Effects of long-term caffeine consumption on the adenosine A_1 receptor in the rat brain: An in vivo PET study with [^{18}F]CPFPX

Abbreviated title: Effects of caffeine intake on A1AR

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ABSTRACT

Purpose: Caffeine, a nonselective antagonist of adenosine receptors, is the most popular psychostimulant worldwide. Recently, a protective role of moderate chronic caffeine consumption against neurodegenerative diseases such as Alzheimer's and Parkinson's disease has been discussed. Thus, aim of the present study was an in vivo investigation of effects of long-term caffeine consumption on the adenosine A_1 receptor (A_1AR) in the rat brain.

Procedures: Sixteen adult, male rats underwent five positron emission tomography (PET) scans with the highly selective A_1AR radioligand [^{18}F]CPFPX in order to determine A_1AR availability. After the 1st baseline PET scan, the animals were assigned to two groups: caffeine-treatment and control group. The caffeine-treated animals received caffeinated tap water (30mg/kg bodyweight/day, corresponding to 4-5 cups of coffee per day in humans) for 12 weeks. Subsequently, caffeine was withdrawn and repeated PET measurements were performed on day 1, 2, 4, and 7 of caffeine withdrawal. The control animals were measured according to the same time schedule.

Results: At Day 1, after 4.4 hours of caffeine withdrawal, a significant decrease (-34.5%, $p<0.001$) of whole brain A_1AR availability was observed. Unlike all other investigated brain regions in caffeine treated rats, hypothalamus and nucleus accumbens showed no significant intraindividual differences between baseline and 1st withdrawal PET scan. After approximately 27 hours of caffeine withdrawal the region- and group-specific effects disappeared and A_1AR availability settled around baseline.

Conclusions: The present study provides evidence that chronic caffeine consumption does not lead to persistent changes in functional availability of cerebral A_1ARs which have previously been associated with neuroprotective effects of caffeine. The acute and region-specific decrease in cerebral A_1AR availability directly after caffeine withdrawal is most likely caused by residual amounts of caffeine metabolites disguising an unchanged A_1AR expression at this early time-point.

KEYWORDS

chronic caffeine, PET, adenosine A_1 receptor, rat, brain

INTRODUCTION

Adenosine is involved in numerous physiological as well as pathophysiological processes and acts as a neuromodulator on all four G protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3) [1]. The A_1 adenosine receptor (A_1AR) has the widest distribution and highest density in the brains of rats [2] and humans [3]. Its primary role in processes like sleep-wake regulation, memory consolidation, epilepsy and schizophrenia (for review see [4,5]) induced great interest in in vivo imaging techniques for the A_1AR with positron emission tomography (PET) in order to visualize molecular processes. The flour-18 labelled A_1AR antagonist ^{18}F-8-cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine ([^{18}F]CPFPX, [6]) has been well characterized as a highly selective A_1AR radioligand in rats [7] and humans [8].

The best-known and frequently consumed adenosine antagonist is caffeine which acts on A_1 and A_{2A} adenosine receptor subtypes in a nearly equipotent manner [9]. Caffeine reduces sleepiness [10] and increases cognitive performance and attention [11,12]. Moreover, epidemiological studies showed that caffeine has a protective role against neurodegenerative diseases such as Alzheimer's and Parkinson's disease [13,14]. Although the beneficial mode of action of chronic caffeine consumption remains hypothetical, an involvement of adenosine and its neuromodulatory properties seems to be most likely. During pathophysiological conditions such as hypoxia and cerebral ischemia, endogenous adenosine is released and subsequently reduces the neuronal damage in tissue primarily mediated via the ubiquitous A_1AR (for review see [15]). Thereby the caffeine-induced receptor blockade – leading to an increase in adenosine concentration (own unpublished data) – may finally contribute to the neuroprotective effects of chronic caffeine consumption. In the past, the molecular effects of chronic oral caffeine consumption on the A_1AR were investigated in various studies with

inconsistent results. Several studies revealed an upregulation [16–18], while other studies reported constant A_1AR density [19–21] after chronic caffeine administration up to 42 days. However, Marangos *et al.* [22] and Boulenger *et al.* [23] already showed that the impact of chronic caffeine ingestion on the A_1AR expression was dependent on the duration of caffeine intake and dosage respectively. All these studies were conducted exclusively in vitro and mainly with agonists as radioligands.

