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Hypothermia in mouse is caused by adenosine A_1 and A_3 receptor agonists and AMP via three distinct mechanisms

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Abstract

Small mammals have the ability to enter torpor, a hypothermic, hypometabolic state, allowing impressive energy conservation. Administration of adenosine or adenosine 5'-monophosphate (AMP) can trigger a hypothermic, torpor-like state. We investigated the mechanisms for hypothermia using telemetric monitoring of body temperature in wild type and receptor knock out (*Adora1*$^{-/-}$, *Adora3*$^{-/-}$) mice. Confirming prior data, stimulation of the A_3 adenosine receptor (AR) induced hypothermia via peripheral mast cell degranulation, histamine release, and activation of central histamine H_1 receptors. In contrast, A_1AR agonists and AMP both acted centrally to cause hypothermia. Commonly used, selective A_1AR agonists, including N^6-cyclopentyladenosine (CPA), N^6-cyclohexyladenosine (CHA), and MRS5474, caused hypothermia via both A_1AR and A_3AR when given intraperitoneally. Intracerebroventricular dosing, low peripheral doses of Cl-ENBA [(±)-5'-chloro-5'-deoxy-N^6-*endo*-norbornyladenosine], or using

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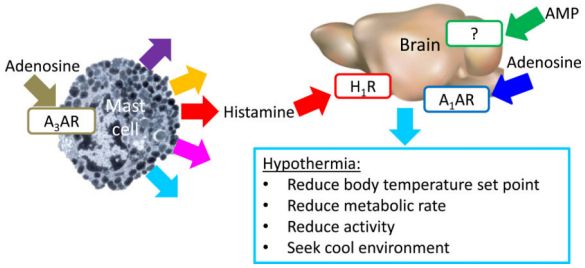
Chemical compounds

AMP, adenosine 5'-monophosphate
CCPA, 2-chloro-N^6-cyclopentyladenosine
CHA, N^6-cyclohexyladenosine
Cl-ENBA, (±)-5'-chloro-5'-deoxy-N^6-*endo*-norbornyladenosine
CPA, N^6-cyclopentyladenosine
MRS5474, (1R,2R,3S,5S)-4-(2-chloro-6-((dicyclopropylmethyl)amino)-9H-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol
R-PIA, N^6-R-phenylisopropyladenosine
SPA, N^6-(p-sulfo-phenyl)adenosine

Adora3$^{-/-}$ mice allowed selective stimulation of A_1AR. AMP-stimulated hypothermia can occur independently of A_1AR, A_3AR, and mast cells. A_1AR and A_3AR agonists and AMP cause regulated hypothermia that was characterized by a drop in total energy expenditure, physical inactivity, and preference for cooler environmental temperatures, indicating a reduced body temperature set point. Neither A_1AR nor A_3AR were required for fasting-induced torpor. A_1AR and A_3AR agonists and AMP trigger regulated hypothermia via three distinct mechanisms.

Graphical Abstract



Keywords

hypothermia; adenosine; A_1AR; A_3AR; AMP; torpor

1. Introduction

Mammals are endotherms, typically maintaining a warm core body temperature (Tb) of ~35 °C to 37 °C, depending on circadian fluctuations (Refinetti, 2010). Some small mammals, including mice, when exposed to an inadequate food supply in a quiet, cool environment use torpor to achieve significant energy conservation (Geiser, 2004; Melvin and Andrews, 2009). Torpor is an example of a regulated hypothermia (anapyrexia), which is characterized by a reduced Tb set point, metabolic rate, and physical activity, with Tb falling close to the environmental temperature. In regulated hypothermia multiple physiologic mechanisms are coordinated to cool the body, including vasodilation, decreased physical activity, reduced brown adipose tissue thermogenesis, and seeking a cool environment (Lute *et al*, 2014). Regulated hypothermia differs from hypothermia caused by cold exposure, where the body attempts, but is unable, to maintain Tb.

Hypothermia can also be caused by a wide variety of drugs and neurotransmitters (Clark and Lipton, 1985), although in most cases it is not documented if there is a reduction in Tb set point. Clinically, hypothermia is routinely employed to minimize tissue damage after hypoxic or ischemic injury (Arrich *et al*, 2012; Azzopardi *et al*, 2014). The hypothermia is typically induced with surface cooling. However, pharmacological reduction of the Tb might minimize undesired compensatory mechanisms (e.g., sympathetic activation, shivering). Development of a drug regimen that produces controlled, regulated hypothermia and avoids activating counter-regulatory responses would likely be of clinical utility (Drew *et al*, 2015; Tupone *et al*, 2014).

The hypothermic effect of adenosine was first reported in 1931 (Bennet and Drury, 1931). Once multiple adenosine receptor (AR) subtypes were identified, the hypothermia was attributed to action at A_1AR, likely within the brain (Anderson *et al*, 1994). Peripheral dosing of N^6-cyclohexyladenosine (CHA; see Fig. S1 for structures) elicited hypothermia, which was lost in A_1AR knock out (*Adora1*$^{-/-}$) mice (Johansson *et al*, 2001). CHA also caused hypothermia when infused directly into the nucleus of the solitary tract in rats (Tupone *et al*, 2013). However, some data suggested a contribution from non-A_1AR mechanisms, as only partial attenuation of hypothermia caused by N^6-R-phenylisopropyladenosine (R-PIA) was seen in *Adora1*$^{-/-}$ mice (Yang *et al*, 2007).

More recently, it has become clear that adenosine agonists also induce hypothermia in mice via A_3AR. A critical observation was that hypothermia caused by R-PIA was attenuated in A_3AR knock out (*Adora3*$^{-/-}$) mice (Yang *et al*, 2010). In rodents, A_3AR is expressed on immune cells (Borea *et al*, 2015) and A_3AR agonists trigger mast cell degranulation (Auchampach *et al*, 1997; Fozard *et al*, 1996; Salvatore *et al*, 2000). We have recently shown that A_3AR agonists activate peripheral mast cells releasing histamine, which then acts on central histamine H_1 receptors to lower the Tb set point (Carlin *et al*, 2016).

Adenosine 5′-monophosphate (AMP) is a proposed natural regulator of torpor and injection of a large dose of AMP (500–3500 mg/kg, i.p.) causes hypothermia (Zhang *et al*, 2006). Given the dose size, one might consider if some of the effects of AMP are due to its conversion to free adenosine (Rittiner *et al*, 2012; Swoap *et al*, 2007). The observation that AMP's hypothermic effects remain intact in mice lacking any one of the four AR subtypes, indicates that AMP is not acting non-redundantly via a single AR (Daniels *et al*, 2010). However, AMP-induced hypothermia was reported to be blocked by infusion of an A_1AR antagonist in the pre-optic area (Muzzi *et al*, 2013). The mechanism by which AMP causes hypothermia is currently unclear.

While studying hypothermia caused by A_3AR agonists, we observed that some nucleoside derivatives commonly used as A_1AR agonists also had activity at the A_3AR. This prompted the current re-examination and comparison of A_1AR agonists, A_3AR agonists, and AMP. Our data suggest that each of these can trigger hypothermia in mice via different mechanisms.

2. Materials and Methods

2.1 Mice

Male C57BL/6J and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Stock #012861) (Grimbaldeston *et al*, 2005; Nigrovic *et al*, 2008) were obtained from the Jackson Laboratory. *Adora1*$^{-/-}$ mice on a C57BL/6J background were provided by Dr. Jurgen Schnermann (Sun *et al*, 2001) and genotyped by PCR (*Adora1* reverse common primer 5'-ACATGGGGGTTGAACAGAGA, *Adora1* forward primer 5'-AGCTGGCTACCGCTACACAT, and Neo forward primer 5'-TCTGGATTCATCGACTGTGG), producing 302 bp wild type and ~900 bp null allele products. *Adora3*$^{-/-}$ mice on a C57BL/6 background made by Merck (Salvatore *et al*, 2000) were provided by Dr. Stephen Tilley and genotyped by PCR (*Adora3* reverse common primer 5'-ACTGGCCCATACACAACCTG, *Adora3* forward primer 5'-

AGACAATGAAATAGACGGTGGTG, and Neo forward primer 5'-ATGGAAGGATTGGAGCTACG), producing 208 bp wild type and ~400 bp null allele products. Mice were singly housed at ~22 °C with a 12:12-h light-dark cycle. Chow (NIH-07, Envigo Inc, Madison, WI) 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 acclimatization. No specific effort was made to acclimatize mice to handling in individual experiments. Studies were approved by the Animal Care and Use Committee of National Institute of Diabetes and Digestive and Kidney Diseases.

2.2 Drugs

The following compounds (vehicle) were purchased from Sigma (St. Louis, MO) or Tocris (Minneapolis, MN): Cl-ENBA, (±)-5'-chloro-5'-deoxy-*N*^{6}-*endo*-norbornyladenosine (Franchetti *et al*, 2009; Trivedi *et al*, 1989) (10% DMSO in saline); CHA, *N*^{6}-cyclohexyladenosine (dissolved in DMSO, then diluted to 10% DMSO with saline); CPA, *N*^{6}-cyclopentyladenosine (saline); pyrilamine (saline); AMP, adenosine 5'-monophosphate (saline); CCPA, 2-chloro-*N*^{6}-cyclopentyladenosine. MRS5474, (1R,2R,3S,5S)-4-(2-chloro-6-((dicyclopropylmethyl)amino)-9H-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol, (dissolved in DMSO, then diluted with 9 volumes 30% PEG400) was synthesized as described (Tosh *et al*, 2012b).

