**ORIGINAL ARTICLE**

# The striatum drives the ergogenic effects of caffeine

Ana Cristina de Bem Alves[1] · Ana Elisa Speck[1] · Hémelin Resende Farias[2] · Leo Meira Martins[3] ·
Naiara Souza dos Santos[1] · Gabriela Pannata[1] · Ana Paula Tavares[1] · Jade de Oliveira[2] · Ângelo R. Tomé[4] ·
Rodrigo A. Cunha[4,5] · Aderbal S Aguiar Jr[1]

## Abstract

Caffeine is one of the main ergogenic resources used in exercise and sports. Previously, we reported the ergogenic mechanism of caffeine through neuronal $A_{2A}R$ antagonism in the central nervous system [1]. We now demonstrate that the striatum rules the ergogenic effects of caffeine through neuroplasticity changes. Thirty-four Swiss (8–10 weeks, $47 \pm 1.5$ g) and twenty-four C57BL/6J (8–10 weeks, $23.9 \pm 0.4$ g) adult male mice were studied behaviorly and electrophysiologically using caffeine and energy metabolism was studied in SH-SY5Y cells. Systemic (15 mg/kg, i.p.) or striatal (bilateral, 15 µg) caffeine was psychostimulant in the open field ($p < 0.05$) and increased grip efficiency ($p < 0.05$). Caffeine also shifted long-term depression (LTD) to potentiation (LTP) in striatal slices and increased the mitochondrial mass ($p < 0.05$) and membrane potential ($p < 0.05$) in SH-SY5Y dopaminergic cells. Our results demonstrate the role of the striatum in the ergogenic effects of caffeine, with changes in neuroplasticity and mitochondrial metabolism.

**Ana Cristina de Bem Alves** graduated with BSc in physical therapy from the Federal University of Santa Catarina (UFSC), with an internship at the University of Northern Iowa and the University of Pittsburgh. She has a master's degree in neurosciences and is currently finishing her Ph.D. at UFSC. She has expertise in neurodegenerative diseases, emphasizing neuroinflammation and the adenosinergic system. Ana studied the potential of adenosine $A_{2A}$ receptors ($A_{2A}R$) antagonism in managing fatigue in the past 2 years. She researched new therapeutic options for neurological patients to improve their quality of life.

✉ Aderbal S Aguiar Jr
aderbal.aguiar@ufsc.br

Ana Cristina de Bem Alves
anacristinadebemalves@gmail.com

Ana Elisa Speck
anaespeck@gmail.com

Hémelin Resende Farias
hemelin.farias@hotmail.com

Leo Meira Martins
leo.meira@ufrgs.br

Naiara Souza dos Santos
naiarassfisio@gmail.com

Gabriela Pannata
gabrielapntt@icloud.com

Ana Paula Tavares
anapaullasanttos36@gmail.com

Jade de Oliveira
deoliveirajade10@gmail.com

Ângelo R. Tomé
atome@ci.uc.pt

Rodrigo A. Cunha
rcunha@fmed.uc.pt

1  LABIOEX-Laboratory of Exercise Biology, Federal University of Santa Catarina-UFSC, Ararangua, SC 88905-120, Brazil

2  Post-graduation Program in Biological Sciences, Department of Biochemistry, Institute of Basic Sciences of Health, Federal University of Rio Grande do Sul-UFRGS, Porto Alegre, RS 90040-060, Brazil

3  Post-graduation Program in Biological Sciences, Department of Physiology, Institute of Basic Sciences of Health, Federal University of Rio Grande do Sul-UFRGS, Porto Alegre, RS 90050-170, Brazil

4  CNC-Center for Neuroscience and Cell Biology, University of Coimbra, 3004-504 Coimbra, Portugal

5  FMUC – Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal

## Introduction

Exercise fatigue is one of the main barriers to exercise and sports performance. Caffeine (1,3,7-trimethyl xanthine) is a non-selective antagonist of adenosine $A_1$ and $A_{2A}$ receptors ($A_1R$ and $A_{2A}R$) that exhibit psychostimulant and ergogenic effects (i.e., increases physical performance) [1]. It is one of the most popular ergogenic aids in exercise and sports, available in coffee, tea, caffeinated soft and sports drinks, sports supplements, and other beverages and foods [2–5] The gastrointestinal system rapidly absorbs caffeine, distributed throughout the body fluids, reaching plasma peak concentrations of 2-15 μM (100 mg caffeine) with a half-life of 2.5-10h [6, 7]. The World Anti-Doping Agency (WADA) detected caffeine in 73.8% of 20,686 urine samples from elite athletes between 2004 and 2008 [8], a percentage close to that reported by Van Thuyne et al. [9]: 73.6% in 11,361 samples between 1993 and 2002. The International Society of Sports Nutrition recommends the oral consumption of caffeine in an anhydrous form in doses between 3 and 6 mg/kg (100–300 mg) [10]. Larger doses (> 9 mg/kg) do not enhance the ergogenic effect and are unsafe as they can trigger adverse effects problematic for athletic performance, such as restlessness, gastrointestinal discomfort, nausea, insomnia, anxiety, and chest pain [10].

Several of the central effects of caffeine can explain the ergogenic effects of caffeine. In fact, caffeine-enhancing effects include improved neurological functions such as typing speed, simple reaction time, sustained attention, memory, and logical reasoning during rest [11]. The physiological targets of caffeine for micromolar concentrations achieved with dietary consumption are $A_1$ and $A_{2A}$ adenosinergic receptors [6, 7]. Davis first demonstrated that the adenosine receptor agonist 5′-N-methyl carboxamide adenosine (NECA), injected into the rat ventricles, increases running performance [12]. We then demonstrated the role of endogenous extracellular adenosine in the development of fatigue in mice [13] and the pivotal role of neuronal $A_{2A}R$ in the mouse forebrain for the ergogenic effects of caffeine [1, 14]. Caffeine increases performance expectancy in cyclists [15], reinforcing the findings of Sun et al. [16] on the role of $A_{2A}R$ in the *nucleus accumbens* (*NAc*) on early fatigue associated with exertion in mice or effort-based cost-benefit decision-making (E-CBDM). Accordingly, the overfunction of $A_{2A}R$ [17, 18] is a hypothetical mechanism for the fatigue symptom (E-CBDM) in neurological diseases [19].

Fatigue recruits corticostriatal loop connections that reduce performance [20] and impairs long-term potentiation (LTP) and long-term depression (LTD) [21]. This exercise-induced alterations of adenosine signaling [1, 13] and of neuroplasticity [20, 21] are candidate mechanisms

responsible for the central component of fatigue. In this work, we investigated the striatal neuroplasticity of caffeine ergogenicity.

## Materials and methods

Four different experiments were conducted in three different laboratories. First, caffeine was administered in vivo through systemic (i.p., experiment #1) or local treatment via stereotaxy (striatum, experiment #2) to assess their impact on animals' fatigue and motor control (Swiss mice, Labioex/UFSC, Brazil). Extracellular electrophysiology analyzed neuroplasticity in striatal slices treated with caffeine (experiment #3, C57BL/6J mice, CNC/U Coimbra, Portugal), and experiment #4 evaluated in vitro the effects of caffeine on the mitochondrial metabolism of SH-SY5Y human neuroblastoma cell lines (UFGRS/ Brazil).

