The striatum drives the ergogenic effects of caffeine

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Meet the Author Guidelines

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 adenosinergic A_{2A} receptors ($A_{2A}R$) antagonism in managing fatigue in the past two years. She researched new therapeutic options for neurological patients and improved their quality of life.

Abstract

Caffeine is one of the main ergogenic resources used in exercise and sports. Previously, we presented the ergogenic mechanism of caffeine through neuronal $A_{2A}R$ antagonism in the central nervous system [1]. We demonstrate here that the striatum rules the ergogenic effects of caffeine through neuroplasticity changes. Thirty-four Swiss (8-10 weeks, 47 ± 1.5 g) and twenty-four C57BL6 (8-10 weeks, 23.9 ± 0.4 g) adult male mice were challenged in behavior and electrophysiology experiments using caffeine and SH-SY5Y cells for energetic metabolism. Systemic (15 mg/kg, i.p.) or striatal (bilateral, 15 µg) caffeine was psychostimulant in the open field ($p < 0.05$) and increased gripping muscle power ($p < 0.05$). Caffeine also induced long-term potentiation (LTP) in striatal slices ($p < 0.05$) and increased mitochondrial mass ($p < 0.05$) and membrane potential $p < 0.05$) in SH-SY5Y dopaminergic cells. In summary, our results demonstrate that caffeine stimulation in the striatum produces ergogenic effects accompanied by an LTP, possibly associated with acute increased mitochondrial metabolism observed in dopaminergic cell lines.

Keywords: caffeine; dopamine; grip strength; mitochondria; neuroplasticity; striatum

INTRODUCTION

Exercise fatigue is one of the main barriers to exercise and sports performance. Caffeine (1,3,7-trimethylxantine) is a non-selective adenosine antagonist for A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) that exhibit psychostimulant and ergogenic (i.e., increases physical performance) effects, one of the most popular ergogenic aids in exercise and sports [2–5]. The central mechanisms of caffeine have robust evidence of ergogenic effects. Caffeine-enhancing effects include improved neurological functions such as typing speed, simple reaction time, sustained attention, memory, and logical reasoning during rest [6]. Davis first demonstrated that adenosine agonist 5′-N-ethylcarboxamide adenosine (NECA) injected into the rat ventricles increases running performance [7]. We then demonstrate the role of extracellular adenosine in the development of fatigue in mice [8] and the pivotal role of neuronal $A_{2A}R$ in the mouse forebrain for the ergogenic effects of caffeine [1, 9]. On the other hand, the increase in $A_{2A}R$ [10, 11] is a hypothetical mechanism for the fatigue symptom in neurological diseases [12].

Fatigue recruits corticostriatal loop connections that reduce performance [13] and impairs long-term potentiation (LTP) and long-term depression (LTD) [14]. This exercise-induced adenosine signaling [1, 8] and neuroplasticity change [13, 14] are responsible for the central component of fatigue. In this work, we investigated the striatal neuroplasticity of caffeine ergogenicity.

MATERIALS AND METHODS

Four different experiments are part of this work. First, caffeine was administered *in vivo* through systemic (i.p., experiment #1) or local treatment via stereotaxy (striatum, experiment #2) to assess the animals' fatigue and motor control. Extracellular electrophysiology analyzed neuroplasticity in striatal slices treated with caffeine (experiment #3), and experiment #4 *in vitro* evaluated the effects of caffeine on the mitochondrial metabolism of SH-SY5Y human neuroblastoma cell lines.

Experimental designs #1 and #2

Animals, caffeine, and stereotaxy

We used the experiment's 34 Swiss 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 am, and room temperature of 21±1°C) and ad libitum access to food and tap water. Housing and handling animals followed the current Brazilian legislation (National

Council for the Control of Animal Experimentation - CONCEA) with a bioethical approval number (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.

Mice were anesthetized with ketamine/xylazine, and two cannulas were implanted in the right striatum (AP 0.5 mm; ML 2 mm and DV -3 mm) and another in the left striatum (AP 0.5 mm; ML -2 mm and DV -3 mm) [15] for stereotaxy. After one week of surgery, 4µL of caffeine (15µg) or saline (0.5µg) was injected into conscious animals using an infusion pump (2 µL/minute, Bonther®, Ribeirão Preto, Brazil) immediately before behavioral tests. The mortality rate was 30% (9 animals).