2.3 Adenosine Receptor Binding Affinities

Binding affinity for mouse A_1AR, A_{2A}AR, and A_3ARs was measured as described (Kreckler *et al*, 2006) using membranes from human embryonic kidney (HEK)-293 cells stably expressing individual recombinant mouse adenosine receptors and using the agonists [^{125}I]*N*^{6}-(4-amino-3-iodobenzyl)adenosine-5'-methyluronamide ([^{125}I]AB-MECA; A_1AR and A_3AR) and [^{3}H]CGS21680 (A_{2A}AR) as radioligands. Nonspecific binding was defined using 100 μM adenosine-5'-N-ethylcarboxamide (NECA). K_i values were obtained using the Cheng-Prusoff equation from IC_{50} values calculated by non-linear regression analysis of specific binding data using GraphPad Prism software (San Diego, CA).

2.4 Central infusions

Mice were anesthetized with ketamine/xylazine (80/10 mg/kg, i.p.). 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, and +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). Cannula positions were verified by postmortem histological analysis.

2.5 Body Temperature and Activity Telemetry and Indirect Calorimetry

Tb and activity were measured continuously by telemetry (Starr Life Sciences, Oakmont, PA) using ER4000 energizer/ receivers, G2 E-mitters implanted intraperitoneally, and VitalView software with data collected each minute. Invalid E-mitter data points (defined as

a change of ≥ 1 °C in one minute) were replaced by interpolation using flanking data. Any Tb ≤ 24 °C were scored as 24 °C. Unless noted otherwise, the average Tb response was calculated using the first 60 min after agonist dosing. Hypothermia duration is the duration of the interval between dosing and 300 min during which core temperature is <35 °C. Activity is the sum of counts from 10–60 min after A_1AR agonist administration. Inhibitors were dosed 15 min before agonists. Indirect calorimetry was performed as described (Carlin *et al*, 2016). Experiments were performed at ~22 °C. Occasionally a mouse did not become hypothermic with a treatment that generally caused hypothermia. In these cases, the same mouse was retested ≥ 7 days later and routinely became hypothermic and the second data set was used.

2.6 Temperature Gradient

Environmental temperature preference was measured using an in-house apparatus by continuous video monitoring of the position of the mouse in a 45 cm thermal gradient, nominally 15 °C to 35 °C (Carlin *et al*, 2016). Data are reported as mean ± SEM. Significance (two-tailed $p < 0.05$) was determined by t test or ANOVA followed by post hoc Holm-Sidak multiple comparison tests.

3. Results

3.1 CHA induces hypothermia via both A_1AR and A_3AR

We investigated the ability of systemically-dosed adenosine agonists to cause hypothermia. CHA is ~290-fold selective for binding to mouse A_1AR over A_3AR (Table 1). With vehicle treatment, dosing-associated handling caused an increase in Tb and activity that lasted about an hour. Treatment of C57BL/6J mice with CHA (0.02–0.2 mg/kg, i.p.) caused a drop in Tb, accompanied by reduced physical activity (Fig. 1A–E). The Tb and physical activity decrease and the increase in duration of hypothermia were all dose-dependent.

The *in vivo* specificity of CHA was examined using *Adora1*$^{-/-}$ and *Adora3*$^{-/-}$ mice. Hypothermia elicited by CHA (0.05 mg/kg, i.p.) was attenuated in both *Adora1*$^{-/-}$ and *Adora3*$^{-/-}$ mice, but was completely lost in *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ double knock out mice (Fig. 1F–K). These data demonstrate that the hypothermic effect of this low dose of CHA is contributed by agonism at both A_1AR and A_3AR.

3.2 MRS5474 induces hypothermia via A_3AR

Truncated nucleoside MRS5474, which contains a bicyclic substitute for ribose, is a moderately selective, full A_1AR agonist that is well tolerated *in vivo* (Tosh *et al*, 2012b) and is ~280-fold selective for binding to mouse A_1AR over A_3AR (Table 1). It caused dose-dependent hypothermia and hypoactivity (Fig. S2A,B and data not shown). Treatment of *Adora1*$^{-/-}$ and *Adora3*$^{-/-}$ mice with MRS5474 (3 mg/kg, i.p.) demonstrated that the hypothermia was via A_3AR, without a clear contribution from A_1AR (Fig. S2C–F). Since centrally-active dopamine D_2-like receptor agonists cause hypothermia (Nunes *et al*, 1991), we investigated the effect of raclopride, a D_2-like receptor antagonist. Hypothermia induced by MRS5474 (3 mg/kg, i.p.) was not inhibited by pretreatment with raclopride (1 mg/kg, i.p.; data not shown).

3.3 CPA induces hypothermia via A_3AR more than A_1AR

CPA is another widely used adenosine agonist with better selectivity (~2400-fold) for binding to mouse A_1AR over A_3AR (Table 1). CPA caused a dose-dependent (0.03–3 mg/kg, i.p.) hypothermia and reduction in physical activity in C57BL/6J mice (Fig. S3A,B and data not shown). However, hypothermia induced by CPA (0.3 mg/kg, i.p.) was not significantly attenuated in *Adora1*$^{-/-}$ mice and was attenuated but still present in *Adora3*$^{-/-}$ mice (Fig. S3C–F). These data suggest that the hypothermic effects of this dose of CPA are via agonism at A_3AR, likely with a lesser contribution from A_1AR.

A related agonist, CCPA, is often used as a more selective A_1AR agonist. However, we found that its binding affinity at the A_3AR would make it even less desirable than CPA for use in mouse (Table 1). In general, there is a need to reexamine the affinities of widely used AR ligand probes across species (Alnouri *et al*, 2015). Therefore, we compare the affinities of various agonists and antagonists at human and mouse receptors. Some previous studies of adenosine and hypothermia used DPCPX as a selective antagonist of the A_1AR. However, the selectivity ratios of DPCPX vary significantly between species (Table 1 and (Alnouri *et al*, 2015)).

3.4 In vivo Cl-ENBA is a more selective A_1AR agonist

Cl-ENBA is the most selective A_1AR agonist described, with ~2600-fold selectivity for binding to human A_1AR vs. A_3AR (Franchetti *et al*, 2009; Trivedi *et al*, 1989) and ~10,000-fold selectivity for mouse A_1AR over A_3AR (Table 1). Cl-ENBA (0.3–10 mg/kg, i.p.) caused dose-dependent hypothermia and hypoactivity (Fig. 2A,B and data not shown). Cl-ENBA (3 mg/kg, i.p.) hypothermia was not affected by pretreatment with raclopride (1 mg/kg, i.p.; data not shown). It was largely abolished in *Adora1*$^{-/-}$ mice (Fig. 2C,D), remained present in *Adora3*$^{-/-}$ mice (Fig. 2E,F), and was completely lost in *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ mice (Fig. 2G,H). These data (and see 3.5) demonstrate that the hypothermic effects of Cl-ENBA are mainly mediated via A_1AR, but at higher doses there is probably also a contribution from A_3AR.

3.5 A_1AR agonist-induced hypothermia occurs via central sites, while A_3AR agonist-mediated hypothermia requires mast cells

The poor *in vivo* A_1AR selectivity of systemic CHA and CPA prompted us to re-examine the role of mast cells and histamine in A_1AR and A_3AR agonist-induced hypothermia. Hypothermia from A_3AR agonists is caused by histamine release from activated mast cells (Carlin *et al*, 2016), while a role for mast cells in A_1AR agonist-induced hypothermia has not been reported. Hypothermia from systemic Cl-ENBA at a dose of 1 mg/kg was largely intact in mast cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 3A,B), while the hypothermia with 3 mg/kg was significantly attenuated (Fig. 3C,D). In contrast, the hypothermic effect of MRS5474, at a dose that is selective for A_3AR, was abolished in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 3E,F). Pyrilamine, an histamine H_1R antagonist, inhibited hypothermia from this dose, but did not block A_1AR agonist-induced hypothermia (Fig. S4A–D). These results show that, in contrast to A_3AR-mediated hypothermia, A_1AR hypothermia does not require mast cells or H_1R, and suggest that Cl-ENBA at 1 mg/kg i.p., but not 3 mg/kg, is relatively selective for A_1AR.

We next examined the site of A_1AR agonist action to induce hypothermia. Cl-ENBA infused intracerebroventricularly (i.c.v.) at 3.4 μg/mouse (~0.12 mg/kg) caused a drop in Tb comparable to 1 mg/kg given systemically (Fig. 4A,B). The effect of i.c.v. Cl-ENBA was greatly diminished in *Adora1*$^{-/-}$ mice (Fig. 4C,D). These results indicate that Cl-ENBA is acting via central A_1AR.

To avoid confounding due to the modest *in vivo* receptor specificity of the A_1AR agonists, we used *Adora3*$^{-/-}$ mice to investigate mechanistic aspects of A_1AR-driven hypothermia. Cl-ENBA administration reduced metabolic rate and physical activity before the Tb nadir (Fig. 5A–C). Additionally, *Adora3*$^{-/-}$ mice treated with Cl-ENBA had a modest preference for a cooler place in a thermal gradient (Fig. 5D–G). These results suggest that there is a reduced Tb set point and coordinated physiologic response to an A_1AR agonist.

