CHAPTER 4

EFFECT OF FENFLURAMINE-INDUCED INCREASES IN SEROTONIN RELEASE ON [^{18}F]-MPPF BINDING: A CONTINUOUS INFUSION PET STUDY IN CONSCIOUS MONKEYS

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Abstract

[^{18}F]-MPPF is a selective and reversible antagonist to the serotonin-1A (5-HT_{1A}) receptor. The aim of the present study was to investigate if the binding of [18F]-MPPF is sensitive to increases in 5-HT levels. We used the 5-HT releasing agent and reuptake inhibitor fenfluramine (FEN) to increase the concentration of 5-HT. [18F]-MPPF binding was assessed using positron emission tomography (PET) in conscious monkeys. Possible effects of blood flow on ligand binding were excluded by using a bolus-infusion paradigm. Control scans were obtained to assess the state of ligand equilibrium. FEN (5 mg/kg or 10 mg/kg i.v.) was administered between 90 and 130 minutes after start of the [^{18}F]-MPPF infusion. The binding potential (BP) was calculated for an early interval (30 minutes preceding FEN administration) and late interval (20-50 minutes after administration of FEN). Microdialyses results showed a 20-fold and 35-fold increase in extracellular 5-HT levels in the prefrontal cortex after injection of FEN 5 mg/kg and 10 mg/kg respectively. However, despite these large increases in 5-HT levels, the change in BP did not significantly differ between the control and FEN scans. These results may imply that the majority of 5-HT_{1A} receptors is in the low affinity state, in vivo.

Introduction

The serotonergic system has been implicated in the pathophysiology and treatment of a variety of psychiatric disorders such as depression, anxiety and schizophrenia (Blier and de Montigny 1998; den Boer 2000; Kapur and Remington 2001; Seeman 2002; Tauscher et al. 2001). Using Positron Emission Tomography (PET) and a serotonergic receptor ligand it may be possible to measure changes in serotonin (5-HT) levels in the living brain. The technique is based on the assumption that the injected radiolabeled ligand competes with the endogenous neurotransmitter for the same receptor. Changes in neurotransmitter release can be detected as changes in the binding potential (BP) of the radioligand. This approach has been successfully used for the dopaminergic system (Laruelle 2000). Results from studies using serotonergic ligands, however, do not always agree, possibly due to methodological differences.

At present, only a few studies have investigated the effect of changes in 5-HT levels on serotonergic ligands. These studies have primarily used [^{18}F]-MPPF (4-(2'-methoxyphenyl)-1-[2'-(N-2''-pyridinyl)-p-[^{18}F]fluorobenzamido] ethylpiperazine) and [^{11}C]-WAY-100635 ([carbonyl-^{11}C-][O-methyl-3H]-N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridinyl) cyclohexane carboxamide. Both ligands are reversible antagonists to the 5-HT$_{1A}$ receptor, but differ in their affinity (Ki of 0.8 nM and 3.3 nM for [^{11}C]-WAY-100635 and [^{18}F]-MPPF, respectively) (Zhuang et al. 1994).

Studies in human subjects have shown that changes in 5-HT in the physiological range did not affect ligand binding. After changing 5-HT levels by means of either tryptophan depletion or tryptophan infusion, [^{11}C]-WAY-100635 binding in the prefrontal cortex and medial temporal cortex was not consistently affected (Rabiner et al. 2002). We have studied the effect of tryptophan manipulation on [^{18}F]-MPPF binding and did not find significant differences between the infusion and depletion condition in the same subject (Udo de Haes et al. 2002).

