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Identification of coffee components that stimulate dopamine release from pheochromocytoma cells (PC-12)

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ABSTRACT

Coffee and caffeine are known to affect the limbic system, but data on the influence of coffee and coffee constituents on neurotransmitter release is limited. We investigated dopamine release and Ca^{2+}-mobilization in pheochromocytoma cells (PC-12 cells) after stimulation with two lyophilized coffee beverages prepared from either *Coffea arabica* (AR) or *Coffea canephora var. robusta* (RB) beans and constituents thereof. Both coffee lyophilizates showed effects in dilutions between 1:100 and 1:10,000. To identify the active coffee compound, coffee constituents were tested in beverage and plasma representative concentrations. Caffeine, trigonelline, *N*-methylpyridinium, chlorogenic acid, catechol, pyrogallol and 5-hydroxytryptamides increased calcium signaling and dopamine release, although with different efficacies. While *N*-methylpyridinium stimulated the Ca^{2+}-mobilization most potently (EC_{200}: 0.14 ± 0.29 μM), treatment of the cells with pyrogallol (EC_{200}: 48 ± 14 nM) or 5-hydroxytryptamides (EC_{200}: 10 ± 3 nM) lead to the most pronounced effect on dopamine release. In contrast, no effect was seen for the reconstituted biomimetic mixture.

We therefore conclude that each of the coffee constituents tested stimulated the dopamine release in PC-12 cells. Since no effect was found for their biomimetic mixture, we hypothesize other coffee constituents being responsible for the dopamine release demonstrated for AR and RB coffee brews.

1. Introduction

Coffee is one of the most consumed beverages worldwide, with an overall consumption of more than 5 million tons per year. Consumers prefer coffee because of its taste and its excitatory effects (Fernstrom, 2000; Geel et al., 2005). In previous studies of our group, coffee was investigated for its antioxidant and chemopreventive activity, and its effects on gastric acid secretion (Boettler et al., 2011; Lang et al., 2010a; Rubach et al., 2008; Somoza et al., 2003). In these studies, the coffee constituent *N*-methylpyridinium (NMP) has been identified as an *in vivo* antioxidant and as a potent inhibitor of gastric acid secretion when added to the coffee brew (Somoza et al., 2003), whereas 5-hydroxytryptamides (5-HTs) and caffeine (CAF) were demonstrated to effectively stimulate gastric acid secretion (Lang et al., 2010a). In the study presented here, we focus on the neurotransmitter excitatory effect of coffee.

It is well-known that coffee, CAF and other coffee components influence brain function (Fernstrom, 2000). CAF is the most frequently consumed dietary stimulant of the central nervous system (CNS) and has been demonstrated to improve mental performance at a bolus dose of 3.3 mg/kg (Battig and Welzl, 1993). As mechanism of action, CAF and other methylxanthines were shown to be nonselective competitive adenosine receptor antagonists (Michaelis et al., 1979; Wood et al., 1989). Also, results from *in vivo* studies in rats indicate that CAF induces dopamine release after 30 min at i.p. doses of 1.3–2.6 mg/kg or after 60 min at doses of 10 and 30 mg/kg (Morgan, 1998; Solinas et al., 2002). Another well-studied coffee component is trigonelline (TRI). TRI is known to influence neuronal functions like neurite outgrowth in human neuroblastoma cells (Tohda, 1999). However, the effect of TRI on neurotransmitter release has not been investigated yet.

Psychostimulants such as cocaine and amphetamine display their impact on the CNS via an increase of the extracellular dopamine concentration in the *nucleus accumbens* (Pontieri et al., 1995). Dopamine exocytosis is regulated by neuronal electrical activity, where the calcium (Ca^{2+}) signal evoked is modulated by protein kinase C (PKC) (Jaffe, 1998; Westerink et al., 2002). Dopa-

Abbreviations: AUC, area under the curve; AR, *coffea arabica*; RB, *coffea canephora var. robusta*; NMP, *N*-methylpyridinium; CA, chlorogenic acid; CAF, caffeine; CAT, catechol; CNS, central nervous system; HBSS, Hank's buffered salt solution; PBS, phosphate buffered saline; PC-12, pheochromocytoma-12 cells; PKC, protein kinase C; PYR, pyrogallol; TRI, trigonelline; 5-HTs, 5-hydroxytryptamides.

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mine fluctuation in the *nucleus accumbens* is involved in the rewarding effects of psychomotor stimulants and correlated with drug and food reward (Di Chiara and Imperato, 1988; Ikemoto et al., 1997). These fluctuations of the dopamine concentration can underlie different mechanisms, for example blocking dopamine reuptake, releasing it from stores, inhibiting autoreceptor-mediated feedback inhibition or directly increasing the phasic activity of dopaminergic neurons (Dayan, 2009). Cocaine, for example, binds to monoamine receptors and blocks the inward transport of dopamine via the neuronal dopamine transporter (DAT) which results in increasing dopamine concentrations in the synaptic cleft (Farnsworth et al., 2009). An additional function of the DAT is the outward transport of dopamine, which can be enhanced by drugs like amphetamines (Eshleman et al., 1994). This reverse transport is regulated by PKCβ_{II} (Johnson et al., 2005) and can be promoted by the interaction of the DAT by proteins, like syntaxin 1A (Binda et al., 2008).

However, with the exception of CAF, little is known about the effects of different coffee compounds on the neurotransmitter release and their contribution to the CNS-stimulating effect of coffee. This raised the question whether coffee or coffee constituents have a direct influence on neurotransmitter release from neural cells. Although, coffee, as a beverage does not reach the neurons, it has been shown for coffee constituents to contribute to the excitatory effect of this beverage (Ferre, 2010). In the work presented here, the time and concentration dependent effects of two lyophilized coffee beverages and multiple compounds thereof (Fig. 1) on dopamine release and Ca^{2+}-mobilization were studied in pheochromocytoma-12 cells (PC-12). The lyophilized coffee beverages were prepared from two commercial coffee roasts, one brewed from arabica (AR), the other one brewed from robusta (RB) beans. PC-12 cells originate from rat adrenal pheochromocytoma cells and display an established *in vitro* model for catecholamine neurotransmitter release such as dopamine and noradrenaline (Greene and Tischler, 1976). In fact, this cell line has already been used to determine the effects of food ingredients such as glucose (Avidor et al., 1994) and CAF (Koshimura et al., 2003) on dopamine release.

