Neuronal adenosine A<sub>2A</sub> receptors signal ergogenic effects of caffeine

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Caffeine is one of the most used ergogenic aid for physical exercise and sports. However, its mechanism of action is still controversial. The adenosinergic hypothesis is promising due to the pharmacology of caffeine, a nonselective antagonist of adenosine A<sub>1</sub> and A<sub>2A</sub> receptors. We now investigated A<sub>2A</sub>R as a possible ergogenic mechanism through pharmacological and genetic inactivation. Forty-two adult females (20.0 ± 0.2 g) and 40 male mice (23.9 ± 0.4 g) from a global and forebrain A<sub>2A</sub>R knockout (KO) colony ran an incremental exercise test with indirect calorimetry (VO<sub>2</sub> and RER). We administered caffeine (15 mg/kg, i.p., nonselective) and SCH 58261 (1 mg/kg, i.p., selective A<sub>2A</sub>R antagonist) 15 min before the open field and exercise tests. We also evaluated the estrous cycle and infrared temperature immediately at the end of the exercise test. Caffeine and SCH 58621 were psychostimulant. Moreover, Caffeine and SCH 58621 were ergogenic, that is, they increased VO<sub>2</sub>max, running power, and critical power, showing that A<sub>2A</sub>R antagonism is ergogenic. Furthermore, the ergogenic effects of caffeine were abrogated in global and forebrain A<sub>2A</sub>R KO mice, showing that the antagonism of A<sub>2A</sub>R in forebrain neurons is responsible for the ergogenic action of caffeine. Furthermore, caffeine modified the exercising metabolism in an A<sub>2A</sub>R-dependent manner, and A<sub>2A</sub>R was paramount for exercise thermoregulation.

The natural plant alkaloid caffeine (1,3,7-trimethylxantine) is one of the most common ergogenic substances for physical activity practitioners and athletes.<sup>1–10</sup> Caffeine increases endurance<sup>1,8–12</sup> and resistance<sup>7,13,14</sup> in humans. In rodents, its ergogenic effects are conserved because caffeine increases running time on the treadmill at constant<sup>16,17</sup> and accelerated speeds<sup>18,19</sup>. Sports sciences promote nonselective phosphodiesterase (PDE) inhibition<sup>7,8</sup> and increased calcium mobilization<sup>2,7,8</sup> as mechanisms for these ergogenic effects. However, the primary pharmacological effect of caffeine is the nonselective antagonism of adenosine A<sub>1</sub> and A<sub>2A</sub> receptors (A<sub>1</sub>R, A<sub>2A</sub>R)<sup>20–23</sup>.

Adenosine can act as an inhibitory modulator of the Central Nervous System (CNS) associated with tiredness and drowsiness<sup>24–28</sup>. During exercise, circulating ADP/AMP/adenosine levels increase due to ATP hydrolysis<sup>30,31</sup>. However, there is still no substantial evidence on the role of adenosine in exercise-induced fatigue. It is just known that the nonselective A<sub>1</sub>R and A<sub>2A</sub>R agonist 5′-(N-ethylcarboxamido)adenosine (NECA), injected into the rat tail, abolishes the ergogenic effects of caffeine.<sup>29</sup>

Since there is increasing evidence that the adenosine modulation system critically controls allostatics<sup>29</sup> and A<sub>2A</sub>R have a crucial role in the ability of caffeine to normalize brain function<sup>20</sup>, we hypothesized that caffeine decreases fatigue during exercise through antagonism of A<sub>2A</sub>R in the CNS. We combined the use of pharmacology (SCH 58261 and caffeine) and transgenic mice with tissue-selective deletion of A<sub>2A</sub>R, to test this hypothesis in an incremental running test with indirect calorimetry (or ergospirometry). A<sub>2A</sub>R knockout (KO) mice allow assessing if the ergogenic effect of caffeine persists in the absence of A<sub>2A</sub>R; the use of SCH 58261, the current reference for A<sub>2A</sub>R antagonists<sup>22,23</sup>, allows directly assessing the ergogenic role of A<sub>2A</sub>R. SCH 58261 has excellent selectivity and affinity for A<sub>2A</sub>R<sup>22,23</sup> and affords motor benefits in animal models of Parkinson’s disease as does caffeine, supporting the recent FDA approval of the A<sub>2A</sub>R antagonist Istradefylline for PD treatment<sup>33</sup>. Our goal is to assess the ergogenicity of A<sub>2A</sub>R using the pharmacological and genetic tools described above.
Methods