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, in controlled temperature (22±1ºC) and luminosity (10 lux). All equipment was cleaned with EtOH 20% after each animal test.

Open field

The circular open field test (30×30cm, Insight® EP154C, Ribeirão Preto, Brazil) estimated locomotion for 5 minutes [16]. The numbers of crossings and rearings were manually analyzed.

Rota rod

The Rotarod test (Insight® EFF411) evaluated motor coordination assessment. The primary inclusion criterion was remaining in the stationary cylinder (0 RPM) for 30 seconds and rotating (5 RPM) for 90 seconds [17]. After 30 minutes, the latency to fall was evaluated in the accelerated cylinder (0.1 RPM/second) with an initial rotation of 5 RPM. Three falls excluded the animal from the evaluation stage. Both 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 it steady, the experimenter gently pulled the tail in the opposite direction of the bar. It was performed in 4 trials with 10 seconds each and 1-minute intervals between trials. It was considered the average of 3 best trials (3 out of 4) [18–20].

Experimental design #3

Electrophysiology

Twenty-four C57BL/6 mice were euthanized through cervical dislocation, and the brain was quickly removed (within 1min) and placed in ice-cold, oxygenated (95% O2 and 5% CO2) artificial cerebrospinal fluid (ACSF; in mM: 124 NaCl, 4.5 KCl, 1.2 Na2HPO4, 26 NaHCO3, 1.2 CaCl2, 1 MgCl2, 10 glucose). Striatal slices (400µm thick) were obtained using a Vibratome 1500 (Leica, Wetzlar, Germany) and allowed to recover for at least 90min before being transferred to a submerged recording chamber and superfused at 3mL/min with oxygenated ACSF kept at 30.5ºC. The corticostriatal transmission was assessed with the stimulating electrode placed in the corpus callosum and the recording electrode filled with ACSF (2–5 MX), placed in the dorsolateral portion of the striatum. The neuronal stimulus was performed using 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 1µM, 3µM, and 10µM of caffeine in ACSF oxygenated solution for twenty minutes before the high-frequency stimulation (HFS). This protocol consists of three trains (100Hz for 1 s with 10-s intervals between trains). The amplitude of population spike response for each concentration was assessed 30min after HFS [21–23].

Experimental design #4

Cell culture and mitochondrial metabolism evaluation

Human neuroblastoma SH-SY5Y cells were maintained in DMEM/F12 supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin at 37ºC, with 5% CO_2, under standard conditions. Cells were incubated with caffeine (1µM, 3µM, and 10µM) or PBS. An MTT assay evaluated the viability of cells 20 minutes after caffeine exposure [24]. Mitochondrial mass and membrane potential were analyzed using green and red mitotracker [24].

MTT assay

SH-SY5Y cells were seeded into 96-well plates in DMEM-F12 with 10% FBS (1□×□104 cells/well). After caffeine treatment, the cells were incubated with 0.5 mg/ml of MTT for two h at 37 °C. The cell viability was measured by quantifying the cellular dehydrogenases activity that reduces MTT (3-4,5-dimethylthiazolyl-2,5-diphenyl-2H-tetrazolium bromide, Sigma Inc.) to a purple formazan salt [Hansen et al., 1989]. 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 [24].

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 (37 °C) pre-warmed DMEM F-12. A bandpass green measured the emission of fluorescence (FL1; 530 nm/30) and red (FL3; 670 nm long pass) in a FACSCalibur using CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA), and data from 10.000 events were acquired using a log scale for all parameters, including FSC and SSC. The fluorescence intensity mean 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) [24].

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) [25]. A one-way ANOVA analyzed electrophysiological and mitochondrial essays; Cohen's effect size (η^2) was calculated as small (0.01), medium (0.09), and large (0.25) (1.3) [25]. Differences were considered significant when $p<0.05$.

Data Availability

This work makes open data available under the creative commons license (CC BY) [26].