### Experimental designs #1 and #2

#### Animals, caffeine, and stereotaxy

We used 34 Swiss naïve male adult mice (8–10 weeks, 47 ± 1.5 g). The animal housing had controlled lighting and temperature (12h light-dark cycle, lights on at 7:00 and off at 19:00, and room temperature of 21±1°C) and *ad libitum* access to food and tap water. Housing and handling of the animals followed the current Brazilian legislation (National Council for the Control of Animal Experimentation - CONCEA) and received bioethical approval (CEUA 1503210519). The allocation of animals to the experimental groups was random. Systemic treatment (in a volume of 10 ml/kg) with caffeine (15 mg/kg, i.p.) or saline (NaCl 0.9%, i.p.) was performed 15 min before the behavior and exercise tests.

Another group of mice were anesthetized with ketamine/ xylazine, and two cannulas were implanted, one in the right striatum (AP 0.5 mm; ML 2 mm and DV -3 mm) and other in the left striatum (AP 0.5 mm; ML −2 mm and DV −3 mm) [22]. One week after surgery, 4 μL of caffeine (15 μg) or saline (0.5 μg) was injected into conscious animals using an infusion pump (2 μL/min, Bonther®, Ribeirão Preto, Brazil) immediately before behavioral tests. The caffeine dose (15 μg) was selected through pilot studies and literature data [23, 24]. The mortality rate was 30% (*N*=9).

#### Behavioral analysis

The behavioral experiments were performed during the light phase of the circadian cycle, after 1 hour of habituation,

between 9:00 and 16:00, under controlled temperature (22±1°C) and luminosity (10 lux). All equipment was cleaned with EtOH 20% after each animal test.

## Open field

We manually recorded crossings and rearings in the circular open field test (diameter 58 cm × height 50 cm, Insight® EP154C, Ribeirão Preto, Brazil) for 5 min [1].

## Rota rod

The Rotarod test (Insight® EFF411) was used to evaluate motor coordination. The primary inclusion criterion was remaining in the stationary cylinder (0 RPM) for 30 s and rotating (5 RPM) for 90 s [25]. After 30 min, the latency to fall was evaluated in the accelerated cylinder (0.1 RPM/s) with an initial rotation of 5 RPM. Three falls excluded the animal from the evaluation stage. All pieces of equipment were cleaned after each animal test.

## Grip strength meter

The grip strength meter test (Bonther® 5 kgf) graded the strength and time of the front paw grip. Each mouse was placed individually in the grip meter bar, and after both paws were holding the bar, the experimenter gently pulled the tail in the opposite direction of the bar. We performed 4 trials with 10 s each and 1-min intervals between trials, and we considered the average of 3 best trials (3 out of 4) [26–28]. The equipment measures the force applied and the grip time, and the software provides the maximum and submaximal values. The decrease in physical performance is one of the characteristics of fatigue, evaluated in this behavioral test by the decrease in strength and grip time. We finalized the fatigue assessment by calculating the impulse by integrating the force × grip time curve.

## Experimental design #3

### Electrophysiology

Twenty-four naïve C57BL/6J mice (male, 23.7 ± 0.5 g, 8–10 weeks old) were euthanized through cervical dislocation, and the brain was quickly removed (within 1 min) and placed in ice-cold, oxygenated (95% $O_2$ and 5% $CO_2$) artificial cerebrospinal fluid (ACSF; in mM: 124 NaCl, 4.5 KCl, 1.2 $Na_2HPO_4$, 26 $NaHCO_3$, 1.2 $CaCl_2$, 1 $MgCl_2$, 10 glucose). Striatal slices (400 µm thick) were obtained using a Vibratome 1500 (Leica, Wetzlar, Germany) and allowed to recover for at least 90 min before being transferred to a submerged recording chamber and superfused at 3 mL/min with oxygenated ACSF kept at

30.5°C. Corticostriatal transmission was assessed with the stimulating electrode placed in the corpus callosum and the recording electrode filled with ACSF (2–5 MΩ) placed in the dorsolateral aspect of the striatum. The neuronal stimulus was delivered with a Grass S44 stimulator (Grass Technologies, RI), and the recordings were obtained by using an ISO-80 amplifier (World Precision Instruments, Hertfordshire, England) and digitized with an ADC-42 system (Pico Technologies, Pelham, NY). Three consecutive population spike responses were averaged and quantified using the Win LTP software v. 2.20b (WinLTP Ltd., Bristol, UK). The relationship between dendritic responsiveness and the synaptic input was determined based on input/output curves in which the population spike amplitude was plotted *versus* the stimulus intensity before and after administering different concentrations of caffeine in the ACSF solution. Stimulation intensity was then selected to yield 40%–50% of the maximum response. Corticostriatal slices were treated with 1 µM, 3 µM, or 10 µM caffeine in ACSF oxygenated solution for 20 min before the high-frequency stimulation (HFS). HFS consists of three trains each with 100 Hz pulses for 1 s with 10-s intervals between trains. The amplitude of synaptic plasticity was evaluated by the alteration of population spike amplitude 30 min after HFS for each concentration of caffeine [29–31]. Importantly, the tested concentrations of caffeine only act through the antagonism of adenosine receptors to impact of synaptic transmission [31].

## Experimental design #4

### Cell culture and evaluation of mitochondrial metabolism

Human neuroblastoma SH-SY5Y cells were maintained in DMEM/F12 supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin at 37°C, with 5% $CO_2$, under standard conditions. Cells were incubated with PBS or caffeine in concentrations (1 µM, 3 µM, or 10 µM) selectively acting as an adenosine receptor antagonist [6, 7]. An MTT assay (see below) evaluated the viability of cells 20 min after caffeine exposure [32]. Mitochondrial mass and membrane potential were analyzed using green and red mitotracker [32].

### MTT assay

SH-SY5Y cells were seeded into 96-well plates in DMEM-F12 with 10% FBS ($1 \times 10^4$ cells/well). After caffeine treatment, the cells were incubated with 0.5 mg/mL of MTT for 2 h at 37 °C. The cell viability was measured by quantifying the activity of cellular dehydrogenases that reduce MTT (3-4,5-dimethyl thiazolyl-2,5-diphenyl-2H-tetrazolium bromide, Sigma Inc.) to a purple formazan salt [33].

The formazan salt formed was dissolved in dimethyl sulfoxide (DMSO), and the absorbance was quantified in a spectrophotometer (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) at 540 nm [32].

### Mitotracker flow cytometry protocol

Mitotracker Green FM (Invitrogen) (MTG) or Mitotracker Red (MTR) are used for staining mitochondria of live cells depending, respectively, on the organelle lipid content (mitochondrial mass) and oxidative activity (mitochondrial membrane potential). Briefly, after caffeine treatment, SH-SY5Y cells were washed on PBS saline and harvested using trypsin. Then, cells were resuspended and incubated for 20 min in the dark with 100 nM of MTG and MTR diluted into pre-warmed (at 37 °C) DMEM F-12. Emitted fluorescence was measured (FL1 "green" 530 nm/30; FL3 "red" 670 nm long pass) in a FACSCalibur using the CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA) and data from 10.000 events were acquired using a log scale for all parameters. The fluorescence mean intensity of FL1-H and FL3-H was evaluated to estimate mitochondrial mass and potential, respectively. All flow cytometry analyses were performed using the FCS Express 4 software (De Novo, Pasadena, CA, USA) [32].

