



# Improved in vivo PET imaging of the adenosine A<sub>2A</sub> receptor in the brain using [<sup>18</sup>F]FLUDA, a deuterated radiotracer with high metabolic stability

Thu Hang Lai<sup>1,2</sup> · Magali Toussaint<sup>1</sup> · Rodrigo Teodoro<sup>1</sup> · Sladjana Dukić-Stefanović<sup>1</sup> · Daniel Gündel<sup>1</sup> · Friedrich-Alexander Ludwig<sup>1</sup> · Barbara Wenzel<sup>1</sup> · Susann Schröder<sup>2</sup> · Bernhard Sattler<sup>3</sup> · Rareş-Petru Moldovan<sup>1</sup> · Björn H. Falkenburger<sup>4</sup> · Osama Sabri<sup>3</sup> · Winnie Deuther-Conrad<sup>1</sup> · Peter Brust<sup>1</sup>

Received: 19 August 2020 / Accepted: 13 December 2020  
© The Author(s) 2021

## Abstract

**Purpose** The adenosine A<sub>2A</sub> receptor has emerged as a therapeutic target for multiple diseases, and thus the non-invasive imaging of the expression or occupancy of the A<sub>2A</sub> receptor has potential to contribute to diagnosis and drug development. We aimed at the development of a metabolically stable A<sub>2A</sub> receptor radiotracer and report herein the preclinical evaluation of [<sup>18</sup>F]FLUDA, a deuterated isotopologue of [<sup>18</sup>F]FESCH.

**Methods** [<sup>18</sup>F]FLUDA was synthesized by a two-step one-pot approach and evaluated in vitro by autoradiographic studies as well as in vivo by metabolism and dynamic PET/MRI studies in mice and piglets under baseline and blocking conditions. A single-dose toxicity study was performed in rats.

**Results** [<sup>18</sup>F]FLUDA was obtained with a radiochemical yield of 19% and molar activities of 72–180 GBq/μmol. Autoradiography proved A<sub>2A</sub> receptor-specific accumulation of [<sup>18</sup>F]FLUDA in the striatum of a mouse and pig brain. In vivo evaluation in mice revealed improved stability of [<sup>18</sup>F]FLUDA compared to that of [<sup>18</sup>F]FESCH, resulting in the absence of brain-penetrant radiometabolites. Furthermore, the radiometabolites detected in piglets are expected to have a low tendency for brain penetration. PET/MRI studies confirmed high specific binding of [<sup>18</sup>F]FLUDA towards striatal A<sub>2A</sub> receptor with a maximum specific-to-non-specific binding ratio in mice of 8.3. The toxicity study revealed no adverse effects of FLUDA up to 30 μg/kg, ~ 4000-fold the dose applied in human PET studies using [<sup>18</sup>F]FLUDA.

**Conclusions** The new radiotracer [<sup>18</sup>F]FLUDA is suitable to detect the availability of the A<sub>2A</sub> receptor in the brain with high target specificity. It is regarded ready for human application.

Thu Hang Lai, Magali Toussaint, Winnie Deuther-Conrad and Peter Brust contributed equally to this work.

This article is part of the Topical Collection on Preclinical Imaging

✉ Thu Hang Lai  
t.lai@hzdr.de

✉ Magali Toussaint  
m.toussaint@hzdr.de

<sup>1</sup> Department of Neuroradiopharmaceuticals, Institute of Radiopharmaceutical Cancer Research, Helmholtz-Zentrum Dresden-Rossendorf, Leipzig, Germany

<sup>2</sup> Department of Research and Development, ROTOP Pharmaka Ltd., Dresden, Germany

<sup>3</sup> Department of Nuclear Medicine, University Hospital Leipzig, Leipzig, Germany

<sup>4</sup> Department of Neurology, Dresden University Medical Center, Dresden, Germany

**Keywords** Adenosine receptors · A<sub>2A</sub> receptor · Neurodegeneration · Positron-emission tomography · Fluorine-18 · FESCH

## Introduction

The signaling molecule adenosine is an important modulator of neurotransmission, regulating physiological processes such as sleep, motor activity, or sensorimotor gating [1]. Consequently, modulating adenosine signaling is an emerging treatment option for neuropsychiatric and neurodegenerative disorders [1]. Adenosine regulates neurotransmission of glutamate, acetylcholine, γ-aminobutyric acid, and dopamine using four G-protein-coupled plasma membrane receptors - adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors [1]. Whereas A<sub>1</sub>, A<sub>2B</sub> and A<sub>3</sub> receptors are widely distributed throughout the brain, the A<sub>2A</sub> receptor is specifically expressed at high densities in the dorsal

and ventral striatum, the main input structures of basal ganglia circuitry [2]. The binding of adenosine to the A<sub>2A</sub> receptor activates protein kinase A (PKA) via G protein-mediated stimulation of adenylyl cyclase and the corresponding increase in cyclic adenosine monophosphate (cAMP). In addition, PKA-independent pathways have also been reported [3, 4]. The A<sub>2A</sub> receptor forms dimers with itself and with further G-protein-coupled receptors, in particular D<sub>2</sub>, mGluR<sub>5</sub>, CB<sub>1</sub>, and A<sub>1</sub> [3], which causes changes in A<sub>2A</sub> density in a wide range of neuropsychiatric and neurodegenerative diseases.

For instance, the measurement of mRNA levels indicated a reduced A<sub>2A</sub> receptor density in the caudate and putamen of patients with Parkinson's disease (PD) [5], while increased A<sub>2A</sub> protein levels were found in PD patients with dyskinesias [6, 7] and in patients with Huntington's disease (HD) [8]. Given that PD is characterized by a reduced mobility whereas PD patients with dyskinesias and HD patients show excessive movements, these findings collectively indicate that A<sub>2A</sub> is an important reporter - and modulator - of mobility in human basal ganglia. Accordingly, the A<sub>2A</sub> antagonist istradefylline has been approved for treatment of PD in the USA and in Japan [3, 9].

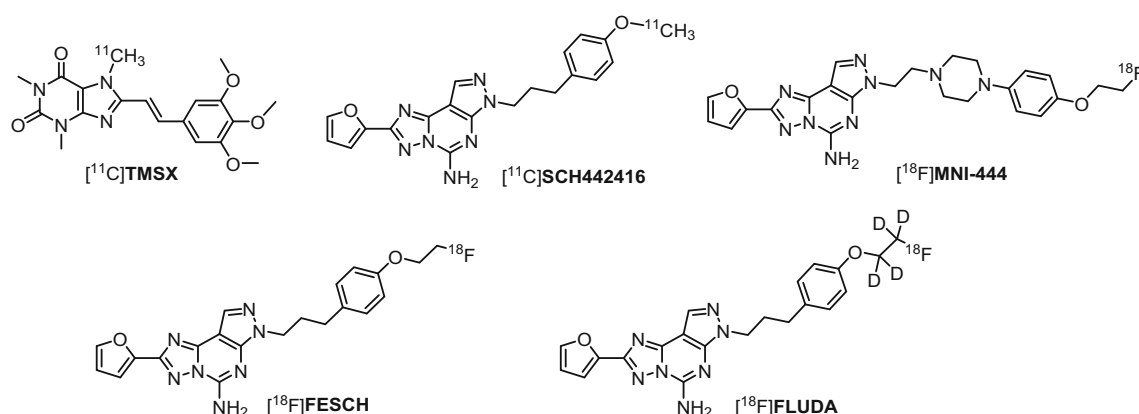
Molecular imaging of the A<sub>2A</sub> receptor by means of positron-emission tomography (PET) has the potential to quantitatively assess the receptor availability and changes thereof during the course of neuropsychiatric diseases' pathological processes and determine optimal dosing regimens for drugs targeting A<sub>2A</sub>. Accordingly, research teams in both academia and industry have been working on the development of suitable PET radiotracers since more than 15 years. Initially, <sup>11</sup>C-labeled caffeine derivatives such as [<sup>11</sup>C]KF17837 [10], [<sup>11</sup>C]CSC [11], or [<sup>11</sup>C]KF21213 [12] were developed and investigated in animal models. With [<sup>11</sup>C]SCH442416, one of the first non-xanthine A<sub>2A</sub> receptor antagonists has been proven as suitable for in vivo imaging [13]. The first studies in humans assessing the distribution of A<sub>2A</sub> receptors in normal human brain with [<sup>11</sup>C]KF18446 (also known as [<sup>11</sup>C]TMSX, Fig. 1) [14] as well as the occupancy of the A<sub>2A</sub> receptor of the