2. Materials and methods

2.1. Materials

Commercial Arabica Brazil (AR) and Robusta (RB) ground roast coffees were obtained from Tchibo GmbH (Hamburg, Germany). Of each coffee, a total amount of 54 g was weighed into a paper filter commonly used for home preparation of coffee beverage (Melitta Gold, Nr. 4, Aldi, Germany) to prepare 1 L of coffee beverage. Portions of freshly boiled tap water ($T \sim 90$–95 °C, approximately 100 mL each) were poured over the powder and the hot filtrate was collected in a volumetric flask of 1000 mL. Exactly 500 mL of the coffee solution was transferred to crystallizing dishes, frozen (−20 °C) immediately, and finally dried by lyophilization (48 h, 0.77 mbar, 25 °C) yielding a fluffy brown lyophilizate. The yields for 500 mL of the AR and RB coffees used in this study were 12.74 and 15.80 g, respectively. For the cell culture experiments, both samples were reconstituted in accordant buffer dilutions of up to 1/10,000 (w/v) freshly for every experiment. All purified compounds tested were dissolved in respective concentrations in buffer prior to every experiment.

CAF, PYR, CAT and TRI were purchased from Sigma Aldrich, USA. NMP iodide was synthesized as reported previously (3). Briefly, methyliodide (60 mmol) was added dropwise to a solution of pyridine (60 mmol) in acetonitrile (15 mL). This mixture was stirred for 20 min at room temperature and then heated for 6 h at 50 °C. The oily mixture was then chilled on ice (200 g), the precipitate obtained was washed with ice-cooled acetonitrile and filtered. After recrystallization from acetonitrile, the target compound was obtained as colorless crystals (3).

Unless stated otherwise, all further chemicals were purchased from Sigma Aldrich, USA. PC-12 cells were obtained from ATCC, as well as the cell culture media. All reagents solutions and buffers were prepared with ultra-pure water.

2.2. Methods

2.2.1. Cell culture

The PC-12 cells were cultivated in Ham's F12-K nutrient mixture containing 15% DHS, 2.5% FBS and 1% P/S at 37 °C, 5% CO_2 and 95% humidity in 75 cm^2 flasks (Sarstedt, Greiner). The cells were tested for mycoplasm contamination on a regular basis.

2.2.2. Dopamine ELISA

For the ELISA, 500,000 PC-12 cells were seeded in a 35 mm culture dish and allowed to settle for 48 h. After a wash with 1 mL PBS, the cells were stimulated with the compound of interest for 3.5 min at 37 °C in Krebs Ringer solution (128 mM NaCl, 1.9 mM KCl, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, pH 5). Of the supernatant, 300 μL were immediately diluted with 30 μL 1 N HCl and the ELISA was performed according to the manufacturer's protocol (dopamine ELISA, DLD Diagnostika, Hamburg, Germany).

2.2.3. Luminescence assay for dopamine release

For the dopamine release assays, 25,000 cells per well were seeded in a white 96-well plate (Nunc, Rochester, NY, USA) and allowed to attach for at least 24 h. The incubation media was discarded and the cells were washed with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2 \cdot 2H_2O$, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 4.6 mM HEPES) twice. Thereafter, the measurement solution (200 μL Locke's buffer, 0.46 U/mL MAO A, 6.4 U/mL horse radish peroxidase, 30 μM luminol, with a total volume of 290 μL) was added to each well.

The assay was performed as described by Shinohara et al. (2008) with slight modifications (Shinohara et al., 2008). After 10 min of incubation at 37 °C, 5% CO_2 and 95% humidity, the plate was stimulated with the compound of interest by injection with a plate reader (infinite M200, Tecan, USA) and the luminescence signal was measured immediately. The following compounds have been tested using this assay: CAF (3 nM to 3 mM), TRI (4.977 nM to 497.7 μM), NMP (14.7 pM to 291 μM), 5HTs (40.5 pM to 4.05 μM) and PYR (3.2 pM to 32 nM). The dopamine standard curve (data not shown) demonstrated a dose-dependent signal for dopamine in the test system.

Protein concentrations of each well were determined using the BCA protein assay kit from Pierce. For this test, cells were washed with PBS three times and lysed in PBS at −20 °C.

2.2.4. Determination of compound efficacy on dopamine release

To compare the different compounds and their efficacy to stimulate the dopamine release from PC-12 cells, the concentrations of the substances at which the neurotransmitter release was doubled compared to non-treated control cells (100%) were calculated (EC_{200}-values). Linear regression for the *T*/*C*-values vs. the concentration was used to determine the *y*-intercept (*b*) and the slope (*m*). Afterwards, the EC_{200}-value was assessed with the following formula: $EC_{200} = (200 - b)/m$.

2.2.5. Fluorescence assay for determination of intra-cellular calcium levels

In a 96-well plate, 100,000 PC-12 cells were seeded per well. After 24 h, the growth media was exchanged by 50 μL/well serum-free media to achieve a synchronization of the cells. After another 24 h, the cells were loaded with 50 μL of the fluo-4 direct dye and incubated for 30 min at 37 °C, followed by a 5 min incubation step at room temperature. Reference compounds, diluted coffee lyophilizates or purified compounds thereof were added by injection using a multimode plate reader (Infinite M200, Tecan, Austria). Fluorescence at 490 nm excitation and 520 nm emissions was measured immediately after addition of the compound, every 2 s for a total of 1 min. The obtained relative fluorescence units (RFUs) were normalized against the DNA concentration of the samples.

