):568–78. Epub 2002/03/30. PMID: 11923614; PubMed Central PMCID: PMCPMC1422473.

81. 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.

82. Kim SM, Eisner C, Faulhaber-Walter R, Mizel D, Wall SM, Briggs JP, et al. Salt sensitivity of blood pressure in NKCC1-deficient mice. Am J Physiol Renal Physiol. 2008; 295(4):F1230–8. Epub 2008/08/15. https://doi.org/10.1152/ajprenal.90392.2008 90392.2008 [pii]. PMID: 18701622; PubMed Central PMCID: PMC2576156.

83. Copeland S, Warren HS, Lowry SF, Calvano SE, Remick D, Inflammation, et al. Acute inflammatory response to endotoxin in mice and humans. Clin Diagn Lab Immunol. 2005; 12(1):60–7. Epub 2005/01/12. https://doi.org/10.1128/CDLI.12.1.60-67.2005 PMID: 15642986; PubMed Central PMCID: PMCPMC540200.

84. Gewirtz H, Brown P, Most AS. Measurement of plasma adenosine concentration: methodological and physiological considerations. Proc Soc Exp Biol Med. 1987; 185(1):93–100. Epub 1987/05/01. PMID: 3554257.

85. Shryock JC, Boykin MT, Hill JA, Belardinelli L. A new method of sampling blood for measurement of plasma adenosine. Am J Physiol. 1990; 258(4 Pt 2):H1232–9. Epub 1990/04/01. https://doi.org/10.1152/ajpheart.1990.258.4.H1232 PMID: 2331011.

86. Veras FP, Peres RS, Saraiva AL, Pinto LG, Louzada-Junior P, Cunha TM, et al. Fructose 1,6-bisphosphate, a high-energy intermediate of glycolysis, attenuates experimental arthritis by activating anti-inflammatory adenosinergic pathway. Sci Rep. 2015; 5:15171. Epub 2015/10/20. https://doi.org/10.1038/srep15171 PMID: 26478088; PubMed Central PMCID: PMCPMC4609967.

87. Slowiaczek P, Tattersall MH. The determination of purine levels in human and mouse plasma. Anal Biochem. 1982; 125(1):6–12. Epub 1982/09/01. PMID: 6897341.

88. Xiao C, Pinol RA, Carlin JL, Li C, Deng C, Gavrilova O, et al. Bombesin-like receptor 3 (Brs3) expression in glutamatergic, but not GABAergic, neurons is required for regulation of energy metabolism. Mol Metab. 2017; 6(11):1540–50. https://doi.org/10.1016/j.molmet.2017.08.013 PMID: 29107299; PubMed Central PMCID: PMCPMC5681273.