RESULTS

Systemic and striatal treatment with caffeine is ergogenic

Systemic treatment with caffeine increased number of 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 test, and motor coordination in the rotarod test (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 Area Under Curve (AUC) from strength vs time results (fig.1f) and demonstrates 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 [15], and fig.2a (bottom) shows a coronal brain view of a mouse brain, confirming cannulas were implanted in the respective location. Caffeine injected directly into the striatum enhanced 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 test. Similarly, as systemic treatment, caffeine in the striatum 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 the impulse magnitude (fig.2f) remains in the topical treatment with caffeine in the striatum (t_8= 3.50, p< 0.05, d = 1.48, fig. 2f').

Caffeine-induced long-term potentiation (LTP) in the striatum

Fig.3a shows the location of the stimulus electrode (top) in the corpus callosum and the recording electrode (bottom) in the dorsolateral region of a slice of the striatum. The first input/output curve (fig.3.b) of increasing electrical stimuli shows the functional slice's viability in the pre-treatment period without caffeine. After 20 min of caffeine

treatment, no concentration changed the basal plasticity of the slices (fig.3c). The second input/output curve (fig.3.D) of increasing electrical stimuli shows the functional viability of the slices after 20 min of treatment with increasing concentrations of caffeine (1.0-10.0 µM). Applying the HFS (high-frequency stimulation) protocol (fig.3e, black arrow) after 10 min of basal synaptic plasticity demonstrates LTD response in control slices, without any treatment, and at 3.0 and 10.0 µM caffeine concentrations. Fig.3f represents the final 10 min of the LTP protocol with a change in the behavior of the slices from LTD to LTP with caffeine 1.0 µM ($F_{1.10} = 4.97$, $p < 0.05$, 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 was not modified in any caffeine concentration (1µm, 3µm, and 10µm) analyzed by the MTT assay ($F_{3,68} = 0.58$, $p = 0.6$, ($\eta^2 = 0.03$, fig.2a). Caffeine at concentration 1 um increased mitochondrial membrane potential (1µm, $F_{3,10} = 14.99$, $p < 0.05$, ($\eta^2 = 0.83$, fig.2b) and mitochondrial mass ($F_{3,11} = 20.87$, $p < 0.05$, ($\eta^2 = 0.81$, fig.2c) of SH-SY5Y cells. Fig.1F shows a fluorescence shift to the right at a concentration of 1µm.

DISCUSSION

This work demonstrated that the ergogenic effect of caffeine on grip strength is associated with an LTP in the striatum, probably with an increase in mitochondrial mass and membrane potential of neurons. Our behavioral data reinforce the evidence that systemic caffeine administration is ergogenic [2–5, 7, 16]. 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 well observed in humans [27, 28]. Also, we demonstrated that local caffeine treatment in the striatum reproduces these ergogenic effects (systemic treatment) for the first time. This ergogenic effect size was large and very large for systemic and striatal treatment, respectively. The modification of force performance was on impulse; the grip strength size did not change, but it was applied for a longer time. Once again, we demonstrate that caffeine modifies a time-derived index of physical performance (force × time), as we previously demonstrated with running power (force ÷ time) [16]. Thus, we have been building a body of evidence reinforcing the neurological mechanisms of the ergogenic effects of caffeine. The ergogenic effects of caffeine depend on the antagonism of neuronal $A_{2A}R$ in the rodent CNS [1, 7], reinforced by the local (striatum)

effect and neuroplasticity change demonstrated in this work, a brain region dense in $A_{2A}R$ [29]. Caffeine improves animal motor coordination (in rota rod) and grip time from shifting striatal neuroplasticity to LTP. In rats, caffeine injected into rat brain ventricles improves treadmill performance, which disappears with the adenosinergic agonist NECA [7].