## Statistical analyses

Statistical analysis was performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). A one-tailed Student's $t$-test analyzed the open field test, rotarod, and grip strength meter; the Cohen's effect size ($d$) was calculated as small (0.2), medium (0.5), large (0.8), and very large (1.3) [34]. A one-way ANOVA analyzed electrophysiological and mitochondrial assays; Cohen's effect size ($\eta^2$) was calculated as small (0.01), medium (0.09), and large (0.25) (1.3) [34]. Differences were considered significant when $p<0.05$.

## Data availability

This work makes open data available under the creative commons license (CC BY) [35].

## Results

### Systemic and striatal treatment with caffeine is ergogenic

Systemic caffeine increased crossings ($t_{22}= 2.16$, $p< 0.05$, $d = 1.0$; Fig. 1A) and rearings ($t_{22}= 2.84$, $p< 0.05$, $d = 1.2$;

Fig. 1B) in the open field, and motor coordination in the rotarod ($t_{28}= 3.08$, $p< 0.05$, $d = 0.45$; Fig. 1C). Caffeine did not alter grip strength peak ($t_{22}= 1.37$, $p= 0.09$; Fig. 1D) but increased grip time ($t_{22}= 3.31$, $p< 0.05$, $d= 1.13$; Fig. 1E). Figure 1F' shows the area under curve (AUC) from strength vs. time results to demonstrate that ergogenic effects of caffeine are due to impulse magnitude ($t_{22}= 2.14$, $p< 0.05$, $d = 0.81$; Fig. 1F–F').

Figure 2A (top) shows the coordinates of the mice striatum according to Paxinos and Franklin [22], and Fig. 2A (bottom) shows a coronal brain view of a mouse brain, confirming that cannulas were implanted in the expected localization, as previously done [36, 37]. Caffeine injected directly into the striatum enhanced the number of crossings ($t_8= 9.81$, $p< 0.05$, $d = 1.82$; Fig. 2B) and rearings ($t_8= 3.66$, $p< 0.05$, $d = 1.50$; Fig. 2C) in the open field. Similarly, as occurred for its systemic treatment, the direct intra-striatal caffeine administration did not alter peak strength ($t_8= 0.85$, $p= 0.20$; Fig. 2D) but improved time grip time ($t_8= 4.08$, $p< 0.05$, $d = 1.56$; Fig. 2E) in the grip strength meter test. The ergogenic effect on impulse magnitude (Fig. 2F) was also observed after the intra-striatal direct administration of caffeine ($t_8= 3.50$, $p< 0.05$, $d = 1.48$; Fig. 2F').

### Caffeine changes LTD to LTP in striatum slices

Figure 3A shows the location of the stimulus electrode (top) in the corpus callosum and of the recording electrode (bottom) in the dorsolateral region of the striatum in a coronal brain slice. The first input/output curve (Fig. 3B) shows that all slices have a similar pattern of induced excitability during the pre-treatment period without caffeine. After 20 min of caffeine treatment, none of the tested concentrations of caffeine (1–10 µM) significantly altered basal synaptic transmission of corticostriatal synapses ($F_{3,16}=0.47$, $p=0.7$, Fig. 3C). The second input/output curve (Fig. 3D) performed after 20 min of exposure to caffeine shows an increased excitability with the higher concentration of caffeine (10 µM), as would be expected based on the ability of caffeine to antagonize $A_1$ receptors controlling basal synaptic transmission [31]. High-frequency stimulation (HFS, black arrow, $F_{29,864}=4.26$, $p< 0.05$, $\eta^2=0.21$, Fig. 3E) triggered an LTD in control slices (without any treatment), as well as in slices treated with 3 and 10 µM caffeine, whereas the exposure to 1 µM caffeine changed the response to HFS from an LTD to an LTP ($F_{3,20} = 11.2$, $p< 0.05$, $\eta^2=0.62$, Fig. 3F).

### Caffeine improved mitochondrial metabolism in SH-SY5Y cells

Caffeine acutely increased the mass and membrane potential of the mitochondria of SH-SY5Y cells. Cell viability
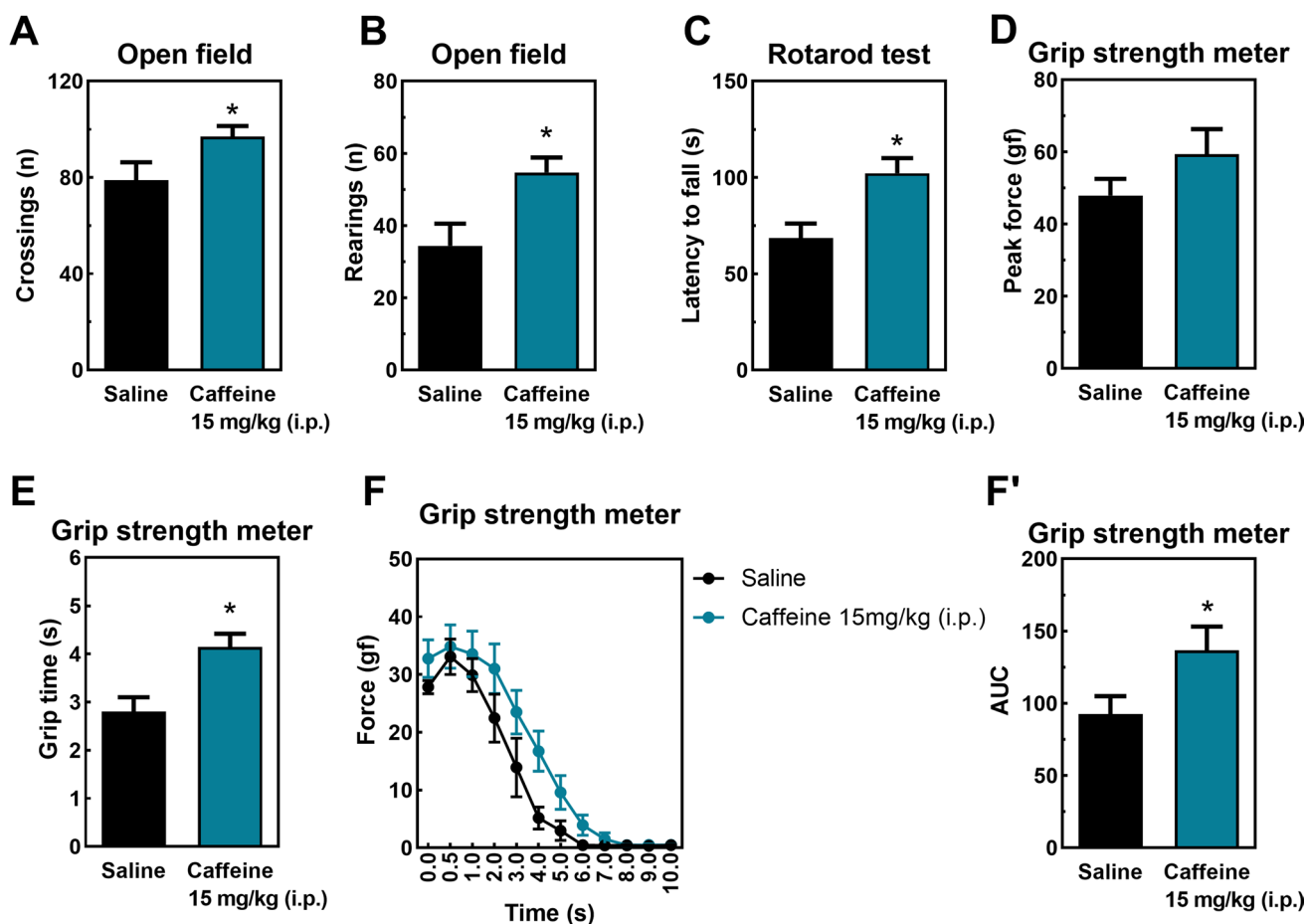
**Fig. 1** The behavioral effects of systemic caffeine in mice, namely psychostimulating (**A** and **B**), improved motor coordination (**C**), no effect on grip strength (**D**), but increase in grip time (**E**). Panel F′ is the integral of strength × time (**F**), representing increased caffeine-induced impulse. Data are mean ± SEM. $N$=7–8 animals/group for three independent experiments. *$P$<0.05 vs. saline (Student's $t$-test)

