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Imaging of the muscarinic acetylcholine neuroreceptor in rats with the M2 selective agonist [¹⁸F]FP-TZTP

L. Ravasi^{1,2}, J. Tokugawa³, T. Nakayama⁵, J Seidel⁶, L. Sokoloff⁵, W.C. Eckelman^{1,4}, and D.O. Kiesewetter^{1,3}

¹PET Dept, CC/NIH, Bethesda, MD, U.S.A

²Service de Médecine Nucléaire et Imagerie fonctionnelle, Hôpital Roger Salengro CHRU Lille, EA 1046

³Laboratory of Molecular Imaging and Nanomedicine, NIBIB, National Institutes of Health, Bethesda, MD, U.S.A

⁴Molecular Tracer LLC, Bethesda MD, U.S.A

⁵LCM, NIMH/NIH, Bethesda, MD, U.S.A

⁶Molecular Imaging Program, CCR, NCI, National Institutes of Health, Bethesda, MD, U.S.A

Abstract

Introduction—[¹⁸F]FP-TZTP is a M2 muscarinic subtype selective receptor-binding radiotracer used *in vivo* to image human and non-human primate brain following both bolus injection and infusion. In order to carry out repeated studies in rodents, the techniques developed for primates must be transferred to rodents with the same precision. This includes obtaining a metabolite corrected input function.

Methods—We compared bolus injection with constant infusion in rats that were awake or under isoflurane anesthesia. Brain-plasma and brain-blood distribution ratios were calculated by dividing brain ¹⁸F concentrations, determined *in vivo* by PET imaging with the ATLAS (Advanced Technology Laboratory Animal Scanner), *ex vivo* by direct counting in excised brain tissue, or quantitative autoradiography, by the plasma or whole blood concentrations that had been corrected for metabolite contents.

Results—Blood volume constraints prevented adequate blood sampling to define a precise input function after bolus injection thus preventing full kinetic analysis. Constant infusion, however, required fewer blood samples to define the input function allowing calculation of distribution ratios, but complete equilibrium between plasma and tissue had not yet been reached after 120 min.

Conclusion—Our results showed that the blood clearance and metabolism was too rapid to obtain a reproducible input function after bolus injection. The equilibrium distribution ratios did not lead to precise biochemical parameters but the constant infusion was more suitable in that distribution ratios between tissue and plasma were statistically more precise. Constant infusion is the better approach for studying [¹⁸F]FP-TZTP by small animal imaging.

Corresponding author: Dale O Kiesewetter, Laboratory of Molecular Imaging and Nanomedicine, NIBIB, National Institutes of Health, Bethesda, MD, U.S.A., dk7k@nih.gov, phone, fax.

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Keywords

Muscarinic receptor; Small animal PET scanner; Distribution ratios; Alzheimer's disease; agonist

Introduction

Quantitative biochemical parameters, including binding potentials, volumes of distribution, and distribution ratios have been extracted from *in vivo* data collected in non-human primates and human subjects by combining external imaging and arterial blood sampling [1]. Serial imaging studies can measure the effect of pharmacologic intervention in the same subject thereby increasing the statistical power. Unfortunately, these same types of studies in small animals have been challenging because of the limited ability to determine the metabolite-corrected input function from arterial sampling coupled with metabolite analysis in these low blood volume species. Thanks to the development of extraction procedures that allow determination of parent concentration with relatively small quantities of blood [2], we evaluated experimental conditions that would enable the calculation of the full kinetic model as well as equilibrium distribution ratios in rats for the M2 muscarinic agonist 3-(3-(3-[¹⁸F]fluoropropyl)thio)-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine ([¹⁸F]FP-TZTP).

Previously, we demonstrated that biochemical parameters could be extracted using the Advanced Technology Laboratory Animal Scanner, ATLAS [3], and [¹⁸F]FDG [4]. FDG metabolism rates obtained from time activity curves (TAC) in rats under isoflurane anesthesia agreed with previous studies using ¹⁴C-deoxyglucose in isoflurane-anesthetized rats using either the Patlak analysis or compartmental analysis. In addition, ratios of mBq/mL obtained from the image and mBq/mL obtained from tissue sampling in the same animal also agreed, which validated the imaging procedure. The input function from plasma was determined by collecting blood samples over time. It was not necessary to analyze the blood for metabolites because metabolites of FDG are not present in plasma.

[¹⁸F]FP-TZTP is a selective radioligand for the M2 muscarinic receptor, as proven through *ex vivo* autoradiography in previous studies with knock-out mice [5]. The muscarinic neurosystem is involved in many diseases such as Alzheimer's disease [6] and depression [7,8]. In particular with regards to Alzheimer's disease, it is crucial to identify *in vivo* methodologies suitable to study the pharmaceutical effects of potential symptomatic drugs, so-called "disease-modifier", in preclinical studies.

The objective of this study was to develop a methodology applicable to rats to measure [¹⁸F]FP-TZTP kinetics in the brain as an index of receptor binding. We used ATLAS to perform *in vivo* and *ex vivo* scans in rats which had received an intravenous injection of [¹⁸F]FP-TZTP. We also investigated the effect of isoflurane on the distribution ratios of [¹⁸F]FP-TZTP since all *in vivo* dynamic scans require the rodents to be anesthetized. Since [¹⁸F]FP-TZTP clears the blood rapidly [9] and is rapidly metabolized [10], we performed 1) bolus injections where blood had to be drawn rapidly and often and 2) constant infusions in which fewer and less frequent blood samplings were required.

Methods

Chemicals were obtained from the following sources: halothane was from Halocarbon, and isoflurane (Forane) was from Baxter. Euthanasia solution was obtained from Schering-Plough Animal Health Corp. (Beuthanasia-D special). [¹⁸F]FP-TZTP was synthesized by a previously reported method [11].