In order to investigate the effect of larger increases in 5-HT levels, several studies have been performed in rats. In most of these experiments, 5-HT levels were increased using the 5-HT releasing agent and reuptake inhibitor fenfluramine (FEN). After administration of FEN at a dose of 10 mg/kg, 10-30 fold increases in extracellular 5-HT levels have been reported (Hume et al. 2001; Maeda et al. 2001; Udo de Haes et al. 2005; Zimmer et al. 2002). Despite these extensive increases in 5-HT levels, the results of the studies were not always consistent. Hume et al. (2001) reported the effect of FEN (10 mg/kg i.p.) on [^{11}C]-WAY-100635 binding. They investigated the effect by means of PET in anesthetized rats and the ex vivo distribution in dissected brain tissues from non-anesthetized rats. The PET results showed a 20% decrease in [^{11}C]-WAY-100635 binding potential in the hippocampus but not in the prefrontal cortex or raphe nucleus. The post mortem dissection studies did not show a statistically significant effect of FEN on [^{11}C]-WAY-100635 uptake, except in the medulla. Using the same radioligand and comparable methods, Maeda et al. (2001) did not find an effect of FEN (10 mg/kg i.p.) in the hippocampus of anesthetized rats. Zimmer et al. (2002) investigated the effect of different doses of FEN on [^{18}F]-MPPF binding in anesthetized rats, using a ß$^{+}$ radiosensitive probe. The authors reported a dose-related decrease of [^{18}F]-MPPF binding in the hippocampus. We studied the effect of FEN (10 mg/kg i.p.) and of a combination of the selective serotonin reuptake inhibitor (SSRI) citalopram (10 µmol/kg, s.c.) with the 5-HT$_{2C}$ antagonist ketanserin (100 nmol/kg, s.c). The effect on [^{18}F]-MPPF binding was assessed in conscious rats using ex-vivo autoradiography. FEN treatment resulted in a significant reduction of [^{18}F]-MPPF binding in the frontal cortex, hypothalamus, amygdala and hippocampus, whereas administration of the combination did not affect [^{18}F]-MPPF binding (Udo de Haes et al. 2005).

The aim of the current study was to investigate if [^{18}F]-MPPF binding in the conscious monkey brain is decreased after a FEN-induced increase in 5-HT levels. We used conscious monkeys since previous studies have shown that ligand binding may be affected by the use of anesthetics (Ginovart et al. 2002; Harada et al. 2004; Hassoun et al. 2003; Momosaki et al. 2004; Seeman and Kapur 2003; Tsukada et al. 2002). So far, all studies have used bolus injections of the ligand. To exclude possible effects of drug-

induced changes in blood flow on ligand binding we used a bolus-infusion paradigm in our study.

Materials and Methods

Animals

Five young-adult male rhesus monkeys (*Macaca mulatta*) (A, B, C, D and E, weighing 6.7 ± 0.9 kg) were used for the PET experiments. The microdialysis experiments were performed with the same group of monkeys (n = 3 per dose). The monkeys were maintained and handled in accordance with the recommendations of the United States National Institutes of Health and the guidelines of the Central Research Laboratory, Hamamatsu Photonics. They were trained to sit on a chair twice a week over a period of more than 3 months. At least 1 month before the PET study, an acrylic plate, with which the monkey could be fixed to a monkey chair, was attached to the head under pentobarbital anesthesia as described previously (Onoe et al. 1994). The monkeys did not receive any pharmacological treatment prior to the present study.

MRI scans

Magnetic resonance images (MRI) of the monkeys were obtained with a Toshiba MRT-50A/II (0.5 T) under pentobarbital anesthesia. The stereotactic coordinates of PET and MRI were adjusted based on the orbitomeatal (OM) line with a specially designed head holder (Takechi et al. 1994).

Microdialysis analysis

A guide cannula was previously implanted during the procedure for attachment of the acrylic plate, 35 mm anterior to the intrameatal line and lateral 10 mm from the midline (A: 35, L: 10) according to the individual MR images and a stereotactic brain atlas (Snider and Lee 1961). The microdialysis experiments were performed in the conscious state, while the monkey was sitting in the monkey chair. A microdialysis probe with a membrane region of 250 μm in diameter and 3 mm in length (Eicom A-I-08-

