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ORIGINAL ARTICLE

In vivo phenotypic validation of adenosine receptor-dependent activity of non-adenosine drugs

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Abstract

Somenon-adenosinergic drugs are reported to also act through adenosine receptors (ARs). We used mouse hypothermia, which can be induced by agonism at any of the four ARs, as an in vivo screen for adenosinergic effects. An AR contribution was identified when a drug caused hypothermia in wild type mice that was diminished in mice lacking all four ARs (quadruple knockout, QKO). Alternatively, an adenosinergic effect was identified if a drug potentiated adenosine-induced hypothermia. Four drugs (dipyridamole, nimodipine, cilostazol, cyclosporin A) increased the hypothermia caused by adenosine. Dipyridamole and nimodipine probably achieved this by inhibition of adenosine clearance via ENT1. Two drugs (cannabidiol, canrenoate) did not cause hypothermia in wild type mice. Four other drugs (nifedipine, ranolazine, ketamine, ethanol) caused hypothermia, but the hypothermia was unchanged in QKO mice indicating non-adenosinergic mechanisms. Zinc chloride caused hypothermia and hypoactivity; the hypoactivity was blunted in the QKO mice. Interestingly, the antidepressant amitriptyline caused hypothermia in wild type mice that was amplified in the QKO mice. Thus, we have identified adenosine-related effects for some drugs, while other candidates do not affect adenosine signaling by this in vivo assay. The adenosine-modulating drugs could be considered for repurposing based on predicted effects on AR activation.

Keywords Purinergic receptors · Nucleosides · Adenosine receptor · Prodrug · Pain · Steatohepatitis · Drug repurposing · Equilibrative nucleoside transport · Hypothermia · Knockout mice

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Introduction

Adenosine acts as an autocrine or paracrine signal by activating four G protein-coupled receptors (adenosine receptors, ARs) that have been the focus of extensive medicinal chemical and drug development efforts [1–7]. Generally, extracellular adenosine elicits protective actions to restore the stability of an organism in response to challenges or stresses. The ARs are distributed

widely throughout the body and mediate local, often tissue-specific, effects [1, 2]. There is a rich history and experience in the development of selective synthetic AR agonists [2, 3, 5, 7].

In addition to drugs developed for their agonism or antagonism at the four ARs, some drugs designed for other actions coincidentally act as AR antagonists [8]. One example of off-target binding of an approved drug is the antimalarial mefloquine, which is an A_{2A} receptor antagonist [9]. In fact, most ligand chemotypes found fortuitously or by computational approaches to bind to ARs do so as antagonists [10–12]. Other drugs with coincidental AR antagonism include experimental Alzheimer's drug etazolate, dopamine agonist (3,4-dihydroxy-phenylamino)-2-imidazoline (DPI) [10, 13], anxiogenic β-carbolines (e.g. methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) [14], flavonoid derivatives hispidol and galangin [15], and 1,4-dihydropyridines such as nicardipine [16]. Drugs listed in databases as binding to the A_{2A}AR include tamoxifen, imiquimod, and sildenafil [17] (Table S1).

Other drugs enhance AR signaling indirectly, by increasing adenosine availability to the ARs. This can occur by inhibiting adenosine's cellular uptake, metabolism, and/or degradation, raising extracellular adenosine levels [18–20]. For example, diverse compounds inhibit nucleoside uptake through the equilibrative transporters (ENT1–3) or concentrative (CNTs) transporters of the SLC29 family. The antithrombotic $P2Y_{12}$ receptor antagonist ticagrelor and ethanol and cannabidiol are all reported to raise adenosine levels by inhibiting its transport [21–24]. In a screen of 1625 diverse molecules, more than half bound to ENT1 with a K_i value < 10 μM [25], suggesting that additional drugs may share this property. Other compounds inhibit intracellular adenosine kinase, thereby reducing cellular uptake of adenosine via equilibrative transporters. The antimetabolite methotrexate increases intracellular adenosine levels (and thus indirectly extracellular levels) by increasing levels of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), and this is proposed to contribute to methotrexate's therapeutic benefit in rheumatoid arthritis [26]. For still other compounds, the mechanism of action (MoA) leading to activation of one or more AR subtypes is unknown [8].

Pharmacological modulation of ARs can be evaluated in vivo using mouse models in which specific components of the signaling pathways are genetically deleted [27, 28]. Mice with one or more of the ARs genetically knocked out (KO mice), either globally or in a tissue-specific manner, are important tools for exploring the interaction of drugs with pathways [27–31]. They are particularly useful for the ARs, since adenosine often acts locally so the relevant primary site of systemic adenosinergic drug action can be difficult to determine. A mouse line in which all four ARs have been globally deleted is useful for investigating adenosine physiology [27]. At baseline these mice resemble wild type mice by most criteria examined, including body temperature regulation (diurnal variation, response to stress, and torpor), suggesting that the ARs are more important in allostatic rather than homeostatic functions.

Adenosine can cause hypothermia (and hypoactivity) in mice by individual activation of each of the four AR [29, 30, 32], and the quadruple AR knockout mice (QKOs) no longer respond to adenosine administration [27]. Here we evaluated compounds that have previously been reported to have adenosinergic actions, using mouse hypothermia as a sensitive, standardized assay, with a goal of identifying drugs that might be repurposed for altering adenosinergic signaling.

Methods

Chemicals and mice

Chemicals were of reagent grade and obtained from Sigma-Aldrich (St. Louis, MO), unless noted. All compounds were administered intraperitoneally (i.p., 10 ml/g body weight). Cannabidiol (CBD, stored at –80 °C) was dissolved freshly in 1:1:18 dimethyl sulfoxide (DMSO):Tween 80:saline prior to the injection (10 mg/kg). Cilostazol (10 mg/kg), dipyridamole (10 and 30 mg/kg), nimodipine (10, 20, and 30 mg/kg), and nifedipine (10 and 20 mg/kg, Tocris), sildefanil citrate (1, 3, 10, and 30 mg/kg), cyclosporine (30 mg/kg, Tocris) were dissolved in 15:15:70 DMSO: Kolliphor EL: saline. Canrenoate (0.3, 1 and 3 mg/kg), $ZnCl_2$ (1, 3, 10 and 30 mg/kg), ethanol (1, 2, and 3 g/kg), and ketamine (Zetamine, VetOne, 1, 3, 10, and 30 mg/kg) were dissolved in saline, ranolazine (25 and 50 mg/kg) in PBS, and amitriptyline (20 mg/kg) in 10% DMSO. The animal protocol for the in vivo studies was approved by the NIDDK Animal Care and Use Committee. All experiments were performed on male mice. QKO mice on a mixed genetic background were generated as reported and compared to male wild type C57BL/6 J mice (Jackson Laboratories, Bar Harbor, ME) as controls [27]. Mice were kept at ~ 21–22 °C in a 12:12-h light–dark cycle, and chow (NIH-07, Envigo Inc., Madison, WI) and water were provided ad libitum.

Body temperature

Surgical operations to implant G2 E-mitters intraperitoneally were performed on the mice at least seven days prior to experimentation. Core body temperature (Tb) and locomotor activity were measured continuously by telemetry (ER4000

energizer/receivers, and VitalView software, Starr Life Sciences, Oakmont, PA) with data collection intervals of 1 min. The Tb response was followed for up to 24 h after drug injection. The indicated drug was injected 20–25 min before adenosine (100 mg/kg, i.p.). Two standard analysis intervals were used to calculate mean Tb. Using 0–60 min after injection (timing from the second injection when two injections were done) includes the increase in Tb and physical activity due to handling and is a sensitive measure, able to detect transient or small hypothermic effects. The mean Tb and time below 34 °C measured 0–300 min after injection better discriminate larger, longer duration effects.

