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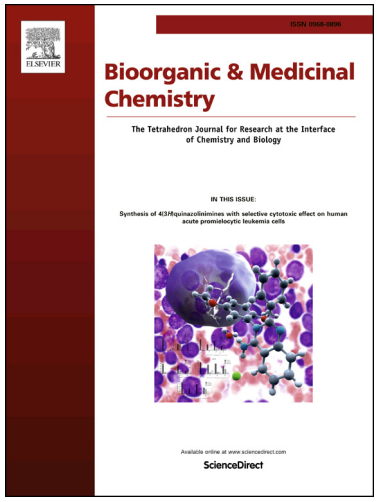

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Development of molecular tools based on the dopamine D_3 receptor ligand FAUC 329 showing inhibiting effects on drug and food maintained behavior

*Anne Stößel[a], Regine Brox[a], Nirupam Purkayastha[a], Harald Hübner[a], Carsten Hocke[b], Olaf Prante[b], Peter Gmeiner[a]**

[a]Department of Medicinal Chemistry, Emil Fischer Center, Friedrich-Alexander University, Schuhstraβe 19, D-91052 Erlangen, Germany

[b]Department of Nuclear Medicine, Ulmenweg 18, D-91054 Erlangen, Germany

***Corresponding Author**. Phone: +49-(0)9131-85-24116. Email: peter.gmeiner@fau.de

Keywords

Dopamine D_3 receptor; selective D_3 antagonist; subtype selectivity; positron emission tomography (PET); fluorine-18; autoradiography; self-administration; drug maintained behavior; food maintained behavior

Abstract

Dopamine D_3 receptor-mediated networks have been associated with a wide range of neuropsychiatric diseases, drug addiction and food maintained behavior, which makes D_3 a highly promising biological target. The previously described dopamine D_3 receptor ligand FAUC 329 (**1**) showed protective effects against dopamine depletion in a MPTP mouse model of Parkinson`s disease. We used the radioligand **[^{18}F]2,** a [^{18}F]fluoroethoxy substituted analog of the lead compound **1** as a molecular tool for visualization of D_3-rich brain regions including the islands of Calleja. Furthermore, structural modifications are reported leading to the pyrimidylpiperazine derivatives **3** and **9** displaying superior subtype selectivity and preference over serotonergic receptors. Evaluation of the lead compound **1** on cocaine-seeking behavior in non-human primates showed a substantial reduction in cocaine self-administration behavior and food intake.

1. Introduction

The neurotransmitter dopamine is synthesized in and released from dopaminergic neurons to stimulate G-protein coupled receptors, thereby controlling movement, cognition and emotion.[1-3] Dysregulation of the dopaminergic system is implicated in various pathophysiological conditions including Parkinson`s disease, schizophrenia, drug abuse and addiction. The dopamine D_3 subtype revealed a restricted distribution in the limbic regions such as nucleus accumbens and the islands of Calleja[4-6] implying an involvement in emotional and cognitive functions and, thus, emerged as an interesting biological target.[7-10] Recently, the D_3 antagonist cariprazine received FDA approval for the treatment of schizophrenia and bipolar disorders (Figure 1).[11-13] Moreover, the regulation of the dopamine system in these mesolimbic brain areas was identified to play a crucial role in reward-related behavior[14] including drug addiction and food reward-linked obesity.[15-20] Recent investigations using [^{3}H]-(+)-7-OH-DPAT for ligand binding and autoradiographic mapping revealed increased D_3 receptor density in the striatum of human cocaine overdose fatalities.[21] Interestingly, the selective D_3 receptor partial agonist BP897 was identified to reduce cocaine cue-controlled seeking behavior

in rats without producing any intrinsic reinforcing effects.[22, 23] There is an increasing evidence that the dopamine D_3 receptor may also be involved in seeking behavior of reinforcing effects of natural rewards like food.[24] Support was provided by the finding that the D_3 receptor antagonist SB-277011A produced a significant decrease in food intake and responses for food in an operant task in Zucker obese and lean rats.[25] Further evidence of the involvement of D_3 receptors in modulating food-seeking behavior was demonstrated by a study of Nathan et al.[26], which examined the effects of the D_3 receptor antagonist GSK598809 on attentional bias to rewarding food cues in overweight to obese individuals. Although GSK598809 had only moderating effect on attentional bias to food cues, individual differences in eating styles impacted on the effect of GSK598809 on attentional bias, with a stronger drug effect observed in those with lower restrained eating. However, the involvement of the dopamine receptor subtypes in reward is more complex and their functional role in drug- and feeding-related behaviors remains unclear due to the lack of highly selective pharmacological tools.

We previously described the synthesis of the dopamine receptor partial agonist **1** (FAUC 329), which displays high affinity and selectivity for the D_3 receptor.[27] Furthermore, we evaluated the effects of **1** in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson`s disease.[28, 29] Compound **1** demonstrated significant neuroprotective effects on dopamine depletion, which was most pronounced in the nucleus accumbens according to the preferential abundance of D_3 receptors in this region. A preparation of **[^{18}F]2** on an analytical scale had been previously published,[30] however, the radiotracer has not yet been investigated for imaging purposes.

In this paper, we describe the evaluation of the ^{18}F-labeled D3 radioligand **[^{18}F]2** by in-vitro and ex-vivo autoradiography and by in-vivo PET imaging studies. Moreover, in vivo studies on drug and food maintained behavior are reported and structural variations are described leading to an enhancement of target selectivity for the pyrimidylpiperazines of type **3**.