Small Animal PET Scanner

All PET images were obtained with the National Institutes of Health Advanced Technology Laboratory Animal Scanner (ATLAS) small animal PET scanner. ATLAS has an 11.8-cm ring diameter, an 8-cm aperture, a 6-cm effective transverse field of view, and a 2-cm axial field of view. The scanner contains 18 dual-layered phoswich detector modules of Ce-doped $\text{Lu}_{18}\text{Gd}_{0.2}$ (7 mm) and GSO (Ce-doped Gd_2SiO_5) (8 mm) (Hitachi). This phoswich design allows depth-of-interaction (DOI) detection while preserving a sensitivity of 2.7% for a window of 100–650 keV. Images were reconstructed with a 3-dimensional ordered-subset expectation maximization (3D OSEM) reconstruction algorithm that includes exact positioning of the lines of response and a model of system resolution [12]. Spatial resolution in the reconstructed images was 1.5 mm at full width at half-maximum (FWHM) when 16 subsets were used and ten iterations taken. The reconstructed voxel size was $0.56 \times 0.56 \times 1.125$ mm. Image data was not corrected for attenuation or scatter. Coronal, sagittal and axial section images were created for subsequent data analysis. For scanner activity calibration, a cylinder 3.1 cm in diameter by 7.5-cm long was used to simulate the rat head and upper torso. This cylinder was filled with ^{18}F in water, centered in the scanner aperture, and imaged. A dose calibrator determined the radioactivity in the cylinder, and this value was divided by the volume of the cylinder to give concentration. Given these data, a calibration factor (C) was determined that related image counts to tracer concentration in the animal:

$$C = (\text{phantom kBq/ml}) / (\text{cps in voxel} / \text{voxel volume in image})$$

Concentrations can then be calculated for any new image by multiplying the counts per second (cps) in a given voxel of that image by the calibration factor C. This calibration factor C was determined periodically and did not vary considerably with time.

Image based tracer concentrations were then calculated by multiplying the counts per second (cps) in a given image voxel by the calibration factor C.

Animal Preparation

Normal adult male Sprague-Dawley rats were purchased from Charles River Laboratories and maintained in a climate-controlled room on a 12-h light: dark cycle with food and water available ad libitum. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Clinical Center Animal Care and Use Committee. Animals were anesthetized with halothane (5% induction and 1%–1.5% for maintenance in 30% O_2 /70% N_2O). Polyethylene catheters (PE 50; Clay Adams) were inserted into left and right femoral arteries and the left femoral vein. One arterial catheter was used for continuous monitoring of mean arterial blood pressure (MABP) and the other was used for sampling of arterial blood. The length of the catheter used for blood sampling was fixed precisely at 16 cm to minimize sampling errors due to catheter delay and dispersion. The venous catheter was used for injection of $[^{18}\text{F}]\text{FP-TZTP}$. After the catheterizations, surgical wounds were treated with 5% lidocaine ointment and sutured. Duration of anesthesia during the surgical preparation was usually about 30 min. Throughout the surgical preparations and the experimental periods, body temperature was monitored by a rectal probe and maintained at 37°C by a thermostatically controlled heating lamp (model 73 A; Yellow Springs Instrument Co., Inc.). At least 3 h were allowed for recovery from the surgery and anesthesia before the PET study. During this recovery time, the rats were awake but restrained from movement of the inferior part of the body to prevent any accidental removal of the catheters.

Physiologic Variables

In all animals, MABP was monitored continuously with a Micro-Med Blood Pressure Analyzer (model 300; Micro-Med) that had been calibrated with an air-dampened mercury manometer; arterial blood partial pressure of CO₂ (pCO₂), partial pressure of O₂ (pO₂), and pH were measured with a blood-gas analyzer (model 288 Blood Gas System; Ciba-Corning Diagnostics Corp.). Hematocrit was determined in arterial blood samples centrifuged in a Microfuge B (Beckman Instruments). Arterial plasma glucose concentration was determined with a Beckman Glucose Analyzer 2 (Beckman Instruments). Physiologic variables other than MABP were measured immediately before radiotracer injection and again approximately every 15 min.

PET Study

PET scans were performed in 19 rats. After at least 3-h recovery from catheterization surgery, the rat was reanesthetized with isoflurane (5% for induction and 1.5% for maintenance in 100% O₂ at a flow rate of 1L/min) using a nose cone. After preparation, the rat was placed in a prone position on the platform of the scanner. With the help of a laser alignment device attached to the scanner, the rat was positioned so that the center of the field of view was 12 mm caudal to the line between the lateral edges of the eyes. For the bolus injection studies, [¹⁸F]FP-TZTP (61 ± 7 MBq; 300–600 µL in volume, injected mass was ca. 1 nmol) was administered via the venous catheter over a period of about 30 seconds, and the catheter was immediately flushed with 100 µL of saline from a syringe connected with a custom-made Y-connector.

For the infusion studies, [¹⁸F]FP-TZTP (148±18 MBq; 2 mL in volume, injected mass was ca. 2 nmol) was delivered constantly to the venous catheter at a rate of 60 µL/min for 30 min-studies, at 40 µL/min for 45 min-studies and at 15µL/min for 120 min-studies. Scanning was initiated at the onset of [¹⁸F]FP-TZTP administration (the specific activity at injection time was 71.8 ± 31.3 GBq/µmol). A sequence of scans with varying duration was acquired; total scanning time was 30 min for one group, 45 min for the second group and 120 min for the third group. After the last scan, the rat was euthanized with a bolus venous injection of euthanasia solution.