03, Eicom, Tokyo, Japan) was inserted into the frontal cortex of the brain via the guide cannula. The probe was perfused with Ringer's solution (147 mM NaCl, 3.4 mM $CaCl_2$ and 4 mM KCl) at a rate of 5 µl/min. 75 µl samples were collected every 15 min, and the content of serotonin was measured by HPLC with electrochemical detection. The averaged data obtained from 0 to 120 min before administration of FEN was used as " baseline" data. FEN 5 mg/kg or 10 mg/kg was administered 120 min after the start of sampling. Sampling continued during 240 minutes after FEN injection. The frontal cortex ECF serotonin level was expressed as percentage of baseline. To verify the exact positioning of the probe, 5 µl of China ink was injected via the guide cannula at the end of the experiments. Animals were anesthetized with sodium pentobarbital and decapitated. The brains were quickly removed, coronal sections were cut on a cryostat, and the location of the probe implantation site was determined visually.

Synthesis of [^{18}F]-MPPF

[^{18}F]-MPPF was prepared by nucleophilic [^{18}F] fluorination of the appropriate nitro precursor (see Shiue et al. 1997 for a comparable method). It was formulated into a NaCl solution. Levels of nitro precursor were << 1 mg/L. The radiochemical purity was greater than 95% and the specific activity > 10 TBq/mmol at the time of injection.

PET scans

Data were collected on a high-resolution PET scanner (SHR-7700, Hamamatsu Photonics K.K., Hamamatsu, Japan) with a transaxial resolution of 2.6 mm full width at half-maximum and a center-to-center distance of 3.6 mm (Watanabe et al. 1997). The PET camera allowed 31 slices for imaging to be recorded simultaneously. After an overnight fast, the animals were fixed to the monkey chair with the stereotaxic coordinates aligned parallel to the OM line. A cannula was implanted into the posterior tibial vein of the monkey for administration of [^{18}F]-MPPF, and another cannula was put into the posterior tibial vein of the other leg to administer FEN. A transmission scan was made for attenuation correction of the dynamic PET scan. [^{18}F]-MPPF (510 MBq) was administered as a bolus injection to study the distribution in the monkey brain (monkey A). In this study, the following time frames were used: 6 frames of 10 seconds, 6

frames of 30 seconds, 12 frames of 1 minute and 25 frames of 3 minutes. In the other experiments [^{18}F]-MPPF (mean ± SD, 560 ± 430 MBq) was administered as a bolus followed by a constant infusion over maximal 180 minutes. Three monkeys (A, B and C), were used for bolus-infusion experiments with the following time frames: 6 frames of 30 seconds, 7 frames of 1 minute and 34 frames of 5 minutes. The Kbol was 84 min. For monkey D and E a “Feedback-controlled infusion system" was used (Ohba et al. 2004). The frame duration was 1 minute for these experiments. Control scans (without FEN administration) were obtained in monkey A, B and C. Monkey A and B both received a FEN dose of 5 mg/kg. A dose of 10 mg/kg was given to monkey A, C (twice), D and E. FEN was injected between 90 and 130 minutes after start of the [^{18}F]-MPPF infusion, at the time of ligand equilibrium (constant ratio between regions of interest and cerebellum). Due to the head fixation, there was no detectable head movement. Regions of interest (ROIs) were identified according to the MR images of each monkey’s brain, for the frontal cortex, hippocampus, striatum and cerebellum. The ROI for the raphe nucleus was placed directly on the PET image. Time activity curves were obtained and the binding potential (*BP*) was calculated by $(C_t\text{-}C_{ref})/C_{ref}$, were C_t is the activity in the region of interest at equilibrium and C_{ref} is the activity in the reference region (Ito et al. 1998). In our study we selected the cerebellum as reference region since the cerebellum is virtually devoid of 5-HT$_{1A}$ receptors (Hall et al. 1997). *BP*s were calculated for two intervals, an early interval: $BP(T_1)$ (mean of the 30 minutes preceding FEN administration) and a late interval: $BP(T_2)$ (mean of the 20-50 minutes after FEN administration).