targeted drug candidate vipadenant with [<sup>11</sup>C]SCH442416 (Fig. 1) were published about 10 years ago [15]. At about the same time, the first <sup>18</sup>F-labeled radiotracers were reported, e.g., analogs of SCH442416, such as [<sup>18</sup>F]MRS5425, (also known as [<sup>18</sup>F]FESCH, Fig. 1) [16, 17]. [<sup>18</sup>F]MNI-444 (Fig. 1) is another PET radiotracer that was used for imaging of the A<sub>2A</sub> receptor in healthy human subjects [18]. To assess the suitability of A<sub>2A</sub> receptor PET for the assessment of changes in the availability of A<sub>2A</sub>/D<sub>2</sub> heterodimeric receptors in neurodegenerative diseases, our group performed dynamic PET studies in a rotenone-based mouse model of Parkinson's disease with [<sup>18</sup>F]FESCH. However, the study yielded inconclusive data, at least in part due to the penetration of a non-negligible fraction of a single radiometabolite into the mouse' brain [19]. Therefore, we intended to enhance the metabolic stability of [<sup>18</sup>F]FESCH by developing a deuterated isotopologue, FLUDA. To test the hypothesis that a selective substitution with deuterium improves the imaging properties of the novel A<sub>2A</sub> receptor-specific radiotracer [<sup>18</sup>F]FLUDA (Fig. 1), we performed a series of preclinical animal studies including in vivo metabolism and dynamic PET/MRI investigations in mouse and dynamic PET investigations in piglets to investigate target specificity and pharmacokinetics in different species. To further support the transfer of [<sup>18</sup>F]FLUDA to first-in-human studies, a preclinical acute toxicity study in rats has been commissioned as well.

## Materials and methods

The full description of all procedures is provided in the Supplementary information (SI).

**Chemical synthesis** FLUDA was synthesized by a microwave-assisted alkylation reaction of 4-[3-(5-amino-2-furan-2-yl-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-7-yl)-propyl]-phenol (desmethyl SCH442416) and 2-fluoroethyl-1,1,2,2-*d*<sub>4</sub> 4-methylbenzenesulfonate (3), that was previously



**Fig. 1** Representative radiotracers for PET imaging of the A<sub>2A</sub> receptor and the herein reported [<sup>18</sup>F]FLUDA

prepared by tosylation of the commercially available ethane- $d_4$ -1,2-diol (**1**) and fluorination of ethane-1,2-diyl- $d_4$  bis(4-methylbenzenesulfonate) (**2**, Scheme 1).

**Radiosynthesis** [ $^{18}\text{F}$ ]FLUDA was prepared by a two-step one-pot manual radiosynthesis of the tosylate precursor **2** and the phenol precursor **desmethyl SCH442416** with [ $^{18}\text{F}$ ]fluoride in anhydrous acetonitrile (MeCN) in the presence of potassium carbonate and Kryptofix 222 ( $\text{K}_{222}$ ) according to our optimized procedure for the radiosynthesis of [ $^{18}\text{F}$ ]FESCH (Fig. 2a) [19].

**Physicochemical properties** The chemical stability of [ $^{18}\text{F}$ ]FLUDA was proven in saline, phosphate-buffered saline (PBS, pH 7.4) and *n*-octanol by incubation at 37 °C up to 60 min followed by radio-TLC and radio-HPLC analyses. The  $\log D_{7.4}$  value was experimentally determined by the conventional shake-flask method using *n*-octanol and PBS as partition system ( $n = 4$ ).

**Animals** The animal experiments were performed with female CD-1 mice (10–12 weeks, 26–38 g) obtained from the Medizinisch-Experimentelles Zentrum (MEZ) at Universität Leipzig (Leipzig; Germany) and female piglets (6–12 weeks old, 14–18 kg) obtained from the Lehr-und Versuchsgut of the Faculty of Veterinary Medicine at Universität Leipzig (LVG; Oberholz; Germany).

**In vitro binding assays** The test compounds (10 mM stock in DMSO) were incubated with crude cell membrane homogenates obtained from CHO cells stably transfected with human  $\text{A}_{2\text{A}}$  receptor or human  $\text{A}_1$  receptor and with  $\text{A}_{2\text{A}}$  receptor-specific [ $^3\text{H}$ ]ZM241385 or  $\text{A}_1$  receptor-specific [ $^3\text{H}$ ]DPCPX in an incubation buffer at room temperature (RT). The  $\text{IC}_{50}$  values were determined by non-linear regression analysis with GraphPad Prism 4.1 (GraphPad Inc.; La Jolla; CA), and  $K_i$  values estimated according to the Cheng-Prusoff equation with  $K_{\text{D,ZM241385}} = 0.8 \text{ nM}$  and  $K_{\text{D,DPCPX}} = 0.45 \text{ nM}$ .

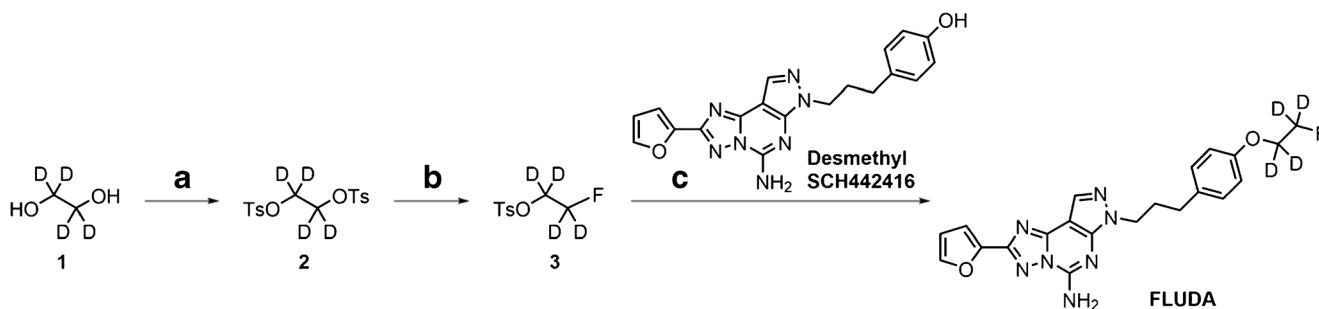
**In vitro autoradiography** Cryosections from mouse and piglet brain were thawed, dried, and pre-incubated with buffer

containing adenosine deaminase (ADA), and then with buffer containing ADA and [ $^{18}\text{F}$ ]FLUDA alone or together with 10  $\mu\text{M}$  ZM241385 to assess non-specific binding of [ $^{18}\text{F}$ ]FLUDA or together with different concentrations of test compounds to estimate their binding affinity towards the  $\text{A}_{2\text{A}}$  receptor. After washing and drying, the slides were exposed to a phosphor imager plate, and after scanning, the autoradiographic images were analyzed with the AIDA 2.31 software and inhibition curves created with GraphPad Prism 4.1 (GraphPad Inc., La Jolla, CA).