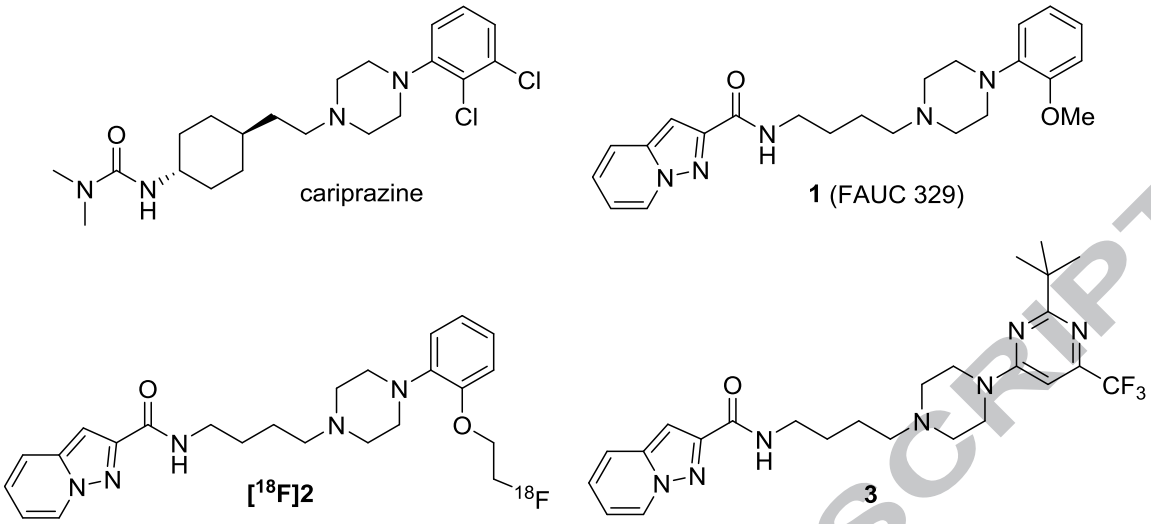


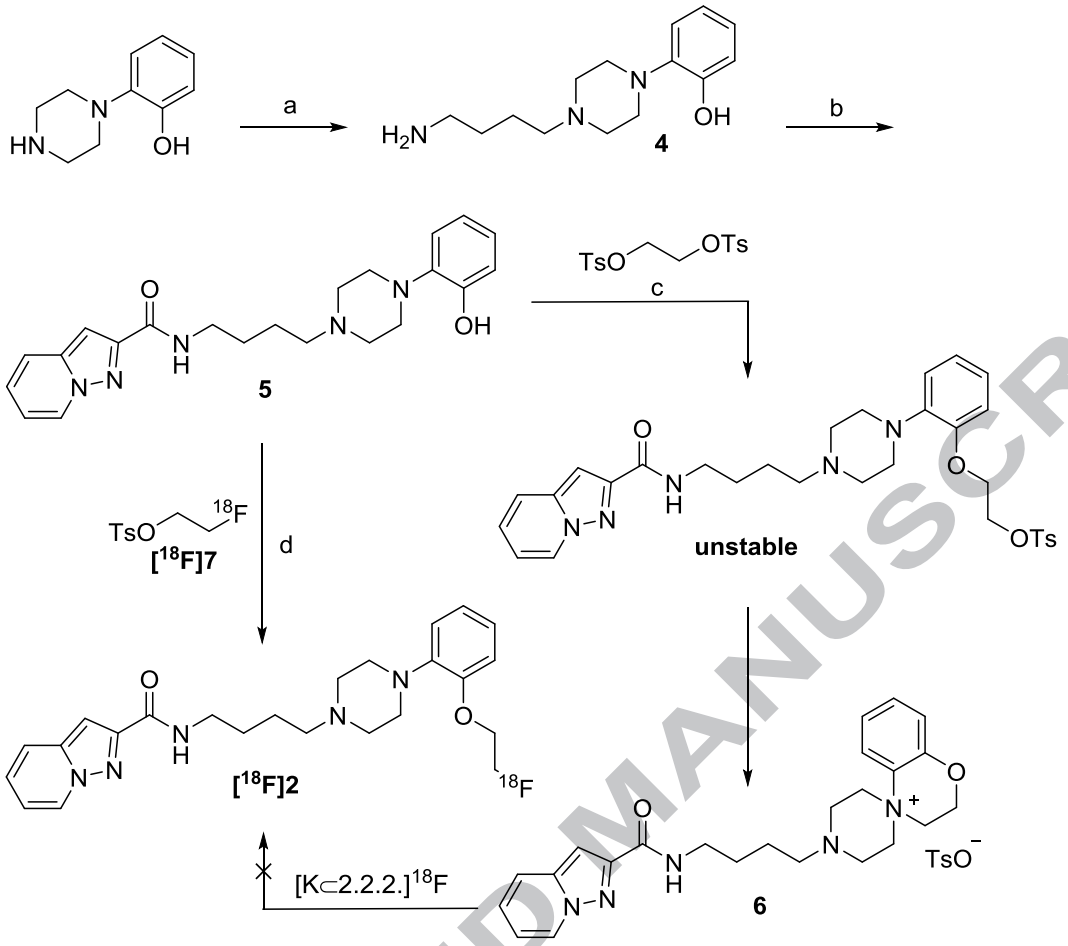
Figure 1. Chemical structures of partial D_3 agonist cariprazine, D_3 antagonist FAUC 329 (**1**), the radiolabeled derivative **[^{18}F]2** and the highly selective D_3 receptor ligand **3**.

2. Results and discussion

2.1 Evaluation of the radioligand [^{18}F]2

2.1.1 Optimization of the radiosynthesis

Based on our previous work on the preparation of **[^{18}F]2** in an analytical scale,[30] it was our intention here to optimize the radiosynthesis for extended in-vitro and in-vivo PET imaging studies. To accomplish this goal, we envisaged the synthesis of a suitable tosylate labeling precursor for an efficient one-step nucleophilic ^{18}F-fluorination that would provide direct access to **[^{18}F]2**. Using potassium carbonate as a base, we applied a three-step synthesis starting with a chemoselective *N*-alkylation of commercially available 2-hydroxyphenylpiperazine with 4-bromobutyronitrile to give the respective nitrile, which was reduced to yield the primary amine **4** (Scheme 1) following a similar strategy as described previously.[31, 32] Subsequently, an amide coupling reaction with pyrazolo[1,5-*a*]pyridinyl-2-carboxylic acid[27] activated by HATU afforded the carboxamide **5** in a more straightforward synthesis than described previously[30] without the need of an *O*-demethylation step.



Scheme 1. Synthesis of radioligand **[^{18}F]2**. Reagents and conditions: (a) 1. 4-bromobutyronitrile, K_2CO_3, KI, acetone, reflux, 16 h; 2. $LiAlH_4$, THF, 0 °C to room temperature, 5 h;[32] (b) pyrazolo[1,5-a]pyridine-2-carboxylic acid[27], HATU, DIPEA, DMF, room temperature, 16 h; (c) Cs_2CO_3, CH_3CN, 60 °C, 2 h; (d) 1 eq. NBu_4OH, DMF, 120 °C, 2 min.

Our initial intention was to synthesize the *O*-(2-tosyloxy)ethyl derivative of **5** and subsequently displace the tosyloxy unit by nucleophilic fluorination. However, after treatment of **5** with ethyleneglycol-1,2-bistosylate under various basic conditions, we observed intramolecular *N*-alkylation resulting in the formation of the quaternary ammonium salt **6** (Scheme 1). Consequently, the *O*-(2-tosyloxy)ethyl derivative of **5** could not be isolated, therefore a one-step nucleophilic ^{18}F-fluorination on a tosylate precursor was not achievable. However, the phenol **5** could be used as an intermediate for both, the preparation of the radioligand **[^{18}F]2** via ^{18}F-fluoroalkylation using 1-[^{18}F]fluoro-2-tosyloxyethane (**[^{18}F]7**) and for the synthesis of the nonradioactive

reference compound **2** (Supporting Information).

The introduction of the fluorine-18 isotope was performed via nucleophilic substitution of ethyleneglycol-1,2-bistosylate with [^{18}F]fluoride under classical aminopolyether-assisted reaction conditions to yield 2-[^{18}F]fluoroethyltosylate **[^{18}F]7** as described previously,[33, 34] followed by ^{18}F-fluoroethylation of **5** in the presence of $N(Bu)_4OH$ to give practical access to the final product **[^{18}F]2** in a preparative scale with a radiochemical yield of 50% (referred to [^{18}F]Fluoride), a radiochemical purity of >99% and in molar radioactivities of 44 ± 30 GBq/µmol after isolation by radio-HPLC and final formulation of **[^{18}F]2** in sterile saline solution.