Statistical analysis

The effect of FEN on *BP* in the frontal cortex, hippocampus, striatum and raphe was calculated using a repeated measures ANOVA with time ($BP(T_1)$ and $BP(T_2)$) as within subject factor and condition (control, 5 mg/kg and 10 mg/kg) as between subject factor. The percent change in $BP: (BP(T_2)\text{-}BP(T_1) / BP(T_1))*100$ in the different regions was calculated for the control and FEN scans.

Drugs

Racemic (±)-Fenfluramine hydrochloride ((±)-N-Ethyl-alpha-methyl-*m*-[trifluoromethyl]phenethylamine hydrochloride) was purchased from Sigma-Aldrich, Tokyo, Japan and dissolved in saline.

Results

Microdialysis

Administration of FEN 5 mg/kg and 10 mg/kg resulted in a 20-fold and 35-fold increase in extracellular 5-HT levels respectively, at 15 minutes after injection (Figure 1).

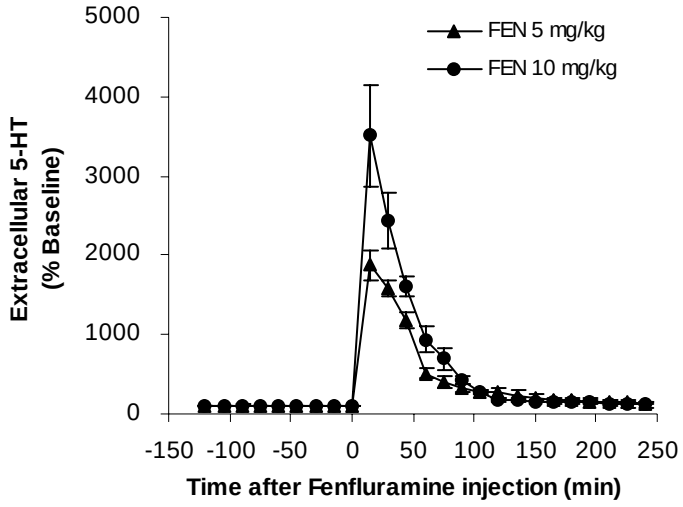

Figure 1: Extracellular 5-HT levels in the prefrontal cortex after fenfluramine administration to conscious monkeys (n=3).

[^{18}F]-MPPF distribution

After administration of [^{18}F]-MPPF, the regional uptake of radioactivity was in agreement with previous results in non-human primates and with known 5-HT_{1A} receptor localization, with the highest uptake in the frontal cortex, cingulate cortex and hippocampus and low uptake in the cerebellum (Kung et al. 1996; Mengod et al. 1996; Plenevaux et al. 2000a; Shiue et al. 1997) (Figure 2).

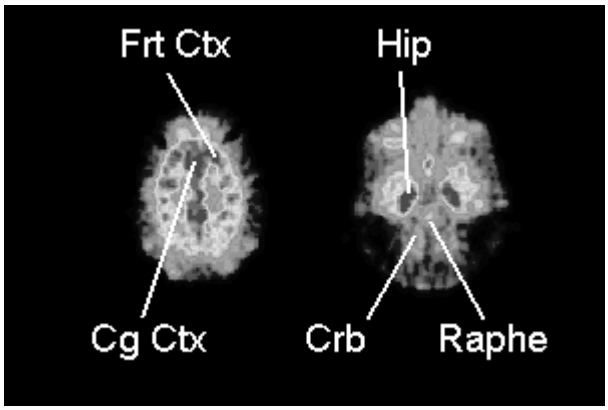

Figure 2: Example of [^{18}F]-MPPF binding in 2 horizontal sections of the conscious monkey brain, at the level of the frontal cortex and hippocampus. Frt Ctx: frontal cortex, Cg Ctx: cingulate cortex, Hip: hippocampus, Crb: cerebellum, Raphe: raphe nucleus.