To visualize the effects of 1.5% isoflurane anesthesia on the uptake and distribution of [¹⁸F]FP-TZTP, we conducted *ex vivo* static scans of the head or directly of the brain, removed from the skull immediately after death, for 15 to 60 min on most of the bolus-injected rats which underwent the scans under isoflurane and all the ones which were awake during uptake. In addition, all infused rats, regardless of the duration of the infusion and their state during uptake underwent an *ex vivo* scan of 15 min starting immediately after death.

Arterial Blood Sampling

To determine the plasma and whole blood time-activity curves, the blood sampling schedule for bolus injection was as follows: 6 continuous samples over increasing intervals (0.5, 0.75, 1, 2, 3, 5, 7.5, 10, 15, 25, 30 min) or 12 over the 1st min and 6 additional samples every 5 minutes. For the infusion studies, when the duration was 30 min, the blood-withdraw scheduling was every 5 minutes with the last sample in triplicates whereas when the study lasted 45 min, the first two samples were withdrawn at a 10 min interval, followed by a 5 min interval after 30 min and a triplicate sampling at 45 minutes. When the studies were 120 min long, the sampling schedule was at 25, 45, 60, 75, 90 and 120 min with a duplicate sample every other time point as well as the last one. Blood samples, approximately 80–100 µL in volume, were withdrawn into polyethylene microcentrifuge tubes coated with heparin-lithium-fluoride (Beckman Instruments, Inc.). Twenty µL of whole blood were used for

metabolite extraction and 10 μL were counted in a model 1480 Gamma Counter; PerkinElmer Life Sciences Inc.). From the remaining whole blood, plasma was separated from red blood cells by centrifugation, and treated similarly to whole blood for counting and metabolite extraction purposes. The remaining plasma was used for the measurement of glucose concentration as an indication of normal physiology [13]. To minimize blood loss, all dead-space volumes of blood collected in the sampling process were reinjected intravenously into the animal.

Direct Tissue Counting and Autoradiography

For the bolus injected studies, immediately after the rat was euthanized or after the 15-min *ex vivo* scan, the brain was removed from the skull and separated into 2 parts, each including one hemisphere and half of the cerebellum and brain stem. One part, further dissected into thalamus, cortex, cerebellum and brain stem, was weighed and counted in a model 1480 Gamma Counter; PerkinElmer Life Sciences Inc.). The remaining part of the brain was counted as one piece. Regional concentration of radioactivity was used for biodistribution purposes. All activities were expressed in MBq/mL, assuming the specific gravity of tissue to be 1.

For the constant infusion studies, one half of the brain was frozen in isopentane maintained at -40°C with dry ice until sectioning. Brains were cut into 20- μm sagittal sections in a cryostat at 22°C . The frozen brain sections were thaw-mounted on glass cover slips, air-dried and exposed to Fuji phosphor imaging plates for a recorded time, about 20 hours. The plates were read with Fuji Film BAS-5000 (FUJI Film Medical Systems USA Inc). Using the Science Lab 2001 Image Gauge Software version 4.0, regions of interest (ROIs) were placed on the digital pictures of the autoradiographic brain slices on hippocampus, amygdala, caudate-putamen, somato-sensory cortex, thalamus, brain stem, cerebellum, inferior and superior colliculi. Radioactive retention was expressed in photo-stimulated luminescence per square mm (psl/mm^2). Psl/mm^2 were converted in MBq/mL by use of a cross-calibration factor that was calculated for each study by drawing an ROI around a known amount of radioactivity dropped on glass cover slip and exposed to the plate simultaneously to the brain sections. The other half brain was weighed and counted in the gamma counter and used for calculation of regional biodistribution.

Analysis of PET Data

Data from the scanner were formatted into 6 to 24 frames according to which administration method was used and the duration of the frames during image acquisition. Counts detected by the scanner were converted into MBq/mL by use of a cross-calibration factor (325.6 scanner cps/mL per MBq/mL) determined by imaging a cylindrical phantom containing a known concentration of our radiotracer. Additionally, for the bolus injection studies, one image containing the sum of all counts collected over the full 30-min interval was constructed for placement of ROIs. One ROI representing the whole brain was also placed on the 17 coronal slices of the summed image. Each slice was 1.125-mm thick and the first coronal slice was 4 ml caudal from the tip of the frontal lobe. The ROIs were then transferred to the image for each time frame of data, and weighted average tissue activity was computed. A background ROI was also drawn outside the brain region. In all cases, the apparent activity in this region reflected the contributions from random coincidence events and from scatter and was found to be very small throughout the course of the studies. Tissue time-activity curves were constructed by subtracting the activity from the background ROI drawn outside the brain from the activity measured in whole brain.

Metabolite extraction: a 2-step procedure

The metabolites of [^{18}F]FP-TZTP in rats have already been described [2] and an extraction procedure for determining parent fraction has also been reported [14]. Briefly, a 20- μL aliquot of whole blood or of plasma (separated from whole blood after centrifugation) was mixed with 200 μL of borate buffer (pH=12.5) and with 500 μL of a solution of 4:1 hexane: ethyl acetate. The mixture was vortexed, centrifuged and the layers separated; the upper organic phase contained the parent compound and a desmethylated metabolite. Twenty μL of acetic anhydride were added to the organic phase followed by 500 μL of 0.1M HCl after a few minutes. The mixture was vortexed and centrifuged. The conversion of the secondary amine of the desmethylated metabolite into an amide allowed isolation of the parent in acidic aqueous media. The activity of this second aqueous phase was divided by the sum of the activities of the 3 phases to determine the percent parent in all plasma and whole blood samples. For accuracy purposes, we also counted a sample of plasma or whole blood and compared the counts to the sum of the counts from the 3 phases. The results did not differ significantly.

Statistical analyses

Unpaired and paired two-tail Student's t tests were used to compare blood radioactive activity and radioactive distribution in cerebral regions under different conditions (awake or under isoflurane). Results were considered significant when $p < 0.005$.