DNA content was determined after washing and lysis of the cells using the Nanoquant plate for the Infinite M200 plate reader (Tecan, Grödig, Austria) by absorbance measurements at 260 and 280 nm.

To compare the effects of the coffees and coffee constituents in different concentrations, the area under the curve (AUC) of the individual experiments was calculated and normalized against the DNA content of each well. Data displayed are expressed as mean AUC values ± SD.

2.2.6. Statistics

All experiments were done in multiple replicates ($n \geqslant 3$). The average of the AUC values was determined and displayed after performing a Nalimov outlier test. Significant differences were determined using the student's *t*-test, when testing against the non-treated control and using a one-way ANOVA with a Holm–Sidak post hoc test to measure concentration dependent effects.

3. Results

3.1. Chemical characterization of Arabica Brazil (AR) and Robusta (RB) coffees

The two coffees, AR and RB, were previously studied for their effects on gastric acid secretion (26). In these studies, both coffees were characterized and their major constituents were quantified (Table 1). Briefly, CAF and CA (Fig. 1) were identified as the two major coffee constituents, and while the CA concentration was comparable between the two coffees, the AR coffee contained only half of the amount of CAF compared to the RB coffee. Further

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constituents that were quantified in the two coffees were NMP, PYR CAT and C5HTs (Fig. 1) (26). The RB coffee exceeded a higher extraction yield than the AR coffee and contained higher concentrations of the two phenolic compounds CAT and PYR, but lower concentrations of NMP and C5HTs.

3.2. Influence of freeze-dried coffee beverages on PC-12 cells' intracellular calcium levels and dopamine release

After dietary intake, a coffee beverage gets diluted upon absorption and only 1/100 to 1/1000 of the amounts of the original coffee constituents can be detected in the plasma. For CAF and CA, e.g., concentrations in regular coffee beverages range between 3 and 7 mM, whereas plasma concentrations after coffee intake of 2–10 μM have been reported for these compounds (Monteiro et al., 2007; Newton et al., 1981). Concentrations of TRI and NMP in coffee beverages range from 1500 to 2300 and 25 to 500 μM (26), respectively, whereas plasma concentrations of up to about 6 and 0.8 μM (Lang et al., 2010b) have been analyzed for these two compounds.

To prove that freeze-dried coffee beverages and dilutions thereof stimulate the neurotransmitter release from PC-12 cells by increasing the second messenger Ca^{2+} in the cells, we measured the intracellular Ca^{2+} levels using a Ca^{2+}-sensitive fluorescent dye. Peak signals were detected within the first 30 s after injection and varied depending on the stimulant. Fig. 2A shows the results of four independent experiments for buffer-treated cells (buffer control) and those stimulated with either the diluted freeze-dried coffee beverages or bradykinin (1 μM) as a well-known stimulant of Ca^{2+}-fluctuation. Lyophilized RB coffee had a more pronounced effect than the AR coffee. However, in these experiments, cells were treated only with 1:1000 diluted freeze-dried coffee beverages. Therefore, coffee beverage dilutions of 1:100 up to 1:10,000 were tested in the following experiments. As can been from Fig. 2B, treatment of the cells with both coffee lyophilizates increased the Ca^{2+}-mobilization in a dose-dependent manner ($p < 0.05$) up to 620 ± 189% (RB). At dilutions of 1:1000, 1:5000 and 1:10,000 the RB coffee showed a significantly stronger effect on the Ca^{2+}-mobilization than the AR coffee (Fig. 2B).

To test whether this Ca^{2+}-signal leads to dopamine exocytosis, an ELISA for dopamine release was performed (Fig. 2C). Both freeze-dried coffee beverages evoked a dopamine release from PC-12 cells compared to the buffer control up to a maximum of 229 ± 65.5% and 269 ± 110% after stimulation with a 1:10,000 dilution of the AR and the RB coffee, respectively. This result raised the question which coffee constituent is responsible for the effect or whether the dopamine release stimulated by reconstituted freeze-dried coffee beverages is based on a synergistic effect of multiple compounds.

3.3. Intra-cellular calcium levels in PC-12 cells after stimulation with coffee constituents

CAF and TRI have already been described in the literature to have an effect on the neuroregulation (Fernstrom, 2000; Hossain et al., 2003a,b; Tohda et al., 1999). C5HTs are also likely candidates to affect neurotransmitter release since these compounds are structural serotonin analogs. We further tested other compounds quantified in coffee beverages in earlier studies (26): TRI and its degradation product formed upon roasting, NMP, as well as the phenolic compounds CA, CAT and PYR.

Table 1
Composition of the tested coffee beverages (Reprinted with permission from Rubach et al. (2010), Copyright 2010, American Chemical Society).

Substances	AR coffee [mg/L]	RB coffee [mg/L]
Chlorogenic acid	1038	975
Caffeine	618	1389
NMP	32.2	22.4
Pyrogallol	4.05	5.63
Catechol	5.73	9.29
C5HT	0.21	0.12
Of which C22-5HT	0.14	0.06