3.6 Neither A_1AR nor A_3AR is necessary for fasting-induced torpor

To better understand the role of adenosine in thermal physiology, baseline Tb and physical activity in light and dark phase were measured in *Adora1*$^{-/-}$, *Adora3*$^{-/-}$, and *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ mice. There were no clear differences between littermate control and *Adora1*$^{-/-}$ or *Adora3*$^{-/-}$ mice or *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ and age-matched C57BL/6J mice (Fig. 6A–F). A 24-hour fast elicited torpor in both *Adora1*$^{-/-}$ and *Adora3*$^{-/-}$ mice (Fig. 6G–H). In a test for receptor redundancy between A_1AR and A_3AR, *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ double knockout mice were found to enter fasting-induced hypothermia (Fig. 6I). These data demonstrate that neither A_1AR nor A_3AR, individually or in combination, is required for fasting-induced torpor.

3.7 AMP does not require A_1AR or A_3AR to induce hypothermia and can act centrally

Large AMP doses (500–3500 mg/kg, i.p.) induce torpor (Zhang *et al*, 2006). We studied a modest dose (100 mg/kg, i.p.) to minimize possible effects of impurities or breakdown products. The AMP-induced hypothermia was intact in *Adora1*$^{-/-}$ and *Adora3*$^{-/-}$ mice (Fig. 7A–D). It was also intact in *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ double knockout and partially intact in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 7E–H). Pretreatment of *Adora1*$^{-/-}$ mice with the brain-penetrant H_1R antagonist pyrilamine (10 mg/kg, i.p.) did not ablate the hypothermia (data not shown). Similarly, pretreatment with raclopride (2 mg/kg, i.p.) did not block the hypothermia (data not shown). These data demonstrate that the hypothermic effects of AMP do not require mast cells, A_1AR, A_3AR, H_1R, D_2R, or the combination of A_1AR/A_3AR.

To investigate the site of action where AMP acts to cause hypothermia, AMP was infused i.c.v. into C57BL/6J mice. A low i.c.v. dose (100 μg/mouse, ~3.3 mg/kg) induced a drop in Tb that was comparable to a 30-fold larger (100 mg/kg) systemic dose and the hypothermia was not attenuated in *Adora1*$^{-/-}$ mice (Fig. 8A,B).

Mice treated with AMP (100 mg/kg, i.p.) demonstrated a drop in metabolic rate that preceded the hypothermia (Fig. 9A-C). We did not observe a clear change in ambient temperature preference at this dose (not shown). With a higher dose (300 mg/kg, i.p.), the mice generally moved to a cooler region of a thermal gradient and became immobile (Fig. 9D–G). When AMP was administered centrally (100 μg/mouse, ~3.3 mg/kg, i.c.v.), there was a similar movement toward cooler environmental temperature but without a significant

drop in activity (Fig. 9H–K). These findings are consistent with AMP acting in the brain to reduce the Tb set point.

4. Discussion

We demonstrate at least three distinct adenosine-related routes to trigger hypothermia in mice (Fig. S5): 1) activation of central A_1AR, 2) peripheral mast cell activation by A_3AR agonists, releasing histamine, which stimulates histamine H_1 receptors, and 3) a central AMP-mediated mechanism. Additionally, we report that some agonists commonly used as A_1AR probes also activate A_3AR *in vivo*.

4.1 Suboptimal *in vivo* A_1AR agonist selectivity

Much understanding of adenosine physiology has been acquired with selective ligands (Fredholm, 2014) (Table 1). CHA and CPA were developed to be selective for A_1AR vs "A_2AR" (Moos *et al*, 1985). The subsequent identification of four ARs and the generation of receptor-ablated animals now permit improved dissection of the specific receptors mediating adenosine actions. To our initial surprise, CHA, MRS5474, and CPA all produced hypothermia via A_3AR, despite their much higher affinity for A_1AR over A_3AR. This is presumably due to higher drug exposure at peripheral mast cell A_3AR, with poor availability at brain A_1AR. This is striking, with a 2000-fold advantage in binding affinity being nullified by pharmacokinetic considerations. We did not measure peripheral A_1AR effects of these agonists (eg., on heart rate, blood pressure, or kidney function (Sun *et al*, 2001; Vallon *et al*, 2006)) for comparison with the peripheral mast cell A_3AR effect.

This observation suggests a reexamination of prior *in vivo* studies using CHA and CPA as A_1AR probes. In mice, CHA is typically used at i.p. doses of 0.2 to 1 mg/kg, with ED_{50}s in locomotor assays of 0.3 to 1.7 mg/kg (Akula and Kulkarni, 2014; Heffner *et al*, 1989; Zarrindast *et al*, 1993). Doses of 0.1 to 1 mg/kg are reported in other species (Jinka *et al*, 2010; Jinka *et al*, 2015; Olson *et al*, 2013). We demonstrate that both A_1AR and A_3AR contribute to hypothermia at a CHA dose of 0.05 mg/kg. Similarly, CPA is typically used in mice at doses of 0.2 to 1 mg/kg i.p., with ED_{50}s in locomotor assays of 1.0 to 1.7 mg/kg (El Yacoubi *et al*, 2000; Heffner *et al*, 1989; Listos *et al*, 2011; Von Lubitz *et al*, 1993). Comparable doses (0.1 to 1 mg/kg) are used in rats (Heurteaux *et al*, 1995; O'Neill *et al*, 2014; Ramos-Zepeda and Herrero, 2013). Our results with 0.3 mg/kg show that both A_1AR and A_3AR contribute to CHA-induced hypothermia. Thus, commonly used i.p. doses of both CPA and CHA stimulate both A_1AR and A_3AR and some effects attributed to A_1AR may actually be due completely, or in part, to A_3AR. This is of particular concern when the pharmacodynamic effect requires brain-penetration for an A_1AR ligand but only peripheral exposure for A_3AR. A non-brain penetrant agonist, SPA, was found to be highly selective for the mouse A_1AR (~3000-fold vs A_3AR) and could be useful in future in vivo studies.

There have been clinical trials of A_1AR agonists for conditions such as diabetic foot pain, diabetes, glaucoma, and cardiac arrhythmias, many of which have failed (Elzein and Zablocki, 2008). Other envisioned therapeutic applications of A_1AR agonists are based on reported neuroprotective, antiseizure, anti-nociceptive, sleep promoting, and antidepressant effects (Tosh *et al*, 2012b). The fact that some *in vivo* actions of agonists, that appear

selective in *in vitro* receptor assays, also might include A_3AR effects, should be considered in future development of such agonists. A_3AR agonists display some similar effects, such as neuroprotection and antinociception (Janes *et al*, 2016), which might contribute to efficacy of nominal A_1AR agonists and would be difficult to separate mechanistically in clinical trials. The A_3AR is expressed at relatively low levels in the mouse hippocampus, cerebellum and medulla (Janes *et al*, 2016; Yaar *et al*, 2005). Thus, although we found no effects of centrally administered A_3AR agonists on Tb, there may be effects of this receptor on CNS function.

Cl-ENBA is a more selective A_1AR agonist than CPA, CHA, and MRS5474 in the mouse for binding (Franchetti *et al*, 2009; Trivedi *et al*, 1989) (Table 1) and in vivo hypothermia. At 1 mg/kg i.p., Cl-ENBA was largely selective for A_1AR, although at 3 mg/kg some of the effect was contributed by A_3AR. Cl-ENBA is anti-nociceptive in the mouse at 0.5 mg/kg (Luongo *et al*, 2012), suggesting that this effect is via A_1AR. Cl-ENBA's greater selectivity makes it a preferred agonist for investigating *in vivo* A_1AR physiology, at least in mice.

The *Adora1*$^{-/-}$ and *Adora3*$^{-/-}$ mice were essential for assessing *in vivo* ligand selectivity. In the absence of sufficiently selective A_1AR agonists, using *Adora3*$^{-/-}$ mice eliminates confounding A_3AR effects. Adenosine agonist affinities and selectivities can vary profoundly among species (Table 1) (Alnouri *et al*, 2015). Adenosine physiology can also vary by species--for example, A_3AR is not present in some human mast cells (Rudich *et al*, 2012). In summary, the results particularly suggest caution when studying *in vivo* effects of A_1AR when using i.p. dosing.

4.2 A_3AR Hypothermia

A_3AR agonists activate peripheral mast cells, releasing histamine, which then acts on central histamine H_1 receptors to lower the Tb set point (Carlin *et al*, 2016). Here we examined the hypothermic effects of MRS5474 (Tosh *et al*, 2012b) and found that at 3 mg/kg i.p., MRS5474 is acting via A_3AR. The hypothermia is lost in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, confirming that A_3AR agonism is acting via mast cells. Since histamine is reported to both increase and decrease Tb (Tabarean, 2016), further study is required.

The kinetics of hypothermia induction via A_3AR and A_1AR are similar, despite the peripheral vs central sites of agonist action. The likely explanation is that heat loss (and thus hypothermia) occurs slowly compared to the rapid increase in circulating histamine and quick neural transmission.

4.3 A_1AR and regulated hypothermia

The poor *in vivo* A_1AR selectivity of peripherally-dosed agonists raises concern that some physiology attributed to A_1AR might actually be due to A_3AR. For hypothermia, the lack of a Tb effect by brain A_3AR agonist indicates that studies using central agonist dosing are probably studying A_1AR effects. Thus, the hypothermia elicited by local CHA delivery to the anterior hypothalamus of Syrian hamsters (Shintani *et al*, 2005) or to the nucleus of the solitary tract of rats (Tupone *et al*, 2013) remain plausibly attributed to A_1AR. A recent rat study used brain-penetrant CHA to produce A_1AR hypothermia combined with a non-brain-penetrant antagonist (8-(*p*-sulfophenyl)theophylline) to block peripheral A_1AR-mediated

bradycardia (Jinka *et al*, 2015). We interpret this experiment as additionally having hypothermia from peripheral CHA activation of rat mast cell A_3AR (Fozard *et al*, 1996), which may not be blocked by 8-(*p*-sulfophenyl) theophylline (Gao *et al*, 2001).