was not modified after exposure to any tested concentration of caffeine (1 μM, 3 μM, or 10 μM), as analyzed by the MTT assay ($F_{3,68} = 0.58$, $p= 0.6$, $\eta^2 = 0.03$; Fig. 4A). Caffeine (1 μM) increased mitochondrial membrane potential ($F_{3,10} = 14.99$, $p< 0.05$, $\eta^2 = 0.83$; Fig. 4B) and mitochondrial mass ($F_{3,11}= 20.87$, $p< 0.05$; Fig. 4C) of SH-SY5Y cells. Figure 4F shows a fluorescence shift to the right upon exposure to 1 μM caffeine.

## Discussion

This work demonstrates that the ergogenic effect of acute systemic and striatal caffeine on grip strength is associated with a change LTD to LTP in the striatum, probably with an increase of the mass and membrane potential of mitochondrial in neurons. Our behavioral data reinforce the evidence that systemic caffeine administration is ergogenic [1–5, 12]. However, we emphasize that this previous knowledge analyzed treadmill running performance, and we demonstrate here for the first time that caffeine increases (grip) muscle strength in mice, an effect also observed in humans [38, 39]. Additionally, we demonstrated for the first time that direct caffeine administration in the striatum reproduces the ergogenic effects upon systemic treatment with caffeine. Statistical effect sizes were large (systemic) and very large (intrastriatal) for caffeine ergogenicity. The modification of force performance was on impulse the grip strength size did not change. Once again, we demonstrate that caffeine modifies a time-derived index of physical performance (force × time), as we have previously demonstrated with running power (force ÷ time) [1].

Overall, we have been building a body of evidence to unravel the neurological mechanisms of the ergogenic effects of caffeine. The ergogenic effects of caffeine
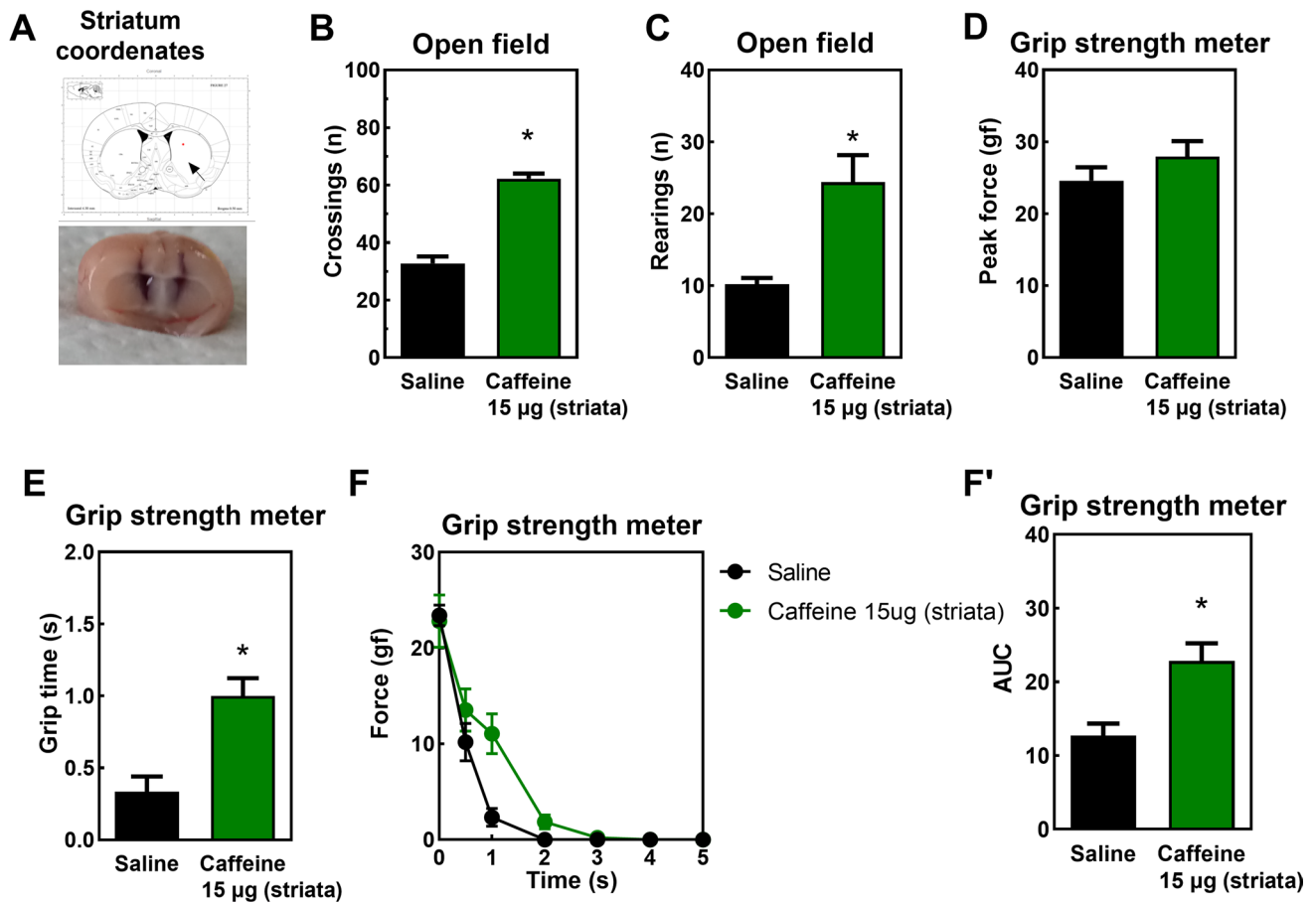
**Fig. 2** The behavioral effects of intrastriatal (**A**) injection of caffeine in mice, namely psychostimulating (**B** and **C**), no effect on grip strength (**D**), but an increase in grip time (**E**). Panel F′ is the integral of strength × time (**F**), representing increased caffeine-induced impulse. Data are mean ± SEM. $N$=7–8 animals/group for three independent experiments. *$P$<0.05 vs. saline (Student's $t$-test)

depend on the antagonism of neuronal $A_{2A}R$ in the rodent CNS [1, 12], and we now show that they involve a local neuroplasticity effect in the striatum, a brain region dense in $A_{2A}R$ [40]. Thus, caffeine improves motor coordination (in the rota rod) and grip time while shifting striatal neuroplasticity from an LTD to a LTP. This joins previous findings that caffeine injected into rat brain ventricles improves treadmill performance, an effect that disappears upon administration of the adenosine receptor agonist NECA [12]. Our results correspond to acute effects of caffeine in naïve animals; importantly, previous evidence suggests additional benefits when caffeine is consumed chronically, namely an increased resistance to stress [41, 42], from social [43] to cold stress [44]. In humans, the ergogenic effects of caffeine do not disappear with chronic consumption [2–5]. However, caffeine might increase anxiety [43] which is associated with impaired exercise performance due to reduced processing efficiency, including

attention, and increased physiological cost [45, 46]. However, caffeine does not impair exercise performance; on the contrary, it enhances it. The key to understand this apparent paradox might be the arousal effect of caffeine [47]. Physiological arousal in exercise bolsters performance when limited by awareness of exertion [45, 46]. Accordingly, caffeine decreases the rating of perceived exertion [48–50].