2.1.2 In-vitro autoradiography

The ^{18}F-labled pyrazolo[1,5-*a*]pyridinyl carboxamide radioligand **[^{18}F]2** was studied for its ability to specifically bind to D_3 receptors by rat brain autoradiography *in vitro*. Due to the subnanomolar D_3 affinity (K_i = 0.31 nM) and excellent D_3/D_2 subtype selectivity (130-fold for D_{2Long} and 68-fold for D_{2short}) we expected the ability of **[^{18}F]2** to specifically label D_3 receptors. The results of the in-vitro autoradiography demonstrated D_3-specific binding of the **[^{18}F]2** in rat brain slices, proven by the displacement with the D_3 receptor ligand BP897 (Figure 2). The most pronounced binding was visible in regions that are known to have high D_3 receptor density such as the islands of Calleja, including the major islands, the lateral septum nuclei as well as striatal and cortical regions.[35] In addition, the distribution pattern of D_3 receptor-rich regions in the rat brain was more differentiated with apparently increased signal-to-noise ratio than for previously reported diazo derivatives.[36]

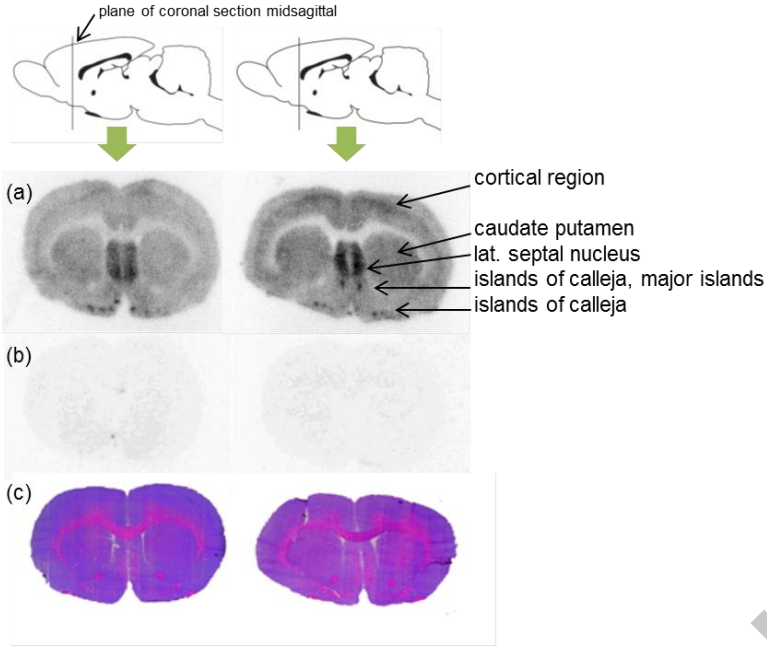


Figure 2. In-vitro autoradiography of coronal rat brain slices. Brain slices were incubated with the (a) radioligand **[^{18}F]2** (b) radioligand **[^{18}F]2** in the presence of BP897 (1 µM) and compared to (c) HE stained slices.

2.1.3 Ex-vivo autoradiography

Ex-vivo rat brain autoradiography of the radioligand **[^{18}F]2** qualitatively showed negligible uptake of the radioligand in the D_3-rich brain regions, such as the islands of Calleja or the nucleus caudatus putamen (striatum). The result are in accordance with those previously reported for ^{18}F-labeled pyridinylphenyl amides and phenyl azocarboxamides.[36, 37] Our experiments showed a significant accumulation of the radiotracer in the ventricles, the ependyma cell layer or plexus tissue (Figure 3). Applying the D_3 ligand BP897, only 7-12% (lateral ventricle, LV) and 5-14% (third ventricle, 3V) of the radioligand was displaced, when the region of interest-to-background (BG)-ratios in the control slices (without BP897) were compared with the sections from animals that were co-injected with BP897.

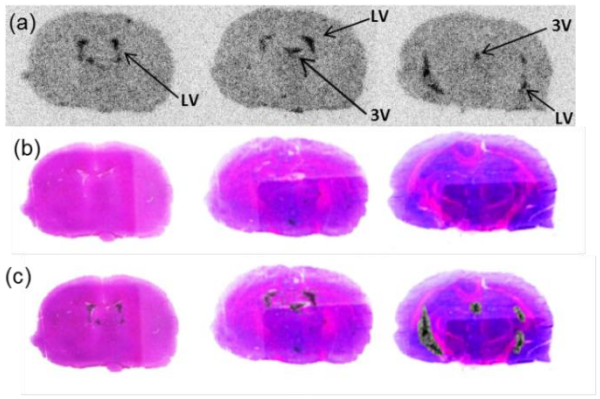


Figure 3. Ex-vivo autoradiography of coronal rat brain sections. Brain sections were obtained from animals that were injected with (a) radioligand **[^{18}F]2** alone and with radioligand containing the D_3 receptor ligand BP897, both at 60 min p.i., (b) HE staining (c) HE stained sections were overlayed with the autoradiography.

2.1.4 PET studies

PET studies were conducted to further evaluate **[^{18}F]2** as a tracer for selective imaging of dopamine D_3 receptors *in vivo*. The results were in accordance with the ex-vivo autoradiography and revealed marginal brain uptake of the radioligand in CNS structures (Figure 4). However, a significant radiotracer uptake in the pituitary gland and in the brain ventricles was observed. The mean whole pituitary gland uptake of the radioligand at 40 min p.i. was 0.394 ± 0.016% ID/g and pretreatment with BP897 moderately reduced the uptake of the radioligand in the pituitary gland by 25%. The in vivo uptake into the ventricular system is not as pronounced as for the azo compound class previously measured.[36] Similar to the previously published data, besides high uptake of **[^{18}F]2** in the pituitary gland, the fourth ventricle also showed uptake as visualized in the PET scan (Figure 4).

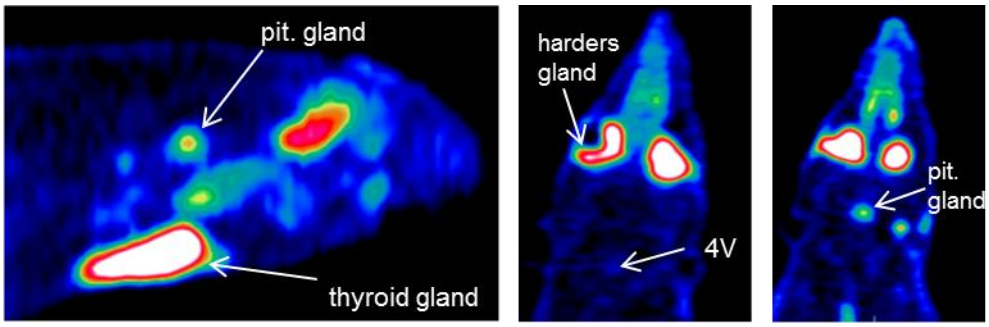


Figure 4. PET imaging study of **[^{18}F]2** in rat, image of frame 30-45 min post radioligand injection (4V = fourth ventricle).