**In vivo metabolism studies** In mice, [ $^{18}\text{F}$ ]FLUDA was administered i.v. as a bolus in the tail vein of awake animals ( $\sim 36 \text{ MBq}$ ,  $n = 3$ ). After 15 min, the animals were slightly anesthetized by isoflurane inhalation, and blood samples were taken by retro-orbital bleeding. The blood plasma was obtained by centrifugation. The brain was isolated immediately after cervical dislocation and homogenized in water. For analysis by RP-HPLC, brain homogenates and blood plasma were mixed with the 4-fold volumes of acetone/water (4/1; v/v), precipitated proteins removed by centrifugation, the supernatants were concentrated and analyzed by analytical RP-HPLC. For analysis by micellar-HPLC (MLC), brain homogenates and blood plasma were mixed with equal volumes of aqueous sodium dodecyl sulfate and directly injected into the MLC system.

In piglets, [ $^{18}\text{F}$ ]FLUDA was administered i.v. as a bolus in the auricular vein of the anesthetized animals ( $\sim 203 \text{ MBq}$ ,  $n = 2$ ). Arterial blood was sampled over 120 min and the blood plasma was obtained by centrifugation. For analysis by semi-preparative RP-HPLC, blood plasma was mixed with the two-fold volumes of acetone/water (4/1; v/v), precipitated proteins were removed by centrifugation, and the supernatants were concentrated and analyzed by semi-preparative RP-HPLC.

**Dynamic PET studies in mice** PET/MRI scans were performed using a preclinical PET/MRI system (PET/MRI 1Tesla; nanoScan<sup>®</sup>; MEDISO Medical Imaging Systems; Budapest; Hungary) in CD-1 mice under baseline ( $n = 4$ ), control (vehicle,  $n = 8$ ), and blocking (pre-administration of 2.5 mg/kg tozadenant (also known as SYN-115) or 1.0 mg/kg



**Scheme 1** Synthesis of FLUDA, reagents and conditions. **a** *p*-TsCl,  $\text{NEt}_3$ ,  $\text{CH}_2\text{Cl}_2$ , 0 °C, 3 h, 68% yield. **b** TBAF, MeCN/THF, 90 °C, 15 min, 36% yield. **c** Desmethyl SCH442416,  $\text{Cs}_2\text{CO}_3$ , MeOH, microwave heating (1 h, 100 °C, 100 W), 37% yield

istradefylline (also known as **KW-6002**) i.v. 15 to 8 min before radiotracer  $n = 4$ , respectively) conditions. Dynamic whole-body animal PET scans were acquired during 60 min after i.v. administration of [ $^{18}\text{F}$ ]**FLUDA** (3.1–9.7 MBq, 0.7–4.5 nmol/kg). T1-weighted imaging was performed afterwards for anatomical orientation and attenuation correction. After reconstruction, PET images were analyzed in PMOD 3.9 (PMOD technologies LLC; Zurich; Switzerland), and volumes of interest were applied to the PET series to extract time-activity curves (TACs). TACs were expressed in standardized uptake value (SUV).

**PET studies in piglets** PET scans were obtained on a clinical PET-System (ECAT Exact HR+; Siemens Healthcare GmbH; Erlangen; Germany) in piglets under control (vehicle,  $n = 1$ ) and blocking conditions (2.5 mg tozadenant /kg i.v. 15 min before radiotracer followed by continuous infusion at 0.9 mg/kg/h for the duration of the study,  $n = 1$ ). Dynamic PET scans were acquired during 90 min after i.v. administration of [ $^{18}\text{F}$ ]**FLUDA** (178–229 MBq; 0.08–0.16 fmol/kg, for control and blocking conditions respectively). After reconstruction, PET images were analyzed in PMOD 3.9 (PMOD technologies LLC; Zurich; Switzerland).

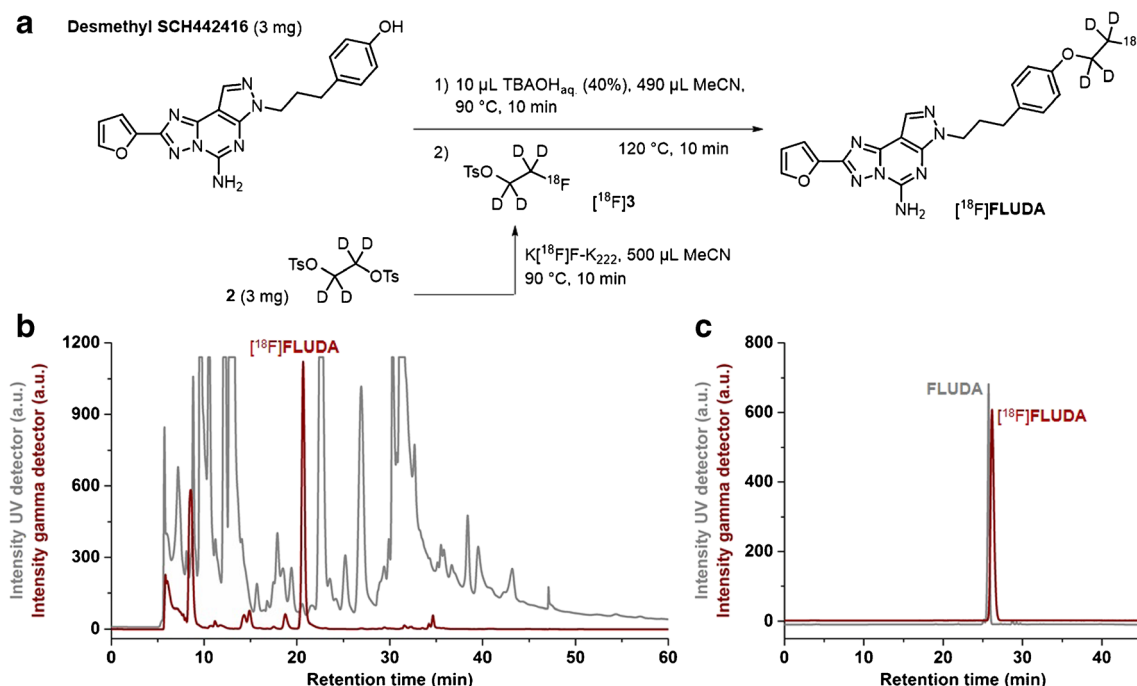
**Toxicity studies in rats** The extended single-dose toxicity studies of **FLUDA** in male ( $n = 45$ ) and female ( $n = 45$ ) outbred Wistar rats were performed in the Biological Testing Laboratory (BTL) in Russia (Study Number 678/19). The test

item **FLUDA** was administered by a single bolus i.v. injection at doses of 6 and 30  $\mu\text{g/kg}$  body weight (bw). Mortality, clinical pathology parameters (hematology and serum chemistry), organ weights, and microscopic tissue parameters were investigated 24 h and 2 weeks after treatment.

## Results

**Synthesis of the reference compound and affinity investigation** The deuterated derivative of **FESCH** was obtained by the use of a deuterated fluoroethyl building block according to the synthesis described in Scheme 1. Binding studies were performed by competitive radiotracer binding assays and revealed high-affinity binding of **FLUDA** towards the human  $A_{2A}$  receptor with a  $K_i$  value of  $0.74 \pm 0.26$  nM and negligible binding towards the human  $A_1$  receptor ( $K_i > 1$   $\mu\text{M}$ ).

**Radiosynthesis, lipophilicity, and chemical stability** [ $^{18}\text{F}$ ]**FLUDA** was obtained by a one-pot two-step radiofluorination as shown in Fig. 2 with a radiochemical yield of  $19 \pm 3\%$  (end of bombardment = EOB), a radiochemical purity of  $\geq 99\%$ , and molar activities in the range of 72–180 GBq/ $\mu\text{mol}$  (end of synthesis = EOS) within a total synthesis time of  $102 \pm 4$  min ( $n = 9$ ). The shake-flask method was used to determine the  $\log D_{7.4}$  value of [ $^{18}\text{F}$ ]**FLUDA** ( $2.01 \pm 0.07$ ). The incubation of [ $^{18}\text{F}$ ]**FLUDA** in saline, PBS (pH



**Fig. 2** **a** Scheme of the two-step one-pot procedure for the radiosynthesis of [ $^{18}\text{F}$ ]**FLUDA**. **b** Representative chromatograms of isolation of [ $^{18}\text{F}$ ]**FLUDA** by semi-preparative HPLC. **c** Identification of

[ $^{18}\text{F}$ ]**FLUDA**. HPLC chromatograms obtained by co-injection with the reference compound **FLUDA**

7.4), and *n*-octanol has shown no degradation or defluorination.

