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ORIGINAL ARTICLE

Effects of animal handling on striatal DAT availability in rats

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Abstract

Objective Positron emission tomography (PET) is a non-invasive technique measuring quantification of physiological and biochemical processes in the living organism. However, there are many considerations including anesthesia and fasting to acquire small animal imaging. We aimed to evaluate the effects of anesthesia and fasting of rats in dopamine transporter (DAT) imaging acquisition.

Methods Male Sprague Dawley (SD) rats aged 7 weeks and weighing 180–260 g were used in this study. Rats were randomly divided by 4 groups. Group A was kept under anesthesia for 40 min and fasted over 12 h. Group B was only fasted over 12 h. Group C was only kept under anesthesia for 40 min. Group D was neither kept under anesthesia nor fasted over 12 h. PET scans were started at 40 min after ^{18}F-FP-CIT injection and obtained for 20 min. Volumes-of-interest for striatum and extrastriatal area were used for ^{18}F-FP-CIT PET analysis. Cerebellum was considered as a reference region. Specific binding ratio (SBR) was calculated as follows: [(uptake of target-uptake of cerebellum)]/(uptake of cerebellum).

Results SBR without fasting and anesthesia (group D) was significantly lower than those of other groups (vs group A, $p = 0.0004$; vs group B, $p = 0.0377$; vs group C, $p = 0.0134$). However, SBRs of extrastriatal area ($p = 0.5120$) were not affected by fasting and anesthesia.

Conclusions In conclusion, the SBR of striatum was increased after anesthesia by isoflurane and fasting. When designing an experiment using DAT imaging, the effects of isoflurane and fasting should be considered.

Keywords Positron-emission tomography · Isoflurane · Fasting · Dopamine transporter · Corpus striatum

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Introduction

Small animal imaging is widely used for its advantage including longitudinal monitoring of disease, drug effectiveness, and potential for translational research [1]. However, there are several factors that affect the condition of small animal imaging such as anesthesia and fasting [2].

In animal experiment, anesthesia is needed to restrain animal to prevent motion which might hamper imaging quality [1]. Anesthesia causes depression of an autonomic nervous system resulting in depression of cardiovascular and respiratory system, and hypothermia [1]. Each anesthetic agent has a different effect on the brain [2]. According to the study by Momosaki et al., dopamine receptor (DR) availability was increased after anesthesia with chloral hydrate and ketamine [3], while DR availability was decreased in rats after anesthesia with pentobarbital [3]. Also, isoflurane had an effect on release of striatal dopamine in a concentration dependent manner [4].

Fasting also has an effect on uptake of radiopharmaceuticals of brain positron emission tomography (PET) in small animals [2]. Fasting causes decrease of blood glucose levels, and prolonged fasting results in a loss of body mass, and decrease of fatty acid [2]. As dopamine is a neurotransmitter modulating reward system, which processes information related to food reward influenced by metabolic and hormonal signals [5], both acute fasting and chronic food restriction might affect the dopaminergic system visualized by small animal imaging [6].

Previously, dopaminergic system in the caudate–putamen complex (CPu) was visualized with PET scan using radiopharmaceuticals of dopamine transporter (DAT) and DR [7]. In this study, we aimed to investigate the effects of anesthesia and fasting on DAT availability with small animal imaging using PET.

Materials and methods

Study design

Male Sprague Dawley (SD) rats aged 7 weeks and weighing 180–260 g were used in this study. Rats were randomly divided into 4 groups according to the states of fasting (over 12 h) and anesthesia during uptake time (40 min) after injection of radiopharmaceutical, before scan; (1) Group A with fasting over 12 h & with anesthesia during uptake time, (2) Group B with fasting over 12 h & without anesthesia during uptake time (kept conscious), (3) Group C without fasting over 12 h (ad libitum feeding) & with anesthesia during uptake time, (4) Group D without fasting over 12 h (ad libitum feeding) & without anesthesia during uptake time (kept conscious) (Table 1, Fig. 1). PET scans were conducted only once per each rat for exclude potential effect of aging on DAT availability. Twenty-four SD rats were categorized into 4 groups with 1 rat from group C excluded for poor image quality. Anesthesia was done by inhalation of isoflurane at a concentration of 1.5% with an oxygen rate of 1.5 L/min. For the western blot analysis, rats from each group ($n = 4$) were sacrificed and the striatum was dissected from the brain. The dissected striatum was frozen and then stored at − 70 °C. The animal experiments were approved by the Institutional Animal Care and Use Committee of Pusan National University Hospital.