Results

a) Physiological parameters and injected radioactivity doses of bolus and constant infusion methods

The mean arterial blood pressure (MABP), the hematocrit, the arterial gas pressures and the pH of the ten rats that received a bolus injection of approximately 62 MBq of [^{18}F]FP-TZTP and of the fifteen rats that received a constant infusion with approximately 138 MBq of [^{18}F]FP-TZTP were measured at the beginning and at the end of each study (Table 1). Similarly, the plasma glucose levels were measured for all rats except the four that received a constant infusion over 45 min due to a technical problem of the Beckman Glucose analyzer on the day of those studies. The values did not differ from the normal range in which they, respectively, should fall. The lower MABP pressures in the anesthetized rats compared to the awake ones were consistent with the effect of the isoflurane and the higher pO_2 blood gas is a result of the isoflurane being administered to the rats in 100% oxygen. The plasma glucose level of the rats under anesthesia for 120 min is greater than the ones in the 30 min group only because the animals were already slightly hyperglycemic at a base line level. The 120 min studies did not induce such stress to be detected by rising glucose levels.

b) Bolus injection method

After a bolus injection, the percent of [^{18}F]FP-TZTP in whole blood of the awake rats was similar to that of the rats under isoflurane. Only over the first minute were the metabolites slightly greater ($p=0.044$ at 0.5 min; $p=0.041$ at 1 min) in the rats that were awake compared to the anesthetized ones (Table 2). Only whole blood was analyzed because the combination of rapid clearance and rapid metabolism would have required 12 samples of blood over the first minute to obtain metabolite-corrected plasma and this decreased the hematocrit dramatically. On the contrary, to obtain metabolite-corrected whole blood required about half the blood volume. The tissue activity used to calculate the distribution ratios (i.e. the numerator) was assessed by either biodistribution (Figure 1) or ATLAS small animal PET imaging (Figure 2). Figure 2 illustrates sagittal slices of *ex vivo* scans of two rats as a

comparison of the [^{18}F]FP-TZTP distribution in the rat brain after 30 min of a bolus injected dose of 62 MBq in the animal that was awake versus 46 MBq in the rat under isoflurane anesthesia.

The biodistribution data of [^{18}F]FP-TZTP in the brain regions of the anesthetized and awake rats during radioactive uptake was statistically different in the thalamus, the brain-stem and the cerebellum (unpaired Student's *t* test $p=0.0012$; 0.0004 and 0.0004 , respectively) as opposed to the caudate, the hippocampus and the cortex where the test was not significant ($p>0.1$). The variation of the data retrieved from the rats for regions dissected 60 min after the death of the animal was larger than the variation among animals dissected immediately after death (Figure 1). We used the data with larger coefficients of variation for our calculation of the distribution ratios so we could compare the tissue activity counted by dissection to the one counted by ATLAS scan imaging.

The distribution ratios of [^{18}F]FP-TZTP were calculated by various methods. The gold standard is to use the plasma input function, i.e., the radioactivity that is correlated to the injected parent compound. As previously mentioned, we substituted whole blood for plasma in the bolus studies; therefore, all of the bolus input functions are based on whole blood. The tissue activity normalized by the injected dose of each animal determined by dissection in the awake rats did not differ among the 6 different regions and the mean of these was in good agreement with the radioactivity detected with the ATLAS when the *ex vivo* scan was performed (Table 3). In the anesthetized rats, the same data was also in good agreement between the ATLAS method and dissection, but we noticed a trend that all activities were lower in the rats under isoflurane compared to the animals that were awake. Similarly, we could not detect any regional differences in the distribution ratios in the rats under either condition although the trend of lower values associated with anesthesia was also present. In either state, the variability of the regions identified through autoradiography tended to be greater when the true input function was used as the denominator for the tissue activity compared to the integral of the whole blood activity from injection time to time of euthanasia. However, the variability of the data is reduced by nearly half in the rats that were awake compared to those under isoflurane.

c) Comparisons between plasma and whole blood input function

For the bolus injection method, the amount of blood required to get a complete plasma curve for the peak was greater than what a rat of 250g can lose in such a short period of time (<3 minutes) without physiologic effects. So we substituted the whole blood input function for the plasma input function since the total volume required for whole blood input function is approximately half of the plasma input function volume. However, in the 5 bolus-injected animals used to assess whole blood as a substitution for plasma, we discovered that the relationship between [^{18}F]FP-TZTP parent in plasma and [^{18}F]FP-TZTP parent in whole blood over time was not constant. Similarly, in the infused animals, which required fewer blood samples for both plasma and whole blood input functions to be measured, the data suggest that [^{18}F]FP-TZTP parent in whole blood also does not have a constant relationship with the one in plasma (Figure 3). Therefore, the whole blood input function cannot substitute for the plasma input function for studies with [^{18}F]FP-TZTP.

d) Constant Infusion method

Since fewer samples were drawn in the constant infusion studies, it was possible to obtain metabolite-corrected data from plasma. The percent [^{18}F]FP-TZTP in plasma after infusion was similar whether the rats were awake or under isoflurane (Table 4).

Tissue activity was assessed by the radioactivity detected by the autoradiography technique (regions of interest were placed on hippocampus, amygdala, striatum, cortex, thalamus, brain stem, cerebellum, inferior and superior colliculi) and the ATLAS scans *in vivo* and *ex vivo* (the whole brain was considered as the region of interest). In both methods, a background ROI drawn outside the brain was subtracted from the tissue activities for accuracy. Dissection studies were not performed on rats that received a constant infusion. The distribution ratios of [^{18}F]FP-TZTP were calculated through methods similar to the ones used for the bolus injection studies (Table 5 and Figures 4,5). Distribution ratios calculated by the autoradiographic method at 30 min did not differ whether the rats were awake or under anesthesia in any region but the inferior colliculus ($p=0.0046$; Figure 4). The tissue activity, normalized by the injected dose of each animal detected by the autoradiographic method in the rats under isoflurane that underwent a constant infusion of 30 min, was significantly decreased in the cerebellum compared to the striatum and the cortex ($p=0.0007$ and 0.0048 , respectively). No other statistical difference was detected among the other regions, as well as whether the constant infusion of radioactivity lasted 45 or 120 min. The distribution ratios calculated over the plasma input function were not significantly different among these regions as well (Table 5).