**In vivo metabolism** At 15 min after i.v. injection of [ $^{18}\text{F}$ ]FLUDA in mice and analysis, the parent fraction accounted for about 100% (RP-HPLC, recovery: 98%) and 95% (MLC) in brain (Fig. S2); and 71% (RP-HPLC, recovery: 86%, Fig. 3) and 56% (MLC, Fig. S1) in plasma. In piglets, the parent fraction accounted for about 47% in plasma at 16 min p.i (recovery: 89%, Fig. S1). The metabolic pattern in mice contained two fractions of radiometabolites, [ $^{18}\text{F}$ ]M1 and [ $^{18}\text{F}$ ]M2, not able to cross the blood-brain barrier (BBB). In contrast to the in vivo stability of [ $^{18}\text{F}$ ]FLUDA in mice, the plasma samples of piglets contained two additional radiometabolites, [ $^{18}\text{F}$ ]M3 and [ $^{18}\text{F}$ ]M4, supposed to have a similar structure to [ $^{18}\text{F}$ ]FLUDA based on their chromatographic behavior as shown in Fig. 3. Due to the PET studies in piglets (Fig. 7), these two radiometabolites are not expected to cross the BBB.

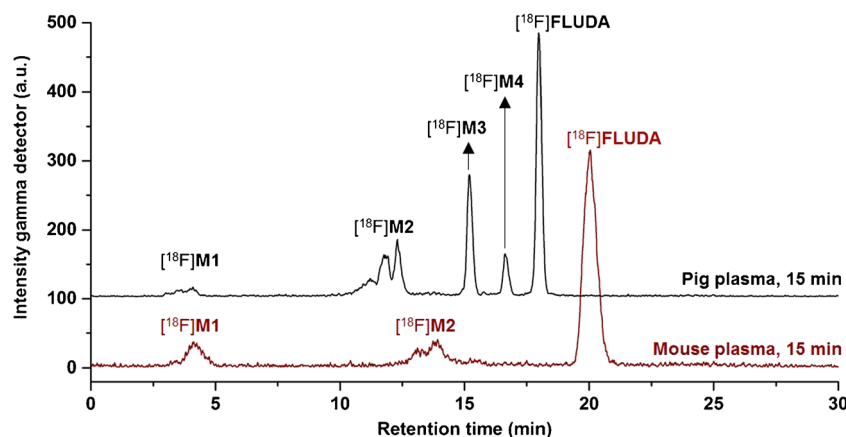
**Quantitative in vitro autoradiography** The binding pattern of [ $^{18}\text{F}$ ]FLUDA in mouse brain is heterogeneous with the highest density of binding sites in the striatum, an  $A_{2A}$  receptor-rich region. In  $A_{2A}$  receptor-poor regions, such as cerebellum, midbrain, cortex, or thalamus, only negligible binding was detected (Fig. 4a, Fig. S3). About 90% of the binding of  $\sim 1$  nM [ $^{18}\text{F}$ ]FLUDA in mouse striatum could be displaced by co-incubation with 10  $\mu\text{M}$  ZM241385 (Fig. 4b). The binding sites in striatum were further characterized by saturation experiments which revealed a  $K_D$  value of  $4.30 \pm 0.73$  nM and a  $B_{\text{max}}$  value of  $556 \pm 143$  fmol/mg wet weight (Fig. 4d). Comparable results were obtained in a single experiment performed in cryosections of the pig brain to promote the preclinical evaluation of [ $^{18}\text{F}$ ]FLUDA in larger species (Fig. 5). The nearly exclusive binding of [ $^{18}\text{F}$ ]FLUDA in the striatum (Fig. 5a) was completely blocked by co-administration of ZM241385 (Fig. 5b). By homologous competition, the binding of FLUDA in the pig striatum was

characterized with a  $K_D$  value of 0.68 nM and a  $B_{\text{max}}$  value of 218 fmol/mg wet weight (Fig. 5c).

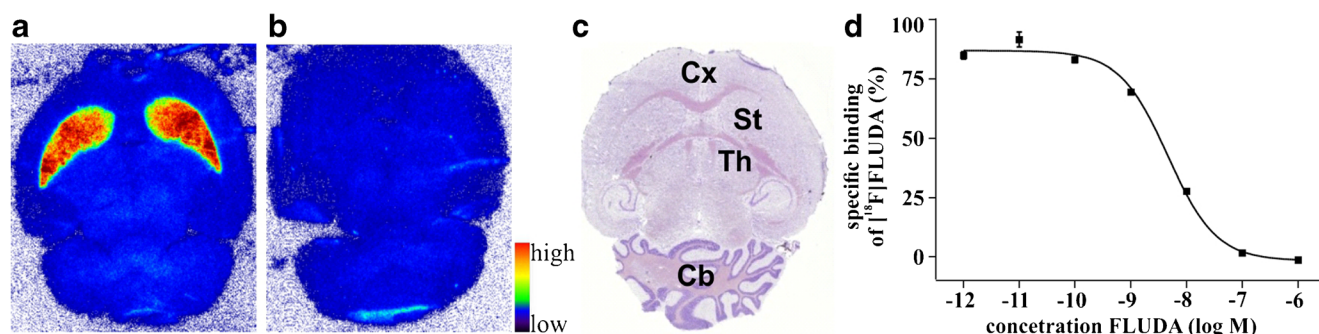
**Dynamic PET studies in mice** Representative PET images of [ $^{18}\text{F}$ ]FLUDA in a mouse brain are shown in Fig. 6a. Corresponding to the in vitro autoradiography, the highest uptake was detected in the  $A_{2A}$  receptor-rich striatum. Also, the negligible accumulation of activity in  $A_{2A}$  receptor-poor regions, such as cerebellum, is in agreement with the in vitro data. These general findings are confirmed by the analysis of the PET-derived regional time-activity curves (TACs) presented in Fig. 6b. In fact, a high specific-to-non-specific binding ratio was reached, with a maximum  $\text{SUV}_{\text{r}/\text{C}_{\text{b}}}$  of 8.3 reflecting a high target selectivity (Fig. 6c). Furthermore, the continuous washout of activity from all brain regions confirms the absence of brain-penetrant radiometabolites. To validate the target specificity of [ $^{18}\text{F}$ ]FLUDA in vivo, the  $A_{2A}$  receptor antagonist istradefylline (1 mg/kg) or tozadenant (2.5 mg/kg) were administered i.v. at 10 min before radiotracer injection. Maximum blocking was obtained by pre-treatment of istradefylline as reflected by the striatal SUV being similar to the cerebellar SUV under blocking conditions during the entire period of investigation (Fig. 6d–e). By tozadenant pre-treatment, the accumulation of activity in the striatum was reduced by 23.5% in comparison to the control (according to the area under the curve (AUC);  $\text{AUC}_{\text{vehicle}}$ :  $24.9 \pm 8.7$  SUV  $\cdot$  min;  $\text{AUC}_{\text{tozadenant}}$ :  $19.0 \pm 4.3$  SUV  $\cdot$  min from 0 to 60 min; Fig. S8). The activity accumulation in the cerebellum was not significantly affected by the blocking compound (Fig. 6e;  $\text{AUC}_{\text{vehicle}}$ :  $5.9 \pm 3.0$  SUV  $\cdot$  min;  $\text{AUC}_{\text{tozadenant}}$ :  $5.4 \pm 1.3$  SUV  $\cdot$  min from 0 to 60 min; Fig. S8), confirming the target specificity of [ $^{18}\text{F}$ ]FLUDA and indicating the suitability of the cerebellum as a reference region for  $A_{2A}$  receptor imaging.