Our results strengthen the hypothesis about the ergogenic mechanism of caffeine through adenosinergic antagonism in the central nervous system [7–9, 16]. Mice treated with caffeine and SCH 58261 demonstrated increased running performance, while caffeine-treated forebrain $A_{2A}R$ knockouts did not demonstrate this effect [16]. The ergogenic effects of caffeine are associated with $A_{2A}R$ antagonism [16]. As the striatum is dense in $A_{2A}R$ [29], we injected caffeine into the dorsolateral striatum of mice and saw psychomotor and ergogenic effects. The psychomotor effects of caffeine on the striatum are associated with allosteric modulation of $A_{2A}R$-D_2R heteromers and presynaptic control glutamatergic neurotransmission [30]. The striatal $A_{2A}Rs$ facilitate neuronal activity in the striatum by increasing dopaminergic signaling, more by increasing the availability of D_2 and D_3 receptors than by increasing dopamine neurotransmission [31]. Thus, the $A_{2A}R$-D_2R allosteric interaction modulates psychostimulant effects, and we believe it may be involved in the ergogenic mechanisms.

Caffeine's control of presynaptic glutamate release and increased NMDA function in the striatum may contribute to the attenuation of fatigue during exercise. Furthermore, the LTP we observed with physiological magnesium concentrations occurs with glutamatergic NMDA receptors' activation [32], suggesting glutamatergic neurotransmission facilitation in caffeine-treated mice. Conversely, exercise-induced fatigue impairs NMDA receptor-dependent LTP in the corticostriatal pathway [14]. The increased mitochondrial metabolism observed in cells supports increased neuronal activity at the site, as previously demonstrated in APPsw transgenic mice [32] and neuroblastoma cells [33].

We demonstrate the psychostimulant and ergogenic effects of caffeine injected locally into the striatum with neuroplasticity modifications that suggest glutamatergic facilitation as an ergogenic mechanism of caffeine.

Funding

Prémio Maratona da Saúde, CAPES (Demanda Social), CAPES-FCT (039/2014), CNPq (302234/2016-0), LaCaixa Foundation (LCF/PR/HP17/52190001), FAPESC (1664/2017), FCT (POCI-01-0145-FEDER-03127 and UIDB/04539/2020), and ERDF through Centro 2020 (project CENTRO-01-0145-FEDER-000008:BrainHealth 2020 and CENTRO-01-0246-FEDER-000010). A.S.A.Jr is a CNPq fellow (310635/2020-9).

Conflict of Interest

RAC is a scientific consultant for the Institute for Scientific Information on Coffee. All other authors declare no conflict of interests.

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.

Data availability

This work makes open data available under the creative commons license (CC BY) [26].