After bolus administration of [^{18}F]-MPPF, the distribution and clearance were slower than reported for the anesthetized cynomologus monkey (Shiue et al. 1997), and more rapid than for [^{11}C]-WAY-100635 and analogues (Farde et al. 1997; Osman et al. 1998; Pike et al. 1998; Tsukada et al. 2001) (Figure 3). The half-life time varied from 27 minutes in the cerebellum to 56 minutes in the hippocampus. After administration of [^{18}F]-MPPF as a bolus followed by a constant infusion, an equilibrium in [^{18}F]-MPPF uptake was achieved in some of the experiments, while activity gradually increased or decreased with time in other experiments. However, the ratio's of the regions of interest to the cerebellum reached stable values approximately

30 minutes preceding FEN administration, assessed by visual inspection of the curves. The *BP* curves in the frontal cortex of the different monkeys are shown in figure 4A. The mean (±SD) *BPs* in the control situation ranged from 0.4 (±0.1) in the striatum, 0.5 (±0.2) in the raphe nucleus, 1.1 (±0.2) in the frontal cortex to 1.2 (±0.4) in the hippocampus.

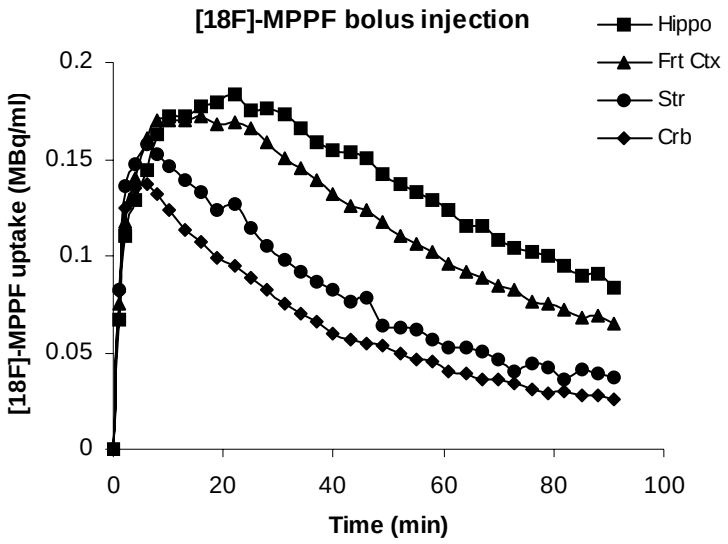

Figure 3: Time-activity curves after bolus injection of [^{18}F]-MPPF (Monkey A).

Effect of fenfluramine treatment on [^{18}F]-MPPF binding

Administration of FEN resulted in variable changes in [^{18}F]-MPPF binding compared to the control situation. Some of the individual time-activity curves seem to show short lasting reductions in [^{18}F]-MPPF binding after FEN administration, however, the reductions were not consistent over the different monkeys. Figure 4B shows the *BP* curves in the frontal cortex of the different monkeys in the FEN (10 mg/kg) condition. The ANOVA results showed that the interaction effects of time*condition and time*condition*region were not significant (p=0.82 and p=0.95 respectively),

thereby indicating that in none of the regions significant differences between the control and FEN conditions were found (Figure 5).