Table 1 Animal conditions

Group	*n*	Fasting (over 12 h)	Anesthesia during uptake time
A	6	Yes	Yes
B	6	Yes	No
C	5	No	Yes
D	6	No	No

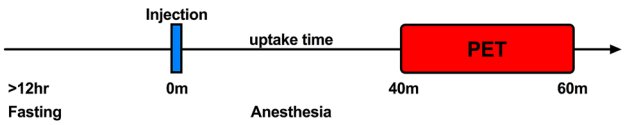

Fig. 1 Study design

Western blot analysis

Tissues were rinsed rapidly in ice-cold PBS and were lysed with a protein extraction solution (PRO-PREP™; iNtRON Biotechnology, Korea). The protein concentration was measured by the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories Inc., Hercules, CA, USA). Proteins were separated by electrophoresis on a 12% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 1 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline (10 mM Tris/HCl, pH 8.0, and 150 mM NaCl) solution containing 0.05% Tween-20. The membranes were incubated with the following specific primary antibodies (Mouse polyclonal, Dilution 1:1000, Abcam) for overnight at 4 °C. The membranes were washed three times for 15 min each in TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham, Little Chalfont, Buckinghamshire, England) for 1 h at room temperature. After washing three times with TBST, the membranes were developed

with an enhanced chemiluminescence detection system (Amersham, Little Chalfont, Buckinghamshire, England). The membrane was subjected to luminescent image analyzer (ATTO, Japan) and analyzed quantitatively.

^{18}F-FP-CIT PET

^{18}F-FP-CIT (18.5–37.0 MBq) was injected into the tail vein within the 1 mL of volume. Forty minutes after injection of ^{18}F-FP-CIT, PET scans were performed using Inveon PET/CT scanner (Siemens Healthcare, Erlangen, Germany) for 20 min. During the acquisition, SD rats were kept under anesthesia by inhalation of isoflurane. Data were reconstructed with three-dimensional ordered-subsets expectation maximization method.

Image analysis

PET image analysis was done with pmod v3.6 (PMOD Technologies LLC, Zürich, Switzerland). Scans were registered and normalized to PET templates constructed from the baseline scans acquired in this study. Using the atlas by Schiffer et al. volumes-of-interest of striatal (CPu) and extrastriatal (brain area other than CPu, cerebellum) areas were adopted for ^{18}F-FP-CIT PET analysis [8]. Cerebellum was considered as a reference region. Specific binding ratio (SBR) was calculated as follows: [(uptake of target-uptake of cerebellum)]/(uptake of cerebellum) (Fig. 2).

Statistical analysis

All data are presented as mean and standard deviation. Changes in SBRs by fasting and anesthesia were analyzed by one-way analysis of variance. Post-hoc analysis was done to perform a test for pairwise comparison of groups using Tukey–Kramer test. To confirm the effect of weight on SBRs, analysis of covariance was conducted. P value less than 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 7 for Mac OS X (GraphPad Software Inc, San Diego, CA, USA).