The aim of the present study was to examine the regional effects of long-term caffeine consumption on A_1ARs in vivo with PET and the highly affine and selective radioligand [^{18}F]CPFPX. After determination of baseline A_1AR availability, caffeine (30mg/kg/day) was administered in drinking water over an application period of 84 days which substantially exceeds previous studies. In human life time, this time span corresponds to approximately 8 years [24] and reflects the chronic consumption behavior of humans adequately.

Since caffeine and the applied radioligand both act on the A_1AR [25], caffeine was withdrawn before PET scanning in order to ensure a valid quantification of the A_1AR. PET measurements were performed on day 1, 2, 4 and 7 of caffeine withdrawal (caffeine-treated group) or normal aging (control group). Previous studies showed that the observed effects of chronic caffeine consumption on the A_1AR persisted at least 2 days after caffeine withdrawal [16,26]. Consequently, the first PET measurement after caffeine withdrawal (Day 1) likely reflects the receptor availability during chronic caffeine application. However, Boulenger *et al*. [16] investigated the effects of caffeine withdrawal with a radioligand unspecific for A_1ARs and A_{2A}ARs. Thus, it is not clear whether the observed relatively long-lasting (15 days) increase in receptor availability is due to radioligand binding to A_1ARs or A_{2A}ARs.

In contrast, Kaplan *et al*. [26] showed a return to baseline A_1AR availabilities after four days – measured ex vivo with an A_1AR specific radioligand. However, in the respective study the total and non-specific binding of the radioligand was not determined in the same but in different animals and specific binding was calculated by total minus mean non-specific

binding. As the uptake of the radioligand might individually vary, values are likely to be more variable than in neuroreceptor quantification with in vivo PET providing estimates of total and non-specific binding within the same animal.

In this regard, the hypothesis of an A_1AR upregulation that may trigger the neuroprotective effects of chronic caffeine consumption is examined in vivo for the first time.

MATERIALS AND METHODS

Animals and study design

Experiments were performed in sixteen adult, male Sprague Dawley rats (age 3-4 months, >400g, Charles River Laboratories) housed in pairs in a 12 hours light/dark cycle (lights on: 7:00 AM to 7:00 PM) at 22±1°C with access to food and water ad libitum. After at least one week of acclimatization, baseline A_1AR availability was determined with PET. Afterwards, rats were assigned to two experimental groups: Caffeine-treated rats (n=8, mean weight at baseline PET: 451±34g) received caffeinated (30mg/kg bodyweight/day, Sigma Aldrich, St. Louis, MO) tap water ad libitum for 12 weeks (Fig.1). Caffeine intake of each pair was weekly determined based on the average bodyweight and fluid intake. After 12 weeks of chronic caffeine administration, repeated A_1AR PET measurements were performed on Day 1 (after 4.4 hours), Day 2 (after 27.8 hours), Day 4 (after 75.8 hours) and Day 7 (after 172.0 hours) of caffeine withdrawal at the same time of day (~6 hours after lights on) in order to control for potential circadian effects. Control rats (n=8, mean weight at baseline PET: 458±16g) receiving pure tap water were housed under the same conditions and were analyzed with an identical time schedule. All experiments were approved by the regional authorities and conducted in accordance with the German Animal Protection Act.

PET Imaging and Image Analysis

PET acquisition as well as image analysis were performed as previously described by Kroll *et al.*[27].