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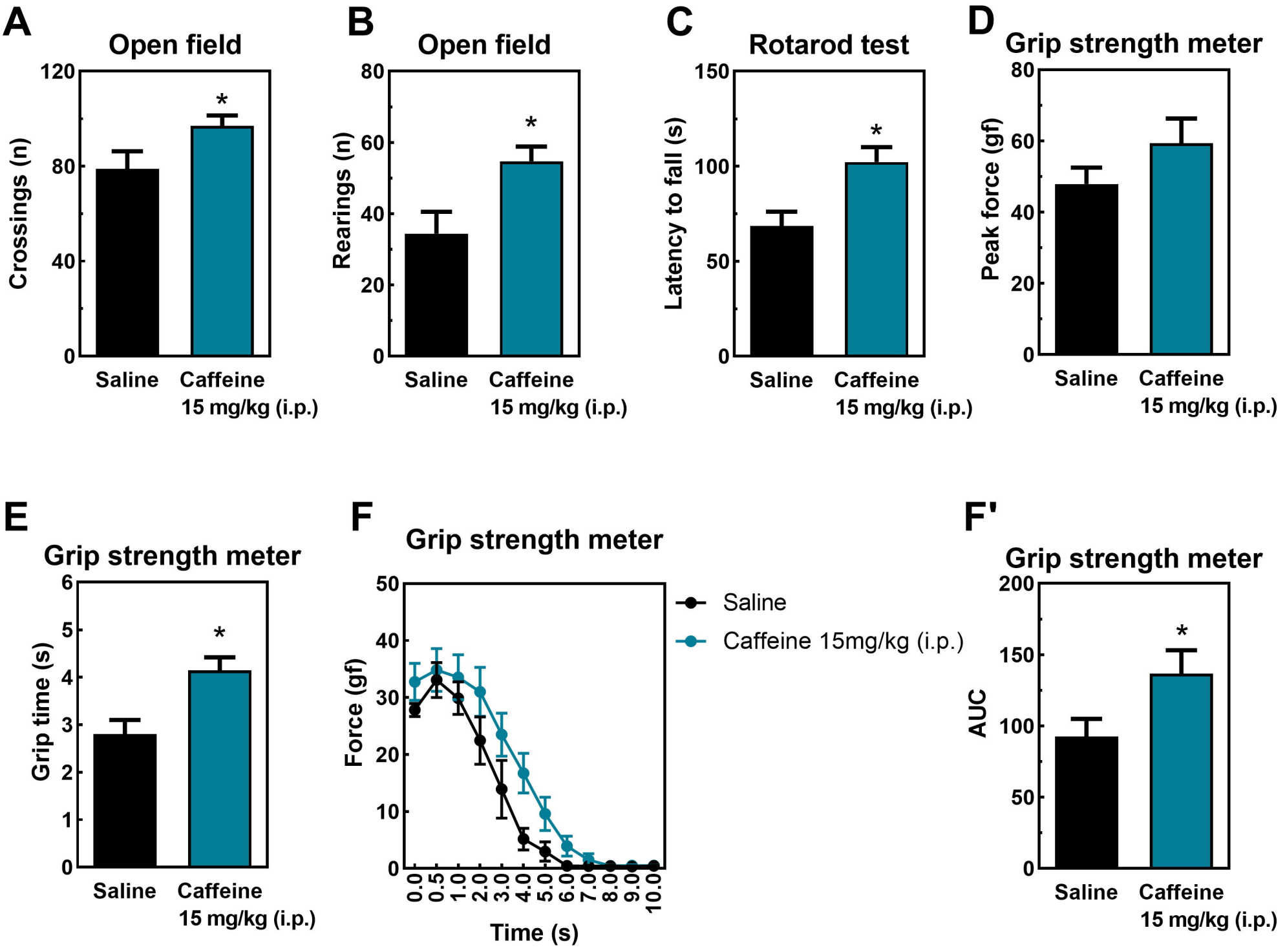
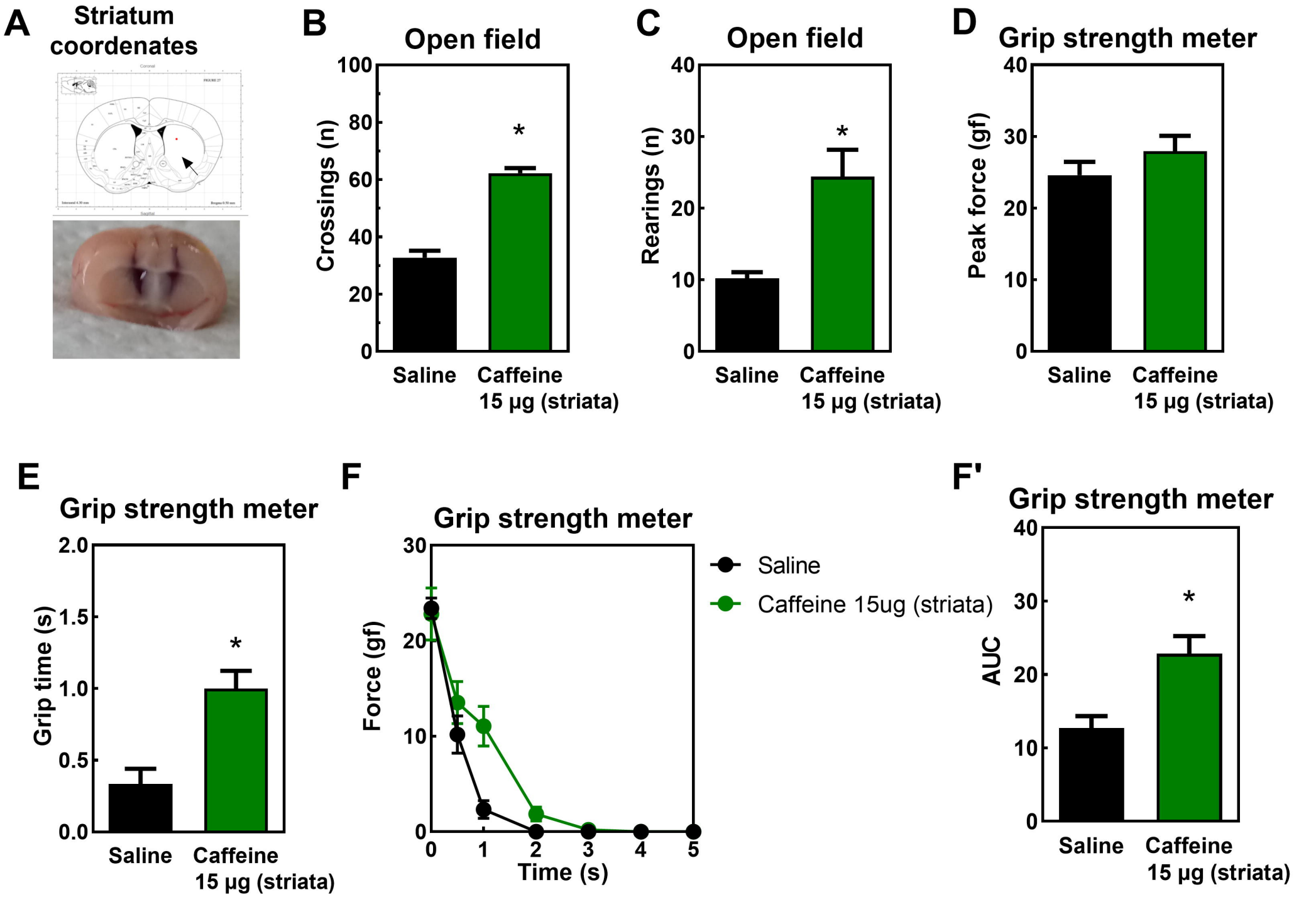