Statistics

All data are expressed as the mean ± SEM. Data were tested for statistical significance.

by two-tailed, unpaired Student's *t* test, or two-way ANOVA followed by post hoc Holm-Sidak multiple comparison tests as appropriate. A *P* value of less than 0.05 was considered significant.

Results

To assess in vivo action of putative adenosinergic drugs, we used mouse hypothermia. Single doses of test compounds were administered i.p., and the effect on Tb and locomotor activity were monitored by telemetry in C57BL/6 J (WT) mice. An AR contribution was identified when a drug caused hypothermia in WT mice that was diminished in mice lacking all four AR receptors (QKO mice). In addition, we also tested the ability of drugs to potentiate adenosine-induced hypothermia in WT mice. The properties of the tested compounds and the proposed interaction with adenosine system are shown in Table 1; the summary of the results is in Table 2.

Drugs inhibiting adenosine transport

Inhibition of adenosine transport (Table 1) increases extracellular adenosine concentrations [2]. We reported previously that the benchmark ENT1 inhibitor 6-*S*-[(4-nitrophenyl)methyl]-6-thioinosine (NBMPR, 1 mg/kg i.p.) induced a slight hypothermia in mice and profoundly increased

Table 1 Compounds examined for effects on adenosinergic signaling

Compound	Use or indication[a]	Main mechanism	Proposed Interaction with adenosine system	Human ENT1, K_i or K_D (nM)	Reference for adenosine interaction
Dipyridamole	Vasodilator	PDE3 inhibitor, ENT1 inhibitor	ENT1 inhibition	2.6	[21]
Nimodipine	Subarachnoid hemorrhage	Ca^{+2} channel blocker	ENT1 inhibition	52	[33]
Cilostazol	Anti-claudication	PDE3 inhibitor	ND[b]	10,000	[25]
Cyclosporine A	Immuno-suppressant	Calcineurin inhibitor	Inhibition of adenosine uptake	ND	*
Cannabidiol	Antiepileptic	unclear	ENT1 inhibition	200	[23, 36–38]
Canrenoate	Diuretic	Mineralocorticoid antagonist	ND	ND	[40]
Nifedipine	Anti-hypertensive	Ca^{+2} channel blocker	ND	13,700	[43]
Ranolazine	Anti-angina	Sodium current inhibition	Increased cardiac adenosine	ND	[44]
Ketamine	Antidepressant	NMDA receptor antagonist	Stimulation of presynaptic A_1AR	ND	[46]
Ethanol	Psychoactive drug	Ion channel modulator	Multiple	200,000,000	[47–49]
Zinc chloride	Zinc deficiency	Unclear	ND	ND	[50]
Amitriptyline	Antidepressant	Serotonin/norepi-nephrine reuptake inhibitor	ND	ND	[51]
Sildenafil	Erectile dysfunction, vasodilator	PDE5 inhibitor	ND	ND	**, ***

[a]All except ethanol are approved by the United States FDA

[b]ND, not determined

*Guieu et al. [34]

**Lee et al. [43]

***Table S1

Table 2 Summary of drugs screened for adenosinergic effects using mouse hypothermia. [a]All drugs given i.p. [b]100 mg/kg adenosine, i.p. [c]Indicates significant drug × adenosine interaction by two-way ANOVA. [d]Indicates additive drug and adenosine effects without significant interaction by two-way ANOVA. [e]ND, not determined. [f]QKO has more hypothermia. Color key: Green indicates significant reduction. Orange indicates the effect in QKO is different from wild type mice. Blue indicates that drug + adenosine has a different effect than either alone. Gray indicates that results demonstrate the drug has an adenosinergic effect

Compound	Figure	Dose (mg/kg)[a]	Tb effect in WT mice	Activity effect in WT mice	Effect in QKO	Pretreatment effect on adenosine-induced hypothermia[b]	Evidence of adenosinergic actions?	Mechanistic interpretation
dipyridamole	**1**	10	no effect	no effect	same as WT (Tb and activity)	potentiated hypothermia and activity decrease (interaction[c])	yes	ENT1 inhibition
nimodipine	**2**	10	initial reduction (possible later increase associated with physical activity)	initial reduction followed by later increase	hypothermia reduced (interaction)	greater hypothermia and activity decrease (additive[d])	yes	consistent with ENT1 inhibition and non-adenosine effect
		20	reduction	reduction	hypothermia reduced (interaction)	ND[e]		
cilostazol	**3**	10	no effect (non-significant decrease)	reduction	ND	greater hypothermia and activity decrease (additive)	yes	
cyclosporine A	**4**	30	reduction	reduction	similar to WT (Tb and activity)	greater hypothermia and activity decrease (interaction)	yes	consistent with transporter inhibition and non-adenosine effect
cannabidiol	**S1**	10	no effect	no effect	ND	no effect	no	
canrenoate	**S2**	0.3, 1, 3	no effect	no effect	ND	ND	no	
sildenafil	**S3**	1, 3, 10, 30	no effect	no effect	ND	ND	no	
nifedipine	**5**	10	reduction	no effect	same as WT	additive hypothermia and activity decrease (have different kinetics)	no	
		20	reduction	no effect	ND	ND		
ranolazine	**6**	12.5, 25	no effect	no effect	ND	ND	no	
		50	reduction	reduction	same as WT (Tb and activity)	no effect		
ketamine	**S4**	1, 3, 10, 30	reduction at 30 mg/kg	no effect	same as WT (Tb and activity)	ND	no	
ethanol	**S5**	1000	no effect	no effect	ND	ND	no	
		2000	slight reduction	no effect	ND	no effect		
		3000	reduction	no effect	same as WT (Tb and activity)	ND		
zinc chloride	**7**	1, 3, 10, 30	dose-dependent reduction, significant at ≥10 mg/kg	dose-dependent reduction, significant at ≥10 mg/kg	10 mg/kg: same decreased Tb; slightly less decrease in activity (interaction) as WT control	ND	yes	
amitriptyline	**8**	20	reduction	reduction	***greater*** Tb decrease (interaction); ***greater*** activity decrease (interaction)	ND	yes	AR contribution appears to lessen the hypothermia (QKO has more hypothermia)

the hypothermic effect of a subsequent dose of adenosine (100 mg/kg i.p.) [27].

The vasodilator dipyridamole inhibits both PDE3 and ENT1 [21]. Dipyridamole (10 mg/kg i.p.) itself produced no hypothermia but increased the hypothermia caused by subsequent adenosine (100 mg/kg i.p.), with a significant adenosine × dipyridamole interaction (Fig. 1, Table S1). The likely explanation for dipyridamole's adenosinergic effects is inhibition of ENT1. However, a higher dipyridamole dose (30 mg/kg i.p.) increased activity (Fig. 1c, f) with a nonsignificant rise of Tb (Fig. 1a, b, d). This dose has not been tested in QKO mice or studied further.

The Ca^{2+} channel blocker nimodipine is coincidentally an ENT1 inhibitor [33]. Nimodipine (10, 20 mg/kg i.p.) caused hypothermia and hypoactivity that were diminished in QKO mice (Fig. 2a–l). Nimodipine treatment also augmented adenosine-induced hypothermia (Fig. 2m–r). Thus, nimodipine has both adenosinergic (such as via ENT1 inhibition) and non-adenosinergic (not lost in the QKO mice) actions.