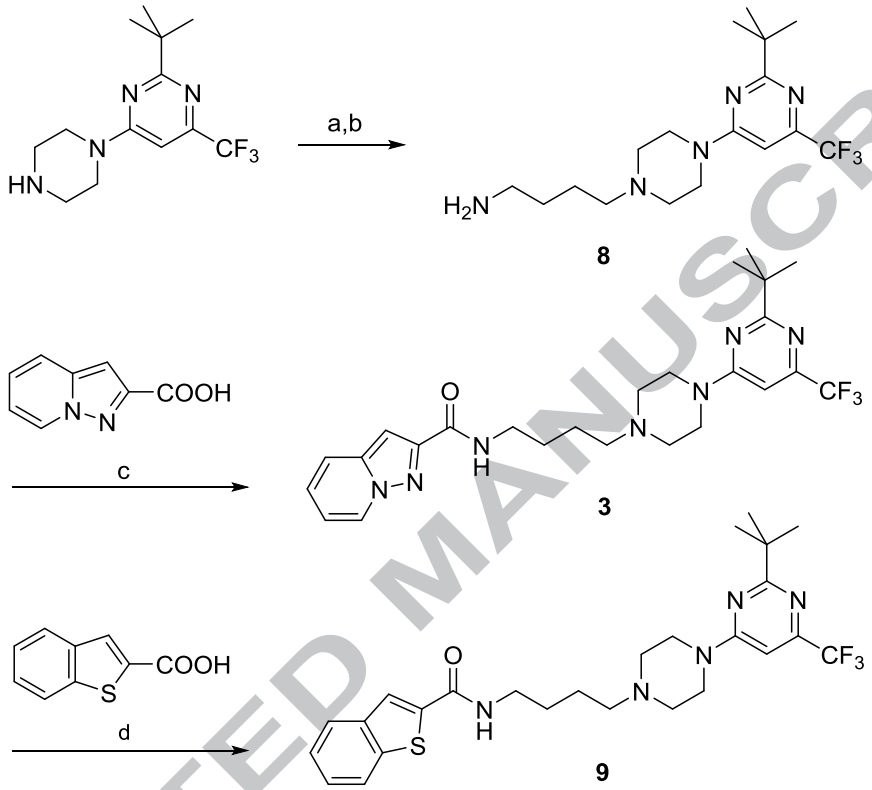
These findings are in accordance with previously reported in vivo PET recordings of structurally related benzamide ligands.[36, 37] We found that **[^{18}F]2** had only low blood-brain-barrier (BBB) penetration and the detection of specific binding of **[^{18}F]2** in CNS structures of the rat brain by PET imaging was not possible. However, the tracer revealed significant penetration of the blood-liquor barrier and uptake into brain ventricles in rats. Moreover, the pituitary gland, lying outside the BBB, showed high specific binding of **[^{18}F]2**, a phenomenon that resembles very much the biodistribution of amisulpiride. It is well known that dopamine receptors are expressed in the pituitary gland, where they regulate hormone synthesis and secretion through binding of dopamine. In conclusion, **[^{18}F]2** can be used for selectively quantifying the distribution of D_3 receptors in rat brain by in vitro autoradiography, but did not fit for detecting D_3 receptors in CNS structures of the living rat brain by PET. The results from our PET imaging study with **[^{18}F]2** suggest that the main mode of action of FAUC 329 in vivo could be induced by binding to dopamine receptors in the pituitary gland.

2.2 Structural modifications

2.2.1 Synthesis

To further fine-tune the selectivity profile of the investigated D_3 ligands, we planned the synthesis of pyrimidylpiperazines of type **3** and **9**, taking advantage of recently described SAR studies.[10, 27, 38] Receptor-ligand interactions that were deduced from the D_3 crystal structure were also helpful for the ligand design.[39] Key step of the syntheses was a HATU and TBTU promoted coupling reaction of the primary amine **8** with

pyrazolo[1,5-*a*]pyridine-2-carboxylic acid and benzothiophene-2-carboxylic acid, respectively (Scheme 2). The intermediate **8** was prepared by *N*-alkylation of the corresponding pyrimidyl-substituted piperazine with 4-bromobutylonitrile and subsequent reduction employing $LiAlH_4$.



Scheme 2. Synthesis of pyrimidylpiperazine derivatives **3** and **9**. Reagents and conditions: (a) 4-bromobutyronitrile, K_2CO_3, KI, MeCN, reflux, 16 h; (b) $LiAlH_4$, THF, 0 °C to room temperature, 5 h; (c) **8**, pyrazolo[1,5-*a*]pyridine-2-carboxylic acid, HATU, DIPEA, DMF, room temperature, 16 h; (d) **8**, benzo[*b*]thiophene-2-carboxylic acid, TBTU, DIPEA, DMF, room temperature, 16 h.

2.2.2 Receptor binding studies

Compound **1** was previously reported as a dopamine D_3 receptor ligand with considerable selectivity over the other dopamine receptor subtypes.[27] To analyze receptor binding and selectivity profiles of the test compounds **3**, **6** and **9**, radioligand displacement assays were employed for the subtypes of dopamine receptors, and for the related serotonergic receptors 5-HT_{1A} and 5-HT_{2A}. The resulting K_i values of the

newly synthesized analogs, compared to the antipsychotic drug cariprazine and the reference agents were determined in parallel under identical conditions. The data are listed in Table 1. Compared to the lead compound **1**, the fluoro substituted analog **2** displayed a similar binding profile indicating that **[^{18}F]2** may act as a valuable imaging agent, which is able to visualize the CNS activity of compound **1**. The spirocyclic-derivative **6** that emerged as a side product was also investigated with regard to its receptor binding profile, when moderate D_3 affinity was detected (K_i = 200 nM).

Aiming to increase selectivity towards 5-HT_{1A} receptors, modifications of the pyrazolo-[1,5-*a*]pyridine moiety and the methoxyphenylpiperazine unit were investigated, when we followed the findings of previous SAR studies.[8, 10, 38, 40-42] The heteroaryl-substituted analog **3** bearing a pyrimidyl-piperazine scaffold, a moiety that was reported for its drug-like properties[38], showed excellent D_3 receptor binding affinity (K_i = 1.2 nM) in the same range as that of compound **1** (Table 1). Interestingly, a huge increase of D_3 selectivity over 5-HT_{1A} was observed for **3** (K_i (D_3/5-HT_{1A}) = 8000). An additional gain in selectivity could be achieved by the exchange of the pyrazolopyridine moiety by a benzothiophene ring system. Thus, the target compound **9** showed 1000-20000 fold D_3 selectivity over all biogenic amine receptors investigated.

Table 1. Receptor Binding for D_3 Receptor Ligands Employing Human D_1, D_5, D_{2long}, D_{2short}, D_3, D_4, 5HT-$_{2A}$ as well as Porcine 5HT-$_{1A}$ Receptors.

	Ki values (nM)[a]							
	[^{3}H]SCH 23990		[^{3}H]spiperone				[^{3}H]WAY600135	[^{3}H]ketanserin
Compd.	hD1	hD5	hD2$_{long}$	hD2$_{short}$	hD3	hD4	p5-HT$_{1A}$	h5-HT$_{2A}$
cariprazine	2100 ± 1200	7900 ± 1700	0.47 ± 0.13	0.41 ± 0.05	0.27 ± 0.07	110 ± 10	0.49 ± 0.13	22 ± 6
1 (FAUC 329)	1600 ± 100	3700 ± 2000	270 ± 30	200 ± 40	3.5 ± 0.4	150 ± 20	10 ± 4	430 ± 50
2[c]	860 ± 100	3800 ± 1500	39 ± 11	21 ± 4	0.31 ± 0.06	160 ± 52	6.5 ± 1.4	520 ± 98
3	7500 ± 4700	5700 ± 2700	430 ± 55	200 ± 61	1.2 ± 0.4	4700 ± 400	9800 ± 3200[b]	2700 ± 1100[b]
6	5700 ± 1700[b]	> 50000[b]	6300 ± 2800	4500 ± 1100	200 ± 31	> 10000	900 ± 400[b]	> 10000[b]
9	> 10000[b]	> 10000[b]	4000 ± 900	2700 ± 500	2.4 ± 0.8	> 10000	> 50000	7100 ± 1900

[a] K_i values in nM ±SEM are based on the mean of 3-12 experiments each performed in triplicate. [b] K_i values in nM ±SD based on two experiments.

[c] previously published in Hocke et al.[30]

2.3 Self-Administration Studies

There is a growing body of evidence that dopamine D_3 receptors are implicated in the reinforcing effects of addictive drugs and food.[17] We examined the ability of our lead compound **1** to modify the reinforcing effects of cocaine and food in non-human primates. Male rhesus monkeys were trained to respond for the delivery of cocaine and food during daily, 2 hour fixed-ratio (FR30) schedule. This experimental setup allowed us to investigate the effects of the D3 ligand **1** on cocaine-self administration as well as food-maintained behavior.