Our results strengthen the hypothesis about the ergogenic mechanism of caffeine involve an antagonism of the adenosine modulation system in the central nervous system [1, 12–14]. Central $A_{2A}R$ are linked to behavioral responses to exertion [16, 51, 52], such as fatigue and decision making, while $A_{2A}R$ antagonists such as caffeine and SCH58261 increase running performance [1, 12], and caffeine-treated forebrain $A_{2A}R$ knockouts mice did not demonstrate this ergogenic effect [1]. Indeed, exertion fatigue and ergogenic effects of caffeine are
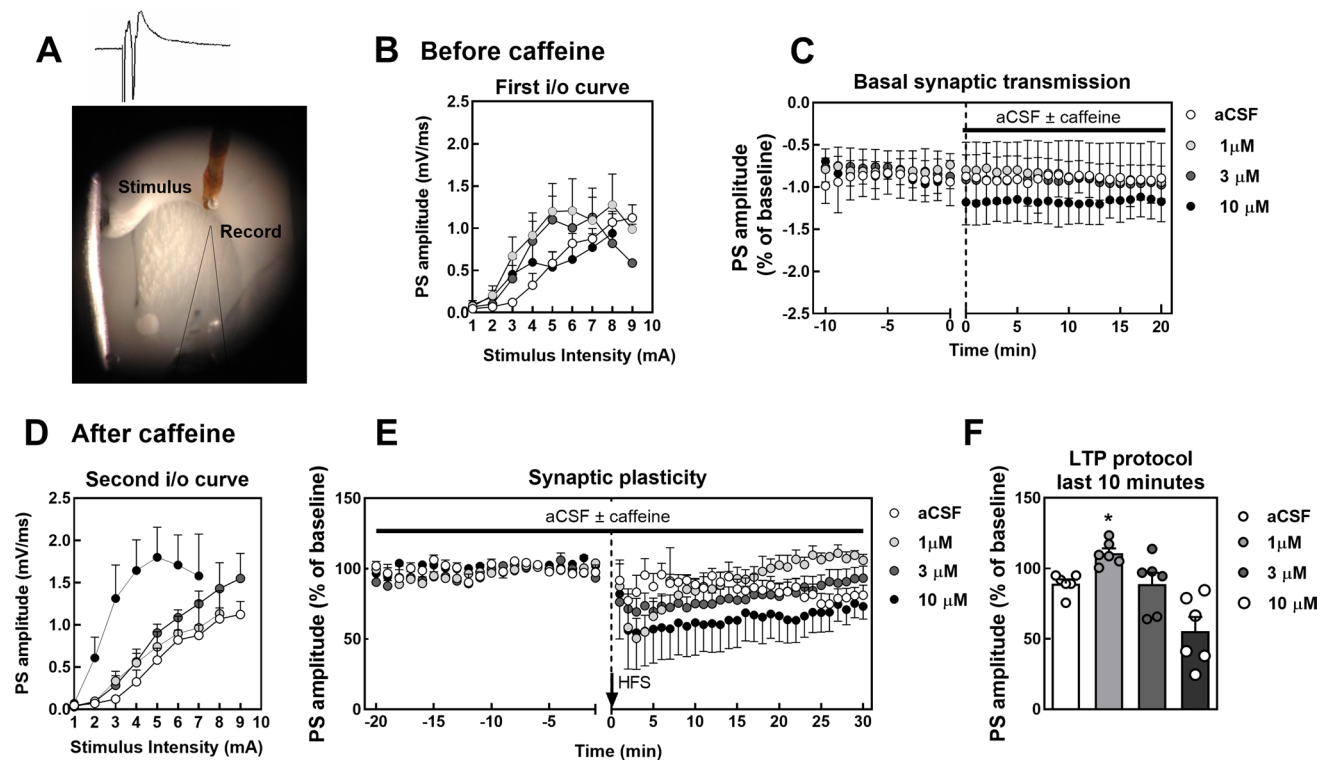
**Fig. 3** Extracellular electrophysiological recordings of population spike responses at corticostriatal synapses in the dorsolateral striatum in brain coronal slices (**A**). All groups displayed superimposable input/output curves, indicating similar efficiency of corticostriatal transmission (**B**). Alteration of basal synaptic transmission after exposure to different concentrations of caffeine (**C**). Twenty minutes after caffeine infusion, only 10 μM caffeine increased synaptic efficiency (**D**). The induction of synaptic plasticity by high-frequency stimulation (HFS) caused a long-term depression (LTD) in corticostriatal synapses (**E**), which was shifted into a long-term potentiation (LTP) upon exposure to 1 μM caffeine, as illustrated in the time course (**E**) and in the average alterations of the amplitude of synaptic plasticity 30–40 min afterHFS (**F**). Data are means ± SEM (*n*=6 slices/group). One-way ANOVA followed by Newman–Keuls post hoc tests). aCSF—artificial cerebrospinal fluid. i/o—input and output. PS—pop spike population

associated with $A_{2A}R$ [1, 16, 51, 52]. As the striatum displays a particularly high density of $A_{2A}R$ [40], we injected caffeine directly into the dorsolateral striatum of mice, which triggered psychomotor and ergogenic effects. The psychomotor effects of caffeine in the striatum are associated with allosteric modulation of $A_{2A}R$-$D_2R$ heteromers and presynaptic control of glutamatergic neurotransmission [53]. Striatal $A_{2A}R$ facilitate neuronal activity in the striatum by increasing dopaminergic signaling, more by controlling the availability of $D_2$ and $D_3$ receptors than by influencing dopamine release [54]. Thus, the $A_{2A}R$-$D_2R$ allosteric interaction modulates psychostimulant responses, which we believe to be involved in the ergogenic effect of caffeine, as discussed above.

Regarding electrophysiological findings, repetition of exercise can modify corticostriatal neuroplasticity, where LTD predominates, which plays an essential role in motor control [55, 56]. Ma et al. [21] demonstrated that exercise-induced fatigue impairs corticostriatal N-methyl-D-aspartate (NMDA) receptor-dependent LTP and endocannabinoid-dependent LTD (eCB-LTD). We observed that caffeine (1.0 μM) modified ex vivo corticostriatal neuroplasticity from LTD to LTP, although this in vivo effect remains to be confirmed. Conversely, the increased mitochondrial metabolism observed in cells supports increased neuronal activity at the site, as previously demonstrated in APPsw transgenic mice [57] and neuroblastoma cells [58].

