Evaluation of drug effects on cerebral blood flow and glucose uptake in un-anesthetized and un-stimulated rats: application of free-moving apparatus enabling to keep rats free during PET/SPECT tracer injection and uptake

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**Objectives** The purpose of this study is the development of novel fluorine-18-fluorodeoxyglucose (18F-FDG)-PET and 99mTc-hexamethylpropylene amine oxime (HMPAO) SPECT methods with free-moving apparatus on conscious rats to investigate brain activity without the effects of anesthesia and tactual stimulation. We also assessed the sensitivity of the experimental system by an intervention study using fluoxetine as a reference drug.

**Materials and methods** A catheter was inserted into the femoral vein and connected to a free-moving cannula system. After fluoxetine administration, the rats were given an injection of 18F-FDG or 99mTc-HMPAO via the intravenous cannula and released into a free-moving cage. After the tracer was trapped in the brain, the rats were anesthetized and scanned with PET or SPECT scanners. Then a volume of interest analysis and statistical parametric mapping were performed.

**Results** We could inject the tracer without touching the rats, while keeping them conscious until the tracers were distributed and trapped in the brain using the developed system. The effects of fluoxetine on glucose uptake and cerebral blood flow were perceptively detected by volume of interest and statistical parametric mapping analysis.

**Conclusion** We successfully developed free-moving 18F-FDG-PET and 99mTc-HMPAO-SPECT imaging systems and detected detailed glucose uptake and cerebral blood flow changes in the conscious rat brain with fluoxetine administration. This system is expected to be useful to assess brain activity without the effects of anesthesia and tactual stimulation to evaluate drug effect or animal brain function.

**Keywords:** conscious, 18F-FDG-PET, free moving, nonanesthesia, rat, 99mTc-HMPAO

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**Introduction** Imaging of the brain activities, including oxygen metabolism, glucose metabolism, and cerebral blood flow (CBF), has been effectively used for diagnosis, brain research, and clinical trials [1–5]. Combination of these imaging techniques and voxel-based comprehensive analysis, such as statistical parametric mapping (SPM), has been used and is useful to understand the pathophysiology of diseases and neural activity about brain function [6–8]. Imaging has been widely recognized as a translational technology because the same tracers and modalities can be used in both preclinical and clinical studies. In addition, the advantage of being noninvasive enables longitudinal evaluation of disease progress or drug effect. However, there are some discrepancies to be matched for the translational research, especially in drug evaluation using imaging biomarkers. The major discrepancy is anesthesia, which is required to immobilize the animals in preclinical studies, whereas clinical brain imaging is performed in the conscious state without anesthesia. Anesthetization before tracer injection can affect brain metabolism and CBF, and also has some interactions with central nervous system-acting drugs [9,10]. It indicates that there might be discrepancies between the imaging data and general pharmacological or pharmacokinetic data also in preclinical research. To avoid this problem, there have been some reports of preclinical imaging using pharmacological MRI, fluorine-18-fluorodeoxyglucose (18F-FDG)-PET and 99mTc-hexamethylpropylene amine oxime (HMPAO)-SPECT in conscious animals [11–14].
However, in most of these studies, the animals are exposed to the stress of physical restraint, which has a huge effect on the brain function [15–17].

In the present study, we tried to develop a new imaging system to deal with these problems, namely, we combined femoral cannulation techniques and a free-moving device commonly used for microdialysis. We implanted polyethylene cannulas into femoral veins of rats and connected them to a free-moving device, which enabled tracer injection without touching conscious rats. Regarding tracers, we used $^{18}$F-FDG and $^{99m}$Tc-HMPAO with a metabolic trapping mechanism so that accumulation of the tracer was kept at a steady level after uptake and distribution in the brain [18,19]. The rats were conscious during the tracer uptake period and were then anesthetized immediately before the scans, so that the image would reflect the brain condition in the conscious state based on the tracer trapping mechanism. Then, we assessed the detectability of the experimental system by the detection of the effect of fluoxetine, a selective serotonin reuptake inhibitor (SSRI). Fluoxetine was used as a reference drug because fluoxetine has been widely used in preclinical and clinical and has been reported to affect glucose metabolism in brain [20–23]. The effects of fluoxetine were investigated by volume of interest (VOI) analysis and SPM. In the $^{99m}$Tc-HMPAO-SPECT study, we also calculated regional cerebral blood flow (rCBF) with VOI analysis and input function data measured by serial blood sampling.