Animals and A2AR KO colony. We used 40 male (23.9 ± 0.4 g, 8–10 weeks old) and 42 female mice (20.0 ± 0.2 g, 8–10 weeks old) from our global-A2AR (A2AR KO) and forebrain-A2AR KO (fb-A2AR KO) inbred colony28,43, and wild type littermates. The sample size for ANOVA comparison had α = 0.05 and β = 0.8.

The inactivation of exon 2 of A2AR in a near congenic (N6) C57BL/6 genetic background was the method of generating A2AR KO mice66,67. We also have good experience with treadmill running in this strain36–40. A2AR KO mice and wild type littermates were matched for sex and age for each experiment. The Cre-loxP strategy, crossing floxed A2AR mice with mice expressing CRE under the forebrain-selective promoter CAM-kinase 2, allows generating fb-A2AR KO mice, as previously described34,41. We used global A2AR KO females and fb-A2AR KO males due to the characteristic of our colony.

Mice were housed in collective cages in HEPA-filtered ventilated racks (n = 3–5) under a controlled environment (12 h light–dark cycle, lights on at 7 AM, and room temperature of 21 ± 1 °C) with ad libitum access to food and water. Housing and handling were performed according to European Union guidelines (2010/63). The Ethical Committee of the Center for Neuroscience and Cell Biology (University of Coimbra) approved the study.

Drugs. 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) was solubilized in 10% dimethyl sulfoxide (DMSO) in 0.9% NaCl – saline. Caffeine was dissolved in saline. SCH 58261 and caffeine were freshly prepared and administered intraperitoneally (volume of 10 mL/kg body mass). Caffeine, DMSO, and NaCl were obtained from Sigma-Aldrich and SCH 58261 from Tocris. The doses used of SCH 58261 (1 mg/kg) and caffeine (15 mg/kg) were based on our previous experience in the use of these compounds42,43 and pilot studies.

Experimental design. Fig.S1 shows the experimental design. The habituation of handling, injections (0.9% NaCl, i.p.), and moving treadmill (15 cm/s) occurred in the first three days of the experiment. The animals were treated with SCH 58261 (1 mg/kg, i.p.) and caffeine (15 mg/kg, i.p.) on days 4 and 5, 15 min before testing in the open field (4th day) and ergospirometry (5th day). The experiments took place between 9 AM and 5 PM, within the light phase of the mouse dark/light cycle, in a sound-attenuated and temperature/humidity controlled room (20.3 ± 0.6 °C and 62.8 ± 0.4% H2O) under low-intensity light (≈10 lx). The open field apparatus and the treadmill were cleaned with 10% ethanol between individual experiments. The allocation for the experimental groups was random. For each test, the experimental unit was an individual animal.

Open field. Mice explored an unaccustomed open field (38 × 38 cm) for 15 min. Locomotion was analyzed using an ANY-Maze video tracking system (Stoelting Co.).