In the analyses performed with the ATLAS data on anaesthetized rats, we calculated the distribution ratios over time in the whole brain considered as one single region (Figure 5). Interestingly, tissue activity and input function were still rising after 30 min of constant infusion, as well as at 45 min. We noticed that they had not reached a plateau even after 120 min of constant infusion. The varying magnitude of the error bars depends of the different number of animals included for each time point (i.e. $n=8$ until 25 min, $n=11$ on time point 27; $n=7$ from 30 to 45 min and $n=3$ for the remaining time points). The magnitude of the standard deviation increases dramatically at late time points due primarily to the decrease in the measured radioactivity, and hence greater error in its measurement in the plasma, due to clearance and decay.

Figure 6 shows the ATLAS images of the same animal which was scanned *in vivo* (left) while receiving a constant infusion of [^{18}F]FP-TZTP for 30 min and *ex vivo* (right). The slices are ca. 6–8 mm from the tip of the brain and the positioning of the animal was identical whether imaged *in vivo* or *ex vivo*.

Discussion

[^{18}F]FP-TZTP is a radiotracer that has *in vivo* affinity for the M_2 muscarinic subtype receptor [5]. To calculate the kinetics of this tracer in rats, we performed *in vivo* and *ex vivo* scans using the ATLAS small animal PET after the animals received a bolus injection of [^{18}F]FP-TZTP. The blood volume needed per sample in order to process the metabolites from the plasma was approximately 80 μl . The blood weighs ca. 8% of the body weight so a rat of 200 g has approximately 16 ml of total blood. It is within the physiological range to withdraw less than 10% of the total blood, which meant for our animals approximately 1.6 ml. Due to the very fast clearance of [^{18}F]FP-TZTP [9], more than two thirds of the samples were withdrawn in the first minute and the hematocrit dropped significantly. Both sampling frequency and total volume drawn affected the physiologic parameters. Therefore, we could not use plasma as the input function in this case. We then used whole blood as the input function, which required half the volume compared to the plasma. Unfortunately, the relationship between [^{18}F]FP-TZTP parent in plasma and [^{18}F]FP-TZTP parent in whole blood was not constant over time.

Since [^{18}F]FP-TZTP is targeting a receptor, which is heterogeneously spread throughout the gray matter it is impossible to use a reference region method to calculate distribution ratios.

Due to blood volume constraints and to the impossibility of substituting the plasma input function with the whole blood input function, the bolus injection method cannot be used to obtain kinetic parameters for [^{18}F]FP-TZTP in rats. Distribution ratios calculated by the autoradiographic method reveal an anesthesia effect only in one region, the inferior colliculus (Figure 4), whereas a more general trend was seen in the bolus injection studies when the distribution ratios were computed based on the whole blood input function (Table 4). Although the difference only became significant in the inferior colliculus, it would mean that the isoflurane has an increasing effect on acetyl choline release that competes with [^{18}F]FP-TZTP at the receptor binding site. This behavior has also been reported by Gomez et al. [15].

In the studies performed with the constant infusion paradigm, the cerebellum tissue activity is significantly smaller than that of the cortex and the striatum after a constant infusion for 30 min under isoflurane, most likely because the cerebellum is the most sensitive region to isoflurane. Variability in the assays will make it difficult to see small differences after pharmacologic intervention and, therefore, the most robust system must be used. In this case, the infusion study produced the more precise results. Other investigators compared the infusion/equilibrium and bolus injection/single time point methods for determining regional binding of (+)-alpha-dihydrotetrabenazine (DTBZ) to the vesicular monoamine transporter of the rat brain [16]. They reported 30–35% variability in the estimates of distribution volume (DV) for separate brain regions using the infusion method. Normalizing these DV to cerebellum values (even if cerebellum is not a non specific binding region) decreased the variability to 10–16%. They also observed larger variability when correcting for metabolites. Kilbourn et al. reported improved statistics based on infusion studies followed by dissection and calculation of distribution ratios [16]. The reproducibility of in vivo specific binding of radiolabeled forms of DTBZ and d-threo-methylphenidate (MPH) in rat brain showed excellent reproducibility (3.62 ± 0.33 ; $n=35$). Striatal binding of MPH to the dopamine transporter (DAT) showed a similar reproducibility over a five-year period. These studies demonstrate that use of a consistent in vivo technique can provide reliable measures of specific binding of radioligands to high affinity sites in the rat brain [17]. Ma et al [18] compared the errors associated with dissection, which has been used for many years, and in vivo imaging studies using a dedicated small animal imager. Treatment with an acetylcholinesterase inhibitor was studied in both awake and isoflurane-anesthetized rats using both ex vivo dissection and in vivo imaging as the analytical tool. Ex vivo dissection showed a statistically significant 10% inhibition of a radiolabeled muscarinic receptor ligand binding in both awake and anesthetized animals, showing no effect of using isoflurane anesthesia. However, small animal imaging in anesthetized animals failed to successfully demonstrate inhibition of radiotracer binding, most likely due to the increased variation [18].