Further analysis of the whole-body PET data obtained in control (vehicle-treated) animals revealed a high initial uptake in the small intestine and the liver, followed by a pronounced accumulation of activity in the former, along with a stable uptake in the kidney over the whole scanning time, indicating

**Fig. 3** Representative RP-HPLC radiochromatograms of plasma samples after administration of [ $^{18}\text{F}$ ]FLUDA to a mouse and piglet







**Fig. 4** Representative in vitro autoradiographic images of the binding pattern of  $[^{18}\text{F}]$ FLUDA (0.93 nM) in horizontal mouse brain slices. The highest accumulation of activity in the striatum (a, red). The binding is completely blocked by co-administration of 10  $\mu\text{M}$  of the  $\text{A}_{2\text{A}}$  receptor antagonist ZM241385 (b). For annotation of the brain regions, the slices were Nissl-stained after autoradiography (c). St striatum,

Cb cerebellum, and Cx cortex, Th thalamus. Representative competition curve (d).  $K_{\text{D}}$  and  $B_{\text{max}}$  calculated from the homologous competition of  $[^{18}\text{F}]$ FLUDA with FLUDA (Cheng-Prusoff equation  $K_{\text{D}} = \text{IC}_{50} - \frac{[\text{specific binding}]}{[\text{total binding}] - [\text{specific binding}]}$  and  $B_{\text{max}} = \text{top} - \text{bottom} \cdot (K_{\text{D}} + [\text{specific binding}]) / [\text{specific binding}]$  [20].

that  $[^{18}\text{F}]$ FLUDA and/or its metabolites mainly undergo hepatobiliary excretion (Table S2; Fig. S9).

**Dynamic PET studies in piglets** To further assist the transfer of  $[^{18}\text{F}]$ FLUDA to clinical studies, we performed a pilot study to evaluate the pharmacokinetic profile of  $[^{18}\text{F}]$ FLUDA by dynamic PET in piglets. The activity distribution in the brain of a control subject resembles the findings obtained in mouse—strong and nearly exclusive accumulation in the striatum and low signal in all other brain regions (Fig. 7a). The pre-administration of tozadenant (2.5 mg/kg) at 15 min before radiotracer followed by continuous infusion inhibited the accumulation of activity in the striatum by 50% ( $\text{AUC}_{\text{vehicle}}$ : 42.1 SUV · min;  $\text{AUC}_{\text{tozadenant}}$ : 21.0 SUV · min from 0 to 60 min), and in  $\text{A}_{2\text{A}}$  receptor-poor regions, such as cerebellum by 17% ( $\text{AUC}_{\text{vehicle}}$ : 24.0 SUV · min;  $\text{AUC}_{\text{tozadenant}}$ : 19.8 SUV · min from 0 to 60 min; Fig. 7b).

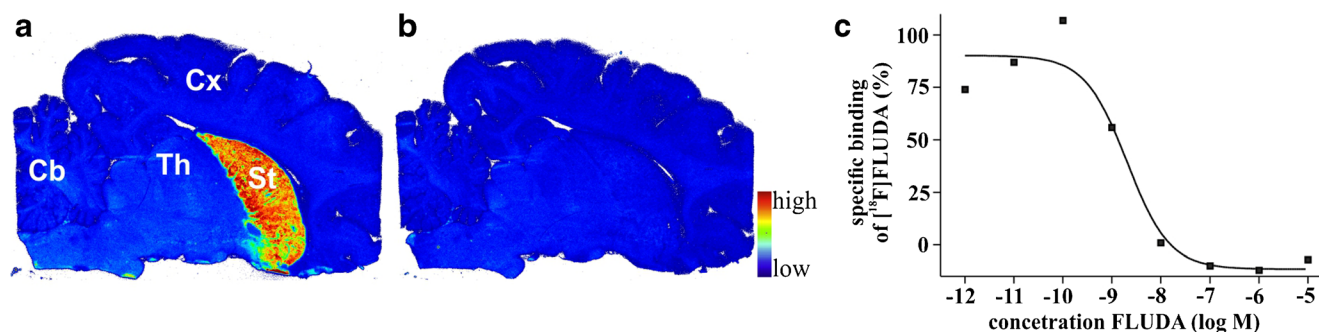
**Single-dose toxicity study** In an extended single-dose toxicity study in rats, administration of FLUDA up to 30  $\mu\text{g}/\text{kg}$  body weight, which is about 4000 times the estimated human dose and thus in accordance with the ICH guideline M3(R2), which

recommends a minimum of a 50-fold the clinical exposure as the maximum dose for general toxicity studies in any species, did not cause any conspicuous features related to the hematology, clinical chemistry, necropsy, and histopathology analyses.

## Discussion

The selective deuteration of the known  $\text{A}_{2\text{A}}$  receptor radio-tracer  $[^{18}\text{F}]$ FESCH significantly improved the metabolic stability of the  $\text{A}_{2\text{A}}$  receptor PET ligand  $[^{18}\text{F}]$ FLUDA. Furthermore, the preclinical evaluation of  $[^{18}\text{F}]$ FLUDA in both small and larger animals demonstrates high specific binding towards the adenosine  $\text{A}_{2\text{A}}$  receptor in the brain, indicating the suitability of  $[^{18}\text{F}]$ FLUDA for first-in-human studies.