4.4 AMP Hypothermia

Circadian fluctuation of plasma AMP concentration is a proposed endogenous regulator of torpor in rodents (Zhang *et al*, 2006). Large exogenous doses of AMP (500 to 3500 mg/kg) cause torpor and have protective effects in various injury models (Miao *et al*, 2015; Miao *et al*, 2012; Tao *et al*, 2011; Wang *et al*, 2014). There is no clearly identified receptor for AMP (although AMP has been proposed to be an A_1AR ligand (Rittiner *et al*, 2012)). In our studies, AMP-induced hypothermia did not require mast cells, A_1AR, A_3AR, H_1R, D_2R-like, or a combination of A_1AR/A_3AR. Other nucleotides, such as ATP, ADP and cyclic-AMP, have hypothermic effects when injected into the hypothalamic region in cats (Dascombe and Milton, 1975), although the mechanisms have not been defined.

Proposed mechanisms for AMP-induced hypometabolism are that AMP uptake into erythrocytes causes increased 2,3-bisphosphoglycerate levels and reduced oxygen transport (Daniels *et al*, 2010) and/or that the AMP activates an intracellular energy depletion sensor, AMP-kinase (Melvin *et al*, 2009). The observation that metabolic rate reduction precedes the drop in Tb (Daniels *et al*, 2010) and our results that relatively small amounts of i.c.v. AMP elicit hypothermia and AMP-treated mice chose a cool environment, all indicate that AMP acts centrally to reduce the Tb set point, suggesting direct or indirect action at the hypothalamus. Muzzi et al. have shown that AMP injected into the mouse preoptic area induces hypothermia and that AMP reduces firing activity of preoptic area neurons in a DPCPX-sensitive manner (Muzzi *et al*, 2013). These observations and the *Adora1*$^{-/-}$ data can be reconciled by hypothesizing that AMP causes hypothermia via both A_1AR-dependent and A_1AR-independent mechanisms.

4.5 Fasting-induced hypothermia does not require A_1AR or A_3AR

Mice have a large surface area/volume ratio and therefore a disproportionally high energetic cost of staying warm, so they enter torpor to conserve energy (Abreu-Vieira *et al*, 2015; Hudson and Scott, 1979; Swoap, 2008). It is likely that the control of torpor is complex, integrating information on nutritional status, energy needs, and environmental situation. For example, leptin deficiency sensitizes to induction of torpor, but replacement of leptin in adipose-deficient mice does not prevent fasting-induced torpor (Gavrilova *et al*, 1999; Himms-Hagen, 1985).

There is much support for A_1AR having a central role in regulating torpor, including daily torpor in mice (Iliff and Swoap, 2012), seasonal torpor in arctic ground squirrel (Jinka *et al*, 2011), and hibernation in Syrian hamsters (Tamura *et al*, 2005). These conclusions are based on pharmacologic manipulation, showing torpor induction with agonist and exit with antagonist treatment. However, the observation that mice lacking A_1AR (or A_3AR, or both) enter torpor upon fasting demonstrates that A_1AR is not required. However, due to the intrinsic variability of fasting-induced hypothermia and the size of our dataset, we cannot rule out quantitative or probabilistic effects of A_1AR and/or A_3AR on this process. This

illustrates the utility of the knock out mice, highlights the limitations of purely pharmacologic approaches, and suggests searching for the other regulators of torpor-related states. Presumably, like leptin, A_1AR signaling is one of multiple inputs that are integrated to cause entry into (or exit from) torpor.

4.6 Limitations

Hypothermia can vary with inter-experiment differences, particularly in duration (with larger standard errors at longer times after dosing). Possible contributing factors include mouse age, weight, and adiposity, acclimation to handling, season, environmental temperature, and the noise/activity level in the animal facility. To assure that this variability does not compromise the conclusions, we focus on the all-or-none effects and have not attempted to quantitate the fraction of a mixed effect that is due to A_1AR versus A_3AR.

While knockout mice are excellent probes of *in vivo* ligand specificity, compensation for receptor loss may occur. No compensatory changes in *Adora1−/−* or *Adora3−/−* mice have been described (Johansson *et al*, 2001; Salvatore *et al*, 2000), but it is possible that undetected compensatory changes do occur. Importantly, there may also be redundant sites of action, for example AMP may act at both A_1AR and other site(s).

4.7 Common themes in A_1AR, A_3AR, and AMP Hypothermia

A_1AR and A_3AR agonists and AMP all trigger a regulated hypothermia that is not inhibited by D_2R antagonist. Yet the three act in different places in the body, with different ligands and ligand affinities. Extracellular adenosine (and AMP) can be thought of as indicators of physiologically unfavorable situations or ‘extreme physiology’ (Fredholm, 2014). It would be advantageous for the body to be able to broadly sense many forms of pathology and funnel the signals to common response mechanisms such as hypothermia. The ability to go into torpor is a widely conserved behavior that reduces injury, conserves energy, and increases survival. Understanding the neural regulation of hypothermia will identify signaling pathways and may lead to improved pharmacologic approaches to hypothermia induction and maintenance in clinical practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

i.p.	intraperitoneal
i.c.v.	intracerebroventricular
AxAR	adenosine x receptor

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Highlights

1. Some commonly used A_1AR agonists are not selective; they act at A_3AR
2. A_1AR, A_3AR, and AMP are three distinct adenosine-related inducers of hypothermia
3. Neither A_1AR nor A_3AR is required for fasting-induced torpor
4. AMP can cause hypothermia via the brain, independent of A_1AR and A_3AR

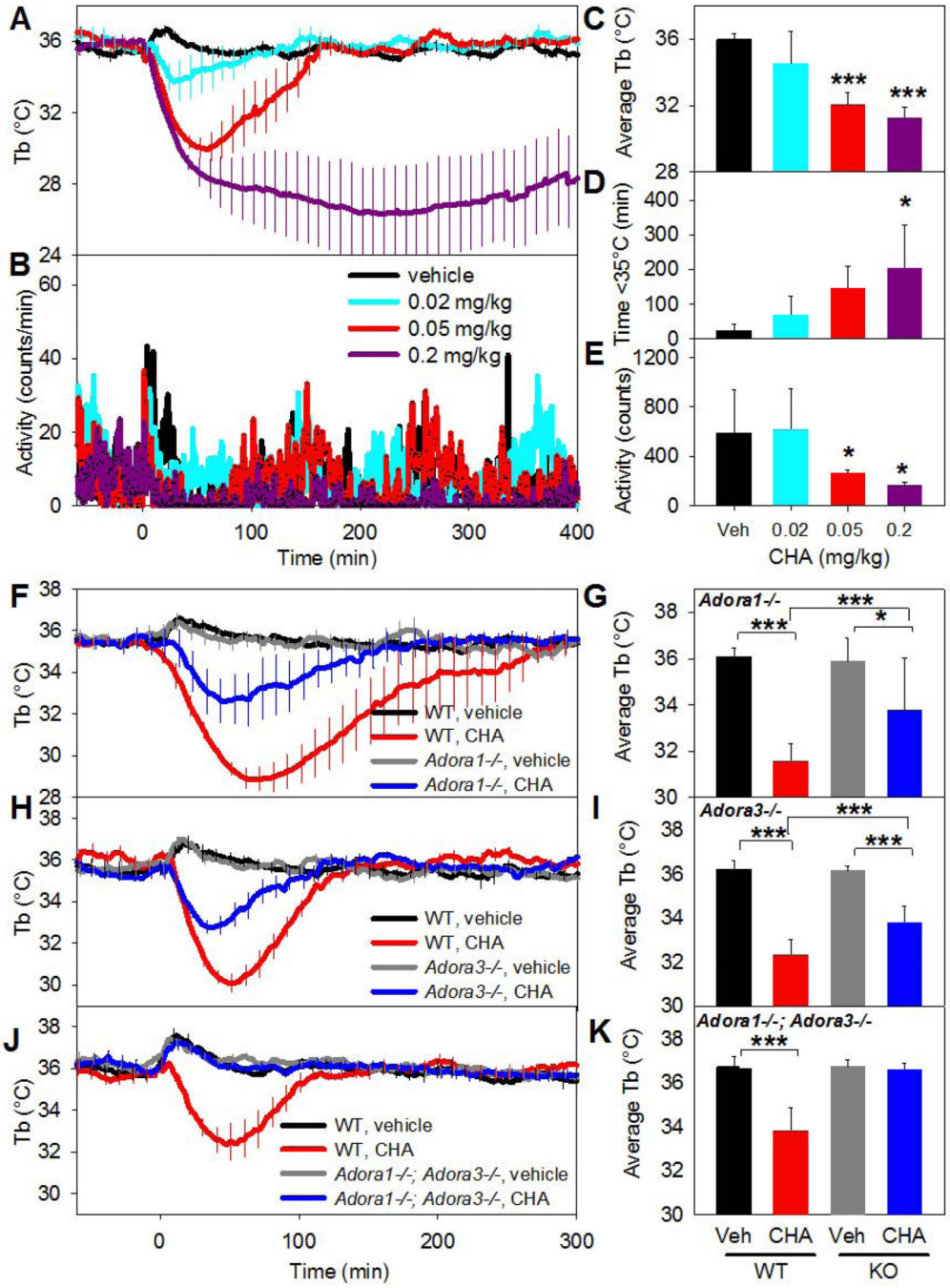

Fig. 1. Systemic CHA causes hypothermia and decreased physical activity through both A_1AR and A_3AR

(A, B) Tb and physical activity response to the indicated CHA doses injected i.p. into C57BL/6J mice. (C–E) Effect of CHA on average Tb (0–60 min), duration of hypothermia, and physical activity (10–60 min). Data are mean ± SEM, n=5/group; every tenth SEM is shown in A and SEMs were omitted in B for visual clarity. (F,G) Tb response to CHA (0.05 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora1*$^{-/-}$ (KO) mice. (H,I) Tb response to CHA (0.05 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora3*$^{-/-}$ (KO) mice. (J,K) Tb response to CHA (0.05 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora1*$^{-/-}$;*Adora3*$^{-/-}$

(DKO) mice. In F–K, data are mean ± SEM, n=5–10/group in a crossover design; every tenth SEM is shown in F, H, and J; * $p<0.05$, *** $p<0.001$.