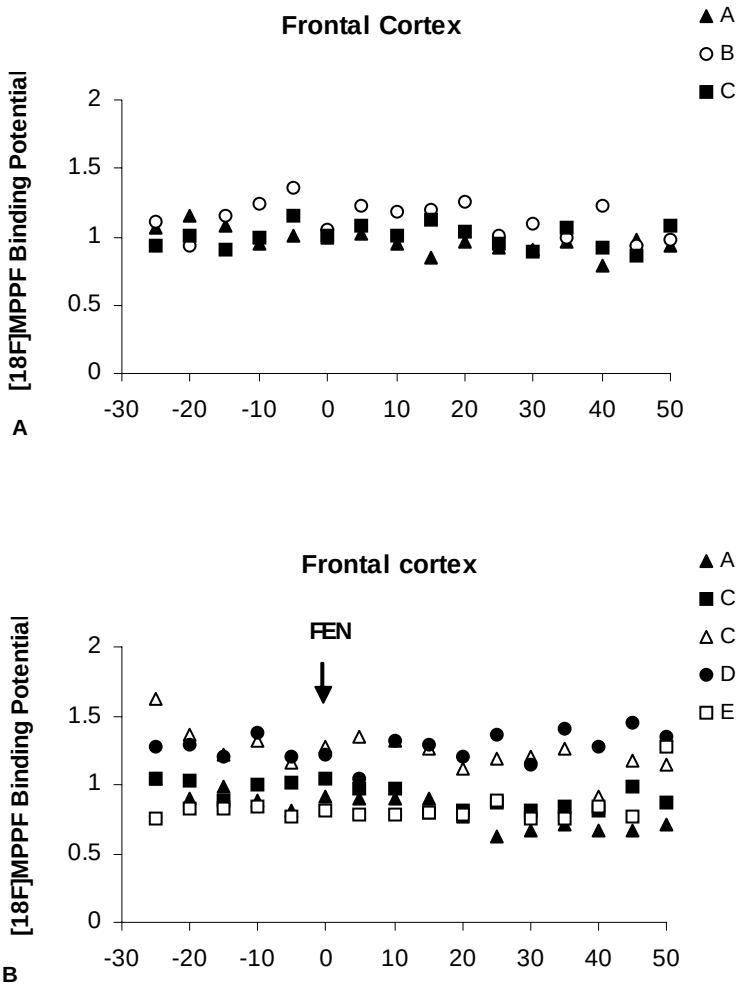

Figure 4: [^{18}F]-MPPF BP in the frontal cortex of the different monkeys after continuous infusion of the ligand, in the control (4A) and fenfluramine (10 mg/kg) condition (4B). Since FEN was administered at different time-points, the time activity curves are relative to FEN administration.

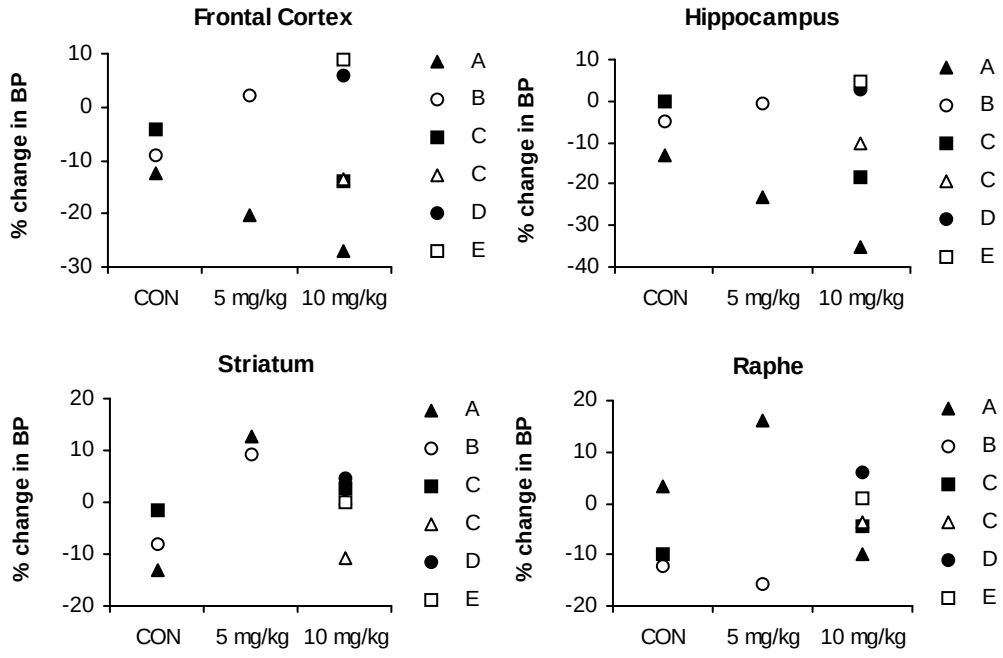

Figure 5: Percent change in *BP* $((BP(T_2)- BP(T_1) / BP(T_1))*100)$ for the different monkeys in the frontal cortex, hippocampus, striatum and raphe nucleus in the control condition (CON) and after administration of fenfluramine 5 mg/kg or 10 mg/kg.