**Materials and methods**

**Animal preparation**

Totally 52 Wistar rats (male, 13 weeks old; Japan SLC Inc., Shizuoka, Japan) were used in this study (Tables 1 and 2). Twenty-six rats were used for the $^{18}$F-FDG-PET study (control, $n=12$; fluoxetine, $n=14$), and 26 rats were used for the $^{99m}$Tc-HMPAO-SPECT study (control, $n=14$; fluoxetine, $n=12$). The rats were kept under environmentally controlled conditions (12-h normal light/dark cycles, 20–23°C and 50% relative humidity) with standard rat chow and water ad libitum. Every rat had surgical catheterization into the right femoral vein under 2–5% isoflurane anesthesia. For $^{99m}$Tc-HMPAO-SPECT, the left femoral artery was also catheterized for serial blood sampling. The catheters were led out to the back of neck via subcutaneous tunnels. At least 2 days were allowed for recovery from the surgery before $^{18}$F-FDG-PET and $^{99m}$Tc-HMPAO-SPECT. After confirming that no abnormal behavior was noticed in the rats, they were fasted 18 h before the tracer injection (Fig. 1). The Animal Care and Use Committee of the Hamamatsu University School of Medicine approved all of the PET and SPECT studies.

**Tracers and drugs**

Fluoxetine hydrochloride (Wako Pure Chemical Industries Ltd, Osaka, Japan, 20 mg/ml/kg suspended in 0.5% methylcellulose saline) was administered intraperitoneally 24, 19 and 1 h before injection of the tracer in both $^{18}$F-FDG-PET and $^{99m}$Tc-HMPAO-SPECT (Fig. 1) [14]. The control group was given 0.5% methylcellulose saline. $^{18}$F-FDG was obtained from Nihon Medi-Physics Co. Ltd (Tokyo, Japan). $^{99m}$Tc-HMPAO solution was synthesized immediately before the injection using a commercial kit (Cerebrotec kit; Nihon Medi-Physics Co. Ltd) and $^{99m}$Tc generator (Nihon Medi-Physics Co. Ltd). The radiochemical purity was measured by thin layer chromatography (Table 2).

**$^{18}$F-FDG-PET scan**

The study protocol is depicted in Fig. 1. Initially, 75 min before $^{18}$F-FDG injection, blood was sampled from the tail vein and the blood glucose was measured by a blood glucose analysis system (ACCU-CHECK compact plus; Roche Diagnostics, Basel, Switzerland). Then the rats were placed in a free-moving catheter system (Sugiyama-Gen Co. Ltd, Tokyo, Japan) and habituated in the acryl cylinder cages (20 cm diameter, 30 cm height) covered with a black sheet. $^{18}$F-FDG (control: $182 \pm 4 \mu$Ci, fluoxetine: $184 \pm 4 \mu$Ci, Table 1) was injected intravenously in each rat from the tip of free-moving cannula outside the cage. Thereafter, 25 min after the $^{18}$F-FDG injection, the rats were anesthetized with propofol (Maruishi Pharmaceutical Co. Ltd, Osaka, Japan) and removed from the free-moving system. Then the head of each rat was fixed to an acrylic head holder (Narishige Japan Co. Ltd, Tokyo, Japan) on the animal bed of an FX preclinical platform scanner (X-O$^{	ext{®}}$-PET•X-SPECT; Gamma Medica-Ides, Northridge, Los Angeles, USA).

**Table 1. Body weight, blood glucose level and injected radioactivity in $^{18}$F-FDG-PET study**

| Animal (N) | Control | Fluoxetine |
|-----------|---------|------------|
| Animal (N) | 12      | 14         |
| Body weight (g) | $268 \pm 5$ | $269 \pm 11$ |
| Blood glucose (mg/dl) | $93 \pm 4$ | $92 \pm 7$ |
| Injected radioactivity ($\mu$Ci) | $182 \pm 4$ | $184 \pm 4$ |

Data are mean ± SEM.