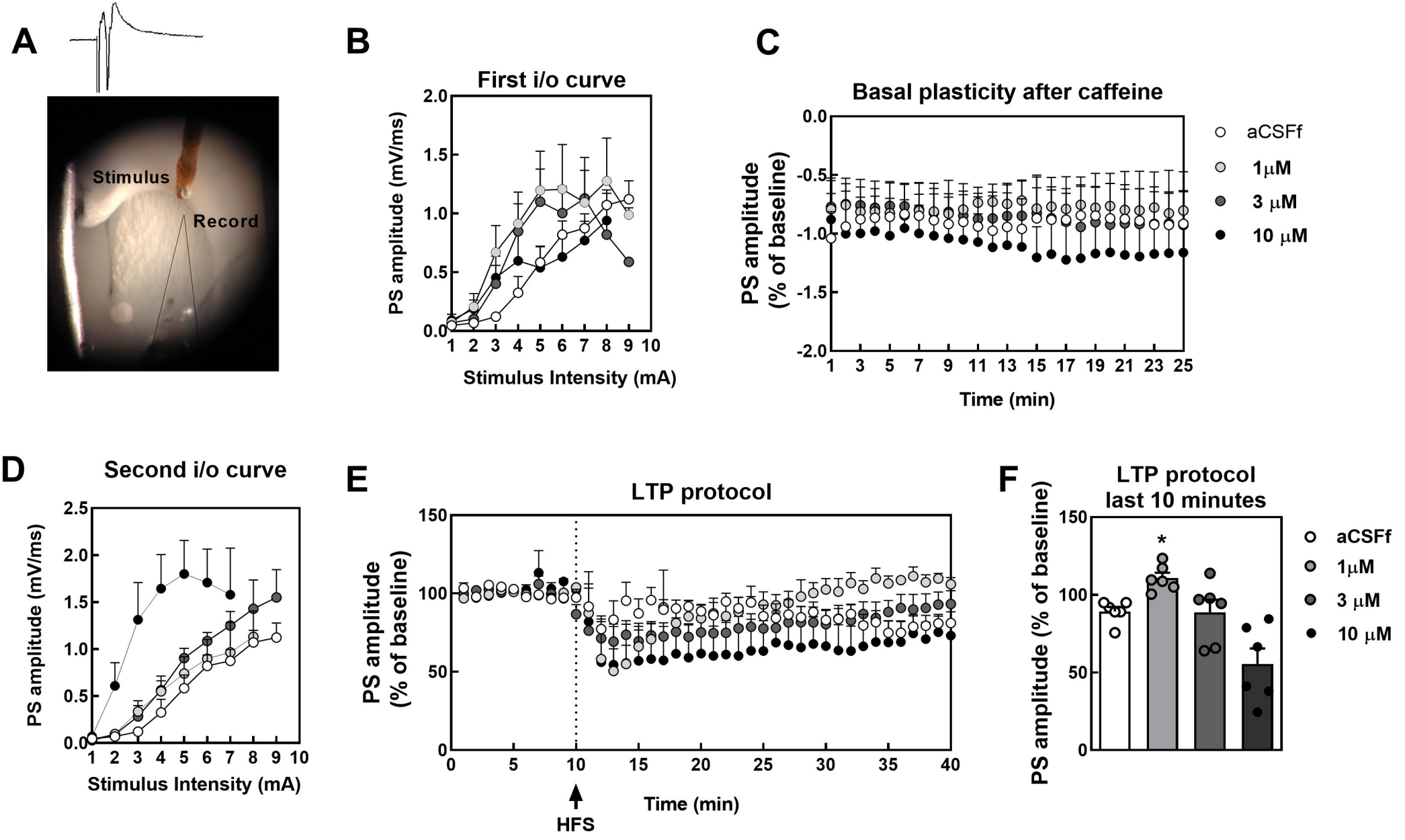
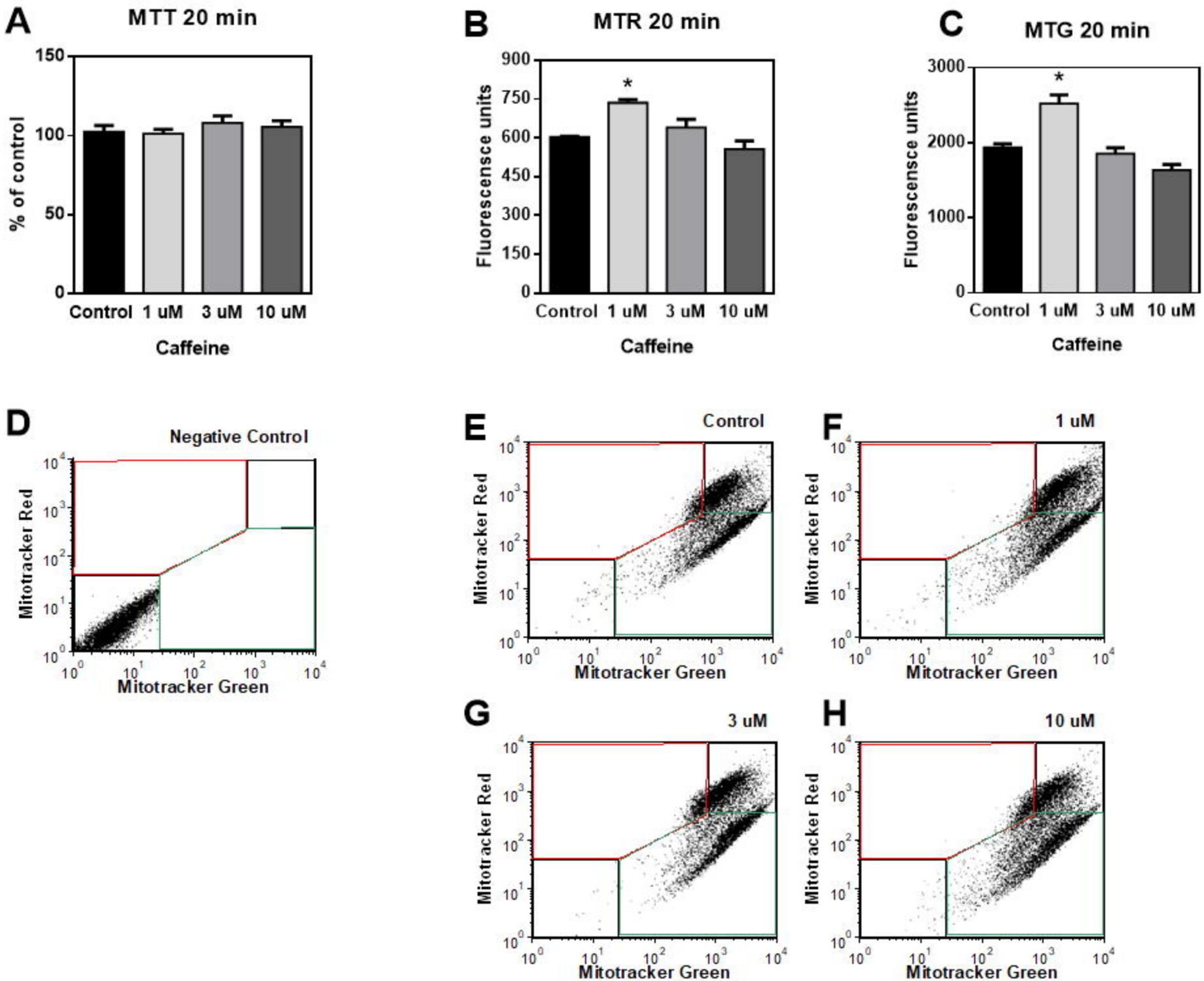