2.3.1 Cocaine self-administration

Cocaine self-administration behavior generally was dose-related and characterized by an inverted U-shaped function that is characteristic for cocaine-maintained behavior under fixed-ratio schedules (supplementary data, Figure S1).[43]

Pretreatment with compound **1** (0.32 mg/kg) produced attenuating effects on the intake of 0.03 mg/kg per injection i.v. cocaine (Figure 5). Specifically, averaged for the group of monkeys, compound **1** decreased cocaine-maintained responding to approximately 20% of control values. Furthermore, pretreatment with various doses of compound **1** showed dose-related decreases in cocaine-maintained responding (supplementary data, Figure S2).

2.3.2 Food-maintained behavior

Averaged data also indicate that compound **1** produced decreases in food-maintained responding prior to the i.v. cocaine self-administration component of the session (food 1), but did not appreciably alter food-maintained responding following the i.v. self-administration component (food 2). Pretreatment with the highest dose of compound **1** (0.32 mg/kg) resulted in an approximately 30% reduction of food-maintained behavior during food 1 session (Figure 5). The low and intermediate pretreatment doses of compound **1** (0.1 and 0.18 mg/kg) did not markedly decrease food 1 and 2 responding (supplementary data, Figure S2). A similar finding was also reported for buspirone that had inconsistent effects on food-maintained responding, but produced substantial decreases in cocaine-maintained behavior.[44] In conjunction, the rate-decreasing effects of compound **1** on i.v. cocaine self-administration behavior and food-maintained responding are not consistent with the idea that its

effects on cocaine-maintained responding are behaviorally selective. In addition, the limited rate-decreasing effects of compound **1** on food-maintained behavior during food 2 components in sessions of i.v. saline availability suggest that behaviorally active doses in the present study had a relatively short (< 2 h) duration of action.

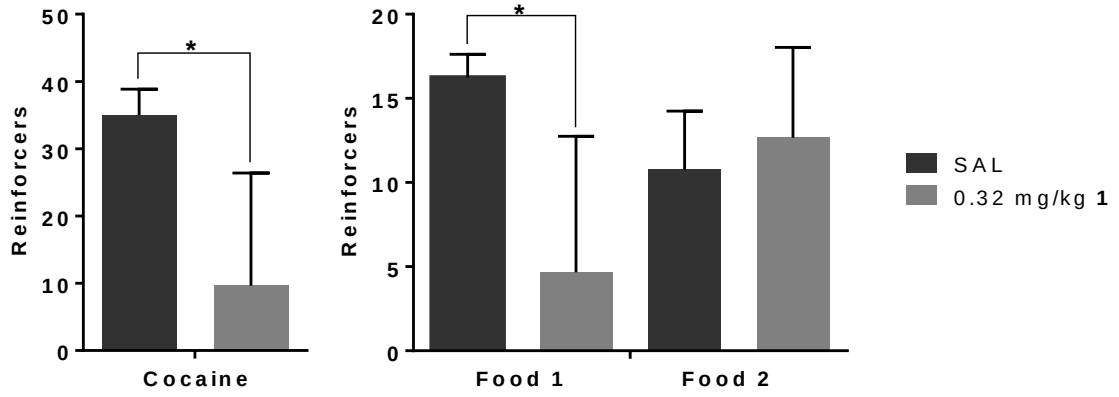


Figure 5. Mean effects of compound **1** (0.32 mg/kg) on behavior maintained by 0.032 mg/kg per injection cocaine. Bars represent the number of total cocaine injections that were self-administered in the 100-min drug component or number of food pellets that were delivered during Food 1 and Food 2. The results shown for "Sal" (black bars) represent the effects of saline pretreatment and serve as baseline values for comparison with the effects of compound **1** (grey bars). Bars represent the mean ± S.E.M. of four individual experiments. * $p < 0.05$, significantly different from baseline values as determined by two-tailed t-test.

3. Conclusions

The exact role of the D_3 receptor in feeding-related behaviors is still an open question. An involvement of D_3 receptors in the regulation of body weight and body fat when consuming diets differing in palatability and fat content was suggested by a study based on the finding that male D_3 receptor deficient mice become obese when fed a high-fat diet.[45] Furthermore, a reduced striatal $D_2/_3$ receptor availability was found in obese compared with non-obese humans confirming a role of the dopaminergic reward system in obesity.[46] Our self-administration studies indicate that our D_3 ligand **1** (FAUC 329) exerts inhibitory effects on cocaine and food maintained behavior of male rhesus monkeys indicating that the compound is a valuable tool for more specific studies investigating the role of D_3 in feeding-related behavior.

A common limitation in clinical and preclinical studies of drug addiction and obesity is

the lack of D_3 selective receptor radioligands to evaluate differences in D_3 receptor expression levels. Until now, the development of suitable receptor PET ligands is still an ongoing challenge because the available agents suffer from selectivity issues and unfavorable lipophilicity.[47, 48] [^{11}C]PHNO is the only radiopharmaceutical in use for imaging of the D_3 receptor, however the selectivity is not very high.[49, 50] To overcome these drawbacks we evaluated the ^{18}F-labeled radioligand **[^{18}F]2** as an analog of the D_3 ligand **1** for imaging purposes. The radioligand **[^{18}F]2** successfully visualized D_3-rich brain regions including the islands of Calleja by *in vitro* autoradiography, However, our PET imaging study revealed that **[^{18}F]2** did not significantly accumulate in CNS structures of the rat brain in vivo. Instead, **[^{18}F]2** showed significant uptake in the brain ventricular system and, more pronounced, in the pituitary gland. Thus, provided that **[^{18}F]2** mimics the biodistribution of **1**, it is tempting to speculate that **1** could induce its effects on cocaine-self administration as well as food-maintained behavior through binding to D3 receptors in the pituitary gland.

To reduce binding affinity towards serotonin 5-HT_{1A} and 5-HT_{2A} receptors in brain tissues,[30, 51, 52] structural modifications were performed resulting in the pyrimidylpiperazine derivatives **3** and **9** with outstanding selectivity over the serotonergic congeners. These compounds may serve as leads for further ligand development.

4. Experimental section

4.1 General

Reagents and dry solvents were of commercial quality and used as purchased. If not stated otherwise, reactions were carried out under nitrogen atmosphere. MS was run on a BRUKER ESQUIRE 2000 using ESI ionization. HRMS-ESI was run on an AB Sciex Triple TOF660 SCiex, source type ESI or at the Chair of Organic Chemistry, Friedrich Alexander University Erlangen-Nürnberg on a Bruker Daltonik micrOTOF II focus or Bruker Daltonik maXis 4G, source type ESI or APPI. NMR spectra were recorded on a Bruker Avance 400 or a Bruker Avance 600 spectrometer at 300 K in the solvents indicated. Chemical shifts are given in ppm (δ) relative to TMS. Purification by column chromatography was performed with silica gel 60. TLC analyses were performed using Merck 60 F254 aluminum plates in combination with

UV detection (254 nm) or ninhydrin staining. Analytical HPLC was conducted on an Agilent 1200 HPLC system employing a DAD detector and a ZORBAX ECLIPSE XDB-C8 (4.6 × 150 mm, 5 µm) column with the following binary solvent systems: System 1: eluent, methanol/0.1% aq formic acid, 10% methanol for 3 min, to 100% in 15 min, 100% for 6 min, to 10% in 3 min, then 10% for 3 min, flow rate 0.5 mL/min, λ = 210 or 254 nm; System 2: CH_3CN/0.1% aq formic acid, 10% CH_3CN for 3 min, to 100% in 15 min, 100% for 6 min, to 10% in 3 min, then 10% for 3 min, flow rate 0.5 mL/min, λ = 210 or 254 nm. Preparative HPLC was performed on an Agilent 1100 Preparative Series, using a ZORBAX ECLIPSE XDB-C8 PrepHT (21.5 x 150 mm, 5 µm, flow rate 10 mL/min) column with the solvent systems indicated.