There are no significant differences between the control and fluoxetine groups. $^{18}$F-FDG, fluorine-18-fluorodeoxyglucose.

**Table 2. Body weight, blood glucose level, injected radioactivity, and radiochemical purity in $^{99m}$Tc-HMPAO-SPECT study**

| Animal (N) | Control | Fluoxetine |
|------------|---------|------------|
| Animal (N) | 14      | 12         |
| Body weight (g) | $272 \pm 2$ | $276 \pm 2$ |
| Blood glucose (mg/dl) | $114 \pm 4$ | $104 \pm 3$ |
| $PCO_2$ (mmHg) | $38.7 \pm 0.4$ | $40.0 \pm 1.0$ |
| pH | $7.48 \pm 0.01$ | $7.49 \pm 0.01$ |
| Injected radioactivity ($\mu$Ci) | $3.82 \pm 0.02$ | $3.79 \pm 0.03$ |
| Radiochemical purity (%) | $92.3 \pm 0.5$ | $91.2 \pm 0.5$ |

Data are mean ± SEM.

There are no significant differences between the control and fluoxetine groups. HMPAO, hexamethylpropylene amine oxime.
The study protocol of (a) $^{18}$F-FDG-PET and (b) $^{99m}$Tc-HMPAO-SPECT. The cannulation surgery was performed into the femoral vein 2 days before the scans. Control or fluoxetine was administered intraperitoneally 24, 19, and 1 h before the tracer injection. Overall, 75 min before the tracer injection, the rats were attached to the free-moving system and habituated, and then tracer was injected. (a) 25 min after $^{18}$F-FDG injection, the rats were anesthetized with propofol and fixed to the animal bed of the PET scanner. Then, 30 min PET scan and sequential X-ray CT scan were performed. (b) Serial arterial blood sampling was performed for 2 min after $^{99m}$Tc-HMPAO injection, and 5 min after $^{99m}$Tc-HMPAO injection, the rats were anesthetized with propofol and fixed to the animal bed of SPECT scanner. Then 60-min SPECT scan and sequential X-ray CT scan were performed. CT, computed tomography; $^{18}$F-FDG, fluorine-18-fluorodeoxyglucose; HMPAO, hexamethylpropylene amine oxime.

Data analyses of $^{18}$F-FDG-PET
$^{18}$F-FDG-PET tomographic images were reconstructed from the projection data using ordered-subset expectation maximization with four iterations and 15 subsets, with image matrix sizes of $256 \times 256 \times 256$ voxels and voxel sizes of $0.4 \times 0.4 \times 0.4$ mm. CT image data were reconstructed with image matrix sizes of $512 \times 512 \times 512$ voxels and voxel sizes of $0.19 \times 0.19 \times 0.19$ mm. Standardized uptake value of $^{18}$F-FDG was calculated according to the following formula: brain radioactivity (kBq/ml)×body weight (g)/injected radioactivity (kBq). For VOI analysis, the radioactivity of the whole brain and each brain region was obtained using PMOD software (version 3.2, PMOD Group, Switzerland, 2009) and the accompanying VOI templates. The VOI template was modified to major 13 regions [whole brain, whole cortex, cortex (anterior), cortex (middle), cortex (posterior), midbrain, cerebellum, brainstem, hippocampus, caudate putamen, thalamus, hypothalamus, and amygdala]. The whole-brain ratio of each VOI was calculated as the ratio of the radioactivity density on each VOI to that for the whole brain. For SPM, the preprocessed PET images were coregistered and extrabrain voxels were masked. After the masking, the images were registered and analyzed using the SPM2 software package (SPM, Welcome Department of Cognitive Neurology, London, UK) with an unpaired t-test (threshold $P < 0.001$, height threshold at 75% of mean, extent four voxels). The anatomical regions where the PET signals changed were determined by coregistered SPM on the MRI template and the rat brain atlas [24,25].