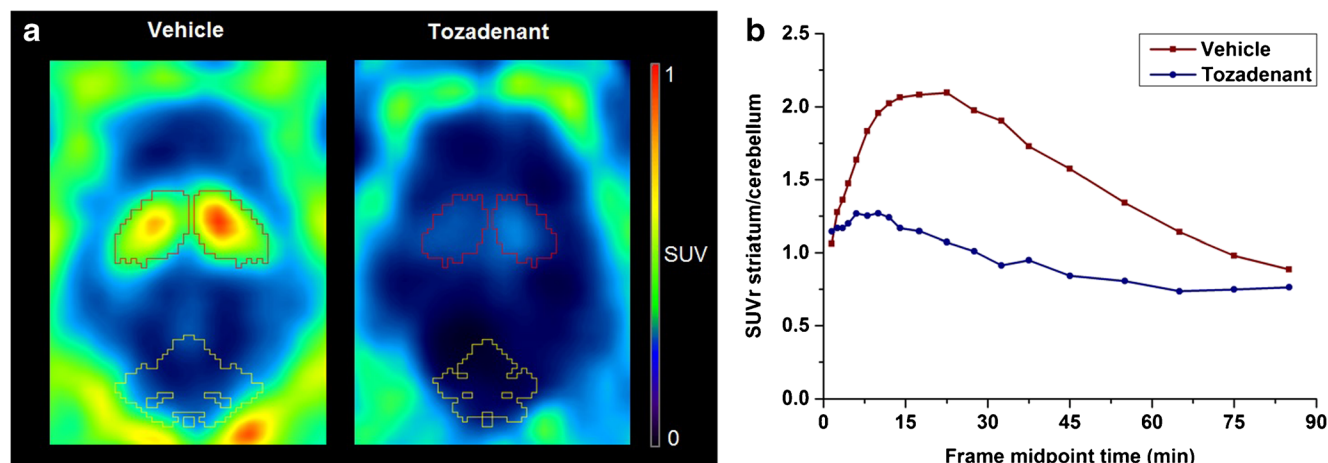
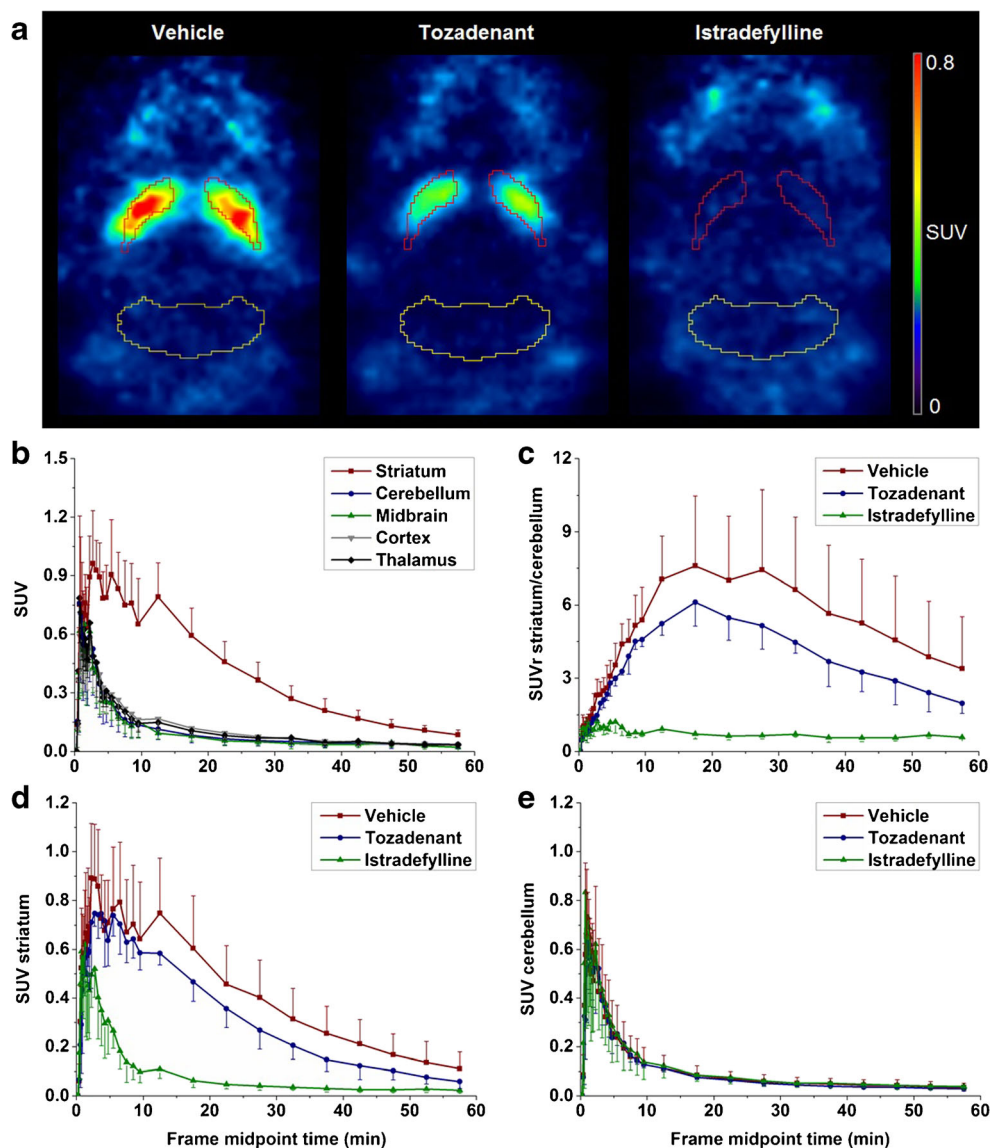
In comparison to  $[^{18}\text{F}]$ FESCH,  $[^{18}\text{F}]$ FLUDA is much less susceptible to biotransformation. This is reflected by the much higher amount of the parent fraction in plasma at 15 min after i.v. injection in mice (41% [19] vs. 71%). The activity accumulating in the brain after i.v. injection of  $[^{18}\text{F}]$ FLUDA corresponds almost completely to the parent compound. This



**Fig. 5** In vitro autoradiographic images of the binding pattern of  $[^{18}\text{F}]$ FLUDA (0.64 nM) in sagittal pig brain slices. The highest accumulation of activity is in the striatum (a, red, St striatum, Cb

cerebellum, Cx cortex, and Th thalamus). The binding is completely blocked by co-administration of 10  $\mu\text{M}$  of the  $\text{A}_{2\text{A}}$  receptor antagonist ZM241385 (b). Homologous competition curve (c)

**Fig. 6** **a** Representative horizontal PET images (0–60 min) of [ $^{18}\text{F}$ ]FLUDA in the brain of CD-1 mice (striatum: red, cerebellum: yellow). **b** TACs at baseline for CD-1 mice ( $n = 4$ ) in different brain regions after injection of [ $^{18}\text{F}$ ]FLUDA; TACs of **c**  $\text{SUV}_{\text{rS/Cb}}$ , **d** SUV striatum, and **e** SUV cerebellum: after pre-treatment with vehicle (red square,  $n = 8$ ), tozadenant (2.5 mg/kg bw, blue circle,  $n = 4$ ) and istradefylline (1.0 mg/kg bw, green triangle,  $n = 4$ )



**Fig. 7** **a** Representative horizontal PET images (0–60 min) of [ $^{18}\text{F}$ ]FLUDA in the brain of piglets (striatum: red, cerebellum: yellow). **b** TACs of  $\text{SUV}_{\text{rS/Cb}}$  of [ $^{18}\text{F}$ ]FLUDA in the pig brain after administration of vehicle (red square,  $n = 1$ ) or blocking with tozadenant (blue circle,  $n = 1$ )

finding is supported by the profile of the region-specific TACs obtained in dynamic PET studies with [ $^{18}\text{F}$ ]FLUDA in both mice (Fig. 6) and piglets (Fig. 7), which do not indicate any confounding accumulation of radiometabolites in the brain. In summary, deuteration of the fluoroethoxy group of [ $^{18}\text{F}$ ]FLUDA eliminates the main obstacle observed in the investigation of disease-related changes of the  $A_{2A}$  receptor availability in preclinical studies using [ $^{18}\text{F}$ ]FESCH: significant amounts of brain-penetrant radiometabolites.

With respect to further more general properties such as affinity, selectivity, blood-brain barrier permeability, and clearance, [ $^{18}\text{F}$ ]FLUDA retains the positive characteristics of [ $^{18}\text{F}$ ]FESCH [16, 17, 19, 21]. The affinity of [ $^{18/19}\text{F}$ ]FLUDA towards human and mouse  $A_{2A}$  receptors ( $K_i \sim 1$  nM vs.  $K_D \sim 4$  nM) corresponds well to the results obtained for [ $^{18/19}\text{F}$ ]FESCH ( $K_i \sim 1$  nM vs.  $K_D \sim 5$  nM; [19]) including a slight discrepancy between the two species that was reported previously (Table S1, Figs. S3, S4, S5, S6, S7) [22]. Regarding specificity, the accumulation of [ $^{18}\text{F}$ ]FLUDA resembles the expression pattern of the  $A_{2A}$  receptor protein in both mouse brain and pig brain and both in vitro and in vivo (Figs. 4, 5, 6, and 7). Moreover, the accumulation of [ $^{18}\text{F}$ ]FLUDA can be blocked completely by structurally different selective  $A_{2A}$  receptor antagonists (Figs. 6 and 7). The presence of an  $A_{2A}$  receptor antagonist inhibited the accumulation of activity in the striatum almost completely but did not affect any other brain region in mice and affected the cerebellum by 17% in piglet. However, the high specificity of [ $^{18}\text{F}$ ]FLUDA, as well as the absence of effect of blocking compound on cerebellum activity accumulation in mice studies, suggests that this effect on the cerebellum of piglet is most likely due to inter-individual variability, and would need a larger-scale study to investigate its significance. Collectively, these findings indicate highly specific in vivo binding of [ $^{18}\text{F}$ ]FLUDA in the striata in both species.