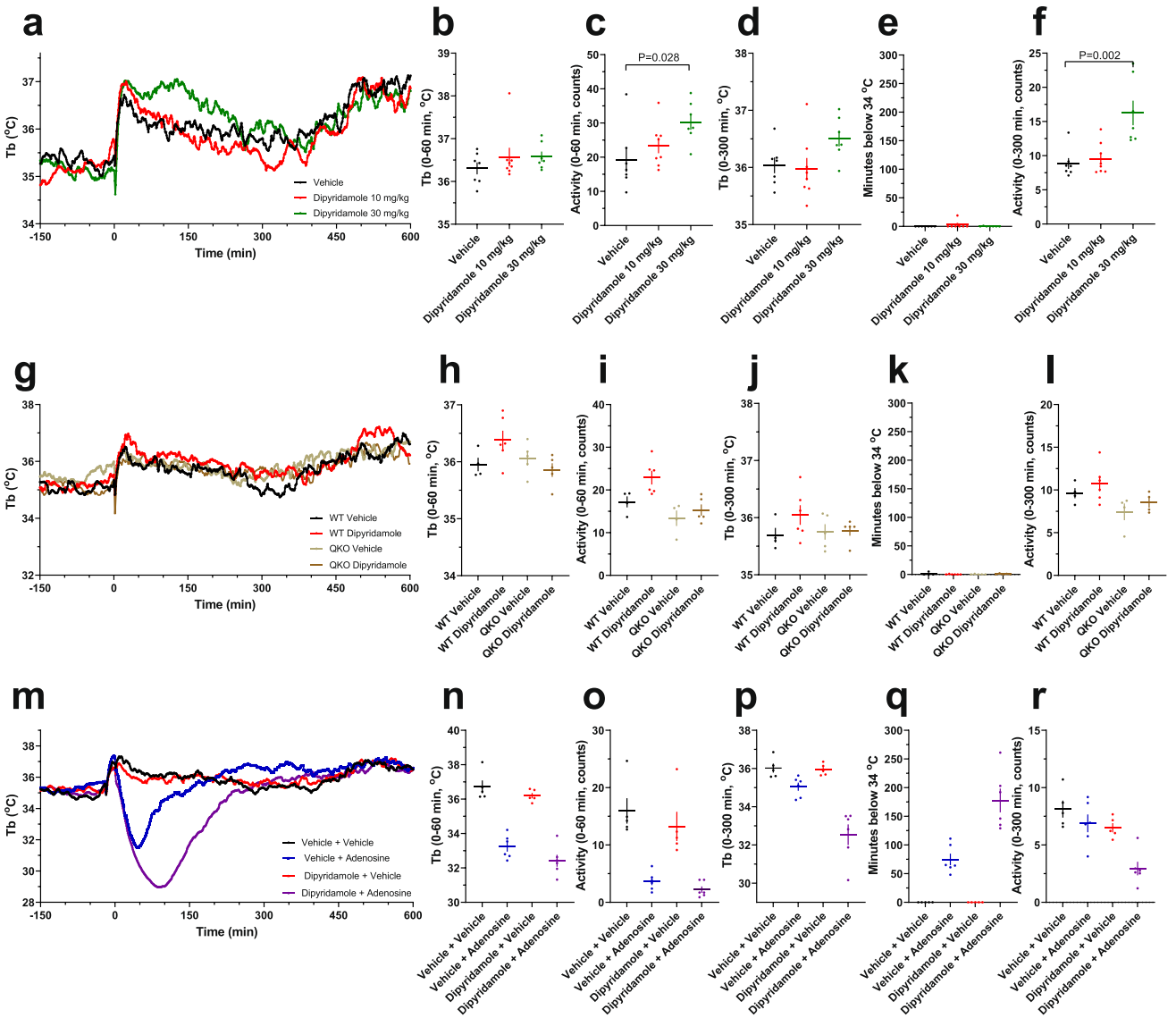

Fig. 1 Effects of dipyridamole. **a** Vehicle or dipyridamole (10 or 30 mg/kg) treatment of WT mice. **b** Mean Tb at 0–60 min, **c** mean activity at 0–60 min, **d** mean Tb at 0–300 min, **e** time below 34 °C, and **f** mean activity at 0–300 min; $n = 7$–8/group. **g** Treatment of QKO and control mice with dipyridamole (10 mg/kg). **h** Mean Tb at 0–60 min, **i** mean activity at 0–60 min, **j** mean Tb at 0–300 min, **k** time below 34 °C, and **l** mean activity at 0–300 min; $n = 4$–6/group. **m** Effect of dipyridamole (10 mg/kg) pretreatment on adenosine (100 mg/kg) induced hypothermia. **n** Mean Tb at 0–60 min, **o** mean activity at 0–60 min, **p** mean Tb at 0–300 min, **q** time below 34 °C, and **r** mean activity at 0–300 min; $n = 5$–6/group. Statistical analyses are in Table S2