Instruments for analysis and preparative radiosynthesis were used as follows: a semi-preparative HPLC system (Agilent 1100) equipped with a quaternary pump, a variable wavelength detector and a radio-HPLC detector D505TR (Canberra Packard) connected to a PC with HPLC data acquisition software (FLO-One, Canberra Packard).

4.2 Syntheses.

The synthesis of pyrazolo[1,5-*a*]pyridine-2-carboxylic as well as FAUC 329 was conducted as previously described.[27] Benzo[*b*]thiophene-2-carboxylic acid was purchased from Acros Organics, 2-(*tert*-butyl)-4-(piperazin-1-yl)-6-(trifluoromethyl)pyrimidine was provided by Abbott.

4.2.1 2-(4-(4-Aminobutyl)piperazin-1-yl)phenol (4)

The synthesis of **4** was performed as reported by Leopoldo et al.[32] with some modifications. To a suspension of 2-(piperazin-1-yl)phenol (1.00 g, 5.61 mmol), K_2CO_3 (1.16 g, 8.42 mmol) and KI (cat.) in dry acetone (30 mL) was added 4-bromobutyronitrile (0.56 mL, 5.61 mmol) and the reaction mixture was refluxed for 16 h. After cooling to room temperature, solids were filtrated off and the solvent was removed under reduced pressure. The crude material was purified by flash chromatography (CH_2Cl_2/MeOH/NH_3 aq., 40:1:0.01) to give 4-(4-(2-hydroxyphenyl)piperazin-1-yl)butanenitrile (1.10 g, 80%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.11 – 7.05 (m, 1H), 6.95 (dd, *J* = 8.1, 1.4 Hz, 1H), 6.86 (td, *J* = 7.7, 1.5 Hz, 1H), 2.90 (app t, *J* = 4.8 Hz, 4H), 2.62 (br s,

4H), 2.55 (t, *J* = 6.8 Hz, 2H), 2.47 (t, *J* = 7.1 Hz, 2H), 1.87 (app quint, *J* = 6.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.5, 138.9, 126.5, 121.4, 120.1, 119.7, 114.0, 56.3, 53.8, 52.5, 22.7, 14.9; ESI-MS *m*/*z* 246.0 $[M+H]^+$. Lithium aluminum hydride (2.23 mL, 4 M in Et_2O) was added dropwise to a solution of 4-(4-(2-hydroxyphenyl)piperazin-1-yl)butanenitrile (728 mg, 2.97 mmol) in anhydrous tetrahydrofuran (40 mL) at 0 °C. After stirring for 10 min at 0 °C the reaction mixture was warmed to room temperature and stirred additional 5 h. The reaction was quenched by adding wet silica gel carefully and portion wise at 0 °C. The solids were filtered and washed (100 mL, CH_2Cl_2/MeOH/NH_3 aq., 10:1:0.05) and the combined filtrates were dried ($MgSO_4$). After removal of solvents under reduced pressure the crude product **4** (571 mg, 77%, colorless foam) was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.06 (td, *J* = 8.0, 1.5 Hz, 1H), 6.93 (dd, *J* = 8.0, 1.4 Hz, 1H), 6.85 (td, *J* = 7.7, 1.5 Hz, 1H), 2.91 (app t, *J* = 4.8 Hz, 4H), 2.73 (t, *J* = 6.8 Hz, 2H), 2.62 (br s, 4H), 2.48 – 2.37 (m, 2H), 1.63 – 1.41 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.5, 139.1, 126.3, 121.4, 120.0, 114.0, 58.5, 53.9, 52.5, 42.1, 31.8, 24.3; ESI-MS *m*/*z* 250.1 $[M+H]^+$.

4.2.2 *N*-(4-(4-(2-hydroxyphenyl)piperazin-1-yl)butyl)pyrazolo[1,5-*a*]pyridine-2-carboxamide (5)

Compound **5** was reported previously.[30] For this study, **5** was resynthesized using a modified synthesis as follows: To a solution of pyrazolo[1,5-*a*]pyridine-2-carboxylic acid (150 mg, 0.93 mmol) and **4** (230 mg, 0.93 mmol) in anhydrous DMF (5 mL) was added DIPEA (0.33 mL, 1.86 mmol) and HATU (372 mg, 0.98 mmol). After stirring the reaction mixture at room temperaturefor 16 h the solvent was removed under reduced pressure. The crude mixture was suspended in aqueous 5% $NaHCO_3$ solution and extracted with ethyl acetate (3x). The combined organic phases were washed with water and brine and were dried ($MgSO_4$). After removal of solvents under reduced pressure the crude material was purified by flash chromatography (CH_2Cl_2/MeOH/NH_3 aq., 20:1:0.01) to give **5** (314 mg, 86%) as a colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.38 – 8.34 (m, 1H), 7.61 – 7.51 (m, 1H), 7.29 (br s, *J* = 5.2 Hz, 1H), 7.18 – 7.12 (m, 2H), 7.09 – 7.04 (m, 2H), 6.94 (dd, *J* = 8.0, 1.4 Hz, 1H), 6.88 – 6.81 (m, 2H), 3.58 – 3.50 (m, 2H), 2.98 – 2.87 (m, 4H), 2.63 (br s, 4H), 2.51 – 2.45 (m, 2H), 1.76 – 1.63 (m, 4H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.2, 151.5, 148.1, 141.4, 139.0, 128.4, 126.4, 123.7, 121.5, 120.0, 119.3, 114.0, 113.6, 98.0, 58.1,

53.9, 52.6, 39.1, 27.6, 24.4; ESI-MS *m*/*z* 394.2 $[M+H]^+$.

4.2.3 4'-(4-(Pyrazolo[1,5-*a*]pyridine-2-carboxamido)butyl)-2,3-dihydrospiro[benzo[*b*][1,4]oxazine-4,1'-piperazin]-4-ium 4-methylbenzenesulfonate (6)