The differences in the efficacy of the two  $A_{2A}$  receptor ligands istradefylline and tozadenant to inhibit the binding of [ $^{18}\text{F}$ ]FLUDA in vitro and in vivo can be explained by known differences in the properties of these antagonists, in particular the remarkably low affinity of tozadenant towards mouse  $A_{2A}$  receptors, which we determined in-house by [ $^{18}\text{F}$ ]FLUDA autoradiography (istradefylline:  $K_i$  (mouse  $A_{2A}$  receptor)  $\sim 60$  nM,  $K_i$  (piglet  $A_{2A}$  receptor)  $\sim 15$  nM; tozadenant:  $K_i$  (mouse  $A_{2A}$  receptor)  $\sim 250$  nM,  $K_i$  (piglet  $A_{2A}$  receptor)  $\sim 10$  nM; Table S1). Most of the preclinical studies that evaluated the specificity of  $A_{2A}$  receptor-targeting PET radiotracers use istradefylline [17, 21, 23], whereas tozadenant has only been applied, to the best of our knowledge, in a single study in rhesus monkeys [24]. A head-to-head comparison as presented here for mice has not been reported so far. The higher efficacy of tozadenant in vivo in piglet in comparison to mouse ( $\sim 50\%$  displacement vs.  $\sim 25\%$  displacement at 2.5

mg/kg followed by 0.9 mg/kg/h infusion and 2.5 mg/kg, respectively) can be explained by the dose-response results reported for the occupancy of [ $^{18}\text{F}$ ]MNI-444-labeled binding sites by tozadenant (47% vs. 95% at 1.5 mg/kg and 10.5 mg/kg, respectively; [24]). The observed difference in the maximum striatum-to-cerebellum ratio between mice (8.3) and piglet (2.1) is regarded to be caused by the different  $A_{2A}$  receptor densities in the striatum, which were autoradiographically determined to be twofold higher in mice than in pigs [25].

By taking into consideration the density of the  $A_{2A}$  receptor in the human striatum under physiologic conditions (e.g., 260–444 fmol/mg protein [26, 27]), we assume that the binding potential of [ $^{18}\text{F}$ ]FLUDA is suitable to quantify the receptor availability in humans. The highly specific binding of [ $^{18}\text{F}$ ]FLUDA towards the  $A_{2A}$  receptor was confirmed by blocking studies conducted in vitro (Figs. 4 and 5) and in vivo (Figs. 6 and 7). The presence of  $A_{2A}$  receptor antagonists inhibited the accumulation of activity in striatum almost completely but did not affect any other brain region. Accordingly, from the herein presented exploratory study, we assume that similar to the already clinically applied non-deuterated [ $^{18}\text{F}$ ]FESCH or [ $^{11}\text{C}$ ]preladenant, the quantification and kinetic modeling of PET data obtained with [ $^{18}\text{F}$ ]FLUDA might be facilitated by the use of the cerebellum as a reference region [21, 28]. However, full kinetic modeling with arterial input functions, ideally performed in a large animal and under conditions comparable to human studies, is required to validate the use of such region for the quantification of the availability of the  $A_{2A}$  receptor by [ $^{18}\text{F}$ ]FLUDA PET.

## Conclusion

[ $^{18}\text{F}$ ]FLUDA is a new  $A_{2A}$  receptor-targeting PET radiotracer with promising preclinical results for clinical translation. The radiotracer can be prepared with high molar activities and in reasonable radiochemical yield (manuscript reporting automated synthesis under preparation). Highly affine and specific binding of [ $^{18}\text{F}$ ]FLUDA in vivo was demonstrated by PET studies performed in different species. The selective deuteration resulted in a high metabolic stability, and the identification of cerebellum as a reference region is assumed to facilitate the quantification of the availability of the  $A_{2A}$  receptor in the brain and disease-related changes thereof in clinical routine. Furthermore, the results of a toxicity and a dosimetry study [29] indicate that the use of [ $^{18}\text{F}$ ]FLUDA in first-in-human imaging studies is safe.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00259-020-05164-4>.



**Acknowledgments** We thank all colleagues of the Institute of Analytical Chemistry, Department of Chemistry and Mineralogy of Universität Leipzig (Leipzig; Germany), for NMR and HRMS measurements; Karsten Franke, Helmholtz-Zentrum Dresden-Rossendorf (HZDR), for providing fluorine-18; Tina Spalholz, HZDR, for technical assistance; and Tatjana Sattler and all colleagues of the Department of Veterinary Medicine and Nuclear Medicine of University Hospital Leipzig (Leipzig; Germany) for the support of pig studies.

**Authors' contributions** All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Thu Hang Lai, Magali Toussaint, Rodrigo Teodoro, Sladjana Dukić-Stefanović, Friedrich-Alexander Ludwig, Barbara Wenzel, Daniel Gündel, Bernhard Sattler, Susann Schröder, and Winnie Deuther-Conrad. The first draft of the manuscript was written by Thu Hang Lai, Magali Toussaint, Winnie Deuther-Conrad, and Peter Brust, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Funding** Open Access funding enabled and organized by Projekt DEAL. This work (project no. 100226753) was funded by the European Regional Development Fund (ERDF) and Sächsische Aufbaubank (SAB).

**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** All animal studies followed the international guidelines of animal care and were approved by Landesdirektion Leipzig (Reg.-Nr.: TVV 18/18; Reference number DD24.1-5131/446/19).

**Consent to participate** Not applicable

**Consent for publication** Not applicable

**Code availability** Not applicable

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

## References

- Cunha RA. How does adenosine control neuronal dysfunction and neurodegeneration? *J Neurochem*. 2016;139:1019–55. <https://doi.org/10.1111/jnc.13724>.
- Borrito-Escuela DO, Fuxe K. Adenosine heteroreceptor complexes in the basal ganglia are implicated in Parkinson's disease and its treatment. *J Neural Transm (Vienna)*. 2019;126:455–71. <https://doi.org/10.1007/s00702-019-01969-2>.
- Domenici MR, Ferrante A, Martire A, Chiodi V, Pepponi R, Tebano MT, et al. Adenosine A<sub>2A</sub> receptor as potential therapeutic target in neuropsychiatric disorders. *Pharmacol Res*. 2019;147:104338. <https://doi.org/10.1016/j.phrs.2019.104338>.
- Fredholm BB, Chen JF, Masino SA, Vaugois JM. Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. *Annu Rev Pharmacol Toxicol*. 2005;45:385–412. <https://doi.org/10.1146/annurev.pharmtox.45.120403.095731>.
- Hurley MJ, Mash DC, Jenner P. Adenosine A<sub>2A</sub> receptor mRNA expression in Parkinson's disease. *Neurosci Lett*. 2000;291:54–8. [https://doi.org/10.1016/S0304-3940\(00\)01371-9](https://doi.org/10.1016/S0304-3940(00)01371-9).
- Ramlackhansingh AF, Bose SK, Ahmed I, Turkheimer FE, Pavese N, Brooks DJ. Adenosine 2A receptor availability in dyskinetic and nondyskinetic patients with Parkinson disease. *Neurology*. 2011;76:1811–6. <https://doi.org/10.1212/WNL.0b013e31821ccce4>.
- Villar-Menéndez I, Porta S, Buira SP, Pereira-Veiga T, Díaz-Sánchez S, Albasanz JL, et al. Increased striatal adenosine A<sub>2A</sub> receptor levels is an early event in Parkinson's disease-related pathology and it is potentially regulated by miR-34b. *Neurobiol Dis*. 2014;69:206–14. <https://doi.org/10.1016/j.nbd.2014.05.030>.
- Varani K, Bachoud-Lévi AC, Mariotti C, Tarditi A, Abbracchio MP, Gasperi V, et al. Biological abnormalities of peripheral A<sub>2A</sub> receptors in a large representation of polyglutamine disorders and Huntington's disease stages. *Neurobiol Dis*. 2007;27:36–43. <https://doi.org/10.1016/j.nbd.2007.03.011>.
- Xu K, Bastia E, Schwarzschild M. Therapeutic potential of adenosine A<sub>2A</sub> receptor antagonists in Parkinson's disease. *Pharmacol Ther*. 2005;105:267–310. <https://doi.org/10.1016/j.pharmthera.2004.10.007>.
- Ishiwata K, Noguchi J, Toyama H, Sakiyama Y, Koike N, Ishii S, et al. Synthesis and preliminary evaluation of [<sup>11</sup>C]KF17837, a selective adenosine A<sub>2A</sub> antagonist. *Appl Radiat Isot*. 1996;47:507–11. [https://doi.org/10.1016/0969-8043\(95\)00295-2](https://doi.org/10.1016/0969-8043(95)00295-2).
- Márián T, Boros I, Lengyel Z, Balkay L, Horváth G, Emri M, et al. Preparation and primary evaluation of [<sup>11</sup>C]CSC as a possible tracer for mapping adenosine A<sub>2A</sub> receptors by PET. *Appl Radiat Isot*. 1999;50:887–93. [https://doi.org/10.1016/S0969-8043\(98\)00162-6](https://doi.org/10.1016/S0969-8043(98)00162-6).
- Wang WF, Ishiwata K, Nonaka H, Ishii S, Kiyosawa M, Shimada J, et al. Carbon-11-labeled KF21213: a highly selective ligand for mapping CNS adenosine A<sub>2A</sub> receptors with positron emission tomography. *Nucl Med Biol*. 2000;27:541–6. [https://doi.org/10.1016/S0969-8051\(00\)00126-8](https://doi.org/10.1016/S0969-8051(00)00126-8).
- Todde S, Moresco RM, Simonelli P, Baraldi PG, Cacciari B, Spalluto G, et al. Design, radiosynthesis, and biodistribution of a new potent and selective ligand for in vivo imaging of the adenosine A<sub>2A</sub> receptor system using positron emission tomography. *J Med Chem*. 2000;43:4359–62. <https://doi.org/10.1021/jm0009843>.
- Mishina M, Ishiwata K, Kimura Y, Naganawa M, Oda K, Kobayashi S, et al. Evaluation of distribution of adenosine A<sub>2A</sub> receptors in normal human brain measured with [<sup>11</sup>C]TMSX PET. *Synapse*. 2007;61:778–84. <https://doi.org/10.1002/syn.20423>.
- Brooks DJ, Papapetropoulos S, Vandenheide F, Tomic D, He P, Coppel A, et al. An open-label, positron emission tomography study to assess adenosine A<sub>2A</sub> brain receptor occupancy of vipadenant (BIIB014) at steady-state levels in healthy male volunteers. *Clin Neuropharmacol*. 2010;33:55–60. <https://doi.org/10.1097/WNF.0b013e3181d137d2>.
- Bhattacharjee AK, Lang L, Jacobson O, Shinkre B, Ma Y, Niu G, et al. Striatal adenosine A<sub>2A</sub> receptor-mediated positron emission