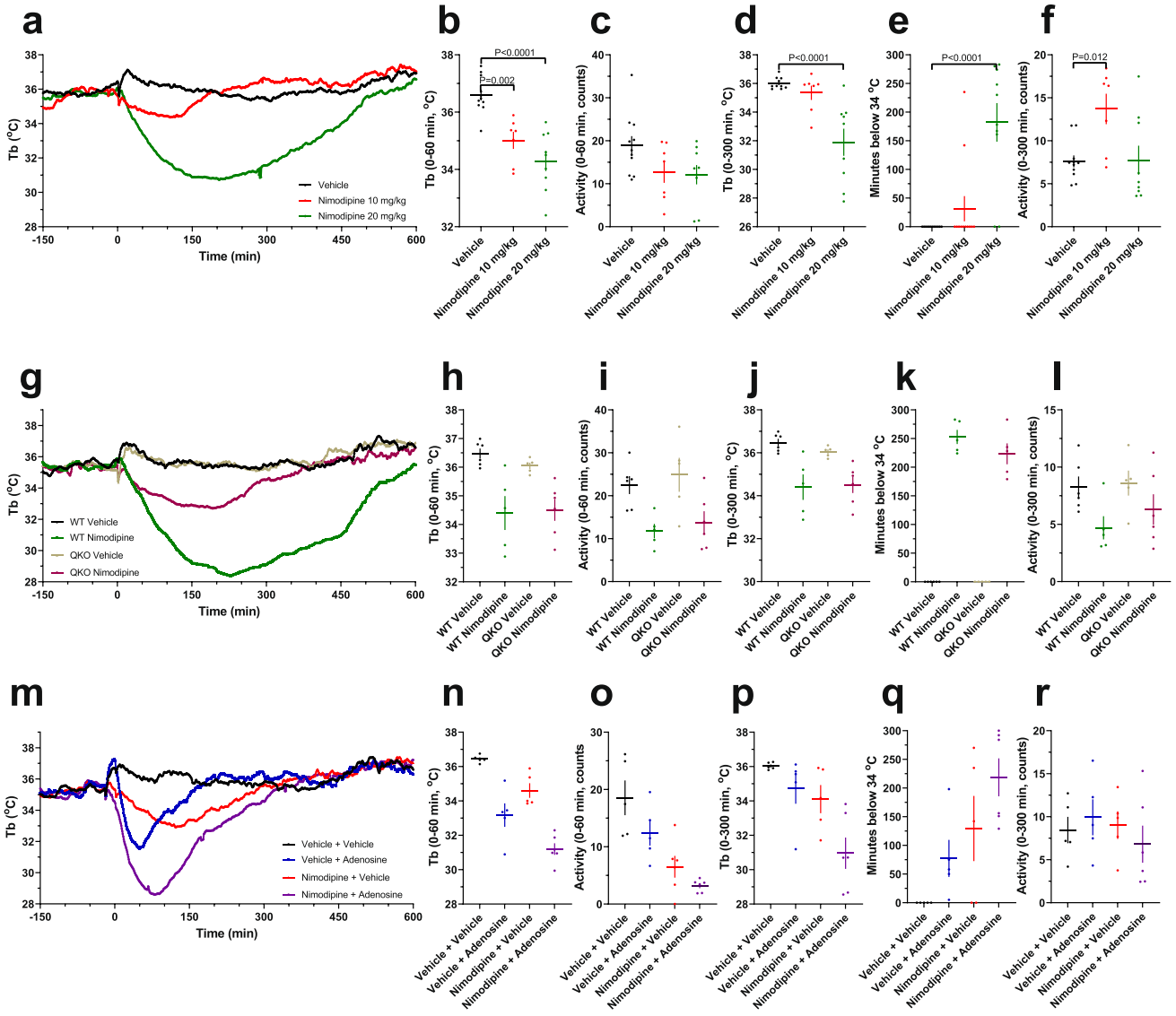

Fig. 2 Effects of nimodipine. **a** Vehicle or nimodipine (10 or 20 mg/kg) treatment of WT mice. **b** Mean Tb at 0–60 min, **c** mean activity at 0–60 min, **d** mean Tb at 0–300 min, **e** time below 34 °C, and **f** mean activity at 0–300 min; $n = 7$–11/group. **g** Treatment of QKO and control mice with nimodipine (20 mg/kg). **h** Mean Tb at 0–60 min, (**i**) mean activity at 0–60 min, **j** mean Tb at 0–300 min, **k** time below 34 °C, and **l** mean activity at 0–300 min; $n = 5$–6/group. **m** Effect of nimodipine (10 mg/kg) pretreatment on adenosine (100 mg/kg) induced hypothermia. **n** Mean Tb at 0–60 min, **o** mean activity at 0–60 min, **p** mean Tb at 0–300 min, **q** time below 34 °C, and **r** mean activity at 0–300 min; $n = 5$–6/group. Statistical analyses are in Table S2

The quinolinone PDE3 inhibitor cilostazol [25] caused hypoactivity and non-significant hypothermia and augmented adenosine-induced hypothermia but is a poor ENT1 inhibitor (Fig. 3). Taken together, these results suggest that at the doses tested, dipyridamole and nimodipine have some adenosinergic effects, likely by ENT1 inhibition, while cilostazol has a different mode of action.

The immunosuppressive drug cyclosporine A, a calcineurin inhibitor, was reported to inhibit adenosine uptake by red blood cells [34] and T-lymphocytes [35]. Cyclosporine A (30 mg/kg, i.p.) caused a slight hypothermia and hypoactivity that were not clearly reduced in QKO mice (Fig. 4a–f). However, cyclosporine treatment significantly increased adenosine-induced hypothermia (Fig. 4g–l). These data suggest that cyclosporine A has both adenosinergic and non-adenosinergic action.

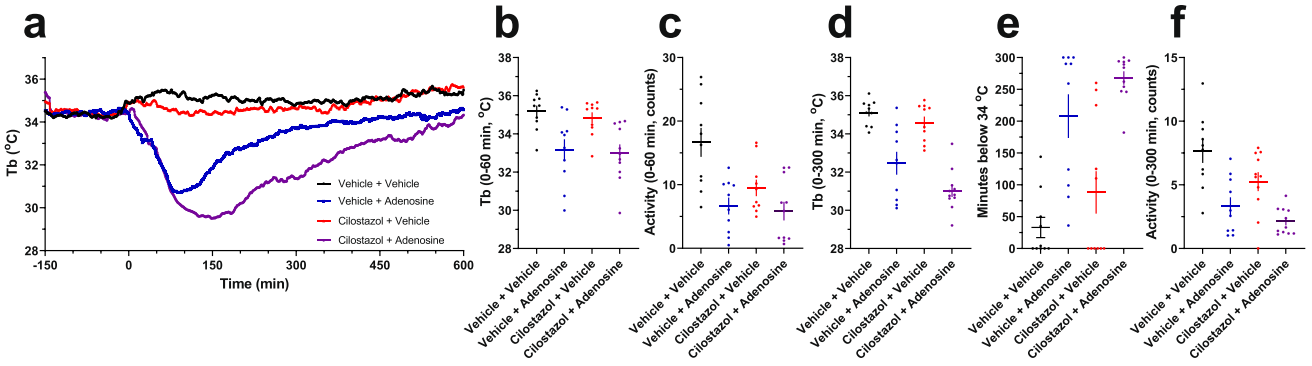

Fig. 3 Effects of cilostazol (10 mg/kg) pretreatment on adenosine (100 mg/kg) induced hypothermia. **a** Time course, **b** mean Tb at 0–60 min, **c** mean activity at 0–60 min, **d** mean Tb at 0–300 min, **e** time below 34 °C, and **f** mean activity at 0–300 min; $n = 10–11$/group. Statistical analyses are in Table S2