$^{99m}$Tc-HMPAO-SPECT scan
The study protocol is depicted in Fig. 1. Initially, 75 min before $^{99m}$Tc-HMPAO injection, blood glucose, pH and partial pressure of carbon dioxide (PCO$_2$) were determined with blood sampling from the tail vein by a blood glucose analysis system and blood gas analyzer (i-STAT; Abbott Laboratories, Abbott Park, Illinois, USA). Then the rats were placed in a free-moving catheter system and...
arterial cannula outside the cage. Then, 5 min after and 120 s after the tracer injection via a free-moving system. The standardized uptake value of the tracer injection, static SPECT scans were performed for ~60 min (1 min × 60 frames) with a 1.5% (v/v) isoflurane anesthesia and subsequent CT scans were performed with the same scanner.

Data analyses in 99mTc-HMPAO-SPECT

99mTc-HMPAO-SPECT images were reconstructed from the projection data using ordered-subset expectation maximization with five iterations and four subsets, with image matrix sizes of 256 × 256 voxels and voxel sizes of 0.92 × 0.91 × 0.91 mm. CT image data were reconstructed with image matrix sizes of 60 × 60 × 60 voxels and voxel sizes of 0.17 × 0.17 × 0.17 mm. SPECT images were smoothed with a Gaussian kernel filter (full width at half maximum = 2.0 mm) and VOI analysis and SPM were performed with the same procedure as the 18F-FDG-PET. rCBF was calculated from the VOI radioactivity and arterial blood radioactivity according to the following formula: regional brain radioactivity (kBq/ml)/blood radioactivity AUC0–26 s (kBq/ml/min) [26]. Blood outflow delay caused by the cannula length was estimated at 8 s and taken into consideration to calculate the AUC.

Statistical analyses

Data were represented as mean ± SD. Statistical analyses were performed with unpaired Student’s t-test. P value of less than 0.05 was considered to be statistically significant.

Ethical approval

The study was approved by the Animal Care and Use Committee of the Hamamatsu University School of Medicine.

Results

Effect of fluoxetine on glucose uptake

There were no differences in the blood glucose levels between either groups immediately before placing in the free-moving system. The standardized uptake value of the whole brain significantly decreased in the fluoxetine group compared with the 0.5% methylcellulose saline-administered control group (5.2 ± 0.2 and 8.5 ± 0.2, respectively). The whole-brain ratio with VOI-based analysis of each brain region is shown in Table 3. Significant 18F-FDG uptake increases were observed in the anterior cortex and hypothalamus (3.4%, both) in the fluoxetine group. There were significant 18F-FDG uptake decreases in the posterior cortex, midbrain, brain stem and hippocampus (2.0, 6.4, 3.7 and 2.6%, respectively) after fluoxetine administration. SPM analysis showed fluoxetine-induced increased signals inside the cerebellum and decreased signals in the large brain region around the midbrain, including the periaqueductal gray (PAG), a part of the hippocampus, the parahippocampal region, and the superior and inferior colliculi (Fig. 2).

Effect of fluoxetine on brain blood flow

There were no differences in the blood glucose levels, pH or PCO2 between either groups (Table 2). Table 4 summarizes the effect of fluoxetine on rCBF calculated with VOI analysis and arterial blood radioactivity. A significant decrease of rCBF was detected in all brain regions. SPM analysis based on global mean scaling showed higher rCBF only in the bilateral small regions around the primary somatosensory cortex after fluoxetine administration (Fig. 3). In contrast, decrease in rCBF was not detected in any cluster of voxels.

Discussion

Clinical PET/SPECT imaging is generally performed without anesthesia and has the discrepancy of differences with preclinical imaging derived from the use of anesthesia. Therefore, we combined 18F-FDG-PET and 99mTc-HMPAO-SPECT methodologies with a free-moving cannula system to avoid the effect of anesthesia on tracer distribution. This allowed injection of the tracers to the rats while keeping the rats conscious and free during the tracer uptake period, and then the brain metabolism or CBF could be assessed with minimum effect of anesthesia and tactile stimulation. In the present study, we evaluated the SSRI (fluoxetine) effect on brain glucose metabolism and CBF using this methodology. SPM analysis showed significant increases in 18F-FDG uptake inside the cerebellum and decreases in the brain region around the midbrain including the PAG, a part of the hippocampus, the parahippocampal region.