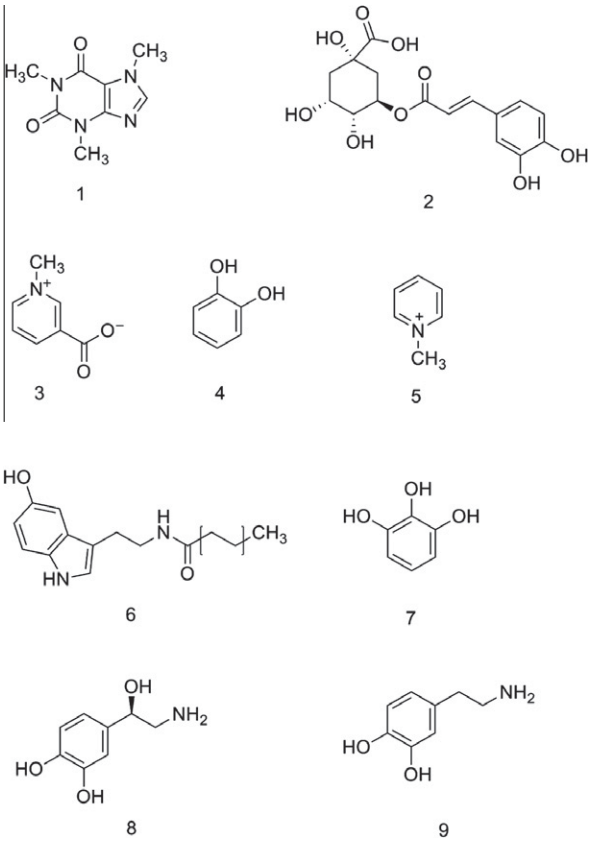

Fig. 1. Chemical structures of the tested coffee compounds (1) caffeine (CAF), (2) chlorogenic acid (CA), (3) trigonelline (TRI), (4) catechol (CAT), (5) *N*-methylpyridinium (NMP), (6) 5-hydroxytryptamides (5HT), (7) pyrogallol (PYR), and the neurotransmitters (8) norepinephrine (NOR) and (9) dopamine (DA).

The effective concentrations to mobilize twice as much Ca^{2+} compared to non-treated control cells (EC_{200}-value) are given in Table 2. The eight tested compounds were analyzed in concentrations found in coffee and plasma representative dilutions thereof. Fig. 3A–D shows the effect measured for these substances in AR representative concentrations and dilutions thereof on Ca^{2+}-mobilization. In contrast to the six other tested compounds ($p < 0.05$), for CAF (Fig. 3A) and CAT (Fig. 3C), no dose-dependent increase in the calcium mobilization was measured. The effect of CAF peaked already at a coffee dilution of 1:300,000 (265 ± 47.7%, data not shown) and varied between 67.4 ± 29.6% and 175 ± 59.0% at dilutions between 1:10 and 1:100 (Fig. 3A). The highest effect of CAT (Fig. 3C) was measured at a dilution of 1:100 (305 ± 81.5%). However, the results presented in Fig. 3 clearly demonstrate that all substances mobilized the intracellular calcium and could, therefore, lead to a signal for a dopamine exocytosis. Based on the concentrations of the individual compounds in the test system, the most effective compounds were determined by the EC_{200}-values as TRI < CA < C22-5HT < PYR < NMP.

3.4. Dopamine release from PC-12 cells after stimulation with coffee constituents

All tested compounds showed a dopamine release excitatory effect (Fig. 4A–D). For bradykinin, a well-studied compound that

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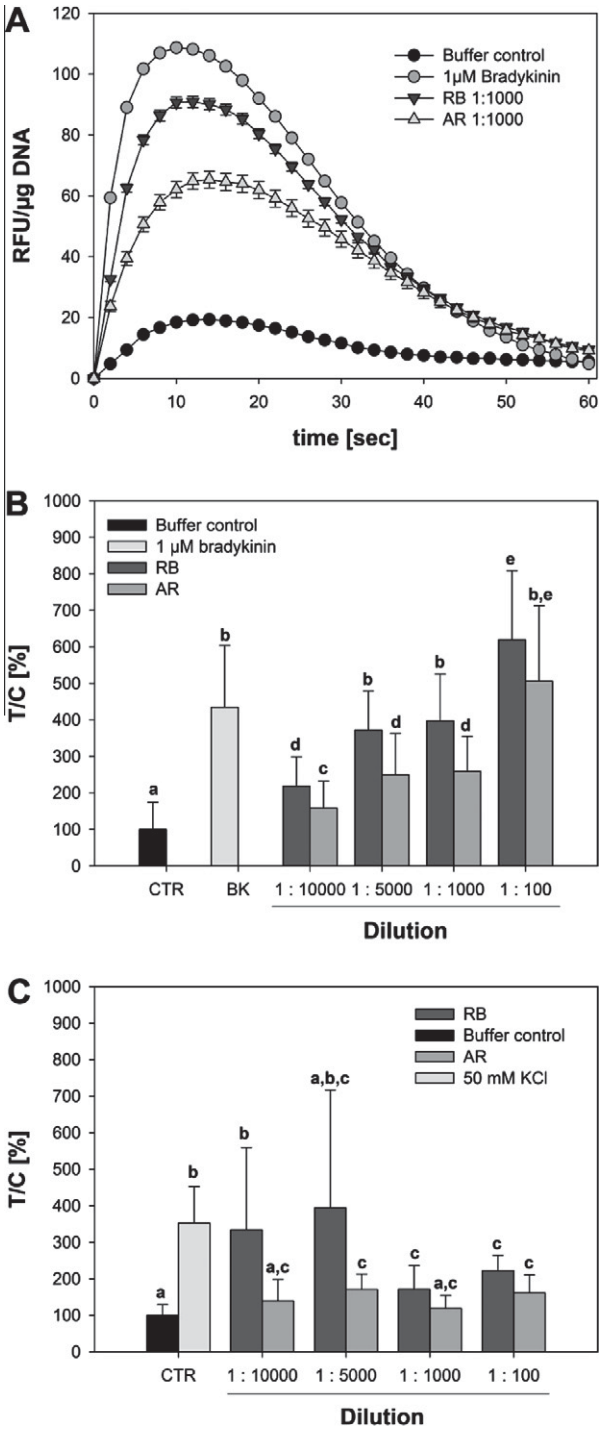

Fig. 2. Effects of the two coffee beverages, AR and RB on the Ca^{2+}-mobilization and the dopamine release by PC-12 cells. (A) Example of a Ca^{2+} curve with 1:1000 dilutions of the two tested coffees compared to the buffer control and the positive control bradykinin, $n = 4$. (B) Ca^{2+}-mobilization by different dilutions of the tested coffees. Results represent mean AUC of the substance vs. mean AUC of the control (%) ± STD normalized to the DNA content of the sample, $n = 4$. (C) Dopamine release by PC-12 cells after stimulation with the two test coffees in different dilutions compared to the buffer control (=100%). Significances were tested according to unpaired student's *t*-test and the differences are indicated with the letters a to e.

induces dopamine fluctuation (Koizumi et al., 2002), a 100% increase of the dopamine release compared to buffer-treated control cells was demonstrated (Fig. 4A–D).