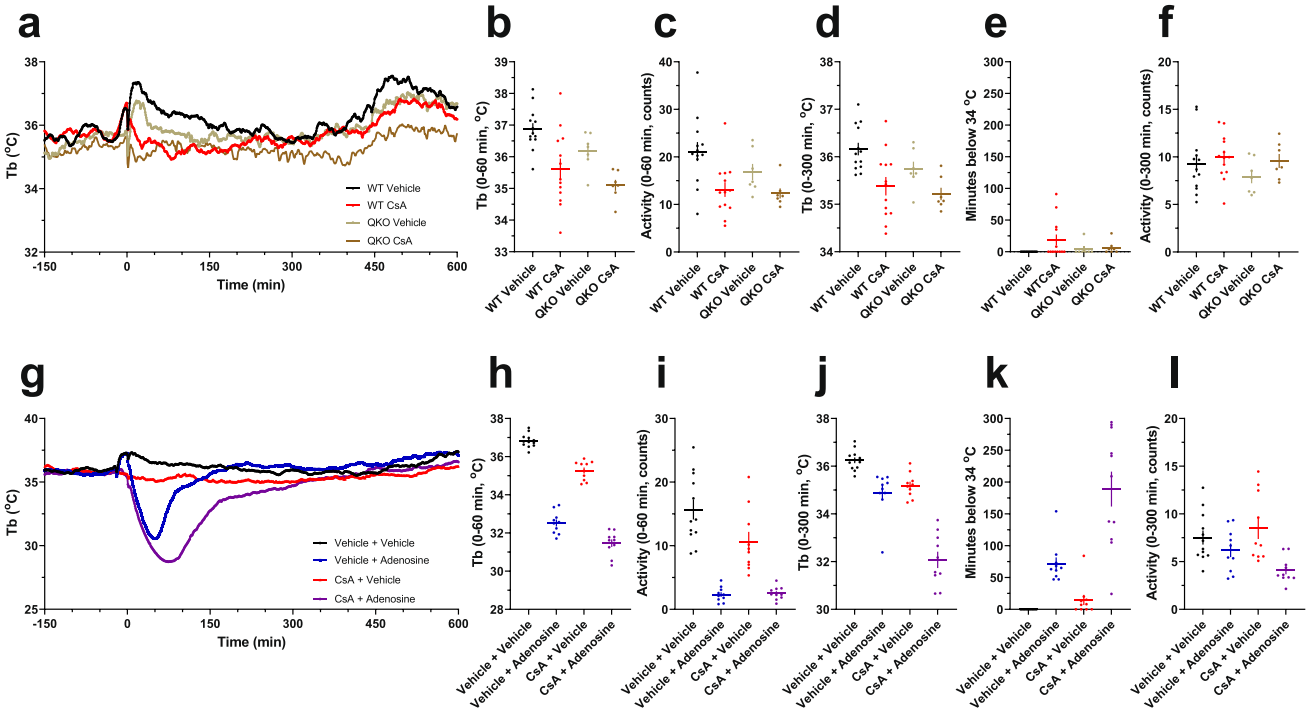

Fig. 4 Effects of cyclosporine A. **a** Treatment of QKO and control mice with cyclosporine A (30 mg/kg). **b** Mean Tb at 0–60 min, **c** mean activity at 0–60 min, **d** mean Tb at 0–300 min, **e** time below 34 °C, and **f** mean activity at 0–300 min; $n = 7–14$/group. **g** Effect of cyclosporine A (30 mg/kg) pretreatment on adenosine (100 mg/kg) induced hypothermia. **h** Mean Tb at 0–60 min, **i** mean activity at 0–60 min, **j** mean Tb at 0–300 min, **k** time below 34 °C, and **l** mean activity at 0–300 min; $n = 10–12$/group. Statistical analyses are in Table S2

Drugs without hypothermic effects

The anti-epileptic drug cannabidiol, a putative ENT1 inhibitor [23, 36–39], had no effect on body temperature and did not augment adenosine-induced hypothermia at 10 mg/kg, i.p. (Fig. S1).

The diuretic canrenoate has cardioprotective effects which are absent in mice lacking either CD73 or the A_{2B}AR, suggesting dependence on extracellular adenosine [40]. However, canrenoate (0.3, 1, and 3 mg/kg, i.p.) did not reduce Tb in WT mice (Fig. S2). At 3 mg/kg, canrenoate tended to increase Tb and, therefore, was not investigated further.

The PDE5 inhibitor sildenafil may modulate antinociception via multiple AR subtypes [41]. However, sildenafil (1, 3, 10, and 30 mg/kg, i.p.) had no effect Tb in WT mice (Fig. S5).

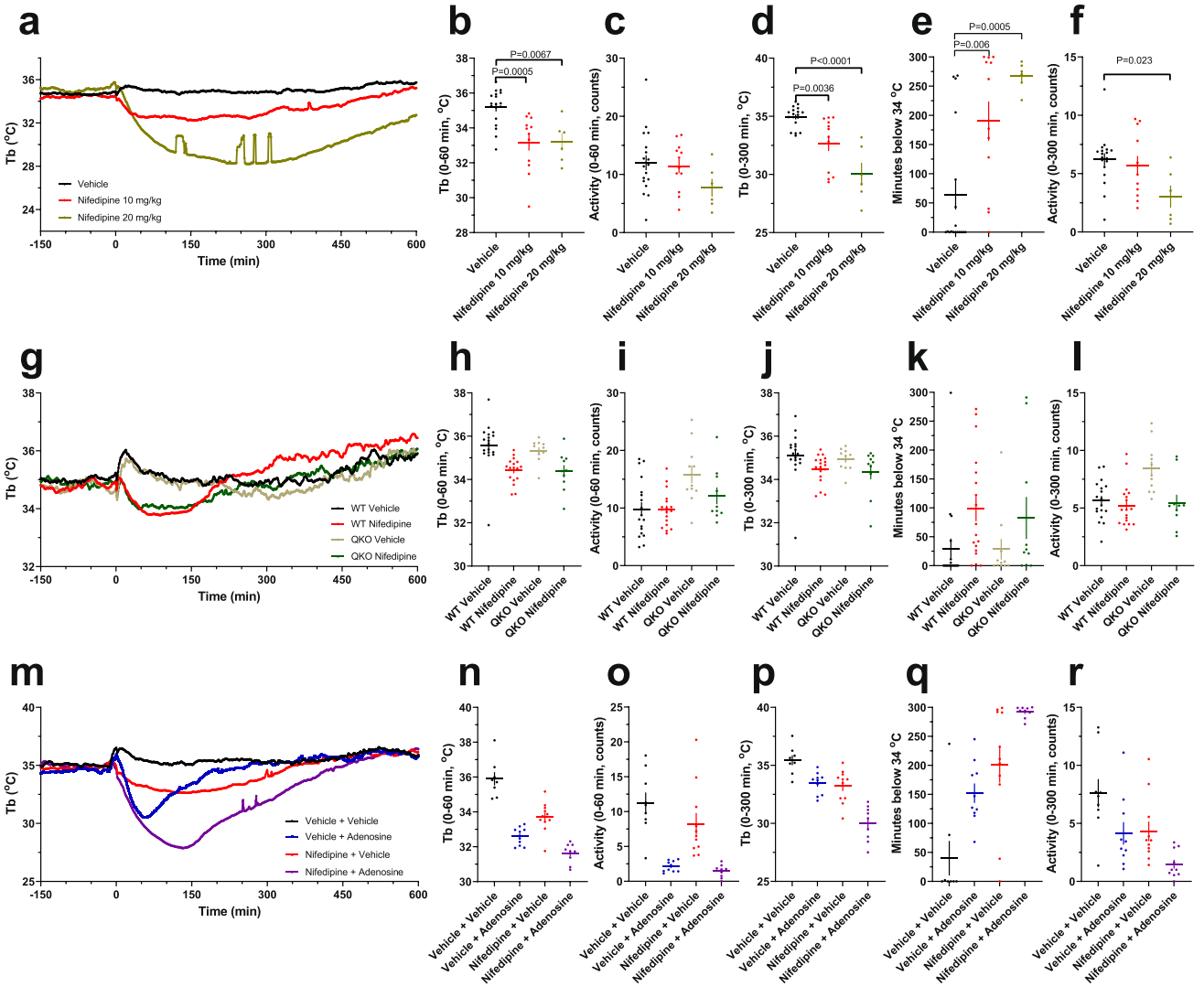

Fig. 5 Effects of nifedipine. **a** Vehicle or nifedipine (10 or 20 mg/kg) treatment of WT mice. **b** Mean Tb at 0–60 min, **c** mean activity at 0–60 min, **d** mean Tb at 0–300 min, **e** time below 34 °C, and **f** mean activity at 0–300 min; $n = 6$–18/group. **g** Treatment of QKO and control mice with nifedipine (10 mg/kg). **h** Mean Tb at 0–60 min, **i** mean activity at 0–60 min, **j** mean Tb at 0–300 min, **k** time below 34 °C, and **l** mean activity at 0–300 min; $n = 10$–18/group. **m** Effect of nifedipine (10 mg/kg) pretreatment on adenosine (100 mg/kg) induced hypothermia. **n** Mean Tb at 0–60 min, **o** mean activity at 0–60 min, **p** mean Tb at 0–300 min, **q** time below 34 °C, and **r** mean activity at 0–300 min; $n = 8$–11/group. Statistical analyses are in Table S2

Therefore, alone it does not appear to have AR agonist activity, but we have not evaluated its possible indirect action.