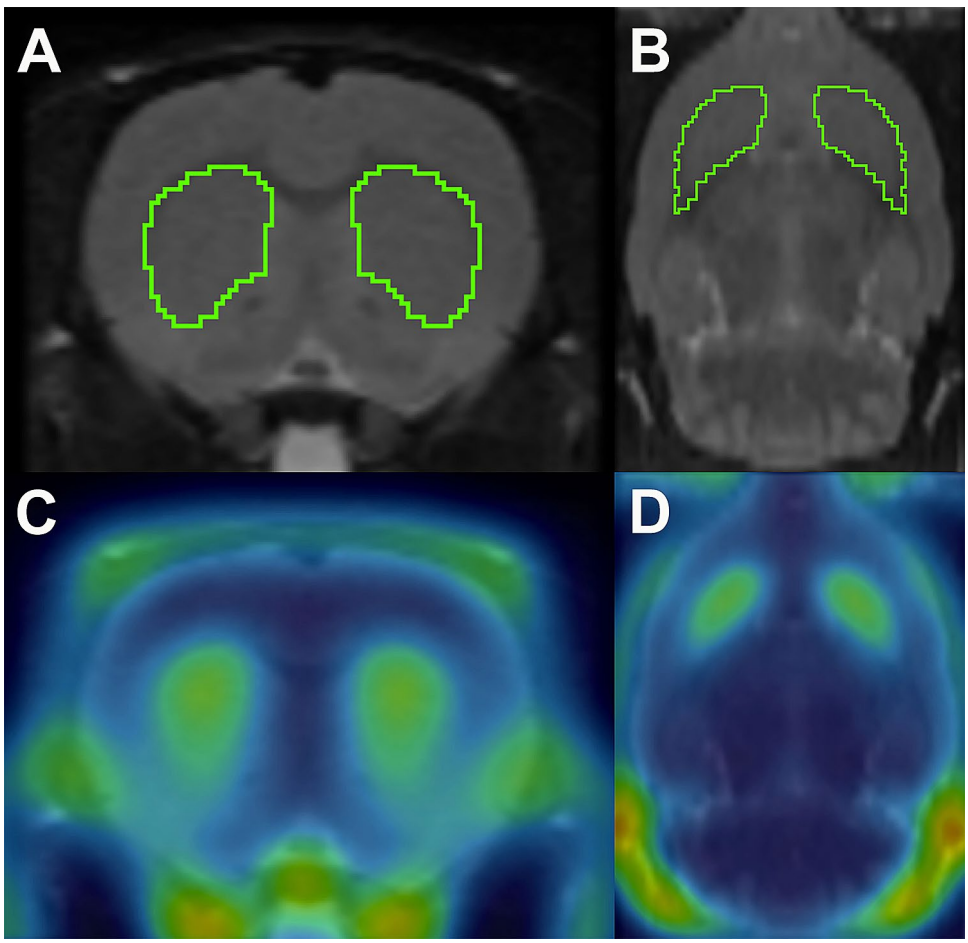

Fig. 2 Representative images of **a**, **b** MRI template, and **c**, **d** Fused ^{18}F-FP-CIT PET and MRI

Results

Mean and standard deviation of SBRs from striatal area was 1.1420 ± 0.1396 for group A, 0.9721 ± 0.0825 for group B, 1.025 ± 0.1231 for group C, and 0.7337 ± 0.1899 for group D. Those from extrastriatal area was 0.2470 ± 0.0615 for group A, 0.2394 ± 0.0282 for group B, 0.2416 ± 0.0472 for group C, and 0.2053 ± 0.0617 for group D (Table 2). Between groups, SBRs of striatal area were significantly different (p = 0.0007). In post-hoc analysis, SBR of group D was lower than those of other groups (vs group A, $p = 0.0004$; vs group B, $p = 0.0377$; vs group C, $p = 0.0134$, adjusted for multiple comparison) (Fig. 3a). However, SBRs from extrastriatal area ($p = 0.5120$) was not different between groups, which means DAT availability of extrastriatal area may not be affected by fasting and anesthesia (Fig. 3b). To confirm the effect of weight on SBRs, analysis of covariance was conducted. The weight of SD rats did not affect the SBRs of the striatum and extrastriatum ($p = 0.686$). Expression levels of DAT of striatum was evaluated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). DAT expression of group A was higher than those of any other groups (Fig. 4). However, the correlation between SBR and DAT/GAPDH ratio of the striatum was not statistically significant ($p = 0.4703$).