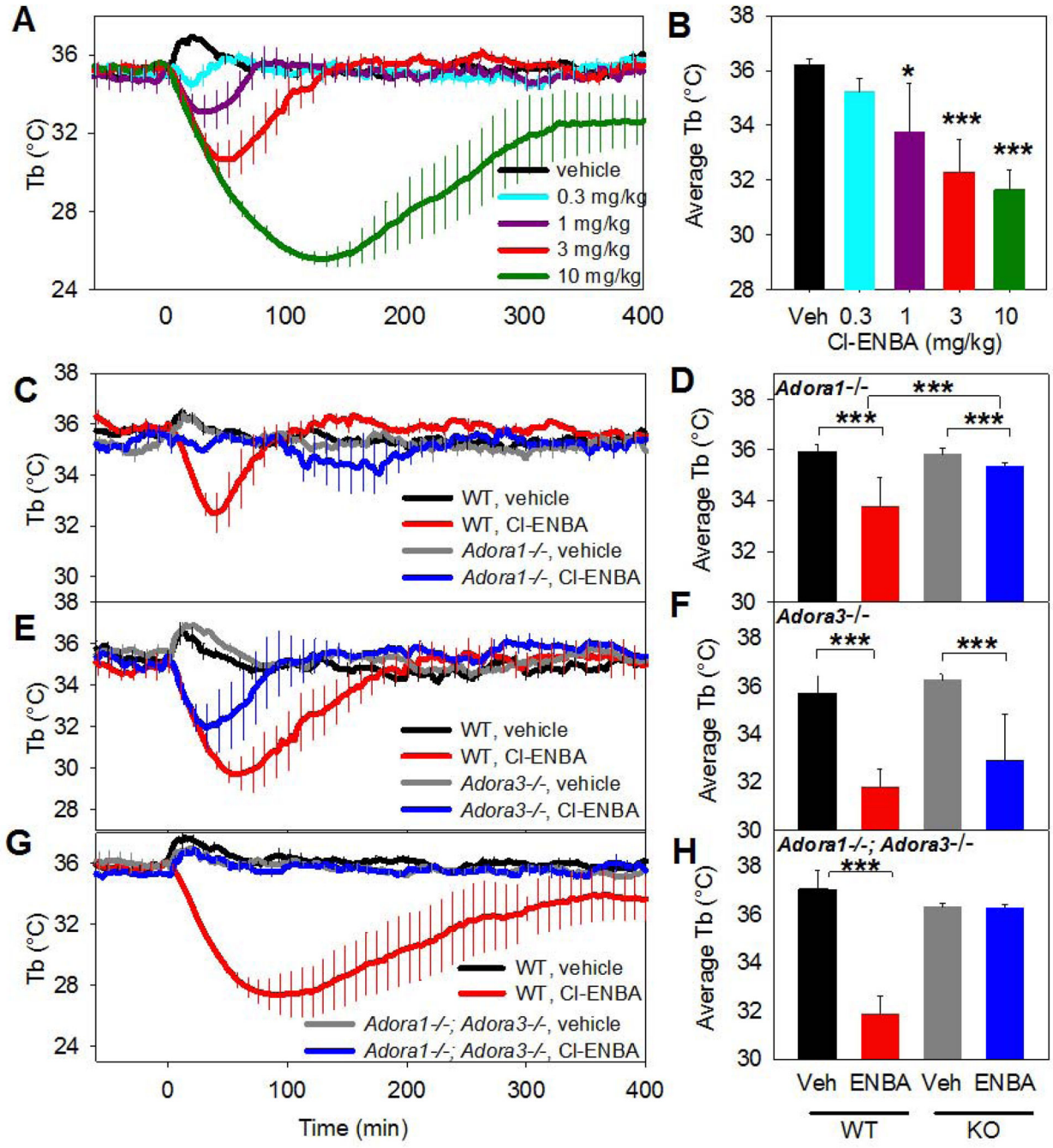

Fig. 2. Systemic Cl-ENBA acts largely via A_1AR to induce hypothermia

(A,B) Tb response to the indicated Cl-ENBA dose injected i.p. into C57BL/6J mice. (C,D) Tb response to Cl-ENBA (3 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora1*$^{-/-}$ (KO) mice. (E,F) Tb response to Cl-ENBA (3 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora3*$^{-/-}$ (KO) mice. (G,H) Tb response to Cl-ENBA (3 mg/kg, i.p.) or vehicle in C57BL/6J (WT) and *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ (KO) mice. Data are mean ± SEM, n=3–6/group in a crossover design; every tenth SEM is shown in C, E, and G; * p<0.05*** p<0.001.

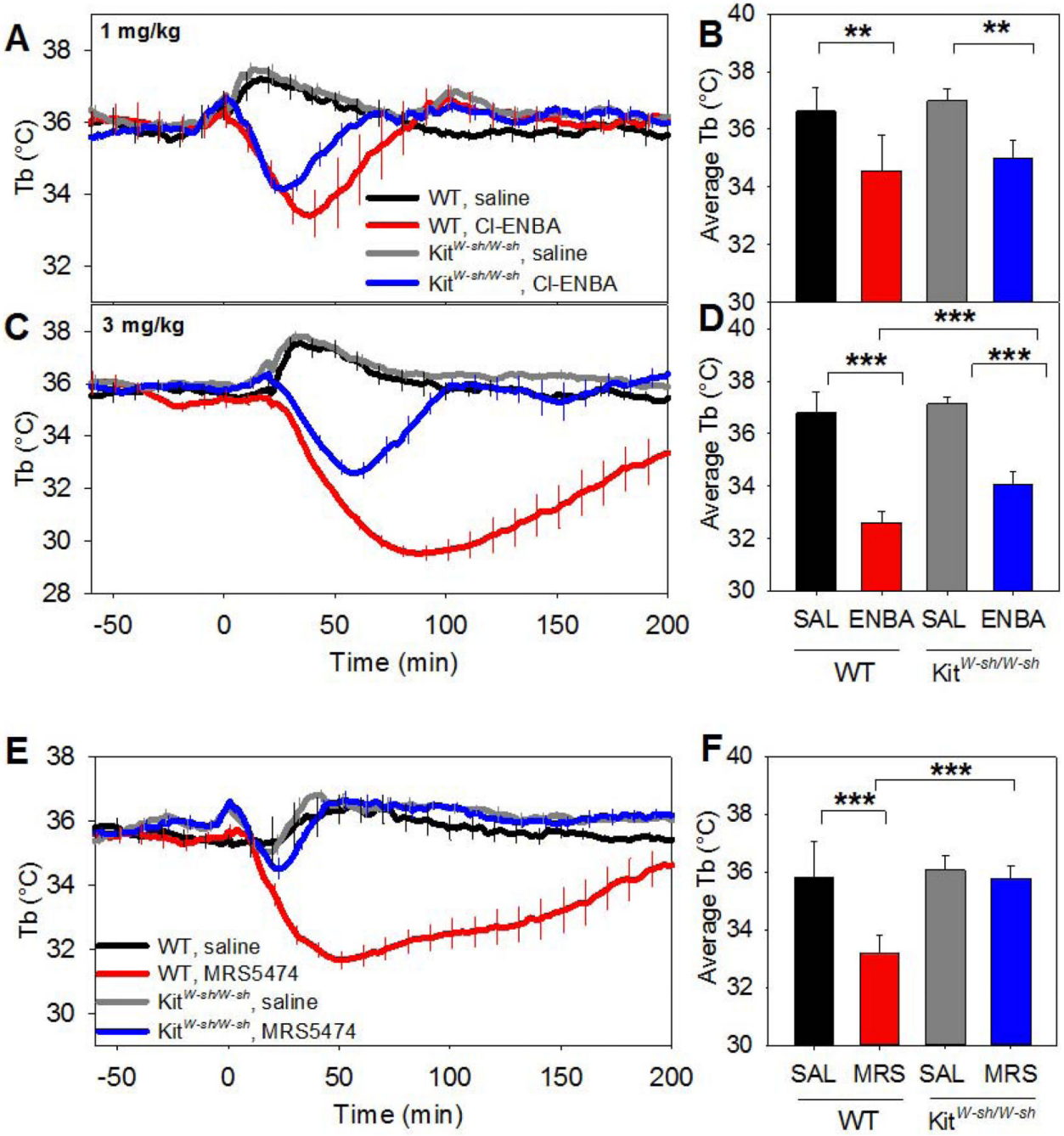

Fig. 3. A_1AR-mediated hypothermia is partially intact and A_3AR-mediated hypothermia is abolished in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice

(A,B) Tb response to Cl-ENBA (1 mg/kg, i.p.) in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. (C,D) Tb response to Cl-ENBA (3 mg/kg, i.p.) in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. (E,F) Tb response to MRS5474 (3 mg/kg, i.p.) in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Data are mean ± SEM, n=4–6/group in a crossover design; every tenth SEM is shown in A, C, and E; ** $p<0.01$.