Discussion

The aim of this study was to investigate if [^{18}F]-MPPF binding to the 5-HT$_{1A}$ receptor is decreased after a pharmacologically-induced increase in 5-HT levels. We have investigated the effect in conscious animals using a constant infusion paradigm. Using this paradigm, a sustained equilibrium in ligand binding is achieved. Under this condition, there is no net radiotracer transfer across the blood-brain barrier and possible effects of FEN-induced changes in blood flow can therefore be excluded (Laruelle 2000). Our study

was performed in conscious monkeys, since anesthetics may have an effect on ligand binding (Ginovart et al. 2002; Harada et al. 2004; Hassoun et al. 2003; Momosaki et al. 2004; Seeman and Kapur 2003; Tsukada et al. 2002). Thus in our study we intended to diminish possible non-specific effects on [^{18}F]-MPPF binding. We have shown that FEN, at the doses as used in our study, induced considerable increases in 5-HT levels. After FEN administration short lasting changes in [^{18}F]-MPPF binding were seen in some of the individual time-activity curves. However, the effect did not significantly differ between the control and FEN conditions.

As mentioned before, previous studies using either [^{11}C]-WAY-100635 or [^{18}F]-MPPF did not show consistent changes in ligand binding. Physiological changes in 5-HT levels did not induce measurable effects in humans (Rabiner et al. 2002; Udo de Haes et al. 2002) and studies in rat have shown that larger increases in 5-HT levels were not always effective either (Hume et al. 2001; Maeda et al. 2001; Udo de Haes et al. 2005).

In contrast to these negative results, some rat studies have shown an effect of 5-HT increases on 5-HT$_{1A}$ ligand binding. As mentioned before, Hume et al. (2001) reported a 20% reduction in [^{11}C]-WAY-100635 binding in the hippocampus after FEN (10 mg/kg i.p.) administration, using PET in anesthetized rats. Zimmer et al. (2002) reported a dose-related decrease of [^{18}F]-MPPF binding in the hippocampus after FEN (1, 2 and 10 mg/kg) administration, also in anesthetized rats. Previously we showed that FEN treatment resulted in a significant reduction of [^{18}F]-MPPF binding in several regions, using ex-vivo autoradiography in conscious rats (Udo de Haes et al. 2005). In addition, in vitro studies have shown that 5-HT is able to compete with [^{11}C]-WAY-100635 or [^{18}F]-MPPF binding (Gozlan et al. 1995; Newman-Tancredi et al. 1996; Watson et al. 2000).

The inconsistencies may be caused by several factors. As mentioned before, the two ligands [^{18}F]-MPPF and [^{11}C]-WAY-100635 differ with respect to their affinity to the 5-HT$_{1A}$ receptor (Ki of 0.8 nM and 3.3 nM for [^{11}C]-WAY-100635 and [^{18}F]-MPPF, respectively). It has been suggested that the lower affinity of [^{18}F]-MPPF could make it more sensitive to changes in 5-HT levels (Plenevaux et al. 2000b). In non-equilibrium situations the

affinity could indeed have an effect on the percent change in ligand binding. Under equilibrium conditions however, the affinity of the ligand does not influence the percentage change in BP (Laruelle 2000).

In addition, differences in timing (Endres and Carson 1998; Yoder et al. 2004) or the extent of increases in 5-HT levels may be an explanation for the inconsistent results of the studies using 5-HT_{1A} ligands. In our rat study (Udo de Haes et al. 2005), the animals were sacrificed at the time of the peak in FEN-induced 5-HT increase, whereas in the study of Maeda et al. (2001) and the post mortem dissection study of Hume et al. (2001), the animals were sacrificed before or after the peak in 5-HT response, respectively. In our monkey study, however, 5-HT increases were even larger than in the rat studies. Therefore, the lack of effect in our study should be explained by other factors.