Animals were scanned in pairs head to head in prone position with a Siemens Inveon multimodality PET scanner (Siemens, Knoxville, TN, USA) under isoflurane anesthesia (1.5-2% isoflurane in 2L O_2/min) guided by a respiratory sequence of ~50/min. Body temperature (rectal probe) and respiratory sequence were monitored (BioVet System, m2m Imaging, Salisbury, QLD, Australia) and kept in physiological ranges. For attenuation correction, a 15-minute transmission measurement was performed with a dual ^{57}Cobalt source prior to emission scans. Subsequently, a bolus of the in-house synthesized [^{18}F]CPFPX [6] diluted in 1mL NaCl was constantly administered over 1 min with a syringe pump (model 44, Harvard Apparatus, Holliston, MA, USA) via a tail vein catheter.

Emission scans were started simultaneously to the tracer application for the first animal. For the second animal, tracer administration started with a time lag of 3 min. For mean injected radioactivity and injected amount of substance per scan and group see Table 1. List mode data were collected for 75 min and data of 70 min (beginning with the radioligand application) were sorted separately for each animal in a dynamic sequence of 12 x 10 s, 3 x 20 s, 3 x 30 s, 3 x 1 min, 3 x 2.5 min and 11 x 5 min frames. Dynamic PET data were reconstructed per time frame by filtered back projection after Fourier rebinning into 2D sinograms. The corresponding voxel size was 0.7764 x 0.7764 x 0.796 m³. Data were corrected for random coincidences, scatter radiation and attenuation.

Individual integrated PET images were manually scaled and co-registered to the slightly modified rat brain atlas implemented in PMOD (Version 3.408, PMOD Group, Zürich, Switzerland). After inspection of dynamic data for potential head movements the co-registration transformation matrix was applied to all frames. Subsequently, all Baseline (BL) images were automatically adjusted (PMOD algorithm “mouse changing”) to a matched average PET template based on 13 [^{18}F]CPFPX PET datasets of Sprague-Dawley rats with corresponding weight. Manually co-registered PET data of the remaining four PET scans

were finally registered to the automatically co-registered BL images applying the same algorithm in order to minimize the bias introduced by variable manual co-registrations to the rat brain template. After visual inspection of exact covering of anatomical structures, time-activity curves (TACs) were defined based on the rat brain atlas for the following regions: Cortex (0.51mL), cerebellum (0.15mL), caudatus putamen (0.09mL), hippocampus (0.07mL), thalamus (0.06mL), hypothalamus (0.04mL), nucleus accumbens (0.02mL) and olfactory bulb (0.05mL). For quantitative analysis of A_1AR availabilities the binding potential (BP_{ND}) – a parameter directly proportional to the maximum number of available receptors [28] – was estimated via the simplified reference-tissue model (SRTM) [29] on the basis of the olfactory bulb as a reference region. The parametric BP_{ND} images (n=8 per group) were generated with SRTM2 and fixed k_2‘ values being calculated by averaging k_2‘ parameters of high binding regions from SRTM.

Statistics

All values are displayed as mean ± standard deviation (SD). Results were generated by calculating the following parameters:

a) relative difference$_{group}$ $[\%] = \frac{caffeine-control}{control} * 100\%$ and

relative difference$_{scan}$ $[\%] = \frac{scan\ 2-scan\ 1}{scan\ 1} * 100\%$.

b) calculated xanthine plasma level = $initial\ plasma\ level\ [\mu M] \times e^{-\ln 2 \times \frac{decaffeinated\ period\ [h]}{half-life\ [h]}}$.

Fluid intake and weight gain of caffeine-treated and control animals during the period of chronic caffeine administration were compared with two tailed t-tests. General parameters of the scan sessions (length of anesthesia, injected activity and injected amount of substance) were analyzed with a univariate analysis of variance (ANOVA). Differences in A_1AR availability between the caffeine-treated and control group were determined by a mixed model ANOVA (rmANOVA) with condition as between-subject factor and the different brain

regions and scan session treated as within-subject factors. Significant results ($p<0.05$) were further evaluated by post-hoc two-tailed t-tests (independent or matched as appropriate) and subsequent Bonferroni correction. All statistical calculations were performed with the SPSS software (version 22, SPSS Inc., Chicago, IL).