- tomographic imaging in 6-hydroxydopamine-lesioned rats using [ $^{18}\text{F}$ ]-MRS5425. *Nucl Med Biol.* 2011;38:897–906. <https://doi.org/10.1016/j.nucmedbio.2011.01.009>.
17. Khanapur S, Paul S, Shah A, Vatakuti S, Koole MJ, Zijlma R, et al. Development of [ $^{18}\text{F}$ ]-labeled pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH442416) analogs for the imaging of cerebral adenosine  $\text{A}_{2\text{A}}$  receptors with positron emission tomography. *J Med Chem.* 2014;57:6765–80. <https://doi.org/10.1021/jm500700y>.
  18. Barret O, Hannestad J, Vala C, Alagille D, Tavares A, Laruelle M, et al. Characterization in humans of  $^{18}\text{F}$ -MNI-444, a PET radiotracer for brain adenosine 2A receptors. *J Nucl Med.* 2015;56:586–91. <https://doi.org/10.2967/jnumed.114.152546>.
  19. Schröder S, Lai TH, Toussaint M, Kranz M, Chovsepian A, Shang Q, et al. PET imaging of the adenosine  $\text{A}_{2\text{A}}$  receptor in the rotenone-based mouse model of Parkinson's disease with [ $^{18}\text{F}$ ]FESCH - synthesized by a simplified two-step one-pot radiolabeling strategy. *Molecules.* 2020;25. <https://doi.org/10.3390/molecules25071633>.
  20. Harvey M. The GraphPad guide to analyzing radioligand binding data. Copyright© 1995-96 by GraphPad Software. <http://www3.uah.es/farmamol/Public/GraphPad/radiolig.htm>. Accessed 17 Aug 2020.
  21. Khanapur S, Van Waarde A, Dierckx RAJO, Elsinga PH, Koole MJB. Preclinical evaluation and quantification of  $^{18}\text{F}$ -fluoroethyl and  $^{18}\text{F}$ -fluoropropyl analogs of SCH442416 as radioligands for PET imaging of the adenosine  $\text{A}_{2\text{A}}$  receptor in rat brain. *J Nucl Med.* 2017;58:466–72. <https://doi.org/10.2967/jnumed.116.178103>.
  22. Alnouri MW, Jepards S, Casari A, Schiedel AC, Hinz S, Müller CE. Selectivity is species-dependent: characterization of standard agonists and antagonists at human, rat, and mouse adenosine receptors. *Purinergic Signal.* 2015;11:389–407. <https://doi.org/10.1007/s11302-015-9460-9>.
  23. Zhou X, Boellaard R, Ishiwata K, Sakata M, Dierckx R, de Jong JR, et al. In vivo evaluation of  $^{11}\text{C}$ -Preladenant for PET imaging of adenosine  $\text{A}_{2\text{A}}$  receptors in the conscious monkey. *J Nucl Med.* 2017;58:762–7. <https://doi.org/10.2967/jnumed.116.182410>.
  24. Barret O, Hannestad J, Alagille D, Vala C, Tavares A, Papin C, et al. Adenosine 2A receptor occupancy by tozadenant and preladenant in rhesus monkeys. *J Nucl Med.* 2014;55:1712–8. <https://doi.org/10.2967/jnumed.114.142067>.
  25. Sihver W, Schulze A, Wutz W, Stusgen S, Olsson RA, Bier D, et al. Autoradiographic comparison of in vitro binding characteristics of various tritiated adenosine  $\text{A}_{2\text{A}}$  receptor ligands in rat, mouse and pig brain and first ex vivo results. *Eur J Pharmacol.* 2009;616:107–14. <https://doi.org/10.1016/j.ejphar.2009.06.025>.
  26. Albasanz JL, Rodríguez A, Ferrer I, Martín M. Adenosine  $\text{A}_{2\text{A}}$  receptors are up-regulated in Pick's disease frontal cortex. *Brain Pathol.* 2006;16:249–55. <https://doi.org/10.1111/j.1750-3639.2006.00026.x>.
  27. Wan W, Sutherland GR, Geiger JD. Binding of the adenosine  $\text{A}_2$  receptor ligand [ $^3\text{H}$ ]CGS 21680 to human and rat brain: evidence for multiple affinity sites. *J Neurochem.* 1990;55:1763–71. <https://doi.org/10.1111/j.1471-4159.1990.tb04967.x>.
  28. Sakata M, Ishibashi K, Imai M, Wagatsuma K, Ishii K, Zhou X, et al. Initial evaluation of an adenosine  $\text{A}_{2\text{A}}$  receptor ligand,  $^{11}\text{C}$ -preladenant, in healthy human subjects. *J Nucl Med.* 2017;58:1464–70. <https://doi.org/10.2967/jnumed.116.188474>.
  29. Sattler B, Kranz M, Lai TH, Gündel D, Toussaint M, Schröder S, et al. Preclinical incorporation dosimetry of [ $^{18}\text{F}$ ]FLUDA - a novel  $^{18}\text{F}$ -labeled tracer for PET imaging of the expression of the adenosine  $\text{A}_{2\text{A}}$  receptor ( $\text{A}_{2\text{A}}\text{R}$ ). *J Nucl Med.* 2020;61:1014.

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.