Drugs with non-adenosinergic hypothermic effects

Nifedipine is a Ca^{2+} channel blocker used as an antihypertensive agent with a lower affinity at ENT1 but reported to have adenosinergic actions [42, 43]. Nifedipine (10 mg/kg) caused hypothermia without significant reduction of activity in both WT and QKO mice, suggesting non-adenosinergic action (Fig. 5a–l). However, nifedipine treatment potentiated adenosine-induced hypothermia in additive manner (Fig. 4m–r).

The anti-angina drug ranolazine may exert its beneficial effects by increasing myocardial adenosine levels [44]. Ranolazine (50 mg/kg, i.p.) itself reduced Tb and activity in both WT and QKO mice and had no effect on adenosine-induced hypothermia (Fig. 6).

The antidepressant ketamine was reported to boost adenosinergic signaling [45, 46]. Ketamine was tested at four doses (1, 3, 10, and 30 mg/kg, i.p.). Only the highest dose caused significant reduction of Tb with no changes in activity (Fig. S4a–f). However, the hypothermic effect was the same in WT and QKO (Fig. S4g–l), suggesting a non-adenosinergic MoA for the ketamine-induced hypothermia.

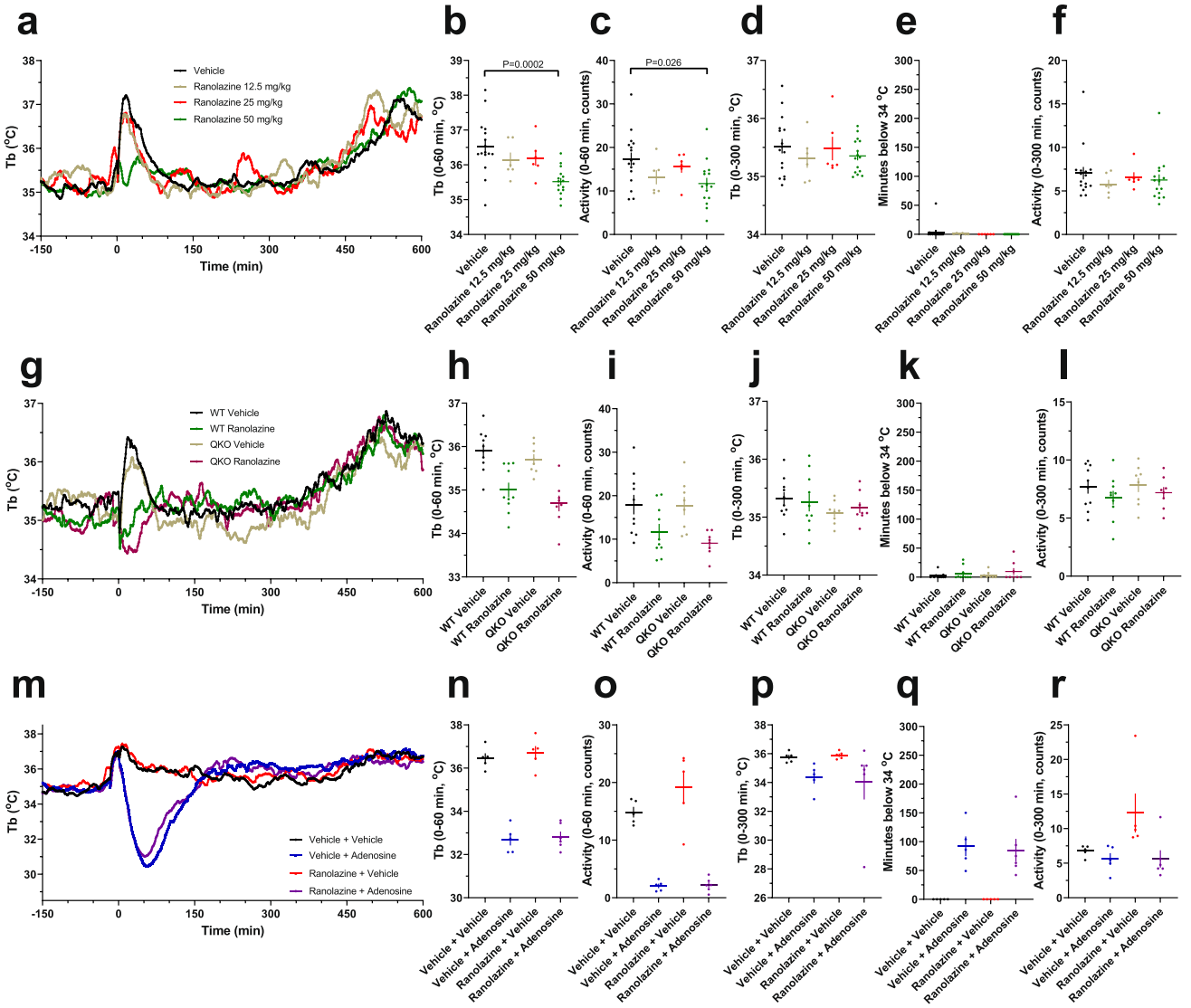

Fig. 6 Effects of ranolazine. **a** Vehicle or ranolazine (12.5, 25, or 50 mg/kg) treatment of WT mice. **b** Mean Tb at 0–60 min, **c** mean activity at 0–60 min, **d** mean Tb at 0–300 min, **e** time below 34 °C, and **f** mean activity at 0–300 min; $n = 6$–16/group. **g** Treatment of QKO and control mice with ranolazine (50 mg/kg). **h** Mean Tb at 0–60 min, **i** mean activity at 0–60 min, **j** mean Tb at 0–300 min, **k** time below 34 °C, and **l** mean activity at 0–300 min; $n = 8$–10/group. **m** Effect of ranolazine (50 mg/kg) pretreatment on adenosine (100 mg/kg) induced hypothermia. **n** Mean Tb at 0–60 min, **o** mean activity at 0–60 min, **p** mean Tb at 0–300 min, **q** time below 34 °C, and **r** mean activity at 0–300 min; $n = 5$–6/group. Statistical analyses are in Table S2

Ethanol may modulate adenosine signaling by multiple mechanisms [47–49]. Ethanol (2 or 3 g/kg) reduced Tb similarly in both WT and QKO mice and did not potentiate adenosine-induced hypothermia, suggesting non-adenosinergic action (Fig. S5).

Zinc chloride elicits an antidepressant-like effect in the mouse model of forced swimming, with some of the effect attributed to enhanced adenosine signaling [50]. Zinc chloride induced a robust hypothermic effect at 10 mg/kg, but not at lower doses; however, the effect was similar in WT and QKO mice (Fig. 7). Of note, at 30 mg/kg, the dose used by Lobato et al. [40], zinc chloride caused death in four out of four WT mice; the difference in the results could be due to the different genetic background, C57BL/6 vs Swiss.

The antidepressant amitriptyline was also reported to modulate adenosine signaling [51]. Amitriptyline (20 mg/kg) reduced both Tb and activity in WT mice, but this reduction was slightly increased in the QKO mice, consistent with non-adenosinergic MoA (Fig. 8).

Taken together, these data show that the hypothermic effects of nifedipine, ranolazine, ketamine, ethanol, zinc chloride, and amitriptyline are independent of AR signaling.