RESULTS

During the administration period (caffeine or tap water respectively) of 12 weeks, there were no significant differences in weight gain and daily water intake between the caffeine-treated and the control group (Table 2). The mean caffeine intake was 29.4±1.4mg/kg bodyweight/day and remained stable over the entire application period. Scan parameters such as length of anesthesia at scan start as well as injected dose were not significantly different between the scans both within and between the groups, whereas the injected amount of substance between scans showed significant differences within the control group (Table 1, $p=0.006$). However, differences did not withstand subsequent post-hoc analysis via unpaired t-tests followed by Bonferroni adjustment with a threshold of $p<0.005$.

Fig. 2 shows mean parametric images of the caffeine-treated and control animals at different time-points reflecting the A_1AR availability in the rat brain. In general, high A_1AR densities were observed in cerebellum, hippocampus, thalamus and cortex. In contrast, nucleus accumbens and hypothalamus showed low A_1AR availabilities. Corresponding whole brain BP_{ND} data for both groups and all investigated time-points are depicted in Fig. 3. Directly after caffeine discontinuation on Day 1 (4.4 hours after withdrawal) whole brain A_1AR availability was significantly reduced by -34.5% ($p<0.001$) in caffeine-treated animals compared to the controls. Regional analysis displayed reductions in A_1AR availability between 31.3% (hypothalamus) and 37.3% (cerebellum). After a caffeine discontinuation of approximately 27 hours (Day 2) these group-specific effects were abolished in all investigated regions and A_1AR availability in caffeine-treated animals settled around baseline values.

Analysis of data with a mixed model ANOVA indicated significant interactions for all parameters ("region x scan x group", $F_{(7,94)}$=4.4, p<0.001) as well as for each individual combination ("region x scan", $F_{(7,94)}$=4.2, p=0.001; "region x group", $F_{(3,44)}$=3.8, p=0.016; "scan x group", $F_{(3,45)}$=9.3, p<0.001). Post-hoc analysis revealed stable BP_{ND} values for all time-points in control animals (Fig. 3). In contrast, in caffeine-treated animals post-hoc analysis indicated significant differences between baseline and Day 1 of caffeine withdrawal in regions with high A_1AR availabilities such as cerebellum and thalamus (33.5±12.5% for cerebellum; 27.5±11.9% for thalamus, p<0.001, Fig. 4). In regions with low A_1AR availabilities like hypothalamus and nucleus accumbens these differences could not be observed.

DISCUSSION

The present in vivo study examined the long-term effects of chronic caffeine administration on A_1AR availability using [^{18}F]CPFPX and small animal PET. Epidemiological studies recently revealed that long-term intake of caffeine might have neuroprotective effects against neurodegenerative diseases such as Alzheimer's and Parkinson's disease [13,14]. Thus, a dose of 3-5 cups of coffee per day reduced the risk of Alzheimer's disease up to 65%. The caffeine dose in the present study was chosen based on intake doses of these epidemiological studies in order to elucidate the neuroprotective molecular mechanisms of caffeine. Considering the faster metabolism of rodents [30], the caffeine intake in the present study corresponded to a human consumption of 4-5 cups of coffee per day (400-500mg caffeine) thus representing a common daily caffeine intake in industrialized countries [9].

In the present study we did not find a long-persistent upregulation of functionally available A_1ARs in vivo as after 27 hours of caffeine withdrawal A_1AR availability settled around baseline values in chronically caffeine-treated animals. At this time-point, the remaining xanthine level is negligible (<0.25%, see below) and any interference at the A_1AR with the