A
Open field
Crossings (n)
120
80
40
0
*
Saline
Caffeine
15 mg/kg (i.p.)
B
Open field
Rearings (n)
80
60
40
20
0
*
Saline
Caffeine
15 mg/kg (i.p.)
C
Rotarod test
Latency to fall (s)
150
100
50
0
*
Saline
Caffeine
15 mg/kg (i.p.)
D
Grip strength meter
Peak force (gf)
80
60
40
20
0
Saline
Caffeine
15 mg/kg (i.p.)
E
Grip strength meter
Grip time (s)
6
5
4
3
2
1
0
*
Saline
Caffeine
15 mg/kg (i.p.)
F
Grip strength meter
Force (gf)
50
40
30
20
10
0
0.0
0.5
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Time (s)
Saline
Caffeine 15mg/kg (i.p.)
F'
Grip strength meter
AUC
200
150
100
50
0
*
Saline
Caffeine
15 mg/kg (i.p.)


A
Striatum coordenates
B
Open field
Crossings (n)
100
80
60
40
20
0
*
Saline
Caffeine 15 µg (striata)
C
Open field
Rearings (n)
40
30
20
10
0
*
Saline
Caffeine 15 µg (striata)
D
Grip strength meter
Peak force (gf)
40
30
20
10
0
Saline
Caffeine 15 µg (striata)
E
Grip strength meter
Grip time (s)
2.0
1.5
1.0
0.5
0.0
*
Saline
Caffeine 15 µg (striata)
F
Grip strength meter
Force (gf)
30
20
10
0
0
1
2
3
4
5
Time (s)
Saline
Caffeine 15ug (striata)
F'
Grip strength meter
AUC
40
30
20
10
0
*
Saline
Caffeine 15 µg (striata)


A
Stimulus
Record
B
First i/o curve
PS amplitude (mV/ms)
Stimulus Intensity (mA)
C
Basal plasticity after caffeine
PS amplitude (% of baseline)
Time (min)
aCSFf
1μM
3 μM
10 μM
D
Second i/o curve
PS amplitude (mV/ms)
Stimulus Intensity (mA)
E
LTP protocol
PS amplitude (% of baseline)
HFS
Time (min)
F
LTP protocol last 10 minutes
PS amplitude (% of baseline)
aCSFf
1μM
3 μM
10 μM


A
MTT 20 min
% of control
150
100
50
0
Control
1 uM
3 uM
10 uM
Caffeine
B
MTR 20 min
Fluorescensce units
900
750
600
450
300
150
0
*
Control
1 uM
3 uM
10 uM
Caffeine
C
MTG 20 min
Fluorescensce units
3000
2000
1000
0
*
Control
1 uM
3 uM
10 uM
Caffeine
D
Negative Control
Mitotracker Red
Mitotracker Green
E
Control
Mitotracker Red
Mitotracker Green
F
1 uM
Mitotracker Red
Mitotracker Green
G
3 uM
Mitotracker Red
Mitotracker Green
H
10 uM
Mitotracker Red
Mitotracker Green