Interestingly, for some compounds, CAF, TRI, NMP and CA, an hormesis effect was observed, with highest effects at low concentrations and that decreased again in a dose-dependent manner at higher concentrations. CAF, e.g., significantly increased the neurotransmitter release in concentrations of 3 µM (1:1000 dilution) and 30 µM (1:100 dilution), although the peak stimulation was analyzed for 100 nM CAF (1:3333 dilution; 158 ± 15.5%, data not shown). Similar effects were found for the induction of neurotransmitter release after stimulation with TRI and NMP. Compared to the buffer control, TRI induced the neurotransmitter release from PC-12 cells significantly to an extent of 136 ± 31.0% at a concentration of 4.977 µM (1:100 dilution). The peak effect (134 ± 3.99%) for NMP, the decarboxylation product of TRI formed upon roasting, was detected at a concentration of 145.7 nM (1:100 dilution). At a concentration of 145.7 µM, neither an induction nor a suppression of the neurotransmitter release was determined.

Table 2
EC_{200} values of the tested coffee compounds of the Ca^{2+}-mobilization in PC-12 cells. EC, effective concentration.

Compounds	EC_{200}-value [µM]
NMP	0.14 ± 0.29
PYR	1.34 ± 0.59
C22-5HT	1.51 ± 0.27
CA	204 ± 108
TRI	439 ± 475

The results obtained for PYR, C5HTs and CAT were used to calculate EC_{200}-values (Table 3). To exclude an over-estimated or artificial effect due to the fact that the serotonin derivatives C5HTs are substrates for the monoamine oxidase A (MAO A), a cell-free assay with the C5HTs was performed and the signal derived was subtracted as background (Fig. 4D). After background correction, the most effective compounds were the C5HTs with an EC_{200} of 10 ± 3 nM. Also, the roasting product PYR showed a strong dopamine excitatory effect at concentrations in the nanomolar range. The order of efficacy from the least to the most effective compound based on the EC_{200}-values was determined thereby as followed: CAT < PYR < C5HTs.

3.5. Calcium mobilization and dopamine release from PC-12 cells after stimulation with coffee constituents in high dilutions

At concentrations representative of a 1:100 dilution of the freeze-dried coffee beverage AR, the most effective compounds were CAT and NMP (Fig. 5A). Treatment of the cells with 450 nM (1:100 dilution) CAT evoked an elevated Ca^{2+}-mobilization of 305 ± 81%. The respective result for NMP is 384 ± 108% (900 nM; 1:100 dilution).

In contrast, at a dilution of 1:100, PYR elevated the dopamine release most effectively (Fig. 5B). PYR increased the DA release to 658 ± 266% at a concentration of 320 nM (Fig 5B). Furthermore, CAT and CA showed a strong tendency to induce dopamine release from PC-12 cells in concentrations of 450 nM and 30 µM, respectively.

3.6. Calcium mobilization and neurotransmitter release from PC-12 cells after stimulation with AR and RB coffee reconstitutes

To test the effect of CAF, CA, PYR, CAT, C5HT, TRI and NMP on Ca^{2+}-mobilization and dopamine in a coffee representative biomimetic mixture, the individual compounds were mixed in amounts representative for the AR and the RB coffee. Each mixture was reconstituted in a 1:100 dilution of the respective coffee with HBSS buffer containing 20 mM HEPES to test the effect on calcium mobilization and with KRS to test the effect on dopamine release. As a result, none of the reconstitutes induced intracellular calcium