Ergospirometry. Mice were accustomed to a single-lane treadmill (Panlab LE8710, Harvard apparatus) at speed 15 cm/s (10 min, slope 5°, 0.2 mA) with a 24 h interval between each habituation session (Fig. S1). The incremental running protocol started at 15 cm/s, with an increment of 5 cm/s every 2 min at 5° inclination40. The exercise lasted until running exhaustion, defined by the inability of the animal to leave the electrical grid for 5 s40,44. Oxygen uptake (VO2) and carbon dioxide production (VCO2) were estimated in a metabolic chamber (Gas Analyzer ML206, 23 × 5 × 5 cm, AD Instruments, Harvard) coupled to the treadmill. The animals remained in the chamber for 15 min before exercise testing. Atmospheric air (=21% O2, =0.03% CO2) was renewed at a rate 120 mL/min, using the same sampling rate for the LASER oxygen sensor (Oxigraf X2004, resolution 0.01%) and infrared carbon dioxide sensor (Servomex Model 15050, resolution 0.1%). We estimated the running and critical power output for a treadmill based on a standard conversion of the vertical work, body weight, and running speed40,45,46. Running power is the sum (Σ) of all stages of the exercise test, and critical power is the running work performed above VO2max.

Vaginal cytology. We evaluated the estrous cycle immediately after the exercise test, through 4–5 consecutive vaginal lavages (with 40–50 μL of distilled H2O) then mounted on gelatinized slides (76 × 26 mm)47,48. These procedures lasted no more than 3–5 min, and there were no significant time delays between behavioral experiments and fluid collection for vaginal cytology.

The vaginal smear was desiccated at room temperature and covered with 0.1% crystal violet for 1 min, then twice washed with 1 mL H2O and desiccated at room temperature47,48. The slides were mounted with Eukitt medium (Sigma-Aldrich) and evaluated under an optical microscope at 1x, 5x, and 20x (Zeiss Axio Imager 2). We evaluated three cell types for determining the estrous cycle: nucleated epithelial cells, cornified epithelial cells, and leukocytes. Cellular prevalence defined proestrus (nucleated), estrus (cornified), metestrus (all types in the same proportion), and diestrus (leukocytes)47,48.

Thermal imaging. An infrared (IR) camera (FLIR C2, emissivity 0.95, FLIR Systems) placed overhead (25 cm height) of a plastic tube (25 cm diameter) was used to acquire a static dorsal thermal image40,45,50. IR images were taken immediately before and after exercise tests, namely at resting and recovery (Fig. 1H), respectively. IR images were analyzed with FLIR Tools software (Flir, Boston).

Statistics. Data are presented as mean ± SEM in graphs built using the GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.
Statistical analyzes were performed according to an intention-to-treat principle using StatSoft, Inc. (2007). STATISTICA (data analysis software system), version 8.0. www.statsoft.com. ANOVA two-way was used to evaluate open field, VO₂max, running power, and metabolic rate (E) until the animals reached fatigue. SCH 58261 was ergogenic in both sexes, as it increased VO₂max (C) and running power (D). The animals presented exercise-induced core and tail hyperthermia (H), which was not 58261 modified by SCH (F–G). Sex was a significant factor in decreasing maximum responses to VO₂ (C) and running power (D), and increasing core and tail temperature in females. Data are presented as mean ± SEM. N = 8–9 animals/group for 12 independent experiments. *P < 0.05 vs. DMSO (Two-way ANOVA followed by Newman-Keuls post hoc test). # P < 0.05 vs. rest (Repeated measures ANOVA followed by Bonferroni post hoc test). DMSO dimethyl sulfoxide. Rec recovery. RER Respiratory Exchange Ratio. VO₂ oxygen consumption.

Results
SCH 58261: pharmacological inactivation of A2A R is ergogenic. SCH 58261 was psychostimulant for males, but not for females, since SCH 58261 only increased male locomotion in the open field (F₁,30 = 4.5, η² = 0.1, β = 0.54, 95% CI 58.8–72.1, P < 0.05, Fig. 1A).