Table 3 Whole-brain ratio of fluorine-18-fluorodeoxyglucose uptake on each brain region

| Brain region     | Control  | Fluoxetine | P-value | %Change |
|------------------|----------|------------|---------|---------|
| Cortex (whole)   | 1.13 ± 0.01 | 1.13 ± 0.00 | 0.199  | 0.78    |
| Cortex (anterior)| 1.28 ± 0.01 | 1.32 ± 0.01 | 0.001  | 3.37    |
| Cortex (middle)  | 1.20 ± 0.01 | 1.22 ± 0.01 | 0.163  | 1.14    |
| Cortex (posterior)| 0.97 ± 0.01 | 0.95 ± 0.00 | 0.012  | −1.95   |
| Midbrain*        | 1.19 ± 0.01 | 1.11 ± 0.00 | 0.000  | −6.42   |
| Cerebellum       | 0.86 ± 0.01 | 0.87 ± 0.01 | 0.014  | 0.61    |
| Brain stem*      | 0.85 ± 0.01 | 0.82 ± 0.01 | 0.043  | −3.67   |
| Hippocampus*     | 1.24 ± 0.01 | 1.21 ± 0.00 | 0.005  | −2.56   |
| Caudate putamen  | 1.50 ± 0.01 | 1.50 ± 0.01 | 0.455  | −0.46   |
| Thalamus         | 1.26 ± 0.01 | 1.28 ± 0.01 | 0.132  | 1.13    |
| Hypothalamus*    | 0.93 ± 0.01 | 0.97 ± 0.01 | 0.037  | 3.41    |
| Amygdala         | 0.91 ± 0.01 | 0.91 ± 0.01 | 0.699  | −0.50   |

Data are mean ± SEM. The regional volume of interest data were compared between the control and fluoxetine groups. *P < 0.05.
function in the cerebellum might be kept to control motor activity under free-moving conditions [30, 31]. Interestingly, the findings in these regions are in disagreement with the serotonin transporter distribution [32, 33]. These results indicate that neuronal activation or deactivation does not always occur in the site of action; therefore, function-based comprehensive analysis like the combination of 18F-FDG-PET and SPM could be useful to target the region related to drug efficacy [34–37]. Furthermore, functional response itself would be an imaging biomarker especially in the case where target-specific ligand is not available [38–41].

There have been some previous reports about the effect of SSRI on rat brain. Freo et al. [22, 23] reported detailed regional cerebral metabolic rates of glucose change by fluoxetine using 14C-2-deoxy-D-glucose. In terms of the relative rate of regional cerebral metabolic rates of glucose to the whole brain, there were some regions of the brain that showed opposite changes compared with our results. Their study was performed in conscious animals, but their experimental procedure was different from our protocol, in that they immobilized the rats. In another report, Jang et al. [14] investigated the effect of fluoxetine on rat brain without anesthesia and physical restraint during 18F-FDG uptake. They detected increased 18F-FDG uptake in the dorsal hippocampus, which was different from our results. Their experimental procedure was similar to our procedure, but they restrained the rat and injected 18F-FDG via the tail vein. The reasons for the differences between our results and the previous reports is not clear; however, there were methodological differences such as animal immobilization and needle stimulation, which might affect the brain activity followed by the difference of regional FDG uptake changes. These discrepancies with our result indicate the effect on brain activity and drug effect by the experimental conditions.

Table 4 Whole-brain blood flow and regional cerebral blood flow in each brain region