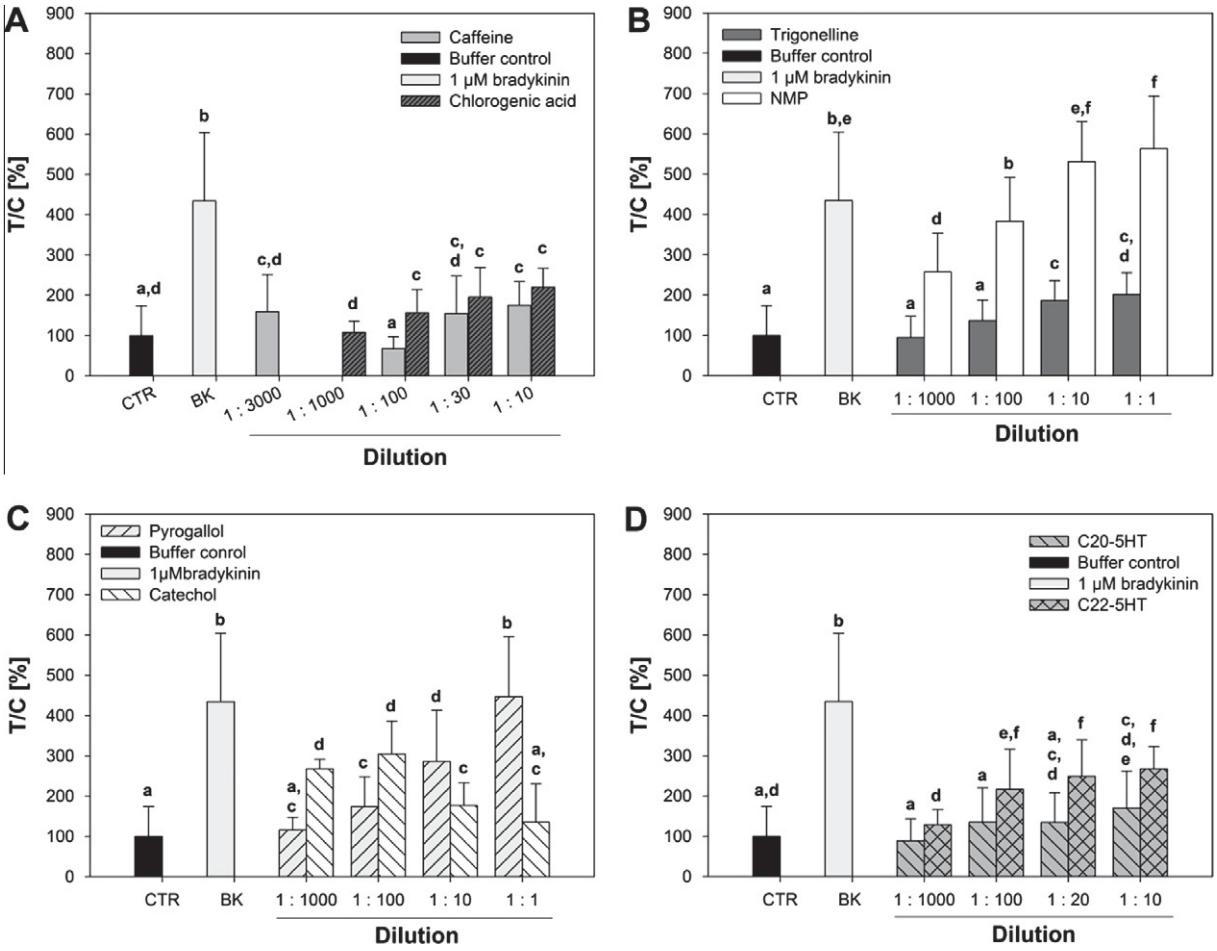

Fig. 3. Ca^{2+}-mobilization by the different coffee constituents shown as AR representative concentration (1:1) and dilutions thereof. Results represent mean AUC of the substance vs. mean AUC of the control (%) ± STD normalized to the DNA content of the sample, $n = 3–6$. (A) CAF and CA, (B) TRI and NMP, (C) PYR and CAT, (D) C5HTs. Significances were tested according to the unpaired student's *t*-test and the differences are indicated with the letters a to f.

mobilization (Fig. 6A) nor dopamine release in PC-12 cells (Fig. 6B). The reconstituted coffees did not stimulate the dopamine release from PC-12 cells.

4. Discussion

We hypothesized that coffee and coffee constituents could excite dopamine release from neuronal cells via calcium signaling. To investigate this hypothesis, we tested two different coffees and seven previously quantified coffee constituents (26) for their potential to induce intra-cellular calcium mobilization and dopamine exocytosis in PC-12 cells. In a first set of experiments, we were able to demonstrate that coffee and components thereof in coffee representative concentrations stimulate mechanisms of neurotransmitter release from PC-12 cells with different efficacies.

Q4 Chu et al. (xxxx) (32) showed that the neuroprotective activity of coffee depends on the roasting process and that a roasted coffee, rich in lipophilic antioxidants and CA lactones, has better neuroprotective properties than a green coffee. Although these results were primarily based on antioxidant effects, Chu et al. (xxxx) (32) demonstrated that the impact of coffee lyophilizates on intracellular signal transduction pathways in primary neuronal cells depends on the chemical composition of the coffee. The two coffees chosen in this study, AR and RB, were roasted to a commercial roast and did not differ in their roasting degree, but varied in their extraction yields (Rubach et al., 2010). We could show that the RB coffee, bearing a higher extraction yield than the AR coffee, had a stronger effect on Ca^{2+}-mobilization and the dopamine release from PC-12 cells than the RB coffee.

To determine the contribution of individual coffee compounds to the neurotransmitter-stimulating effect of the tested coffees, mobilization of intra-cellular calcium was analyzed. Comparing the mean AUC-values, NMP was identified as the most potent compound, followed by the efficacies of PYR > C22-5HT > CA > TRI. Dopamine release, however, was stimulated most effectively by C5HTs, followed by PYR and CAT. This means that, although NMP leads to the strongest effect regarding the Ca^{2+}-mobilization, PYR and C5HTs elicit the strongest effect on the dopamine release in nanomolar concentrations and are thereby the most potent coffee compounds tested to stimulate neurotransmitter release from PC-12 cells. Thus, both C5HTs and PYR, although found in coffee only in micromolar concentrations, may contribute to the effect of coffee.

The most prominent coffee compound CAF is also known to influence the neurological system (Kaasinen et al., 2004; Nehlig et al., 1992). However, for most of the tested compounds, only limited data is available on their influence on the neurotransmitter release. The results of the present study show that there are coffee constituents that may have a stronger neurotransmitter excitatory effect than CAF.

To transfer these results from an *in vitro* model to *in vivo* conditions, the bioavailability of the compounds has to be considered. In addition, to stimulate the neurotransmitter release in the human