radioligand is unlikely. Nevertheless, our results are contradictory to some previous in vitro studies showing a persistent upregulation of the A_1AR after short-term caffeine withdrawal [16,26,31]. However, in these studies shorter time intervals and much higher dosages of caffeine as well as partly rather unphysiological application routes were chosen. Thus, conditions of normal daily caffeine intake in humans were not ideally reflected. Moreover, in all studies homogenates, representing the whole amount of receptors in a certain brain region, were investigated instead of the functionally available portions of receptors. Finally agonists [31], detecting changes in affinity states of the A_1AR, or relatively unspecific A_1AR/A_{2A}R antagonists [16] were used which might further explain inconsistent results. Interestingly, Kaplan *et al.* [26] showed a return to baseline A_1AR availability after high-dosage caffeine administration and subsequent 4 days of withdrawal by using the highly specific A_1AR antagonist [^{3}H]DPCPX which is structurally closely related to the currently used radioligand [^{18}F]CPFPX. These findings further suggest that incongruent results might be due to higher dosages of caffeine administered and the use of different radioligands.

Directly after caffeine discontinuation (4.4 hours of withdrawal) we observed a significant decrease in A_1AR availability which is contradictory to previous reports. In the past, either an upregulation [16–18] or constant levels [19–21] of A_1AR expression in rodents after chronic oral caffeine consumption were reported. Since caffeine, its metabolites and [^{18}F]CPFPX competes for the A_1AR [25], residual xanthine levels in plasma would explain such a decrease in A_1AR availability. As caffeine concentrations in plasma were not determined in this study, the caffeine plasma level was estimated based on similarly treated animals. The plasma caffeine concentration in these animals was 7.29±1.41µg/mL (37.54µM) 4-6 hours before the first withdrawal scan (Day 1). Estimation of the residual plasma levels were performed [32] considering the dose-dependent half-life $t_{½}$ of caffeine (1-4 hours) [32–35] and the decaffeinated period (4-6 hours). Calculated plasma concentration on Day 1 of caffeine

withdrawal was between 4.18µM (6 hours decaffeinated, $t_{½}$=1h) and 18.77µM (4 hours decaffeinated, $t_{½}$=4 hours). These plasma concentrations corresponded to an estimated receptor blockade between 4% and 11% [36]. Consequently, the highly reduced [^{18}F]CPFPX binding on Day 1 after caffeine withdrawal is unlikely attributable to remaining caffeine.

However, a blockade of A_1ARs can also be caused by the metabolites of caffeine. In rodents the main pharmacologically active caffeine metabolites are theophylline, paraxanthine and theobromine with ratios of caffeine to its metabolites of 1:1.5 for theophylline, 1:1.25 for paraxanthine and 1:3 for theobromine [37]. Accordingly, the calculated plasma concentrations of caffeine metabolites were 11.21µM for theophylline, 9.04µM for paraxanthine and 21.24µM for theobromine 4-6 hours before the first withdrawal scan. In contrast to caffeine, the plasma concentration of its metabolites is not equal to the concentration in the cerebrospinal fluid. Thus, a correction factor of 0.7 for theophylline and 1.3 for theobromine was considered [38]. For paraxanthine no correction factor has been determined yet, therefore the corresponding residual concentrations might be slightly biased. Regarding caffeine metabolites' half-lives [32] and their specific binding to the A_1AR [36], minimum binding on the A_1AR (6 hours decaffeinated) was 38% by theophylline, 7% by paraxanthine (uncorrected) and 3% by theobromine. Consequently, the reduced [^{18}F]CPFPX binding (-34.5%) observed in the first scan after caffeine withdrawal is presumably due to a remaining A_1AR blockade by the metabolites of caffeine, especially by theophylline.

Hence, at early time-points after withdrawal residual xanthine concentration would disguise potential short-term caffeine-induced molecular effects on the A_1AR. However, most likely reduced BP_{ND} values on Day 1 reflects masked baseline values and no short-term upregulation, since previous studies showed A_1AR changes of about 15-35% [16–18]. Thus, such an upregulation would result in baseline values under conditions of residual xanthine binding.

In the past, a divergent response of various regions on caffeine was described by Hawkins *et al.* [39] and Marangos *et al.* [22] which is in line with the current results showing divergent responses to caffeine treatment and subsequent withdrawal in the nucleus accumbens and hypothalamus. Potentially in these regions, chronic caffeine administration leads to a lower receptor occupation or to a faster unblocking of receptors after caffeine withdrawal. However, both regions are of small size and showed low tracer accumulation resulting in low signal-to-noise ratios. Accordingly, quantification of A_1AR availability might be slightly biased although stable test-retest results could be generated [27].