A solution of **5** (21.0 mg, 53 µmol) in anhydrous acetonitrile (1.5 mL) was treated with Cs_2CO_3 (26.0 mg, 80 µmol), heated to 60 °C and a solution of bistosyloxyethan (59.0 mg, 159 µmol) in anhydrous acetonitrile (1.5 mL) was added dropwise. The reaction mixture was stirred at 60 °C for 2 h. After removal of the solvent the crude product was purified by short flash chromatography (CH_2Cl_2/MeOH/25% aq. NH_4OH; 5:1:0.1) to give **6** (19.0 mg, 85%) as a colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.36 (d, *J* = 7.0, 1H, pyrazolopyridine-H7), 7.73 (d, *J* = 8.1 Hz, 2H, tosylate-H2/H2'), 7.62 (d, *J* = 8.2 Hz, 1H, H20), 7.56 (d, *J* = 8.9 Hz, 1H, pyrazolopyridine-H4), 7.46 (br t, *J* = 5.5 Hz, 1H, NH), 7.38 – 7.34 (m, 1H, H18), 7.15 – 7.05 (m, 6H, pyrazolopyridine-H5, tosylate-H3/H3', pyrazolopyridine-H3, H17, H19), 6.83 (td, *J* = 6.9, 1.2 Hz, 1H, pyrazolopyridine-H6), 4.71 – 4.63 (m, 2H, CH_2O), 4.52 – 4.46 (m, 2H, CH_2N^+), 4.20 – 4.11 (m, 2H, 2 x N^+CH_{ax}), 3.91 – 3.82 (m, 2H, 2 x N^+CH_{eq}), 3.52 (q, *J* = 6.6 Hz, 2H, CH_2NH), 3.26 – 3.15 (m, 2H, 2 x NCH_{ax}), 3.04 – 2.96 (m, 2H, 2 x NCH_{eq}), 2.62 (t, *J* = 6.9 Hz, 2H, CH_2N), 2.31 (s, 3H, CH_3), 1.74 – 1.66 (m, 2H, CH_2CH_2N), 1.64 – 1.55 (m, 2H, CH_2CH_2NH); ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.3 (CO), 149.5 (Cq), 148.0 (Cq), 143.7 (Cq), 141.3 (Cq), 139.2 (Cq), 131.8 (CH, C18), 129.4 (Cq), 128.6 (2 x CH), 128.5 (CH), 125.9 (2 x CH), 123.8 (CH), 122.6 (CH), 121.1 (CH), 120.6 (CH), 119.2 (CH), 113.7 (CH), 97.9 (CH), 65.0 (CH_2, C14/C14'), 60.6 (CH_2, C16), 56.6 (CH_2, C12), 54.8 (CH_2, C15), 46.5 (CH_2, C13/C13'), 38.9 (CH_2, C9), 27.4 (CH_2, C11), 23.6 (CH_2, C10), 21.3 (CH_3); ESI-MS *m*/*z* 420.3 $[M]^+$; HRMS-ESI (*m*/*z*): $[M]^+$: calcd. for $C_{24}H_{30}N_5O_2$: 420.2394, found: 420.2394; HPLC: System 1: t_R = 13.5 min, purity 96%, System 2: t_R = 11.2 min, purity 97%.

4.2.4 4-(4-(2-(*tert*-Butyl)-6-(trifluoromethyl)pyrimidin-4-yl)piperazin-1-yl)butan-1-amine (8)

Compound **8** was reported previously.[53] For this study, **8** was prepared by a modified synthesis as follows: To a suspension of 4-(*tert*-butyl)-6-(piperazin-1-yl)-2-(trifluoromethyl)pyrimidine (1.0 g, 3.46 mmol), K_2CO_3 (0.96 g, 6.92 mmol) and KI

(cat.) in anhydrous acetonitril (30 mL) was added 4-bromobutyronitrile (3.46 mmol, 0.35 mL) and the reaction mixture was refluxed for 16 h. After cooling to room temperature it was quenched by the addition of water and the aqueous phase was extracted with DCM (3x). The combined organic layers were dried (Na_2SO_4) and solvents were removed under reduced pressure. The crude material was purified by flash chromatography (EtOAc/Cyclohexane, 2:8) to give 4-(4-(6-(*tert*-butyl)-2-(trifluoromethyl)pyrimidin-4-yl)piperazin-1-yl)butanenitrile (1.26 g, 97%) as a colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.59 (s, 1H), 3.71 (br s, 4H), 2.55 – 2.50 (m, 6H), 2.48 (t, *J* = 7.0 Hz, 2H), 1.87 (app p, *J* = 6.8 Hz, 2H), 1.34 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 178.0, 162.3, 155.1 (q, *J* = 33.7 Hz), 121.5 (q, *J* = 274.7 Hz), 119.8, 95.8 (q, *J* = 3.2 Hz), 56.4, 52.8, 44.0, 39.7, 29.6, 22.9, 15.1; ESI-MS *m*/*z* 355.1 $[M+H]^+$. Lithium aluminum hydride (6.33 mL, 1 M in THF) was added dropwise to a solution of 4-(4-(6-(*tert*-butyl)-2-(trifluoromethyl)pyrimidin-4-yl)piperazin-1-yl)butanenitrile (1.50 g, 4.22 mmol) in anhydrous tetrahydrofuran (100 mL) at 0 °C. After stirring for 10 min at 0 °C the reaction mixture was warmed to room temperature and stirred additional 5 h. The reaction was quenched by dropwise addition of sat. $NaHCO_3$ at 0 °C. The solids were filtered of and the solution was dried ($MgSO_4$). After removal of solvents under reduced pressure the crude product **8** (1.21 mg, 80%, colorless oil) was used in the next step without further purification. ESI-MS *m*/*z* 360.2 $[M+H]^+$.

4.2.5 *N*-(4-(4-(2-(*tert*-Butyl)-6-(trifluoromethyl)pyrimidin-4-yl)piperazin-1-yl)butyl)pyrazolo[1,5-*a*]pyridine-2-carboxamide x HCOOH (3)

To a solution of pyrazolo[1,5-*a*]pyridine-2-carboxylic acid (2.40 mg, 14.8 µmol) and **8** (6.00 mg, 14.8 µmol) in anhydrous DMF (1 mL) was added DIPEA (5.00 µL, 29.6 µmol) and HATU (5.90 mg, 15.5 µmol). After stirring the reaction mixture at room temperature for 16 h the solvent was removed under reduced pressure. The crude mixture was suspended in aqueous 5% $NaHCO_3$ solution and extracted with ethyl acetate (3x). The combined organic phases were washed with water and brine and were dried ($MgSO_4$). After removal of solvents under reduced pressure the crude material was purified by preparative HPLC (acetonitrile in 0.1% aqueous HCOOH, 5% to 95%) to give **3** (2.25 mg, 28%) as a colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.38 – 8.34 (m, 1H), 7.61 – 7.57 (m, 1H), 7.31 (br t, *J* = 5.2 Hz, 1H), 7.16 (ddd, *J* = 8.9, 6.7, 1.0 Hz, 1H), 7.07 (d, *J* = 0.6 Hz, 1H), 6.87 (td, *J* = 6.9, 1.3 Hz, 1H), 6.60 (s,

1H), 3.75 (br s, 4H), 3.55 (app q, *J* = 6.6 Hz, 2H), 2.55 (br s, 4H), 2.48 (br s, 2H), 1.77 – 1.61 (m, 4H), 1.36 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.7, 162.2, 154.9 (q, J_{CF} = 33.7 Hz), 148.0, 141.4, 128.3, 123.7, 121.3 (q, J_{CF} = 275 Hz), 119.3, 113.6, 98.0, 95.6 (q, J_{CF} = 3.4 Hz), 57.9, 52.7, 43.8, 39.5, 39.1, 29.7, 29.4, 27.6, 24.2; ESI-MS *m/z* 504.3 $[M+H]^+$; HRMS-ESI (*m/z*): $[M+H]^+$: calcd. for $C_{25}H_{33}F_3N_7O$: 504.2693, found: 504.2688; HPLC: System 1: t_R = 16.3 min, purity 99%, System 2: t_R = 16.2 min, purity 99%.