The running power of females (F₁,27 = 221, P < 0.05, Fig. 1B) and males (F₁,34 = 183, P < 0.05, Fig. 1B’) increased at each stage of the exercise test. Submaximal VO₂ also increased to the maximum (VO₂max, dotted line) of females (F₈,77 = 168, P < 0.05, Fig. 1B) and males (F₇,84 = 14.3, P < 0.05, Fig. 1B’). Female (F₈,70 = 180, P < 0.05,
Fig. S2A) and male (F8,70 = 164, \( P < 0.05 \), Fig. S2B) submaximal VO2 kinetics was similar to VO2. SCH 58261 had no effect on these submaximal values. We demonstrated for the first time that SCH 58261 is ergogenic since SCH 58261 increased VO2max (F1,36 = 27.7, \( \eta^2 = 0.44 \), \( \beta = 1.0 \), 95% CI 1.0–1.3, \( P < 0.05 \), Fig. 1C) and running power (F1,35 = 55, \( \eta^2 = 0.61 \), \( \beta = 1.0 \), 95% CI 1.0–1.3, \( P < 0.05 \), Fig. 1D) in both sexes. SCH 58261 had no effect on increasing RER of females (F7,70 = 6.9, \( P < 0.05 \), \( \eta^2 = 0.43 \), \( \beta = 0.99 \), 95% CI 28.7–29.3, \( P < 0.05 \), Fig. 1E) and males (F7,84 = 9.4, \( \eta^2 = 0.57 \), \( \beta = 0.99 \), 95% CI 28.7–33.9, \( P < 0.05 \), Fig. 1E′). Exercise test raised the animals’ core (F1,26 = 5.5, \( \eta^2 = 0.17 \), \( \beta = 0.62 \), 95% CI 28.7–29.39, \( P < 0.05 \), Fig. 1F) and tail temperature (F1,22 = 81, \( \eta^2 = 0.78 \), \( \beta = 0.99 \), 95% CI 24.2–25.6, \( P < 0.05 \), Fig. 1G), with no effect of SCH 58261. Exercise test raised the animals’ core (F1,26 = 5.5, \( \eta^2 = 0.17 \), \( \beta = 0.62 \), 95% CI 28.7–29.39, \( P < 0.05 \), Fig. 1F) and tail temperature (F1,22 = 81, \( \eta^2 = 0.78 \), \( \beta = 0.99 \), 95% CI 24.2–25.6, \( P < 0.05 \), Fig. 1G), without effect of SCH 58261. Figure 1 shows the increase in core temperature in post-exercise recovery (rec) in relation to rest. Three females at estrus (Fig. S3C) were excluded from temperature experiments due to large exercise-induced tail hyperthermia40. The previous results refer to females in diestrus (Fig. S3A), proestrus (Fig. S3B), and metestrus (Fig. S3D).

Caffeine is not ergogenic in global A2AR knockouts. Caffeine was psychostimulant based on its ability to increase locomotion in the wild type mice (F1,36 = 5.8, \( \eta^2 = 0.13 \), \( \beta = 0.64 \), 95% CI 55.9–71.6, \( P < 0.05 \), Fig. 2A), an effect not seen in A2AR KO mice.

Figure 2B shows the progressive increase in submaximal VO2 (F7,210 = 255, \( P < 0.05 \)), VCO2 (F7,210 = 189, \( P < 0.05 \), Fig. S2C) and running power (F7,210 = 6,243, \( P < 0.05 \)) at speeds 35–50 cm/s, with less VO2 for A2AR KO mice. Caffeine was ergogenic but only in mice expressing A2AR. Caffeine improved VO2max (F1,33 = 12.6, \( \eta^2 = 0.28 \), \( \beta = 0.93 \), 95 CI 0.12–0.17, \( P < 0.05 \), Fig. 2C) and running power (F1,32 = 22.3, \( \eta^2 = 0.4 \), \( \beta = 0.99 \), 95% CI 0.84–1.09, \( P < 0.05 \), Fig. 2D) of wild type mice. The increase in critical power was 43.1 ± 7.1% concerning controls. A2AR KO did not display the ergogenic effects of caffeine.