This study was performed with the radioligand [^{18}F]CPFPX showing a high affinity [6] and selectivity for the A_1AR [7]. Moreover, neither weight, daily water intake nor the scan parameters were significantly different between both groups. Exclusively the injected amount of substance showed a trend towards significance in the control group notwithstanding post-hoc analysis. Nevertheless, the injected amount of substance corresponded in both groups and at each scan to a negligible receptor blockade of <5% [7] according to the tracer principle making an influence on A_1AR quantification unlikely. For the determination of the outcome parameter (BP_{ND}) via SRTM, the olfactory bulb does not represent an ideal reference region, since it displays a specific binding of ~45% [7]. However, the non-invasive quantification of A_1AR densities with the olfactory bulb as a reference region was shown to be reliable and highly correlated with parameters determined with an arterial input function [7,27]. Nevertheless, it should be kept in mind that the reference region might be affected by the application of caffeine, thus differences in BP_{ND} might be under- or overestimated. As effects of caffeine application on specific radioligand binding in the reference region cannot be directly estimated in the current study, we assessed the impact of changes by values obtained in a previous study [7] as well as tissue activity concentrations both in a region with high specific [^{18}F]CPFPX binding and the reference region.

Previous data [7] showed a V_T of 0.65 mL/cm³ in the olfactory bulb derived from a metabolite corrected plasma input function and modelling via the two tissue compartment model. In comparison, V_T in high binding regions like the thalamus was 1.85 mL/cm³.

To estimate the impact of chronic caffeine consumption in the reference region we tried to calculate the influence on the outcome parameter BP_{ND} with the above-mentioned values.

The BP_{ND} is defined as $BP_{ND} = V_T\text{-}V_{ND}/V_{ND} = V_T/V_{ND}\text{-}1$ [28].

For a reference region not void of specific binding this formula can be rearranged to

$BP_{ND} = V_T\text{-}V_{Tref}/V_{Tref} = V_T/V_{Tref}\text{-}1 = (V_S+V_{ND})/(V_{Sref}+V_{ND})\text{-}1$.

By filling in the concrete values, BP_{ND} can be estimated to be 1.85 in the thalamus.

In case of a reference region with certain amounts of specific binding (V_S) this portion will change in the target region as well as in the reference region under the situation of a competition at the receptor level. For a blockade of e.g. 50% this will result in a BP_{ND} = 1.1.

Accordingly, extent of receptor blockade by caffeine is not completely mirrored by BP_{ND} as this outcome parameter is less affected and might even slightly underestimate the situation.

This is in line with our estimation of the impact of possible changes in the reference region olfactory bulb by tissue activity concentrations and previous simulation in humans. Uptake of radioligand into tissue both in a region with high specific [^{18}F]CPFPX binding (thalamus) and the reference region showed that chronic caffeine application led to the following changes in TACs: ~-28% in the reference region and ~-52% in the thalamus resulting in an estimated net change of ~-20% specific binding in the thalamus.

Simulations in humans showed that such a change resulted in a change of BP_{ND} of ~-15% when equal variations in the target and reference region are assumed [40]. This again implies that the presented decrease of BP_{ND} in the first withdrawal scan of the current study might be slightly underestimated by approximately 5%.

Nevertheless, this relatively little methodological bias does not change overall results of the study despite the region-specific intraindividual changes in the low-binding regions

hypothalamus and ncl. accumbens which became significant when data were exploratory corrected for the calculated underestimation. However, as already discussed above these regions has to be regarded with caution due to low binding and relatively high noise.