This work is a proof of concept developed in naïve animals that reinforces our working hypothesis that $A_{2A}R$ in the corticostriatal pathway play a key ergogenic role [1, 13, 14]. However, some limitations of our work need to be considered. The experiments were carried out in males, a limitation [59] since there is evidence demonstrating that the ergogenic effects of caffeine and $A_{2A}R$ antagonists are similar [1, 60–63] or lesser in females [63–65], while the adverse effects are more significant[66]. Moreover, future investigations should evaluate the tolerability developed by
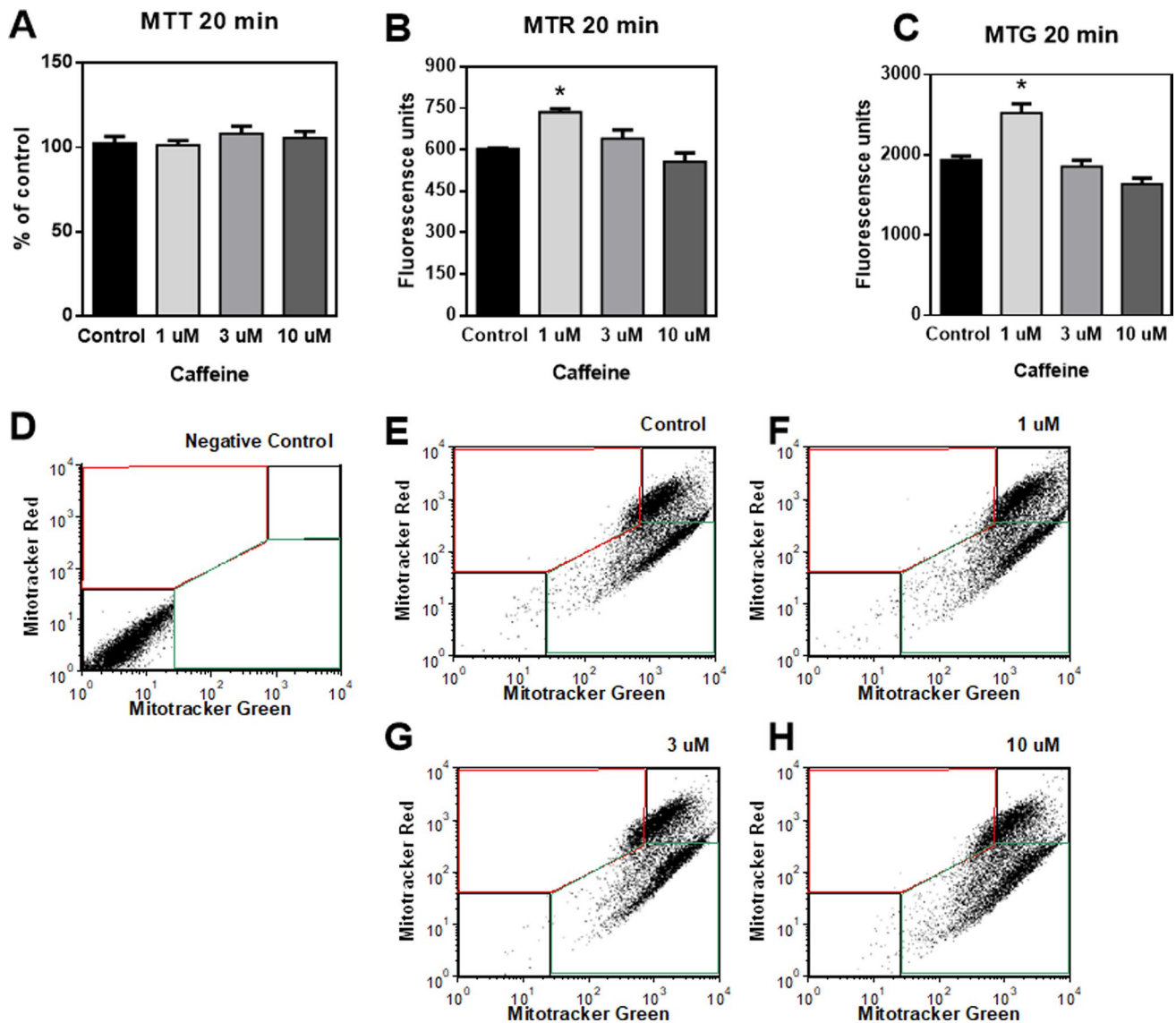
**Fig. 4** Caffeine improved mitochondrial metabolism in SH-SY5Y cells. Whereas it did not change cell viability (**A**), caffeine (1 μM) increased mitochondrial membrane potential (**B**) and mitochondrial mass (**C**). Panels **D–H** represents the flow cytometry analyses. Data are means ± SEM. One-way ANOVA followed by Newman–Keuls post hoc tests). MTG—mitotracker green. MTR—mitotracker red. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

regular consumption of coffee and caffeine on ergogenicity. Finally, it should be noted that the present evidence was gathered using different models, whereby behavior was assessed in Swiss mice, slice electrophysiology in C57BL/6J mice and metabolism in human neuroblastoma SH-SY5Y cells. The few studies on the ergogenicity of caffeine do not yet allow us to conclude on the eventual existence of differences between mouse strains, whereas the use of cell culture is expected to allow unveiling metabolic determinants of brain metabolism underlying the ergogenic effect of caffeine.

In summary, our results demonstrate that striatal $A_{2A}R$ contribute to the neurophysiology of the ergogenic effects of caffeine.

## Declarations

**Ethical approval** The ethics committees approved animal experiments in Brazil (Federal University of Santa Catarina, CEUA 1503210519) and Portugal (University of Coimbra, ORBEA 138-2016/1507201) according to specific legislation.

**Competing interests** RAC is a scientific consultant for the Institute for Scientific Information on Coffee (ISIC). ASAjr is the co-founder of Cannabisports, a sports supplement company. All other authors declare no competing interests.