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Caffeine slowed the progression of RER in the wild type mice ($F_{7,133} = 3.5$, $\eta^2 = 0.15$, $\beta = 0.96$, $P < 0.05$, Fig. 2E). Resting core (Fig. 2F) and tail (Fig. 2G) temperatures were similar between groups. Exercise increased the core (F1,24 = 0.16, $\eta^2 = 0.99$, $\beta = 0.99$, 95% CI 29.5–30.2, $P < 0.05$, Fig. 2F') and tail (F1,25 = 82, $\eta^2 = 0.73$, $\beta = 0.99$, 95% CI 26.2–27.6, $P < 0.05$, Fig. 2G') temperature of wild type animals. Caffeine did not change the exercise-induced core and tail heating, which was lower in the $A_2AR$ KO mice. Core temperature even dropped in caffeine-treated $A_2AR$ KO mice, as expected from the participation of $A_1R$, also targeted by caffeine, on the control of body temperature51.

Knocking out neuronal $A_2AR$ abrogates the ergogenic effects of caffeine. The psychostimulant effect of caffeine was operated by $A_2AR$ since caffeine increased the locomotion of wild type males in the open field ($F_{1,34} = 8.6$, $\eta^2 = 0.11$, $\beta = 0.55$, 95% CI 65.5–78.3, $P < 0.05$, Fig. 3A), but not in $A_2AR$ KO mice. Submaximal VO$_2$ (F7,147 = 329, $P < 0.05$, Fig. 3B) and VCO$_2$ (F7,154 = 359, $P < 0.05$, Fig. S2D) increased during the exercise test without caffeine and genotype effects. Caffeine was ergogenic but only in mice expressing $A_2AR$ KO mice. The increase in critical power was 31.9 ± 4.7% concerning controls. Most importantly, caffeine was not ergogenic in $A_2AR$ KO mice, as expected from the participation of $A_2AR$, also targeted by caffeine, on the control of body temperature51.

The increase in RER during the exercise test was lower in animals treated with caffeine ($F_{2,119} = 3.6$, $\eta^2 = 0.17$, $\beta = 0.97$, $P < 0.05$, Fig. 3E), wild type, and $A_2AR$ KO. Resting and recovery core temperatures were similar in all groups (Figs. 3F and Fig. 3G). The exercise test did not change the core temperature (Fig. 3F). However, exercise heated the mice's tail in a similar way between groups ($F_{1,22} = 102$, $\eta^2 = 0.69$, $\beta = 0.99$, 95% CI 24.9–26.4, $P < 0.05$, Fig. 3G').
Discussion

Neuronal A2AR antagonism is ergogenic. Caffeine increases exercise performance in rodents and humans. Our results show the key role of A2AR in the ergogenic effects of caffeine using pharmacological and genetic tools. Thus, the potent and selective A2AR antagonist SCH 58261 displayed an ergogenic effect similar to that of caffeine, and the ergogenic effect of caffeine was abrogated in A2AR KO mice.

SCH 58261 and caffeine improved VO2max, running power, and critical power of wild-type mice. These results are in line with the improved running time observed in caffeine-treated rats and mice. Further evidence for the ergogenic effect of caffeine is based on its ability to increase muscle power and endurance output in rodents. For the first time, we demonstrated that the selective antagonism of A2AR is ergogenic. Also, for the first time, we demonstrated that the genetic inactivation of A2AR impaired the ergogenic effects of caffeine. Tissue-specific A2AR KO selectively in forebrain neurons further allowed showing that these ergogenic effects of caffeine are due to the antagonism of A2AR in forebrain neurons. Thus, we suggest that caffeine decreases central fatigue during exercise. Moreover, caffeine decreased RER in the submaximal stages of the exercise test, an effect also abrogated in A2AR KO mice. However, exercise-induced core and tail hyperthermia were similar among animals treated with SCH 58261 or caffeine, except for A2AR KO mice, suggesting possible A1R-A2AR-mediated interactions in the temperature control.