In conclusion, this study provides first in vivo evidence that there is no long-persistent upregulation of functional A_1AR expression after chronic caffeine treatment. Transient decreases in A_1AR availability directly after caffeine withdrawal observed in the current study were most likely caused by an A_1AR occupation by residual amounts of caffeine metabolites masking an unchanged A_1AR expression at this early time-point. Altogether, current data excludes a long-persistent upregulation of functional A_1AR availability as the molecular basis for the neuroprotective effects of caffeine. Nevertheless, it cannot be excluded that the adenosinergic system with its neuromodulatory properties is involved in the neuroprotective effects of caffeine maybe as proposed by [41] via changes in affinity states of A_1ARs.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to this article.

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TABLES

Table 1: **Scan parameters of caffeine-treated and control animals at different time-points**

Parameter	Group	PET measurements					*p* value	
		BL	**Day 1**	**Day 2**	**Day 4**	**Day 7**	**between scans**	**between groups**
Length of anesthesia at scan start [hh:mm]	Caffeine	1:05 ± 0:27	0:56 ± 0:15	0:59 ± 0:23	0:47 ± 0:10	0:46 ± 0:11	0.193	0.125
	Control	0:56 ± 0:11	0:50 ± 0:10	0:46 ± 0:10	0:50 ± 0:20	0:41 ± 0:10	0.268	
Injected radioactivity [MBq]	Caffeine	20.63 ± 2.28	18.82 ± 2.73	19.33 ± 3.53	20.37 ± 1.16	20.56 ± 2.39	0.515	0.645
	Control	20.16 ± 1.51	19.12 ± 1.53	20.35 ± 1.81	20.34 ± 2.28	18.59 ± 1.39	0.164	
Injected amount of [^{18}F]CPFPX [nmol]	Caffeine	0.28 ± 0.09	0.42 ± 0.22	0.34 ± 0.11	0.33 ± 0.02	0.34 ± 0.08	0.247	0.182
	Control	0.44 ± 0.25	0.39 ± 0.20	0.16 ± 0.07	0.23 ± 0.09	0.25 ± 0.08	0.006*	

Parameters are given as means ± standard deviation, n=8 per group; statistics: univariate analysis of variance, * indicate significance on a level of $p<0.05$, BL, baseline

Table 2: **Parameters of caffeine-treated and control animals during caffeine administration**

Parameter	**Group**		***p* value**
	Caffeine-treated	**Control**	
Weight gain [%]	20.5 ± 9	21.4 ± 7.5	0.84
Fluid intake [mL/day]	40.3 ± 3.5	42.3 ± 3.3	0.44
Caffeine intake [mg/kg bodyweight/day)	29.4 ± 1.4	-	-

Parameters are given as means ± standard deviation, n=8 per group; statistics: two tailed unpaired t-test

FIGURE LEGENDS

Figure 1:

Study design

Figure 2:

Mean parametric images displaying the A_1AR availability of the baseline and the subsequent four withdrawal scans of the caffeine-treated (n=8) and control animals (n=8). Coronal (left, top), sagittal (left, bottom) and horizontal (right) planes are depicted. BP_{ND}, binding potential; SRTM2, simplified reference tissue model 2

Figure 3:

Mean A_1AR availability of the whole rat brain (grey and white matter with olfactory bulb as reference region) after 12 weeks of chronic caffeine consumption with subsequent withdrawal in caffeine-treated animals (dashed line, n=8). Animals in the control group (solid line, n=8) received tap water and were analyzed with the same time schedule. Error bars denote standard deviation, * indicates significant differences between both groups (mixed model ANOVA with subsequent Bonferroni correction on a threshold of $p<0.01$). BL, baseline; BP_{ND}, binding potential; SD, standard deviation

Figure 4:

Region-specific differences in A_1AR availability between baseline and 1st caffeine withdrawal scan (Day 1) within the caffeine-treated animals in cerebellum, hippocampus, hypothalamus and nucleus accumbens. Error bars denote standard deviation, * indicate significant results ($p<0.002$, mixed model ANOVA with subsequent Bonferroni correction). BL, baseline; BP_{ND}, binding potential; Ncl, nucleus; SD, standard deviation