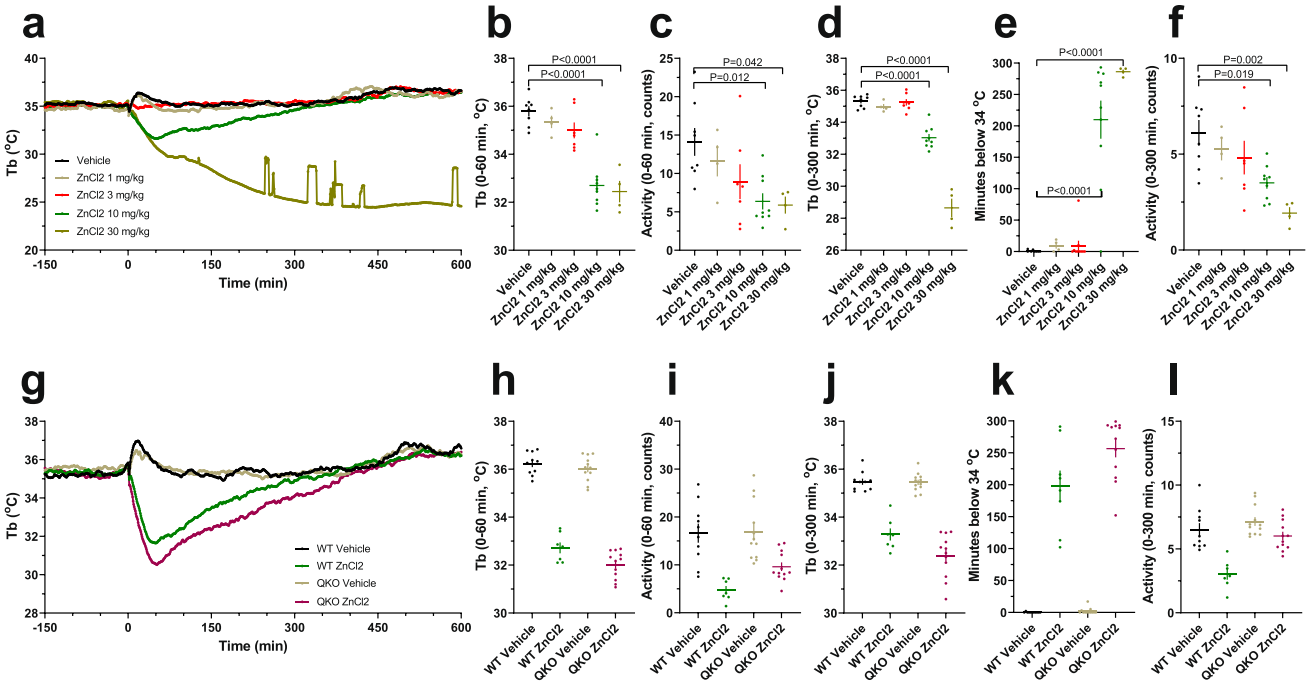

Fig. 7 Effects of zinc chloride. **a** Vehicle or zinc chloride (1, 3, 10, or 30 mg/kg) treatment of WT mice. **b** Mean Tb at 0–60 min, **c** mean activity at 0–60 min, **d** mean Tb at 0–300 min, **e** time below 34 °C, and **f** mean activity at 0–300 min; $n = 4$–9/group. **g** Treatment of QKO and control mice with zinc chloride (10 mg/kg). **h** Mean Tb at 0–60 min, **i** mean activity at 0–60 min, **j** mean Tb at 0–300 min, **k** time below 34 °C, and **l** mean activity at 0–300 min; $n = 10$–12/group. Statistical analyses are in Table S2

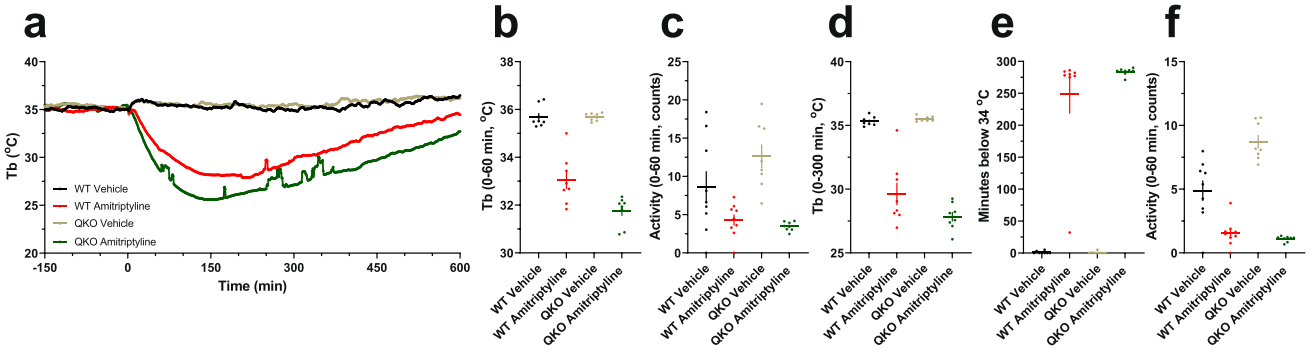

Fig. 8 Effects of amitriptyline (20 mg/kg) treatment of QKO and control mice. **a** Time course, **b** mean Tb at 0–60 min, **c** mean activity at 0–60 min, **d** mean Tb at 0–300 min, **e** time below 34 °C, and **f** mean activity at 0–300 min; $n = 8$/group. Statistical analyses are in Table S2

Discussion

Following up on indirect evidence and prior hypotheses, we have directly tested pharmacologically important substances for adenosinergic effects using mouse hypothermia as an in vivo assay. The thirteen drugs examined can mainly be divided into three groups, (1) evidence for adenosinergic effect, (2) hypothermia via non-adenosinergic mechanisms, and (3) no effect at all in this assay.

A major strength of using hypothermia as an in vivo screen for adenosinergic actions is that it detects agonism at any of the four ARs. The detailed mechanisms are not characterized in all cases. For example, A_3AR agonists activate peripheral mast cells in mice, causing degranulation and histamine release, increased vascular permeability, and hypotension [52]. The hypothermia is caused by the histamine acting via H_1 receptors [53]. Classically, central A_1AR were identified as mediators of adenosine hypothermia [54–56]. Additional studies suggested that activation of A_1AR on neurons both within and outside the blood–brain barrier can cause hypothermia [57]. Within the brain, activation of A_1AR-expressing neurons in the dorsomedial hypothalamus,

but not the preoptic area, causes hypothermia [57]. Agonism at peripheral A_{2A}AR causes hypothermia, possibly via vasodilation and hypotension [32, 58]. Finally, agonism of central A_{2B}AR also causes hypothermia, with the mechanistic details not yet determined [32]. It is conceivable that that some of the activities observed here for drugs that permeate the BBB occur at the CNS level.

Since hypothermia is caused by at least five different AR/site combinations, demonstration of hypothermia and its lack in the QKO mice is a first step in characterizing the adenosinergic actions of a drug. Further experiments are needed to determine if the drug is a direct agonist or antagonist at one or more of the AR, or if it modulates a different target, changing adenosine levels either at particular anatomic sites or throughout the body. Non-receptor targets could include adenosine transporters [18], adenosine deaminase [59], and adenosine kinase inhibitors [27, 60]. Hypothermia can be a sensitive test for some drugs, but might miss potent adenosinergic actions, such as central adenosinergic effects for a drug that does not pass the blood–brain barrier. Even for compounds that do reach the relevant ARs, hypothermia may occur at a higher dose than required for other actions via that AR. It is important to remember that the hypothermia screen depends on the affinity of the tested drug for mouse AR and that a drug's affinity can vary widely among species [61]. There may also be species differences in the biology, with one example being mast cell expression (or not) of A_3AR [62].