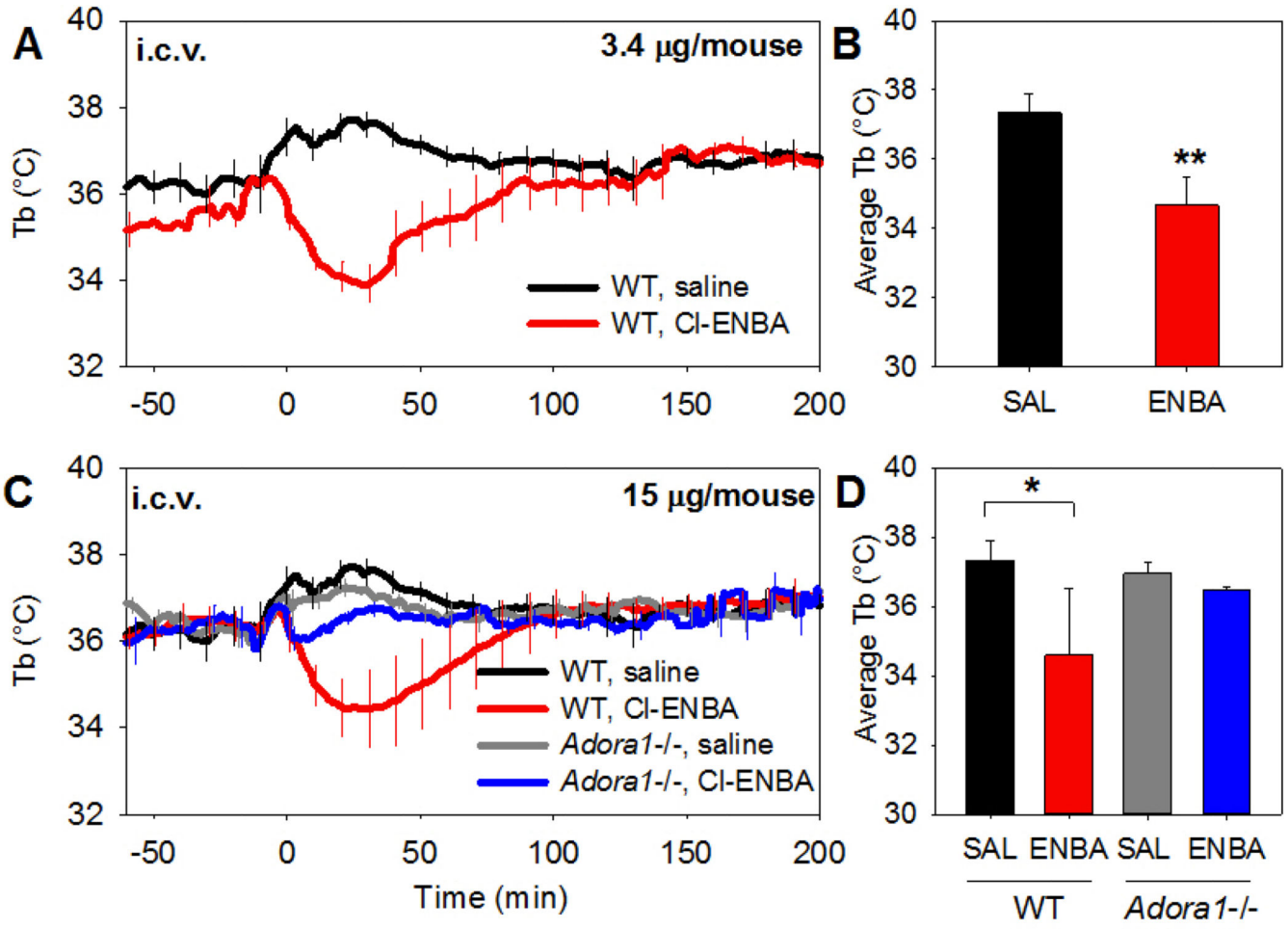

Fig. 4. Cl-ENBA causes hypothermia via central A_1AR

(A,B) Tb response to Cl-ENBA (3.4 µg/mouse) or vehicle injected i.c.v. into C57BL/6J. (C,D) Tb response to Cl-ENBA (15 µg/mouse) or vehicle injected i.c.v. into C57BL/6J or *Adora1*$^{-/-}$ mice. Data are mean ± SEM, n=3–6/group; every tenth SEM is shown in A, C; * p<0.05.

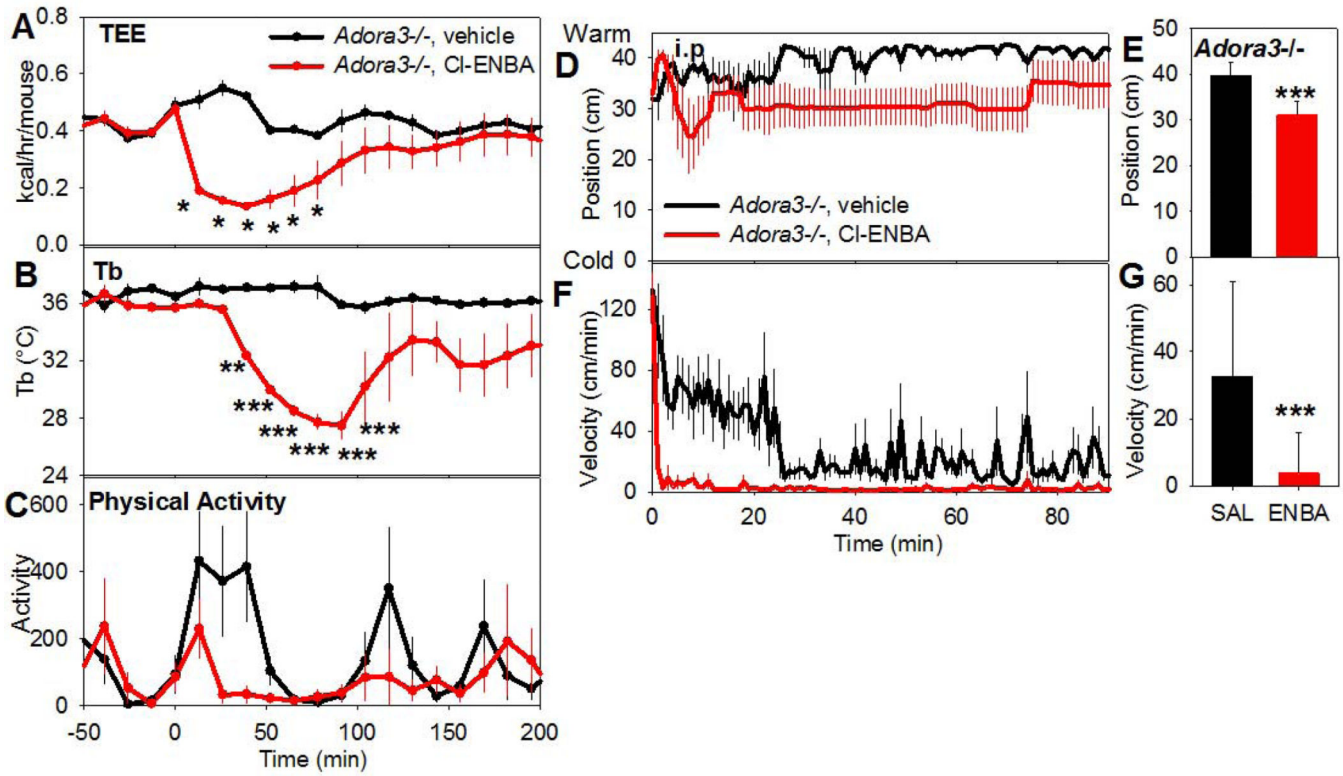

Fig. 5. Systemic Cl-ENBA-induced hypothermia is accompanied by a reduced metabolic rate and preference for a cooler environment

The effect of Cl-ENBA (3 mg/kg, i.p.) or vehicle in *Adora3*$^{-/-}$ mice on (A) total energy expenditure (TEE), (B) Tb, and (C) physical activity. The TEE falls before Tb (nadir ~50 min vs ~100 min for Tb). Data are mean ± SEM, n=5/group. Position (D,E) and activity (F,G) of *Adora3*$^{-/-}$ mice treated with Cl-ENBA (3 mg/kg, i.p.) or vehicle and placed in a thermal gradient. Mean ± SEM, n=6/group, *p<0.05, ***p<0.001.