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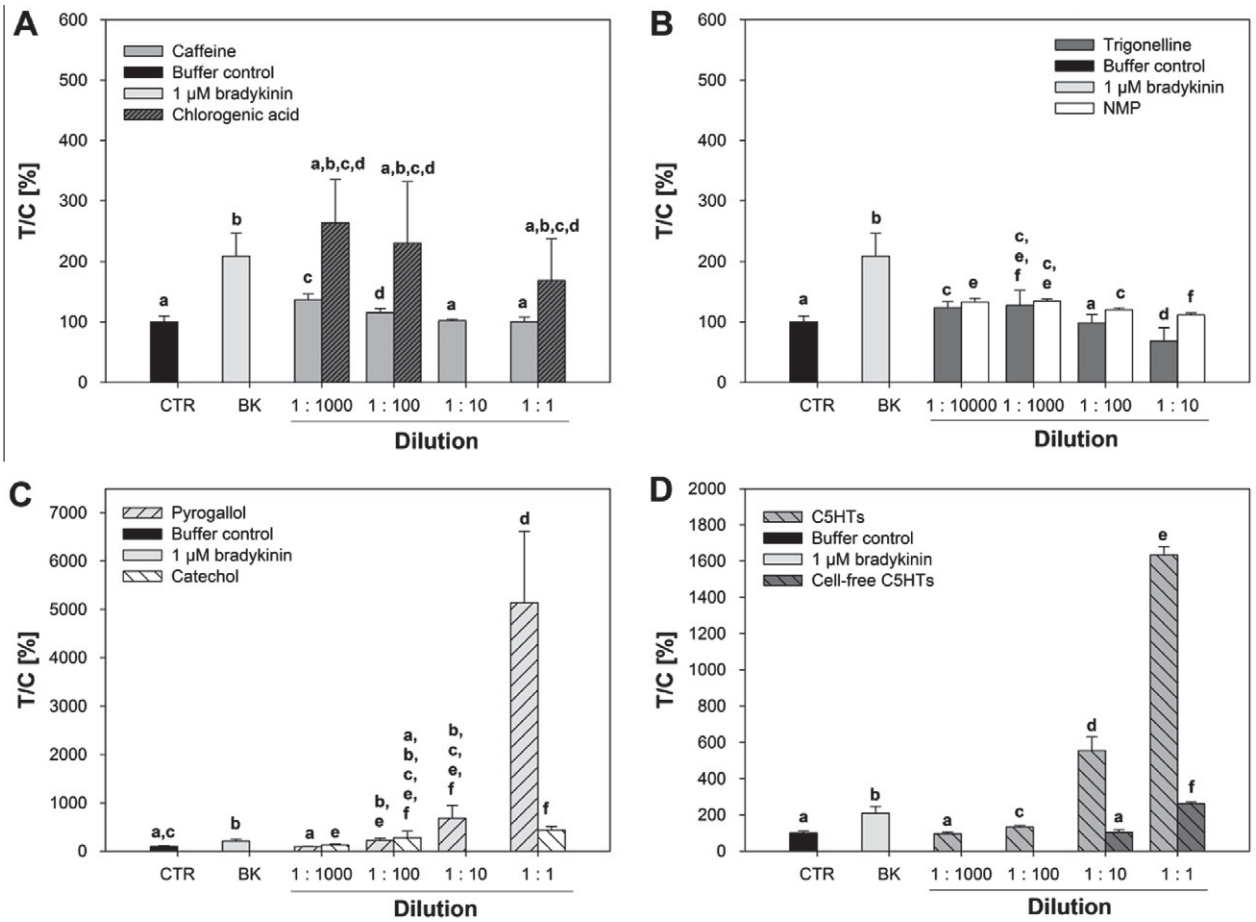

Fig. 4. Dopamine release by PC-12 cells after stimulation with the different coffee constituents compared to the buffer control (=100%). The results represent means ± STD, normalized to the protein content of the sample, $n = 3–4$. (A) CAF and CA, (B) TRI and NMP, (C) PYR and CAT, (D) C5HTs significances were tested according to the unpaired student's *t*-test and the differences are indicated with the letters a to f.

Table 3
EC_{200} values of the tested coffee compounds on the dopamine release from PC-12 cells.

Compounds	EC_{200} [µM]
C5HTs	0.01 ± 0.003
Pyrogallol	0.048 ± 0.014
Catechol	5.32 ± 9.46

brain, the coffee compounds have to cross the blood brain barrier. Substances can pass this barrier via transmembrane diffusion or via a substrate-specific, saturable transport (31). To diffuse through the membrane, a molecule needs to have a low molecular weight and a high degree of lipophilicity.

Very limited data can be found on the bioavailability and passage of the blood brain barrier of most compounds tested in this study. CAF is well described to be highly bioavailable in humans (Blanchard and Sawers, 1983). Also, the transport of CAF and TRI through the blood brain barrier is well characterized (Banks, 2009; Cornford et al., 1992; McCall et al., 1982; Pop et al., 1989). Our results indicate that TRI did not significantly contribute to the effect of the coffee beverages, whereas CA, CAT, PYR, and C5HTs were more active, although data on the bioavailability of these compounds and their ability to cross the blood brain barrier are limited. After the consumption of CA-rich foods like coffee, CA is absorbed and rapidly metabolized, with caffeic acid being reported as the major metabolite found in the plasma (Lafay et al., 2006; Monteiro et al., 2007). In addition, CAT and PYR are also described to be highly bioavailable and metabolized by biotransformation enzymes (Balant et al., 1979; Goldberg et al., 2003; Holt et al., 2002; Lautala et al., 2001; Zhu et al., 1995). No data is available yet whether phenolic compounds, such as CAT, PYR or CA can cross the blood brain barrier in humans. However, the tea polyphenol epigallocatechin gallate (EGCG) has been described to be bioavailable and to be able to cross the blood brain barrier (Lin et al., 2007). This compound has a higher molecular weight than the tested compounds and is comparably lipophilic, so that it is likely that also CAT, PYR and the CAs might be able cross the blood brain barrier and have an influence on the neurotransmitter release in the human brain. In rat brain slices, significant effects of CAT and PYR on neurotransmitter uptake were found (Minchin and Pearson, 1981). For PYR, our data indicate that this compound might be a potent drug targeting the neurotransmitter signaling, leading to an increased neurotransmitter release and inhibiting the degradation of the signal molecules.

To investigate the overall effects of the tested compounds, experiments with biomimetic mixtures were performed. These reconstituted mixtures, containing the compounds tested in coffee representative concentrations, did not bear an effect on neither the Ca^{2+}-mobilization nor the dopamine release from PC-12 cells, showing that the effect of the individual compounds was abolished. This could be due to cross-reactivity of the compounds with each other or through different receptor binding affinities. It has been shown for CAF, CAT and catechins that these compounds possess different binding affinities to the GABA (A) receptor, a brain receptor that is targeted by mood defining drugs (Hossain, 2003). In contrast to the biomimetic mixtures, the here tested AR and RB coffee possessed an effect tested at a 1:100 dilution, raising