## References

1. Aguiar AS, ASPCRC, Aguiar AS Jr, Speck AE et al (2020) Neuronal adenosine A2A receptors signal ergogenic effects of caffeine. Sci Rep 10:13414. https://doi.org/10.1038/s41598-020-69660-1
2. Jackman M, Wendling P, Friars D, Graham TE (1996) Metabolic, catecholamine, and endurance responses to caffeine during intense exercise. J Appl Physiol. https://doi.org/10.1152/jappl.1996.81.4.1658
3. Spriet LL, MacLean DA, Dyck DJ et al (1992) Caffeine ingestion and muscle metabolism during prolonged exercise in humans. Am J Physiol Endocrinol Metab. https://doi.org/10.1152/ajpendo.1992.262.6.e891
4. Grgic J, Mikulic P (2017) Caffeine ingestion acutely enhances muscular strength and power but not muscular endurance in resistance-trained men. Eur J Sport Sci. https://doi.org/10.1080/17461391.2017.1330362
5. Van Soeren MH, Sathasivam P, Spriet LL, Graham TE (1993) Caffeine metabolism and epinephrine responses during exercise in users and nonusers. J Appl Physiol. https://doi.org/10.1152/jappl.1993.75.2.805
6. Fredholm BB (1995) Adenosine, adenosine receptors and the actions of caffeine. Pharmacol Toxicol 76:93–101. https://doi.org/10.1111/J.1600-0773.1995.TB00111.X
7. Fredholm BB (1979) Are methylxanthine effects due to antagonism of endogenous adenosine? Trends Pharmacol Sci 1:129–132. https://doi.org/10.1016/0165-6147(79)90046-4
8. del Coso J, Muñoz G, Muñoz-Guerra J (2011) Prevalence of caffeine use in elite athletes following its removal from the World Anti-Doping Agency list of banned substances. Appl Physiol Nutr Metab 36:555–561. https://doi.org/10.1139/h11-052
9. van Thuyne W, Delbeke FT (2006) Distribution of caffeine levels in urine in different sports in relation to doping control before and after the removal of caffeine from the WADA doping list. Int J Sports Med 27:745–750. https://doi.org/10.1055/S-2005-872921/ID/25
10. Goldstein ER, Ziegenfuss T, Kalman D et al (2010) International society of sports nutrition position stand: Caffeine and performance. J Int Soc Sports Nutr 7(1):5
11. Costenla AR, Cunha RA, De Mendonça A (2010) Caffeine, adenosine receptors, and synaptic plasticity. Am. J. Alzheimer's Dis 20:S25–S34. https://doi.org/10.3233/JAD-2010-091384
12. Davis JM, Zhao Z, Stock HS, Mehl KA, Buggy J, Hand GA (2003) Central nervous system effects of caffeine and adenosine on fatigue. Am J Physiol Regul Integr Comp Physiol 284:399–404. https://doi.org/10.1152/ajpregu.00386.2002
13. Aguiar AS, Speck AE, Canas PM, Cunha RA (2021) Deletion of CD73 increases exercise power in mice. Purinergic Signal 17:393–397. https://doi.org/10.1007/S11302-021-09797-4/FIGURES/1
14. Aguiar JAS, Speck AE, Canas PM et al (2018) Oral communication: the ergogenic effects of caffeine depend on A2A receptors. Purinergic Signal. https://doi.org/10.1007/s11302-018-9637-0
15. Franco-Alvarenga PE, Brietzke C, Canestri R et al (2019) Caffeine improved cycling trial performance in mentally fatigued cyclists, regardless of alterations in prefrontal cortex activation. Physiol Behav. https://doi.org/10.1016/j.physbeh.2019.02.009
16. Sun X, Liu M, Xu X et al (2023) Accumbal adenosine A2A receptor inactivation biases for large and costly rewards in the effort- but not delay-based decision making. Neuropharmacology 222. https://doi.org/10.1016/j.neuropharm.2022.109273
17. Gomes CV, Kaster MP, Tomé AR et al (2011) Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochim Biophys Acta Biomembr 1808:1380–1399. https://doi.org/10.1016/J.BBAMEM.2010.12.001
18. Cunha RA (2016) How does adenosine control neuronal dysfunction and neurodegeneration? J Neurochem 139:1019–1055. https://doi.org/10.1111/JNC.13724
19. Alves ACDB, Bristot VJDO, Limana MD, Speck AE, Barros LSD, Solano AF, Aguiar AS (2019) Role of adenosine A2A receptors in the central fatigue of neurodegenerative diseases. J Caffeine Adenosine Res 9:145–156. https://doi.org/10.1089/caff.2019.0009
20. Schiff ND (2008) Central thalamic contributions to arousal regulation and neurological disorders of consciousness. Ann N Y Acad Sci 1129:105–118. https://doi.org/10.1196/ANNALS.1417.029
21. Ma J, Chen H, Liu X et al (2018) Exercise-induced fatigue impairs bidirectional corticostriatal synaptic plasticity. Front Cell Neurosci 12:14. https://doi.org/10.3389/FNCEL.2018.00014/BIBTEX
22. Paxinos G, Franklin KBJ (2012) The mouse brain in stereotaxic coordinates. Springer, US
23. Josselyn SA, Beninger RJ (1991) Behavioral effects of intrastriatal caffeine mediated by adenosinergic modulation of dopamine. Pharmacol Biochem Behav 39:97–103. https://doi.org/10.1016/0091-3057(91)90403-O
24. van Dort CJ, Baghdoyan HA, Lydic R (2009) Adenosine A1 and A2A receptors in mouse prefrontal cortex modulate acetylcholine release and behavioral arousal. J. Neurosci. Res. 29:871. https://doi.org/10.1523/JNEUROSCI.4111-08.2009
25. Aguiar AS Jr, Araújo AL, da-Cunha TR et al (2009) Physical exercise improves motor and short-term social memory deficits in reserpinized rats. Brain Res Bull 79. https://doi.org/10.1016/j.brainresbull.2009.05.005
26. Takeshita H, Yamamoto K, Nozato S et al (2017) Modified forelimb grip strength test detects aging-associated physiological decline in skeletal muscle function in male mice. Sci Rep 7:1–9. https://doi.org/10.1038/srep42323
27. Luca A De (2008) Use of grip strength meter to assess the limb strength of mdx mice. TREAT-NMD Neuromusclar Network DMD_M.2.2.:1–11
28. Personius KE, Jayaram A, Krull D et al (2010) Grip force, EDL contractile properties, and voluntary wheel running after

postdevelopmental myostatin depletion in mice. J Appl Physiol 109:886–894. https://doi.org/10.1152/japplphysiol.00300.2010

29. Leal G, Bramham CR, Duarte CB (2017) BDNF and hippocampal synaptic plasticity, 1st edn. Elsevier Inc.

30. Paillé V, Picconi B, Bagetta V et al (2010) Distinct levels of dopamine denervation differentially alter striatal synaptic plasticity and NMDA receptor subunit composition. J. Neurosci. 30:14182–14193. https://doi.org/10.1523/JNEUROSCI.2149-10.2010

31. Lopes JP, Pliássova A, Cunha RA (2019) The physiological effects of caffeine on synaptic transmission and plasticity in the mouse hippocampus selectively depend on adenosine A1 and A2A receptors. Biochem Pharmacol 166:313–321. https://doi.org/10.1016/j.bcp.2019.06.008

32. De Oliveira J, Moreira ELG, Mancini G et al (2013) Diphenyl diselenide prevents cortico-cerebral mitochondrial dysfunction and oxidative stress induced by hypercholesterolemia in LDL receptor knockout mice. Neurochem Res 38:2028–2036. https://doi.org/10.1007/s11064-013-1110-4

33. Hansen MB, Nielsen SE, Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119:203–210. https://doi.org/10.1016/0022-1759(89)90397-9

34. Sullivan GM, Feinn R (2012) Using effect size—or why the P value is not enough. J Grad Med Educ:279–282. https://doi.org/10.4300/JGME-D-12-00156.1

35. Speck AE, de Bem Alves AC, Aguiar Jr AS (2022) Open data. https://osf.io/w65eq/

36. Gonçalves N, Simões AT, Cunha RA, de Almeida LP (2013) Caffeine and adenosine A2A receptor inactivation decrease striatal neuropathology in a lentiviral-based model of Machado–Joseph disease. Ann Neurol 73:655–666. https://doi.org/10.1002/ANA.23866

37. Matheus FC, Rial D, Real JI et al (2016) Temporal dissociation of striatum and prefrontal cortex uncouples anhedonia and defense behaviors relevant to depression in 6-OHDA-lesioned rats. Mol Neurobiol 53:3891–3899. https://doi.org/10.1007/S12035-015-9330-Z/FIGURES/5