The effect of a serotonergic challenge on ligand binding, may also be influenced by the baseline serotonergic tone. Baseline 5-HT levels may differ between species or conditions, or even between individual animals. These differences in baseline state have already been shown to play a role in the dopaminergic system. The extent of dopamine release, and as a result the change in ligand binding, is reported to be dependent on the level of anxiety and mood at baseline, probably reflecting baseline dopaminergic tone (Volkow et al. 1994). Previous studies have shown that 5-HT levels are influenced by subjective effects such as stress or activity status (Amat et al. 1998; Bland et al. 2003; Kalen et al. 1989). In our study, the monkeys were trained for several months to minimize possible effects of stress. However, differences in baseline 5-HT levels can not be excluded.

Another important factor is the fact that the 5-HT_{1A} receptor can exist in a high and low affinity state (Gozlan et al. 1995; Khawaja 1995; Mongeau et al. 1992; Nénonéné et al. 1994; Watson et al. 2000). Antagonists, such as [^{18}F]-MPPF and [^{11}C]-WAY-100635 will bind with equal affinity to either state whereas agonists such as 5-HT will preferentially bind to the high affinity state (Gozlan et al. 1995; Khawaja 1995; Mongeau et al. 1992; Nénonéné et al. 1994; Watson et al. 2000). It can be argued that changes in 5-HT will therefore mainly effect [^{18}F]-MPPF and [^{11}C]-WAY-100635 binding at the

receptors in the high affinity state. Consequently, changes in binding can only be detected when a large enough proportion of 5-HT_{1A} receptors is in the high agonist affinity state. The proportion of receptors in the high affinity state may depend on the brain area examined (Khawaja 1995) or the method used (Richfield et al. 1986). Furthermore, the affinity state of G-protein coupled receptors may depend on the use of anesthetics (Seeman and Kapur 2003) and may differ between species (Klotz et al. 1991). It is possible that the proportion of 5-HT_{1A} receptors in the high affinity state differed between the various experiments that have investigated the effect of 5-HT increases on ligand binding, thereby leading to differences in the results.

The relative insensitivity of the 5-HT_{1A} ligands to increases in 5-HT levels contrasts with the results from experiments using dopamine D_2 ligands. Ligand binding at the 5-HT_{1A} receptor is only affected after substantial increases in 5-HT levels. In contrast, previous results have shown that D_2 ligand binding is not only affected after pharmacological challenges but also by physiological changes in dopamine levels (de la Fuente-Fernandez et al. 2002; Pruessner et al. 2004; Zald et al. 2004). It is currently unknown how these differences can be explained. One of the major differences between the serotonergic 5-HT_{1A} and dopaminergic D_2 receptor systems, concerns the proportion of receptors in the high affinity state. Previous dopaminergic studies have calculated that approximately 40% of the receptors is in the high affinity state and vulnerable to competition with the endogenous neurotransmitter (Cumming et al. 2002; Narendran et al. 2004). In contrast, the proportion of 5-HT_{1A} receptors in the high affinity state is probably much lower (Nénonéné et al., 1994; Udo de Haes et al. 2005). These data may offer an explanation for the differences in sensitivity to changes in neurotransmitter concentration. The finding of Bantick et al. (2004) that other 5-HT_{1A} agonists than 5-HT affected ligand binding only at relatively high doses is in agreement with this concept.

Labeled agonists to the 5-HT_{1A} receptor could possibly be used to show effects of 5-HT increases on ligand binding at receptors in the high affinity state. This approach has already been used for the dopaminergic system. Results from these studies show that the agonist tracer was indeed more

sensitive to changes in dopamine as compared to the antagonist tracer. (Cumming et al. 2002; Narendran et al. 2004). In addition, it may be interesting to study possible effects of anesthesia and blood flow on 5-HT_{1A} ligand binding.

In conclusion, [^{18}F]-MPPF binding is not significantly affected in conscious monkeys, even after relatively large increases in 5-HT levels. These results may imply that the majority of 5-HT1A receptors is in the low affinity state, in vivo.

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