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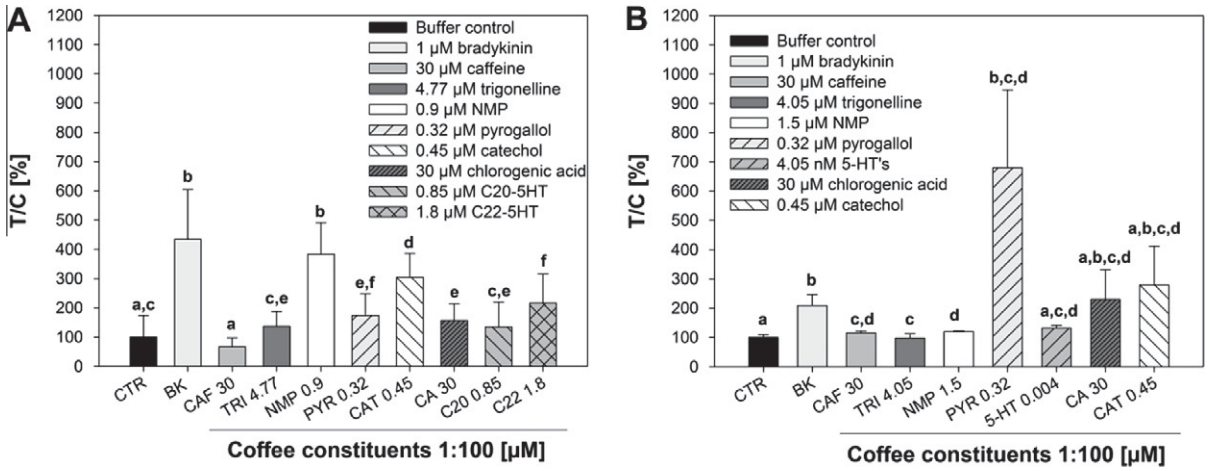

Fig. 5. Effects of the coffee compounds in concentrations corresponding a 1:100 of AR on (A) the Ca^{2+}-mobilization, results represent mean AUC of the substance vs. mean AUC of the control (%) ± STD normalized to the DNA content of the sample, $n = 4$, and (B) the dopamine release by PC-12 cells. The results represent means ± STD. Significances were tested according to the unpaired student's *t*-test and the differences are indicated with the letters a to f.

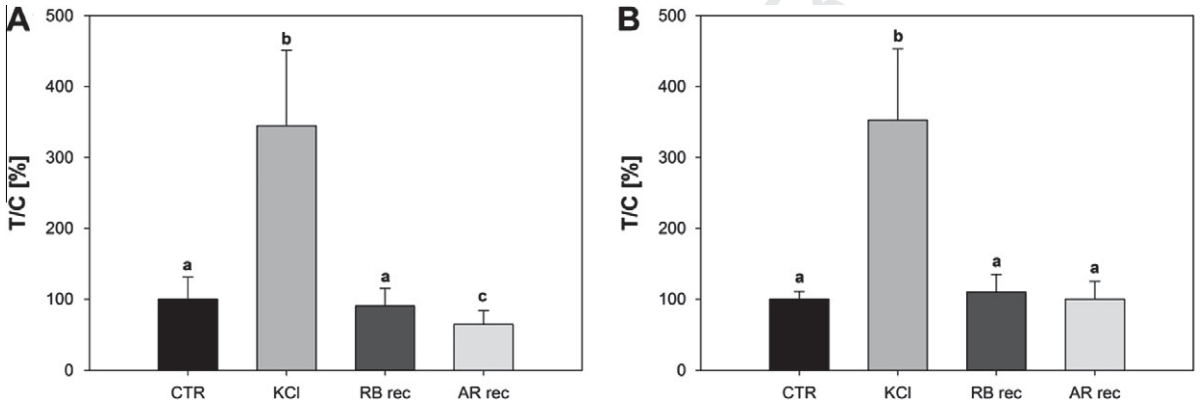

Fig. 6. Effects of the two reconstituted coffees, AR_{rec} and RB_{rec} on (A) the Ca^{2+}-mobilization, results represent mean AUC of the substance vs. mean AUC of the control (%) ± STD normalized to the DNA content of the sample, $n = 4$, and (B) the dopamine release by PC-12 cells. The results represent means ± STD. Significances were tested according to the unpaired student's *t*-test and the differences are indicated with the letters a, b, and c.

the question why the coffee reconstitutes did not. Compared to the reconstitutes, the coffees contained a large variety of other compounds, like melanoidins, lactones or volatiles. Chemical characterization of melanoidins revealed that low molecular melanoidins incorporate CA, leading to a lower concentration of free CA (Bekedam et al., 2008). In addition, CA serves as precursor for different lactones, formed upon roasting of the beans and present as bitter flavor compounds in the coffee beverage (Bruhl et al., 2007; Frank et al., 2007). Thus, other compounds than those investigated in this study, might have contributed to the effect demonstrated for the lyophilized AB and RB coffee. On the other hand, the lack of effect shown for the biomimetic mixture might also be due to antagonistic effects from the here tested compounds. Since their mode of action might be different when they are absorbed from a complex coffee beverage matrix, further investigations need to identify the most relevant compounds in coffee which stimulate mechanisms of neurotransmitter release in the human brain.

5. Conclusion

We were able to prove that coffee and coffee constituents mobilize intracellular calcium, leading to dopamine release from PC-12 cells. Most of the active compounds tested are bioavailable and our results show that these compounds stimulated the neurotransmitter release already in nanomolar concentrations and could therefore be bioactive in humans. Furthermore, one of the most potent compounds tested, PYR, has been shown to stimulate the release of neurotransmitters in the human brain and could therefore contribute to the excitatory effect of coffee. The mechanism of action of PYR as well as of another potent class of compounds, e.g., C5HTs, have to be identified in future studies. In contrast to the lyophilized and then reconstituted beverages, the biomimetic mixtures of CA, CAF, TRI, NMP, PYR, CAT and C5HTs were not effective. Here, we hypothesize (1) antagonistic effects of these compounds and (2) that there are other compounds in coffee that induce the DA release from PC-12 cells and contribute to the effect demonstrated for coffee beverage.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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