38. Delleli S, Ouergui I, Messaoudi H et al (2022) Acute effects of caffeine supplementation on physical performance, physiological responses, perceived exertion, and technical-tactical skills in combat sports: a systematic review and meta-analysis. Nutrients 14. https://doi.org/10.3390/NU14142996/S1

39. Grgic J (2022) Effects of caffeine on isometric handgrip strength: a meta-analysis. Clin Nutr ESPEN 47:89–95. https://doi.org/10.1016/J.CLNESP.2021.12.007

40. Gangarossa G, Espallergues J, Mailly P et al (2013) Spatial distribution of D1R- and D2R-expressing medium-sized spiny neurons differs along the rostro-caudal axis of the mouse dorsal striatum. Front Neural Circuits. https://doi.org/10.3389/FNCIR.2013.00124

41. Fang C, Hayashi S, Du X et al (2021) Caffeine protects against stress-induced murine depression through activation of PPARγC1α-mediated restoration of the kynurenine pathway in the skeletal muscle. Sci Rep 11:7287. https://doi.org/10.1038/s41598-021-86659-4

42. Kaster MP, Machado NJ, Silva HB et al (2015) Caffeine acts through neuronal adenosine A2A receptors to prevent mood and memory dysfunction triggered by chronic stress. Proc Natl Acad Sci U S A. https://doi.org/10.1073/pnas.1423088112

43. Ibrahim MK, Kamal M, Tikamdas R et al (2020) Effects of chronic caffeine administration on behavioral and molecular adaptations to sensory contact model induced stress in adolescent male mice. Behav Genet 50:374–383. https://doi.org/10.1007/s10519-020-10003-1

44. Estler CJ, Ammon HP, Herzog C (1978) Swimming capacity of mice after prolonged treatment with psychostimulants. I. Effects of caffeine on swimming performance and cold stress. Psychopharmacology (Berl) 58:161–166. https://doi.org/10.1007/BF00426900

45. Hardy L (1999) Stress, anxiety and performance. J Sci Med Sport 2:227–233. https://doi.org/10.1016/S1440-2440(99)80175-3

46. Hardy L, Beattie S, Woodman T (2007) Anxiety-induced performance catastrophes: investigating effort required as an asymmetry factor. Br J Psychol 98:15–31. https://doi.org/10.1348/000712606X103428

47. Nehlig A, Daval JL, Debry G (1992) Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Res Rev 17(2):139–170

48. Cole KJ, Costill DL, Starling RD et al (1996) Effect of caffeine ingestion on perception of effort and subsequent work production. Int J Sport Nutr Exerc Metab. https://doi.org/10.1123/ijsn.6.1.14

49. Doherty M, Smith PM, Hughes MG, Davison RCR (2004) Caffeine lowers perceptual response and increases power output during high-intensity cycling. J Sports Sci. https://doi.org/10.1080/02640410310001655741

50. Ivy JL, Kammer L, Ding Z et al (2009) Improved cycling time-trial performance after ingestion of a caffeine energy drink. Int J Sport Nutr Exerc Metab 19:61–78. https://doi.org/10.1123/IJSNEM.19.1.61

51. Mingote S, Font L, Farrar AM et al (2008) Nucleus accumbens adenosine A2A receptors regulate exertion of effort by acting on the ventral striatopallidal pathway. J Neurosci 28:9037–9046. https://doi.org/10.1523/JNEUROSCI.1525-08.2008

52. Font L, Mingote S, Farrar AM et al (2008) Intra-accumbens injections of the adenosine A2A agonist CGS 21680 affect effort-related choice behavior in rats. Psychopharmacology (Berl) 199:515–526. https://doi.org/10.1007/s00213-008-1174-z

53. Ciruela F, Casadó V, Rodrigues RJ et al (2006) Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. J. Neurosci. 26:2080–2087. https://doi.org/10.1523/JNEUROSCI.3574-05.2006

54. Chen JF, Moratalla R, Impagnatiello F et al (2001) The role of the D2 dopamine receptor (D2R) in A2A adenosine receptor (A2AR)-mediated behavioral and cellular responses as revealed by A2A and D2 receptor knockout mice. Proc Natl Acad Sci U S A 98:1970–1975. https://doi.org/10.1073/PNAS.98.4.1970/ASSET/47A065D8-92E2-4687-BD9E-7E79CF248371/ASSETS/GRAPHIC/PQ0313667005.JPEG

55. Gerdeman GL, Ronesi J, Lovinger DM (2002) Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. Nat Neurosci 5:5(5):446–451. https://doi.org/10.1038/nn832

56. Nambu A, Tokuno H, Takada M (2002) Functional significance of the cortico–subthalamo–pallidal 'hyperdirect' pathway. Neurosci Res 43:111–117. https://doi.org/10.1016/S0168-0102(02)00027-5

57. Dragicevic N, Delic V, Cao C et al (2012) Caffeine increases mitochondrial function and blocks melatonin signaling to mitochondria in Alzheimer's mice and cells. Neuropharmacology 63:1368–1379. https://doi.org/10.1016/J.NEUROPHARM.2012.08.018

58. Giunta S, Andriolo V, Castorina A (2014) Dual blockade of the A1 and A2A adenosine receptor prevents amyloid beta toxicity in neuroblastoma cells exposed to aluminum chloride. Int J Biochem Cell Biol 54:122–136. https://doi.org/10.1016/J.BIOCEL.2014.07.009

59. Aguiar AS, Speck AE, Amaral IM, Canas PM, Cunha RA (2018) The exercise sex gap and the impact of the estrous cycle on

exercise performance in mice. 8:10742. https://doi.org/10.1038/s41598-018-29050-0

60. Chen H-Y, Wang H-S, Tung K, Chao H-H (2015) Effects of gender difference and caffeine supplementation on anaerobic muscle performance. Int J Sports Med 36:974–978. https://doi.org/10.1055/s-0035-1550048

61. Skinner TL, Desbrow B, Arapova J et al (2019) Women experience the same ergogenic response to caffeine as men. Med Sci Sports Exerc 51:1195–1202. https://doi.org/10.1249/MSS.0000000000001885

62. Butts NK, Crowell D (2013) Effect of caffeine ingestion on cardiorespiratory endurance in men and women. Res Q Exerc Sport 56:301–305. https://doi.org/10.1080/02701367.1985.10605333

63. Grgic J (2021) Effects of caffeine on resistance exercise: a review of recent research. Sports Med 51:2281–2298. https://doi.org/10.1007/S40279-021-01521-X

64. Sabblah S, Dixon D, Bottoms L (2015) Sex differences on the acute effects of caffeine on maximal strength and muscular endurance. Comp. Exerc. Physiol. 11:89–94. https://doi.org/10.3920/CEP150010

65. Mielgo-Ayuso J, Marques-Jiménez D, Refoyo I et al (2019) Effect of caffeine supplementation on sports performance based on differences between sexes: a systematic review. Nutrients 11. https://doi.org/10.3390/nu11102313

66. Salinero JJ, Lara B, Abian-Vicen J et al (2014) The use of energy drinks in sport: perceived ergogenicity and side effects in male and female athletes. Br J Nutr 112:1494–1502. https://doi.org/10.1017/S0007114514002189