4.2.6 *N*-(4-(4-(2-*tert*-butyl-6-(trifluoromethyl)pyrimidin-4-yl)piperazin-1-yl)butyl)benzo[*b*]thiophene-2-carboxamide (9)

To a solution of benzothiophene-2-carboxylic acid (50.0 mg, 0.28 mmol) and **8** (120 mg, 0.33 mmol) in anhydrous DMF (5 mL) was added DIPEA (0.10 mL, 0.56 mmol) and TBTU (90.0 mg, 0.28 mmol). After stirring the reaction mixture at room temperature for 16 h the solvent was removed under reduced pressure. The crude mixture was suspended in aqueous 5% $NaHCO_3$ solution and extracted with ethyl acetate (3x). The combined organic phases were washed with water and brine and were dried ($MgSO_4$). After removal of solvents under reduced pressure the crude material was purified by flash chromatography (CH_2Cl_2/MeOH, 20:1) to give **9** (97.0 mg, 67%) as a colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.86 (s, 1H), 7.86-7.80 (m, 2H), 7.43 – 7.37 (m, 2H), 6.99 (br s, 1H), 6.60 (s, 1H), 3.87 (br s, 4H), 3.52 (app q, *J* = 6.2 Hz, 2H), 2.86 (app t, *J* = 5.0 Hz, 4H), 2.78 – 2.70 (m, 2H), 1.82 – 1.67 (m, 4H), 1.34 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 178.2, 166.4, 162.6, 162.0, 155.3 (q, J_{CF} = 34.1 Hz), 140.8, 139.2, 138.6, 126.3, 125.3, 125.0, 124.9, 121.1 (q, J_{CF} = 275 Hz), 95.7 (q, J_{CF} = 3.0 Hz), 57.1, 51.7, 42.16, 39.5, 39.2, 29.3, 26.8, 22.3; ESI-MS *m/z* 520.3 $[M+H]^+$; HRMS-ESI (*m/z*): $[M+H]^+$: calcd. for $C_{26}H_{33}F_3N_5OS$: 520.2352, found: 520.2341; HPLC: System 1: t_R = 19.6 min, purity 98%, System 2: t_R = 16.8 min, purity 99%.

4.2.7 *N*-(4-(4-(2-(2-Fluoroethoxy)phenyl)piperazin-1-yl)butyl)pyrazolo[1,5-*a*]pyridine-2-carboxamide x HCOOH (2)

Compound **2** was synthesized in accordance to Hocke et al.[30] with slight modifications: To a suspension of **5** (10.0 mg, 25 µmol), Cs_2CO_3 (20 mg, 62.5 µmol) in dry acetonitrile (2 mL) was added 2-fluoroethyl-4-methylbenzenesulfonate **7** (8.30 mg, 38 µmol) and the reaction mixture was refluxed for 14 h. After cooling to room

temperature the solvent was removed under reduced pressure and the crude mixture was suspended in aqueous 5% $NaHCO_3$ solution and extracted with ethyl acetate (3x). The combined organic phases were washed with brine and were dried ($MgSO_4$). After removal of solvents under reduced pressure the crude material was purified by preparative HPLC (acetonitrile in 0.1% aqueous HCOOH, 5% to 95%) to give **2** (6.50 mg, 54%) as a colorless solid. ^{1}H NMR (600 MHz, MeOD) δ 8.56 (d, *J* = 7.0 Hz, 1H), 7.70 (d, *J* = 8.9 Hz, 1H), 7.25 (dd, *J* = 8.9, 6.7 Hz, 1H), 7.03 – 6.92 (m, 6H), 4.80 – 4.69 (m, 2H), 4.29 – 4.21 (m, 2H), 3.49 (t, *J* = 6.6 Hz, 2H), 3.32 – 3.28 (m, 2H), 3.23 (br s, 4H), 3.08 (br s, 2H), 2.94 – 2.86 (m, 2H), 1.81 – 1.69 (m, 4H); ^{13}C NMR (150 MHz, MeOD) δ 151.3, 147.3, 140.6, 128.3, 124.0, 123.4, 121.6, 118.8, 118.3, 113.8, 113.7, 97.0, 81.9 (d, J_{CF} = 169 Hz), 67.8 (d, J_{CF} = 19.6 Hz), 57.1, 52.5, 48.7, 38.3, 26.7, 22.1, 2C were not observed; ESI-MS *m/z* 440.2 $[M+H]^+$; HRMS-ESI (*m/z*): $[M+H]^+$: calcd. for $C_{25}H_{30}FN_5O_2$: 440.2465, found: 440.2459; HPLC: System 1: t_R = 16.2 min, purity 95%, System 2: t_R = 13.6 min, purity 95%.

4.3 Radiosynthesis of [^{18}F]2

After evaporation of the solvent from the reaction vessel containing [^{18}F]fluoride, the K_2CO_3 base and the aminopolyether 2.2.2, residual traces of water were removed by azeotropic drying after the addition of acetonitrile at 80 °C. The labeling precursor (bistosyloxyethan, 10 µmol) was dissolved in anhydrous acetonitrile (0.4 ml) and added to the vial containing [^{18}F]fluoride. The reaction mixture was heated at 85 °C for 3 min. [^{18}F]Fluoroethyltosylate **[^{18}F]7** was obtained in a radiochemical yield (RCY) of 65-80% and isolated by HPLC (isocratic conditions, 65:35 acetonitrile / 0.1 M ammonium acetate, C-18 column, 4 mL/min, R_t = 7.0 min). The product fraction was collected, diluted with water (10 mL) and passed through a C18 cartridge (Sep-Pak, Waters). After washing the cartridge with water (10 mL), the Sep-Pak cartridge was dried in a He-gas stream. [^{18}F]Fluoroethyltosylate **[^{18}F]7** was eluted with 300 µL DMF for the direct use in the second reaction step with phenol **5** (10 µmol in 200 µL DMF) in the presence of tetrabutyammonium hydroxide (10 µmol, 10 µL, 1N). The ^{18}F-fluoroalkylation was achieved within 2 min at 120 °C. The reaction mixture was diluted with 10 mL of water and passed through a C18 cartridge. The cartridge was eluted with 500 µL of acetonitrile and the eluate diluted with 500 µL of water and subjected to the 1 mL sample loop of the HPLC instrument. The preparative

separation was carried out on the same column as described above with isocratic conditions of 25:75 acetonitrile / 0.1 M ammonium acetate, 4 mL / min, providing the final product **[^{18}F]2** in 50% RCY. After HPLC isolation and collection of the product-containing fractions by solid-phase extraction with C18 cartridge (Sep-Pak, Waters), the ethanol solution was evaporated on a rotary evaporator in a glass flask and 0.9% sterile saline solution (2 mL) was added to redissolve the product. *N*-[4-[4-(2-[^{18}F]fluoroethoxyphenyl)-piperazin-1-yl]-butyl]-pyrazolo-[1,5-*a*]pyridin-2-carboxamide **[^{18}F]2** was identified by analytical radio-HPLC (isocratic conditions, 25:75 acetonitrile / 0.1 M ammonium acetate, Luna C-18 column 150 × 4.6 mm, 1 mL/min, R_t = 14.5 min). **[^{18}F]2** was isolated in a radiochemical purity of >99% and in molar radioactivities of 44 ± 30 GBq/µmol. The final solution was used for PET imaging studies on rats and ex-vivo autoradiography.

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Supplementary data

Supplementary data includes effects of compound **1** on cocaine dose effect function as well as dose-ranging studies, experimental procedures for known derivatives and analytical data for newly synthesized compounds and descriptions of the assays.

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Development of molecular tools based on the dopamine D_3 receptor ligand FAUC 329 showing inhibiting effects on drug and food maintained behavior

*Anne Stößel[a], Regine Brox[a], Nirupam Purkayastha[a], Harald Hübner[a], Carsten Hocke[b], Olaf Prante[b], Peter Gmeiner[a]**

[a]Department of Medicinal Chemistry, Emil Fischer Center, Friedrich-Alexander University, Schuhstraβe 19, D-91052 Erlangen, Germany

[b]Department of Nuclear Medicine, Ulmenweg 18, D-91054 Erlangen, Germany

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