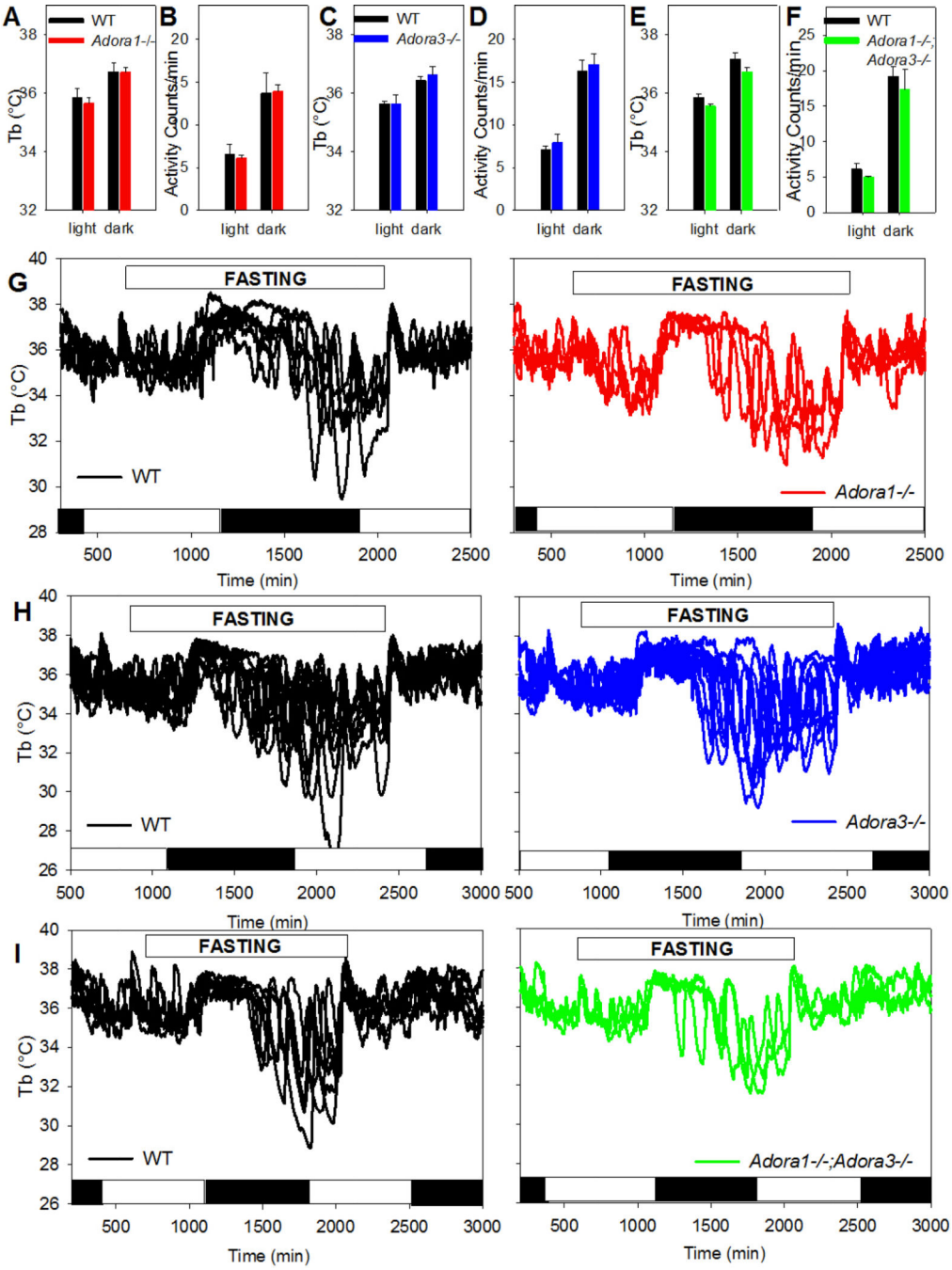

Fig. 6. A_1AR or A_3AR is not required for fasting-induced hypothermia

Baseline Tb (A) and activity (B) in WT (n=6) and *Adora1*$^{-/-}$ (n=4) littermates during the light or dark phase. Baseline Tb (C) and activity (D) in WT (n=5) and *Adora3*$^{-/-}$ (n=4) littermates during the light or dark phase. Baseline Tb (E) and activity (F) in C57BL/6J (n=6) and *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ (n=3) mice. during the light or dark phase. Individual traces of Tb in (G) WT and *Adora1*$^{-/-}$ littermates, (H) WT and *Adora3*$^{-/-}$ littermates, and (I) C57BL/6J and *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ mice during a 24 hour fast. White and black bars indicate light and dark phases, respectively. Data are mean ± SEM.

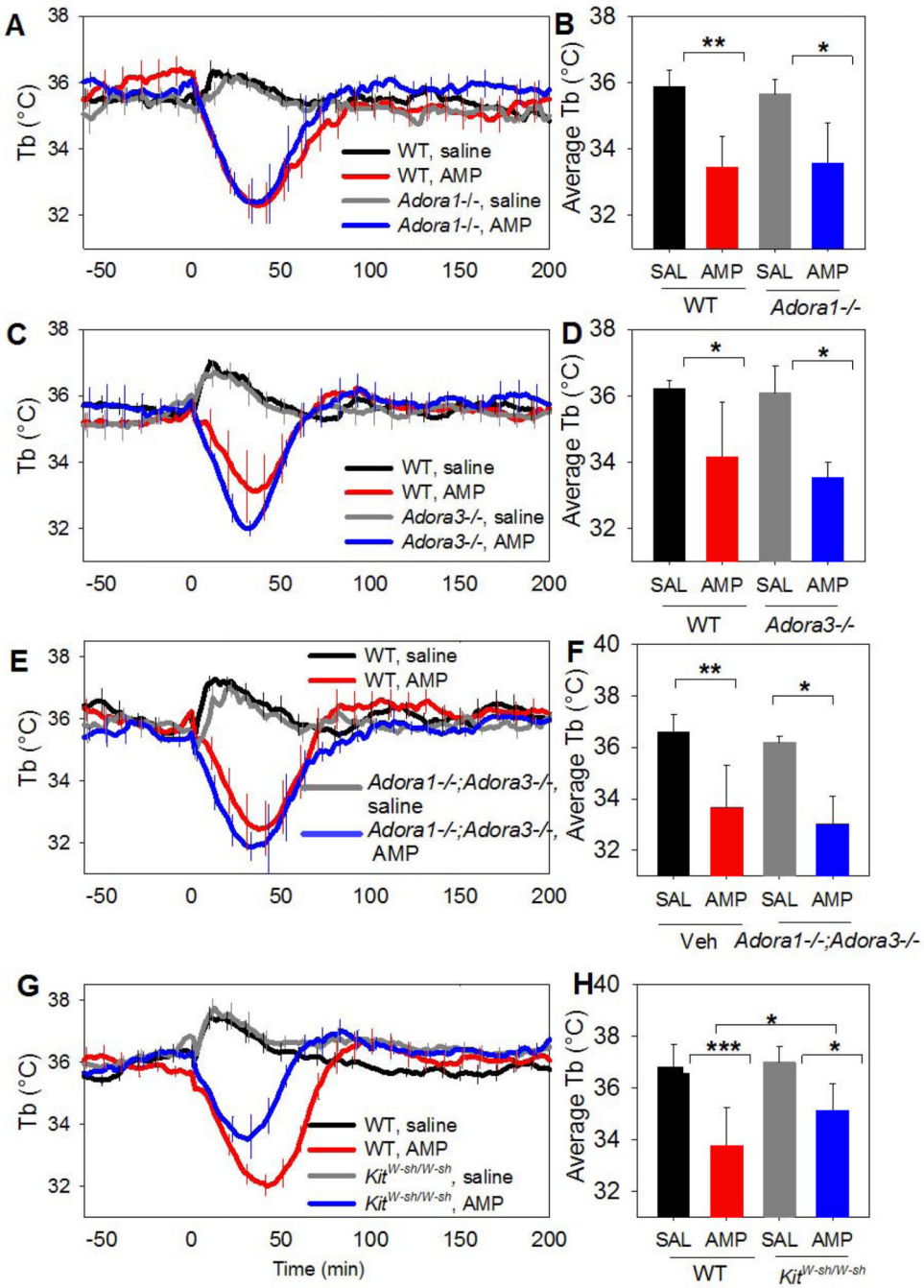

Fig. 7. Systemic AMP causes hypothermia independent of A_1AR, A_3AR, and mast cells

(A,B) Tb response to AMP (100 mg/kg, i.p.) in C57BL/6J (WT) and *Adora1*$^{-/-}$ (KO) mice (n=6/group). (C,D) Tb response to AMP (100 mg/kg, i.p.) in C57BL/6J (WT) or *Adora3*$^{-/-}$ mice (n=4/group). (E,F) Tb response to AMP (100 mg/kg, i.p.) in C57BL/6J and *Adora1*$^{-/-}$;*Adora3*$^{-/-}$ mice (n=5–6/group). (G,H) Tb response to AMP (100 mg/kg, i.p.) in C57BL/6J (WT) or *Kit*$^{W\text{-}sh/Wsh}$ mice (n=7–8/group). Data are mean ± SEM; every tenth SEM is shown in A, C, E, and G; * p<0.05, ** p<0.01, *** p<0.001.