The variability derived from the metabolite-extraction method is an additional factor that leads us to conclude that such extractions are not as reliable in rats as it is in larger animals (monkeys or humans). In fact, in rats, the volumes from which the extractions are made are much smaller because of total blood volume constraints. Since any inaccuracy in pipetting smaller volumes provokes large errors, this leads to greater errors in the percentages of metabolites for rats rather than larger mammals. In addition, Ma et al. [14] observed that the desmethyl metabolite of [^{18}F]FP-TZTP in humans is less than 2% and suggested that it could almost be neglected whereas in rats, the same metabolite represents more than 6 % in the early time points.

Blood sampling and extractions were carried out with our recently developed techniques. Instruments with increased sensitivity are continuing to be developed and could lead to smaller samples and, as a result, fewer physiologic effects after bolus injection of [^{18}F]FP-TZTP (e.g. accelerator mass spectrometry [19] or Laser Resonance Ionization Mass

Spectrometry [20]. Nevertheless, until new instruments enable the retrieval of the full kinetics of [^{18}F]FP-TZTP following bolus-injection, the infusion method that we utilized for this study leads to ratios of volumes of distribution without interfering on physiological parameters. It can therefore be used to study pharmacological intervention on the acetylcholine neuroreceptor system.

A third possible way to inject the radiolabeled tracer could have been the bolus + infusion (B/I) paradigm. However, Carson et al. [10] already discarded such combination in a study with monkeys. In fact, too much variability was found in the cerebral blood flow of the anesthetized animals and since [^{18}F]FP-TZTP extraction is high, delivery is flow-limited so the flow sensitivity leads to great difficulties in finding the infusion parameters to achieve rapid equilibrium for B/I delivery. The authors suggested that the B/I paradigm could be a potential approach in humans where anesthesia could be avoided.

Finally, a recent study from Ichise et al [21] has suggested a way to estimate a normalized distribution volume without the need of arterial data in cases of tracers targeting receptors with no suitable receptor free reference region. Such a parameter could serve to calculate differences in receptor binding in different regions. It would be interesting to compare the results obtained with this method to those obtained from arterial input function and tissue TACs in small animals.

Conclusion

In rats, when 1) a tracer is binding to a neuroreceptor which is located throughout the brain (i.e. there is no specific-binding-free region), and 2) the tracer is metabolizing and clearing very fast, the bolus injection method is not a suitable method to calculate distribution ratios because it does not allow the necessary measurements for the full kinetic model.

The constant infusion method is a better paradigm to obtain the distribution ratios as long as tissue/plasma equilibrium can be reached in a reasonable amount of time before labeled-metabolites can perfuse the blood-brain-barrier. At present, studies in rats using [^{18}F]FP-TZTP should be carried out by infusing the radiotracer and determining the distribution ratio using a PET scan and metabolite-corrected plasma.

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Figure 1. [¹⁸F]FP-TZTP biodistribution data after bolus injection, obtained by dissection and γ -counting either right after or 60 min after death, expressed as percentage of injected dose per regional body weight (%ID/g), in awake and under isoflurane anesthesia (ISO) rats.

Figure 2. $[^{18}\text{F}]$ FP-TZTP distribution in the rat brain viewed sagittally through ATLAS ex-vivo 15-min scans of conscious (A) and anesthetized rat (B) 30 minutes after a bolus injection of $[^{18}\text{F}]$ FP-TZTP (respectively 62 and 46 MBq).

Figure 3.

Plasma and whole blood (WB) input function from 0 to 30 min in awake and under isoflurane rats while ca. 140 MBq of [^{18}F]FP-TZTP is infused at 60 $\mu\text{L}/\text{min}$. Neither plasma nor whole blood was corrected for metabolites but both were normalized by injected dose.

Distribution ratios of [^{18}F]FP-TZTP: tissue activity from autoradiography over metabolite corrected plasma input function at 30 min

Figure 4. Effect of isoflurane anesthesia (ISO) on [^{18}F]FP-TZTP distribution ratios in rat brain calculated by the autoradiographic method after the rats received a constant infusion of ca. 140 MBq of [^{18}F]FP-TZTP for 30 min.

Figure 5.

In vivo distribution ratios of $[^{18}\text{F}]$ FP-TZTP in whole brain defined on the ATLAS images over the metabolite corrected radioactivity in plasma over 120 min. Error bars (negative direction only displayed for clarity) represent the standard deviation of the averaged distribution ratios of $n=8$ (■), $n=11$ (●), $n=7$ (◆) and $n=3$ (—).

Figure 6. $[^{18}\text{F}]$ FP-TZTP distribution illustrated by an ATLAS in vivo brain scan of an anesthetized rat from 25 to 30 min (left) and the summed image of 15 min of the ex-vivo scan (right). The coronal slices shown are ca. 6–8 mm from the tip of the brain

Table 1

Physiological Parameters

Injected radioactivity and physiological parameters of rats that received a bolus injection of ca. 62 MBq of [¹⁸F]FP-TZTP while awake or under isoflurane anesthesia (ISO) for 30 min and of rats which underwent a constant infusion of ca. 138 MBq of [¹⁸F]FP-TZTP awake for 30 min or under isoflurane anesthesia (ISO) for 30, 45 or 120 min.