| Brain region      | Control    | Fluoxetine | P-value | %Change |
|-------------------|------------|------------|---------|---------|
| Whole-brain blood flow* | 52.8±1.7  | 43.7±1.5  | 0.001   | −17.2   |
| Cortex (whole)*  | 54.9±1.7  | 46.5±1.6  | 0.002   | −15.2   |
| Cortex (anterior)* | 59.9±1.9  | 51.0±1.7  | 0.003   | −14.9   |
| Cortex (middle)* | 55.8±1.7  | 48.5±1.6  | 0.005   | −13.1   |
| Cortex (posterior)* | 50.9±1.8  | 42.4±1.5  | 0.002   | −16.7   |
| Midbrain* | 61.0±2.3  | 49.8±1.9  | 0.001   | −18.4   |
| Cerebellum* | 43.2±1.5  | 36.6±1.2  | 0.003   | −15.3   |
| Pons and medulla* | 41.3±3.0  | 28.9±1.8  | 0.002   | −30.0   |
| Hippocampus* | 63.3±2.1  | 53.3±1.9  | 0.002   | −15.8   |
| Caudate putamen* | 75.3±2.6  | 65.7±2.3  | 0.012   | −12.8   |
| Thalamus* | 68.6±2.3  | 57.3±2.1  | 0.001   | −16.5   |
| Hypothalamus* | 66.5±2.2  | 52.9±2.4  | 0.000   | −20.4   |
| Amygdala* | 60.8±2.4  | 49.1±2.1  | 0.002   | −19.1   |

Data are mean±SEM values of cerebral blood flow (ml/min 100 g). The data were compared between the control and fluoxetine groups.

*P<0.05.
SPM analysis of $^{99m}$Tc-HMPAO-SPECT showed different activated regions from the $^{18}$F-FDG study. We detected global CBF changes in the whole brain and relatively high rCBF only in bilateral regions around the primary somatosensory cortex, and these changes were not related to pH or PCO$_2$. The noncorrespondence between $^{18}$F-FDG-PET and $^{99m}$Tc-HMPAO-SPECT may be explained by drug-induced uncoupling [42–44]. It might reveal that region-specific $^{18}$F-FDG uptake change was caused by factors other than rCBF change, such as hexokinase activity. In the $^{99m}$Tc-HMPAO-SPECT study, we performed blood sampling to calculate the absolute value of CBF using blood input function. In all regions of the brain, the absolute value of rCBF decreased with fluoxetine administration. On the contrary, we used global mean scaling for SPM analysis to detect detailed comparative rCBF changes. Comparative values, such as ratio to whole brain or some reference region, are convenient to detect local changes and easy to perform, although absolute values are necessary to detect global changes. It is important to use both simple methods and rigorous methods as necessary, and our free-moving system enables use of both methods.

The effect of anesthesia and stimulation on brain activity is thought to be huge as described before. Some groups tried to minimize such effect, namely using short-time anesthesia or customized animal holders. For example, Mizuma et al. [12] used a customized head holder with an adapter attached to the skull and reduced the effect of stress by habituation to the experimental device. They performed $^{18}$F-FDG-PET in conscious animals and obtained different images from those under anesthesia. Imaging of conscious rats immobilized with a head holder has an advantage that dynamic scanning can be performed, because the rat is restrained in the scanner during the whole experimental procedure. On the contrary, the advantage of our method is that the rats can move freely during the tracer uptake period. Actually the rats might not be completely free in aspect of catheterization and harness connection; however, we confirmed no abnormal behavior or active movement was not observed during the experiments. As there has been no gold standard imaging method under normal physiological conditions, further studies such as head-to-head comparison with other nonanesthesia method would be required to characterize our method. In spite of the future task, our free-moving methodology yet has potentials to be combined with other experimental methodologies, such as behavioral tasks, sensory stimulations, electroencephalogram or microdialysis. It would be useful to reproduce the condition of the pharmacological study to detect brain activation in specific conditions.

We used cannulation in the femoral vein and artery for tracer injection and blood sampling in the present study. Furthermore, we can easily combine additional cannulation for subcutaneous and/or intraperitoneal injection based on the same free-moving concept. In addition, this free-moving methodology has the potential to be applied for other tracers or evaluation of other drugs. Such extendibility potential is one of the appealing aspects of our methodology.
Conclusion
We successfully developed a free-moving PET and SPECT imaging system to evaluate rat brain function in conscious rats, with minimum effect of anesthesia and tactual stimulation. Using this methodology, we demonstrated that we could evaluate cerebral glucose metabolism and rCBF change by drug administration. This methodology has large extendibility such as a combination study with behavior study or other tracer application. Thus, our platform is expected to be useful to investigate drug effect and brain function.

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Conflicts of interest
There are no conflicts of interest.

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