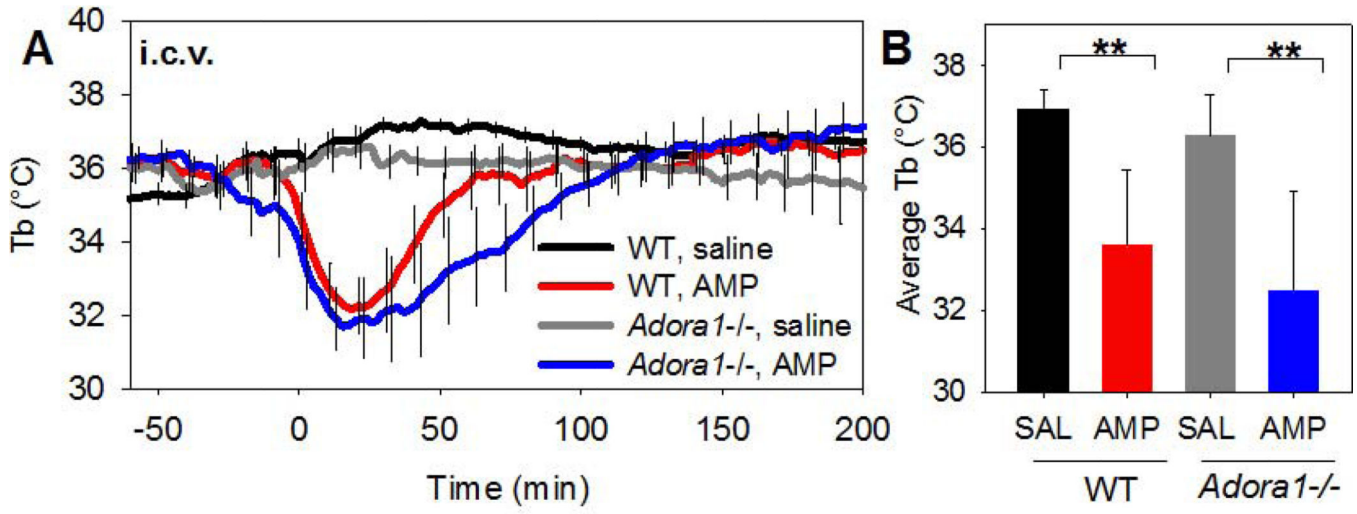

Fig. 8. Centrally-administered AMP induces hypothermia

(A,B) Tb response to 100 μg (~3.3 mg/kg) AMP injected i.c.v. into C57BL/6J mice or *Adora1*$^{-/-}$ (n=4/group). Data are mean ± SEM; every tenth SEM is shown; * $p<0.05$, ** $p<0.01$.

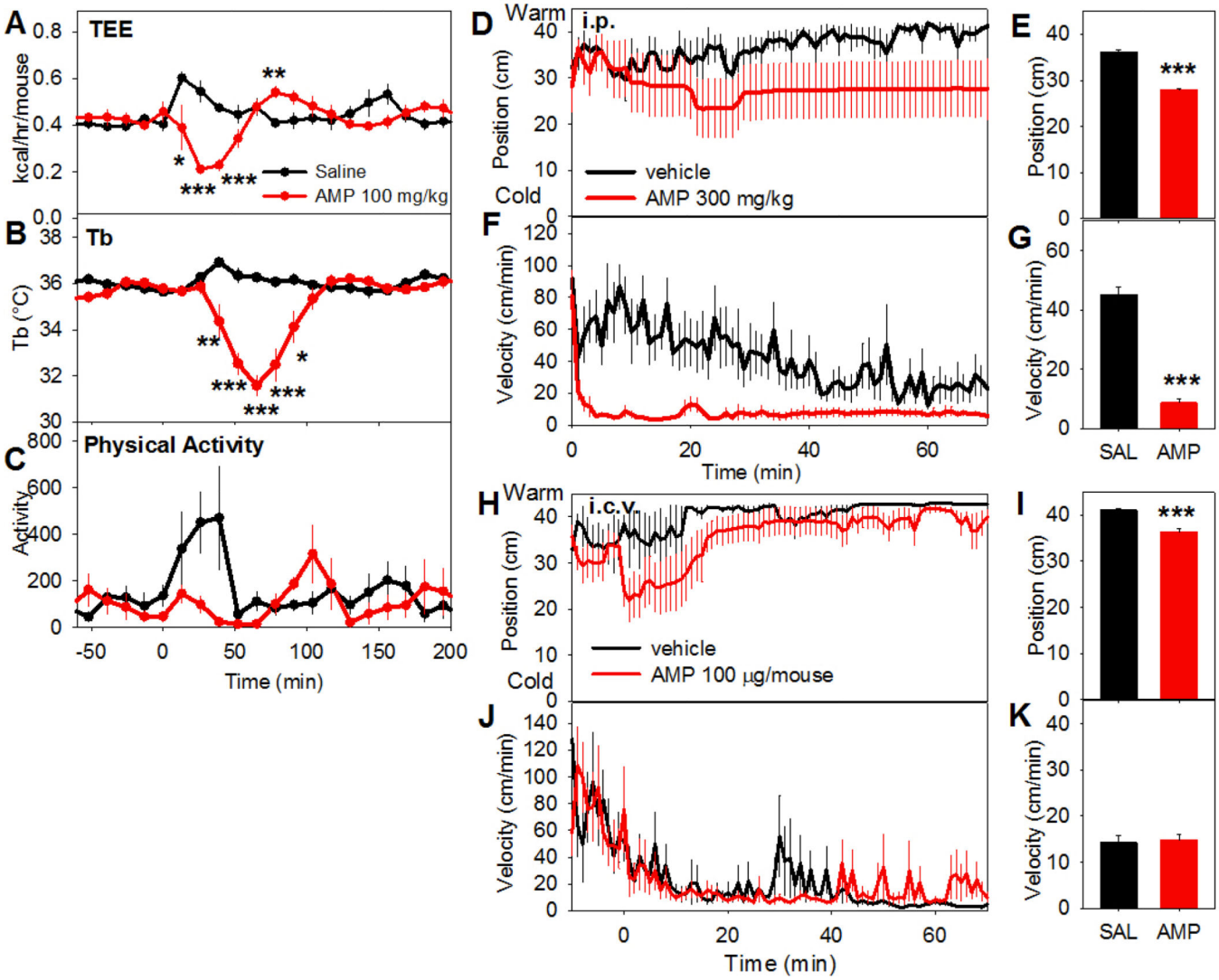

Fig. 9. AMP-induced hypothermia is accompanied by a reduced metabolic rate and preference for a cooler environment

The effect of AMP (100 mg/kg, i.p.) or vehicle in C57BL/6J mice on (A) total energy expenditure (TEE), (B) Tb, and (C) physical activity in a calorimetry chamber. The TEE falls before Tb (nadir ~40 min vs ~60 min). Data are mean ± SEM, n=5/group. Position (D,E) and activity (F,G) of C57BL/6J mice treated with AMP (300 mg/kg, i.p.) or vehicle and placed in a thermal gradient. Position (H,I) and activity (J,K) of C57BL/6J mice treated with AMP (100 µg, i.c.v.) or vehicle and placed in a thermal gradient. Mean ± SEM, n=5–6/group, *p<0.05, ***p<0.001

Table 1

Ligand binding affinity at adenosine receptors.

K_i, nM (*or % inhibition at 10 μM*)

	Mouse			Human		
	A_1AR	**A_{2A}AR**	**A_3AR**	**A_1AR**	**A_{2A}AR**	**A_3AR**
Agonists						
IB-MECA[a,b]	5.9	~1000	0.087	51	2900	1.8
Cl-IB-MECA[a,b]	35	~10,000	**0.18**	220	5400	**1.4**
MRS5474[c,d]	**3.20±0.05**	*34±9%*	1056±251	50	3950	470
MRS5698[e,e]	*14%*	*27%*	**3.1**	*6%*	*41%*	**3.5**
MRS5841[f,f]	*15%*	*1%*	**11**	*16%*	*7%*	**1.9**
MRS5980[g,g]	*38%*	*7%*	**36**	*6%*	*24%*	**0.70**
CCPA[h,i]	0.27	988	16	0.83	2270	43
CPA[c,i]	**0.22±0.01**	808±89	534±14	2.3	794	43
CHA[c,j]	**2.15±0.37**	1695±60	611±12	2.4	1390	73
SPA[c,c]	**1.08±0.04**	*33±2%*	3111±183	7.92±1.55	3778±890	246±21
Cl-ENBA[c,k]	**0.20±0.01**	3985±358	2414±325	**0.51**	1340	1290
CGS21680[c,i]	193±38	10±2	48±11	289	27	67
Antagonists						
MRS1523[n,l]	5,330	*0%*	702	>10,000	3660	**19**
MRS1191[c,l]	*0%*	*0%*	*32±3%*	>10,000	>10,000	**31**
SCH442416[c,m]	765±36	**1.27±0.04**	*6±2%*	*35%*	**4.1**	*67%*
DPCPX[n,i]	**1.5**	598	*0%*	3.9	129	3960

Bold indicates a K_i ≥100-fold lower than the other two receptors of the same species. Superscripts indicate the references for literature data (mouse, human). SEMs are included for experimental results.

[a](Ge *et al*, 2006)

[b](Tchilibon *et al*, 2005)

[c] current results

[d] (Tosh *et al*, 2012b)

[e] (Tosh *et al*, 2012a)

[f] (Paoletta *et al*, 2013)

[g] (Tosh *et al*, 2014)

[h] (Alnouri *et al*, 2015)

[i] (Klotz *et al*, 1998)

[j] (Ferkany *et al*, 1986; Gao *et al*, 2003; Stone *et al*, 1988)

[k] (Franchetti *et al*, 2009)

[l] (Liang *et al*, 2010)

[m] (Kumar *et al*, 2011)

[n] (Kreckler *et al*, 2006)