Parameters	Bolus injection method		Constant infusion method			
	Awake mean ± STD n=5	ISO mean ± STD n=5	Awake for 30 min mean ± STD n=4	ISO for 30 min mean ± STD n=4	ISO for 45 min mean ± STD n=4	ISO for 120 min mean ± STD n=3
Injected activity (MBq)	64 ± 7.4	61 ± 3.7	132 ± 11	147 ± 15	136 ± 30	140 ± 15
Weight (g)	205 ± 28	214 ± 38	311 ± 25	287 ± 9	327 ± 31	353 ± 31
Body temperature (°C)	36.7 ± 0.3 ^a	36.5 ± 1.0 ^a	37.2 ± 0.4 ^a	36.6 ± 0.5 ^a	36.9 ± 0.1 ^a	36.6 ± 0.4 ^a
	36.6 ± 0.4 ^b	36.1 ± 0.1 ^b	37.2 ± 0.4 ^b	37.1 ± 1.1 ^b	36.8 ± 0.4 ^b	36.8 ± 0.1 ^b
Mean Arterial Blood Pressure (mmHg)	164 ± 8 ^a	88 ± 8 ^a	110 ± 9 ^a	81 ± 4 ^a	99 ± 11 ^a	99 ± 3 ^a
	159 ± 9 ^b	86 ± 15 ^b	107 ± 9 ^b	76 ± 3 ^b	99 ± 11 ^b	95 ± 1 ^b
Hematocrit	0.43 ± 0.04 ^a	0.43 ± 0.04 ^a	0.5 ± 0.01 ^a	0.46 ± 0.04 ^a	0.47 ± 0.01 ^a	0.47 ± 0.05 ^a
	0.38 ± 0.06 ^b	0.39 ± 0.01 ^b	0.7 ± 0.05 ^b	0.43 ± 0.03 ^b	0.43 ± 0.01 ^b	0.41 ± 0.05 ^b
Plasma glucose	84 ± 10 ^a	82 ± 2 ^a	100 ± 22 ^a	123 ± 28 ^a	Not measured	172 ± 12 ^a
	91 ± 19 ^b	84 ± 8 ^b	93 ± 17	123 ± 29		188 ± 7
pCO ₂ (mmHg)	42 ± 4 ^a	53 ± 10 ^a	35 ± 2 ^a	50 ± 3 ^a	64 ± 7 ^a	49 ± 15 ^a
	41 ± 3 ^b	54 ± 6 ^b	33 ± 2 ^b	48 ± 2 ^b	61 ± 5 ^b	56 ± 9 ^b
pO ₂ (mmHg)	76 ± 3 ^a	160 ± 17 ^a	78 ± 4 ^a	213 ± 19 ^a	186 ± 43 ^a	180 ± 3 ^a
	78 ± 5 ^b	145 ± 19 ^b	83 ± 7	221 ± 24	237 ± 10	256 ± 14
pH	7.33 ± 0.2 ^a	7.21 ± 0.7 ^a	7.41 ± 0.2 ^a	7.29 ± 0.2 ^a	7.26 ± 0.01 ^a	7.35 ± 0.09 ^a
	7.32 ± 0.02 ^b	7.20 ± 0.05 ^b	7.41 ± 0.04 ^b	7.26 ± 0.03 ^b	7.24 ± 0.01 ^b	7.28 ± 0.05 ^b

^a at injection time,

b at end of study

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Table 2
[¹⁸F]FP-TZTP as a percentage of total radioactivity in whole blood after a bolus injection
in ISO and Awake rats

Percent of [¹⁸F]FP-TZTP over total radioactivity in whole blood as a function of time after bolus injection in awake and under isoflurane anesthesia (ISO) rats.

Time (min)	ISO rats mean ± STD, n=5	Awake rats mean ± STD, n=5
0.5	*83.39 ± 4.47 %	*76.08 ± 5.20 %
1	*53.39 ± 8.34 %	*40.81 ± 8.03 %
2	27.14 ± 6.92 %	26.08 ± 7.94 %
5	13.95 ± 2.16 %	14.47 ± 3.23 %
10	7.84 ± 1.18 %	9.18 ± 1.85 %
15	6.84 ± 0.91 %	7.05 ± 0.92 %
20	5.08 ± 1.13 %	5.74 ± 1.03 %
25	3.94 ± 0.54 %	4.73 ± 0.91 %
30	3.71 ± 0.72 %	3.79 ± 0.86%

* p<0.05 of unpaired Student's t test

Table 3
Distribution Ratios in Awake and ISO rats after bolus injection of [¹⁸F]FP-TZTP

Mean and percent coefficient of variation of ratios of [¹⁸F]FP-TZTP tissue activities (TA) - obtained from autoradiographic studies (discrete regions) and from ATLAS scans (whole brain) - over the respective injected dose, the total whole blood (WB) activity at killing time, the metabolite corrected whole blood activity at killing time or the integral whole blood activity since injection time to killing time, in rats awake (n=5) and under isoflurane anesthesia (ISO) (n=4).

Awake rats	¹ Normalized tissue activity (mL ⁻¹)	² Distribution ratios (mL/mg)	³ Distribution ratios (mL/mg)
Brain regions	Mean ± STD; % coefficient of variation (n=5)		
⁴ whole brain (first 10 min of ex-vivo scan)	7.204 ± 1.474; 20%	101 ± 21; 21%	0.50 ± 0.12; 24%
Caudate	9.734 ± 1.668; 17%	140 ± 45; 33%	0.68 ± 0.13; 19%
Hippocampus	8.454 ± 1.616; 19%	120 ± 30; 25%	0.59 ± 0.10; 18%
Thalamus	9.489 ± 2.024; 21%	135 ± 41; 31%	0.65 ± 0.10; 16%
Brain stem	9.517 ± 1.596; 17%	136 ± 34; 26%	0.66 ± 0.12; 18%
Cerebellum	8.006 ± 1.549; 19%	114 ± 30; 26%	0.56 ± 0.11; 20%
Cortex	10.618 ± 2.096; 20%	152 ± 42; 28%	0.74 ± 0.18; 24%
Mean from regions obtained by autoradiography	9.007 ± 1.981; 22%	129 ± 36; 38%	0.6 ± 0.13; 23%