Selective A2AR antagonism is psychostimulant in males, not females. We assessed the baseline motor behavior due to the motor nature of the running test, without any motor impairment found related to the different genotypes and treatments. Thus, the observed differences were not due to impaired animals’ motor behavior. We also assessed the psychostimulant effects of caffeine and SCH 58261. Notably, the effects of caffeine were abrogated in A2AR KO mice, and SCH 58261 did not modify locomotion in female mice. These results corroborate the robust evidence showing the psychostimulant effects of caffeine in male rodents. However, little is known about the role of sexual dimorphism in adenosine signaling. The absence of a psychostimulating effect of SCH 58261 in females is on step ahead, in notable agreement with the reported ability of the anxiolytic effect of SCH 58261 in males but not in females. However, these differences did not disturb the ergogenic effects of SCH 58261 on females. Future studies will better understand sex differences in adenosine signaling, which was not the aim of this study.

The neuropharmacology of the ergogenic effects of SCH 58261 and caffeine. Adenosine is a potent purine that modulates CNS signaling and functions from its main A1R and A2AR. Here, caffeine (nonspecific A1R and A2AR antagonist) and SCH 58261 (selective A2AR antagonist) similarly increased the VO2max, running power, and critical power of exercising male and female mice. Most importantly, these ergogenic effects were abrogated by the selective deletion of A2AR in forebrain neurons, which indicates the key role of CNS A2AR as an ergogenic mechanism. The basal nuclei, namely the striatum, is the brain region with the highest density of A2AR, which prompts the hypothesis that the A2AR antagonism in the basal ganglia might mediate the ergogenic effect of SCH 58261. In resting and running rodents, caffeine intake can result in a concentration of caffeine of 50 mM in the brain. This concentration is close to the EC50 of caffeine (40 mM) to antagonize A1R and A2AR in the CNS. Since caffeine was not ergogenic in fb-A2AR KO mice, it is concluded that forebrain A2AR signal the ergogenic effects of caffeine. This provides a direct demonstration of the involvement of neuronal A2AR in the ergogenic effects of caffeine, as suggested by two previous reports showing that NECA prevented the ergogenic effects of caffeine in rats and, conversely, that systemic caffeine reversed the poor running performance of NECA-treated rats. Although nonspecific, the pharmacological use of NECA demonstrated that adenosine receptors are crucial for the ergogenic effects of caffeine. We now identified A2AR, specifically located in forebrain neurons, as responsible for this ergogenicity of caffeine.

The neurological effects of caffeine highlight its action on the CNS. Caffeine decreases the rate of perceived exertion, central and mental fatigue during exercise, indicating that caffeine attenuates mental fatigue during exercise. Caffeine also improves performance expectations, cognitive and executive functions, and vigor in exercising subjects. The contribution of the CNS-mediated effects on exercise-induced fatigue conceptualizes central fatigue. Caffeine reduces saccadic eyes fatigue, also, the cortical silence of fatigued ankle muscles. Moreover, caffeine increases spinal excitability and cortical motor area potentiation after exhausting exercise.