An adenosinergic effect of a drug can also be identified by its ability to potentiate adenosine-induced hypothermia, an important approach for screening compounds blocking adenosine transport. While the inhibition of transport alone might not produce significant hypothermia, co-administration of adenosine with the test substance increases the sensitivity of the assay. We previously demonstrated that the ENT1 inhibitor NBMPR by itself produced a hint of hypothermia, but it greatly augmented adenosine-induce hypothermia [27]. Here we show that four drugs reported to inhibit adenosine transport (dipyridamole [21], nimodipine [33], cilostazol [63], and cyclosporine A [34, 35]) also increased the hypothermia caused by adenosine; these results are consistent with inhibition of adenosine transport. In vivo studies indicate that dipyridamole alone, as an ENT2 inhibitor, can increase A_{2B}AR activation, e.g. in colitis and lung injury mouse models [64, 65]. Dipyridamole and nimodipine probably increased adenosine levels through inhibition of adenosine clearance via ENT1. In contrast to dipyridamole which itself did not reduce Tb, nimodipine alone caused hypothermia in WT mice that was diminished in QKO mice. This indicates that nimodipine has both adenosinergic (such as via ENT1 inhibition) and non-adenosinergic actions.

CBD is reported to be a sub-micromolar ENT1 inhibitor [23, 36–38], but CBD did not enhance adenosine-induced hypothermia. Thus, CBD does not appear to be sufficiently efficacious as an ENT1 inhibitor under the in vivo conditions tested. Similarly, no adenosinergic effects were detected for canrenoate or sildenafil.

Cyclosporine A has been shown to increase plasma adenosine levels in kidney transplant recipients and inhibit adenosine uptake in red blood cells [34]. In T lymphocytes, it had dual action and inhibited both adenosine transport and adenosine kinase activity [35]. The enhancement of adenosine-induced hypothermia by cyclosporine A is consistent with inhibition of adenosine transport; however, it is unknown if this effect is mediated by ENT1.

The PDE3 inhibitor cilostazol is an anti-claudication drug that has been reported to inhibit adenosine transport in vitro and in vivo [63]. That MoA is thought to contribute to its cardioprotective and anti-ischemic neuroprotective effects [66]. Cilostazol did not induce hypothermia itself, but enhanced adenosine-induced hypothermia in wild type mice. Since cilostazol is a poor human ENT1 inhibitor (ref. 27 in Table 1), it might act at different targets. Its potency at mouse ENT1 is not reported. Taken together, these results suggest that at the doses tested, dipyridamole and nimodipine have some adenosinergic effects, likely by ENT1 inhibition, while cilostazol may have a different mode of action. Nevertheless, the relationship of cilostazol to potential human adenosinergic signaling was strengthened by a report that in acute coronary syndrome patients it raised plasma adenosine levels [67].

Ethanol has been hypothesized to act in the brain by reducing adenosine uptake [68]. $ENT1^{-/-}$ mice were less sensitive to acute effects of ethanol and showed an increase in alcohol consumption. While the hypothermic effects of ethanol were partially blunted in the A_{2A}AR KO mouse [69], we have not detected any adenosinergic effects in the hypothermia assay. Unexpectedly, both ethanol and cannabidiol, that weakly block ENT1, did not cause adenosine-induced hypothermia. This likely reflects an insufficient degree of ENT1 inhibition under these conditions.

Nifedipine is a calcium channel blocker used as an antihypertensive. It caused hypothermia by itself and enhanced adenosine-induced hypothermia. However, nifedipine hypothermia was not altered in the QKO mice. Nifedipine may potentiate adenosine hypothermia via its hypotensive actions. Our results do not support the proposed adenosinergic effects of nifedipine [42].

The antidepressant, amitriptyline, also has antinociceptive actions reported to depend on A_3AR, because this effect was attenuated by a co-administered A_3AR antagonist MRS1191 [51]. Amitriptyline itself induced hypothermia, but there was no reduction (actually a slight enhancement) in the QKO mice. Therefore, in vivo activation of A_3AR by amitriptyline was not detectable and whether loss of ARs

enhances amitriptyline-induced hypothermia requires further investigation.

There are many drugs and chemicals that can cause hypothermia, potentially for therapeutic application [70]. Here we have identified an adenosinergic mechanism for some compounds and found that others caused hypothermia via non-adenosinergic mechanisms because their effect remained in QKO mice. We have not investigated the mechanisms of these non-adenosinergic drugs.

Adenosine and adenosine receptor signaling has been implicated in many biological processes [1–7]. Extracellular adenosine can be elevated during disease conditions by generation from nucleotides or by transcriptional control of the hypoxia-inducible factor 1α (HIF1A) pathway during hypoxia [71]. Numerous exogenous AR agonists and antagonists have beneficial therapeutic effects in animal models, and many have been tested in clinical trials. However, currently, short-acting, parenteral agonists, adenosine and regadenoson, are the only AR agonists approved for human use [5]. Regadenoson is also being examined for treatment of sickle cell disease, glioblastoma (opening the blood brain barrier) and other conditions (ClinicalTrials.gov Identifier: NCT03971734, accessed November 15, 2022) [72, 73]. Thus, identification of approved drugs that elevate adenosine in vivo could lead to expanded indications for these compounds. Dipyridamole has been repurposed as a potential treatment of Covid19 (ClinicalTrials.gov Identifiers: NCT04391179, NCT04424901, accessed November 15, 2022) based on the anti-inflammatory effects of adenosine elevation [74]. Other conditions in which adenosine could have a beneficial effect include pain, inflammation, steatohepatitis, and seizures. Identifying and characterizing adenosinergic actions is a promising approach for repurposing approved drugs.

Abbreviations AR: Adenosine receptor; CBD: Cannabidiol; ENT: Equilibrative nucleoside transporter; GPCR: G protein-coupled receptor; MoA: Mechanism of action; NBMPR: 6-*S*-[(4-Nitrophenyl) methyl]-6-thioinosine; NMDA: N-methyl-D-aspartate; PDE: Phosphodiesterase; SLC: Solute carrier; Tb: Core body temperature; QKO: Quadruple knockout of adenosine receptors; WT: Wild-type

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Author contribution K.A.J., O.G., and M.L.R. conceptualized the experiments, analyzed data, and wrote the main manuscript text; C.X., O.G., N.L., and S.A.L. performed experiments, analyzed data, and prepared figures and tables. All authors reviewed the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval All animal procedures were conducted with approval of the NIDDK Institutional Animal Care and Use Committee (IUCAC), protocol number K016-DEOB-23. Standards of the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) were followed, and work was performed in an AAALAC-accredited facility. Human studies—not applicable.

Conflict of interest Cuiying Xiao declares that he/she has no conflict of interest. Oksana Gavrilova declares that he/she has no conflict of interest. Naili Liu declares that he/she has no conflict of interest. Sarah A. Lewicki declares that he/she has no conflict of interest. Marc L. Reitman declares that he/she has no conflict of interest. Kenneth A. Jacobson declares that he/she has no conflict of interest.

Competing interests The authors declare no competing interests.

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