ISO rats	¹ Normalized tissue activity (mL ⁻¹)	² Distribution ratios (mL/mg)	³ Distribution ratios (mL/mg)
Brain regions	mean; % coefficient of variation (n=4)		
⁴ whole brain (last 5 min of alive scan)	6.884 ± 1.622; 24%	98 ± 66; 68%	0.33 ± 0.018; 5%
⁴ whole brain (first 10 min of ex-vivo scan)	5.238 ± 1.436; 27%	75 ± 49; 67%	0.26 ± 0.054; 21%
Caudate	8.329 ± 1.289; 15%	119 ± 72; 61%	0.42 ± 0.084; 20%
Hippocampus	7.759 ± 1.682; 22%	102 ± 34; 34%	0.40 ± 0.166; 41%
Thalamus	5.287 ± 1.484; 26%	71 ± 31; 44%	0.28 ± 0.146; 52%
brain stem	5.542 ± 0.793; 46%	79 ± 44; 56%	0.28 ± 0.102; 36%
Cerebellum	4.678 ± 0.621; 36%	65 ± 30; 47%	0.24 ± 0.085; 35%
Cortex	10.352 ± 0.932; 48%	148 ± 83; 57%	0.52 ± 0.140; 27%
Mean from regions obtained by autoradiography	6.834 ± 1.503; 22%	96 ± 53; 56%	0.35 ± 0.13; 37%

¹ Tissue activity, decay corrected at time of injection (kBq/mL) divided by injected dose (kBq).

² Distribution ratios are calculated as tissue activity (kBq/mg) over metabolite-corrected whole blood input function (kBq/mL).

³ Distribution ratios are calculated as tissue activity (kBq/mg) over integral from 0 to 27.5 min of whole blood [¹⁸F]FP-TZTP (kBq/mL).

⁴ data from ATLAS imaging.

Table 4
[¹⁸F]FP-TZTP as % of total radioactivity in plasma after constant infusion in isoflurane anesthetized (ISO) rats and awake rats

Percent [¹⁸F]FP-TZTP in plasma as a function of time over 30 min while rats are receiving a constant infusion of ca.132 MBq and are awake (AWAKE) or ca. 147 MBq and are under isoflurane (ISO).

Time (min)	ISO rats mean ± STD, n=5	Awake rats mean ± STD, n=4
5	39.94 ± 3.28%	40.13 ± 4.38%
10	28.40 ± 2.50%	29.96 ± 2.23%
15	23.05 ± 2.28%	21.44 ± 1.06%
20	19.17 ± 4.53%	18.97 ± 1.99%
25	15.88 ± 4.56%	15.85 ± 1.21%
30	12.65 ± 4.39%	13.90 ± 1.03%

Table 5
[¹⁸F]FP-TZTP in brain of isoflurane-anesthetized rats at times after start of infusion (mean ± STD; coefficient of variation)

Mean, STD and percent coefficient of variation of ratios of [¹⁸F]FP-TZTP tissue activities (TA) in the regions of interest identified by autoradiography technique over the respective injected dose (left) and the metabolite corrected plasma activity (right) at killing time (30, 45 and 120 min) in 11 rats under isoflurane anesthesia.

Brain regions	At 30 min			At 45 min			At 120 min		
	<i>I</i> Normalized tissue activity (mL ⁻¹)	² Distribution ratios (mL/mg)		<i>I</i> Normalized tissue activity (mL ⁻¹)	² Distribution ratios (mL/mg)		<i>I</i> Normalized tissue activity (mL ⁻¹)	² Distribution ratios (mL/mg)	
Hippocampus	7.805 ± 1.334; 17%	26.2 ± 6.6; 25%	(n=4)	5.192 ± 2.027; 39%	26.5 ± 9.3; 35%	(n=3)	3.277 ± 0.551; 17%	48 ± 7.6; 16%	
Amygdala	8.239 ± 0.950; 12%	27.1 ± 5.4; 20%		4.994 ± 1.807; 36%	25.1 ± 7.7; 31%		2.959 ± 0.201; 7%	44 ± 6.1; 14%	
Striatum	8.930 ± 0.814; 9%	29.6 ± 4.4; 15%		6.180 ± 2.368; 38%	31.3 ± 10.4; 33%		3.614 ± 0.520; 14%	54 ± 10.4; 19%	
Cortex	8.358 ± 1.006; 12%	28.2 ± 4.5; 16%		5.846 ± 2.000; 34%	30.1 ± 9.4; 31%		3.649 ± .689; 19%	54 ± 11.5; 21%	
Thalamus	7.013 ± 1.207; 17%	23.2 ± 4.9; 21%		4.420 ± 1.992; 45%	22.3 ± 8.9; 40%		2.664 ± 0.806; 30%	40 ± 13.4; 34%	
Brain Stem	9.204 ± 1.575; 17%	30.6 ± 6.9; 22%		5.380 ± 2.359; 44%	27.2 ± 10.6; 39%		3.351 ± 0.546; 16%	50 ± 10.3; 21%	
Cerebellum	5.856 ± 0.641; 11%	19.5 ± 3.5; 18%		3.373 ± 1.427; 42%	17.1 ± 6.4; 37%		2.068 ± 0.250; 12%	31 ± 4.3; 14%	
Inferior colliculus	7.655 ± 1.201; 16%	25.5 ± 5.5; 21%		4.439 ± 1.660; 37%	22.4 ± 7.2; 32%		2.610 ± 0.460; 18%	39 ± 8.1; 21%	
Superior colliculus	8.181 ± 1.130; 14%	26.6 ± 5.0; 19%		4.317 ± 1.608; 37%	22.1 ± 6.9; 31%		2.367 ± 0.523; 22%	35 ± 8.8; 25%	

I Tissue activity, decay corrected at time of injection (kBq/mL) divided by injected dose (kBq).

² Distribution ratios are calculated as tissue activity (kBq/mg) over metabolite-corrected plasma input function (kBq/mL).