Caffeine decreases RER in mice expressing peripheral A2AR. Caffeine decreases the RER in submaximal exercise in humans and rats. For the first time, we provide evidence that this metabolic effect involves a modification of the A2AR function. In the past, the inhibition of phosphodiesterase and increased intracellular calcium mobilization were the proposed mechanisms. However, these proposals are inconsistent with pharmacological data: caffeine has an EC50 for adenosine antagonism of 40 mM, 1,000 mM for phosphodiesterase inhibition, and 3,000 mM for Ca2+-triggered muscle contraction. Higher caffeine concentrations cause toxicity (above 200 mM) and lethality (above 500 mM). Thus, biological effects for caffeine must be in the range below 100 µM. We have previously shown that caffeine reaches a plasma peak of 10 µM after caffeine intake (6 mg/kg) in running mice. The metabolic effects of caffeine during exercise are currently associated with increased activity of the autonomic nervous system (ANS), including high blood adrenaline and lactate levels, tachycardia and increased blood pressure. However, we must recognize the limitations of lung-based RER measures and their effects on metabolism, due to the possible artifacts such as hyperventilation and disturbances in the acid–base balance.
Adenosine receptors are crucial for exercise-induced hyperthermia. The temperature changes observed were dependent on sex and genotype. The exercise test improved VO$_2$, an index of heat production$^{26}$ but the core temperature increased only in females. The tail temperature, an index of heat loss$^{26}$, increased in both sexes. These results are in line with previous results from our group$^{26}$. Caffeine and SCH 58261 did not modify these thermal responses to the exercise test. In males, tail heating of fb-A2AR KO mice was also similar to that of the wild type mice. However, the thermal response of the global A2AR KO females was different, indicating a peripheral role of these receptors, known to regulate body temperature$^{41}$.

NECA (nonspecific A1R and A2AR agonist) causes core hypothermia in rats and rabbits$^{26,76}$, an effect inhibited by 8-phenyltheophylline (a potent and selective antagonist for A1R and A2AR that crosses the blood–brain barrier, BBB)$^{76}$ but unaffected by 8-（p-sulfophenyl)theophylline (another potent adenosine receptor antagonist with little BBB penetration)$^{76}$, indicating a centrally-mediated effect. In the case of A2AR, its role in regulating body temperature is controversial$^{81}$. The selective A2AR agonist 2-p-(2-carboxyethyl)-phenethylamino-5′-N-ethylcarboxamidoadenosine-hydrochloride (CGS 21680) induces hyperthermia in rats$^{82}$ and mice$^{83}$. We now show that SCH 58261 does not change resting and recovery temperature. This evidence suggests that the peripheral activation of A2AR can induce hypothermia in rodents, but this mechanism does not seem to be physiologically engaged as gauged by the lack of changes from the pharmacological or genetic blockade of A2AR. The previous data are from animals at rest—animals with CNS A2AR deletion present normal hyperthermia response during exercise. However, global A2AR KO displays a decreased response, even hypothermia, when treated with caffeine. Thus, A2AR seems to undergo a gain of function in the periphery during exercise. This data reinforces the well-known role of A1R in hypothermia$^{81}$. Circulating adenosine levels increase during exercise$^{30,31}$, and global A2AR KO imbalance appears to increase the A1R role, signaling hypothermia even after exercise. These results are limited to the use of infrared temperature, as we have not measured rectal temperature due to interference (vaginal manipulation) in the evaluation of the estrous cycle of females. Also, we kept the same methodology in males.

Conclusion. In summary, we have now demonstrated that A2AR antagonism is a mechanism of action for ergogenicity, as SCH 58261 was ergogenic. Furthermore, we showed that the antagonism of forebrain A2AR was the mechanism underlying the ergogenic effect of caffeine since caffeine was not ergogenic in fb-A2AR KO. The use of selective A1R KO in forebrain neurons further reinforces the ergogenic role of caffeine in decreasing central fatigue, with possible involvement of decreased perceived exertion, pain, and mental fatigue in humans. Despite methodological limitations, our data further suggest that caffeine modified the exercising metabolism in an A2AR-dependent manner and that A2AR is essential for exercise thermoregulation.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
A.S.A.Jr designed and performed the experiments, prepared the figures, and wrote the manuscript. A.E.S performed the experiments. P.M.C. designed the experiments and wrote the manuscript. R.A.C. designed the experiments and wrote the manuscript. All authors revised the manuscript.
Competing interests
RAC is a scientific consultant for the Institute for Scientific Information on Coffee (ISIC). All other authors declare no conflict of interest.

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