Table 2 Specific binding ratios of striatal and extrastriatal areas

Group	*n*	Striatal area	Extrastriatal area
A	6	1.1420 ± 0.1396	0.2470 ± 0.0615
B	6	0.9721 ± 0.0825	0.2394 ± 0.0282
C	5	1.025 ± 0.1231	0.2416 ± 0.0472
D	6	0.7337 ± 0.1899	0.2053 ± 0.0617

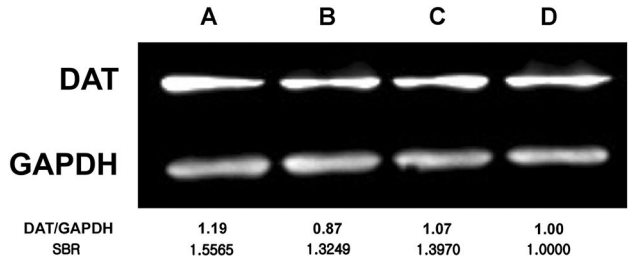

Fig. 4 Western blot of DAT

Discussion

In this study, both fasting and anesthesia with isoflurane during uptake time affected striatal DAT availability measured by PET in rats, suggesting that researchers need to be cautious to handle the animals for investigating DAT availability by PET.

The mechanism of action of inhalation agents can be explained in several ways. In the macroscopic level, inhalation agents globally decrease cerebral blood flow (CBF) and glucose metabolism, especially in thalamus and midbrain [9]. In the synaptic level, inhalation agents inhibit excitatory presynaptic channel activity and augment inhibitory post-synaptic channel activity [9]. In the molecular level, inhalation agents prolonged the $GABA_A$ receptor-mediated inhibitory Cl^- current, which inhibits post-synaptic neuronal excitability [9]. Also, inhalation agents enhance the activity of two-pore domain potassium channels result in hyperpolarization of the plasma membrane [9].

CBF could be measured by various methods including xenon-enhanced computed tomography, single photon emission computed tomography (SPECT), PET, magnetic resonance imaging, and transcranial Doppler ultrasonography [10]. SPECT is one of the most commonly used imaging modality to measure CBF [10]. As ^{99m}Tc-ECD and ^{99m}Tc-HMPAO can be accumulated in the entire brain through the blood brain barrier, they can be used for evaluating

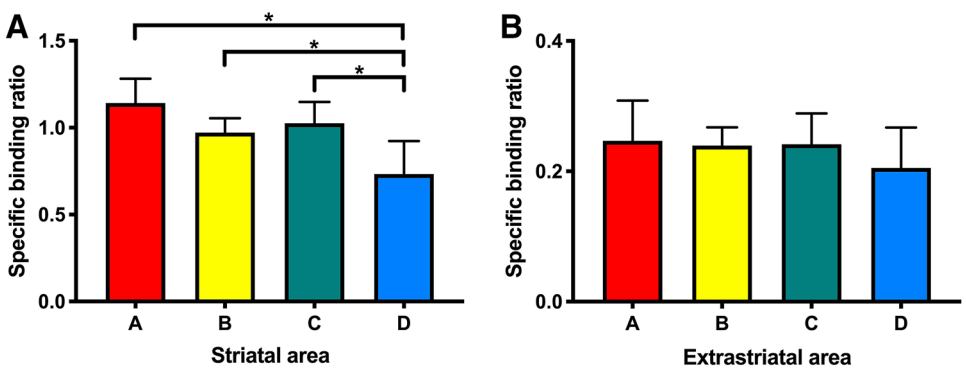

Fig. 3 Comparison of specific binding ratios of **a** striatal and **b** extrastriatal areas (*$p < 0.05$)

distribution of regional CBF [11]. However, as tracer activity is subject to K1 which is the multiplication of CBF and extraction, because of limited first-pass extraction fraction of ^{99m}Tc-ECD and ^{99m}Tc-HMPAO, brain SPECT using ^{99m}Tc-labeled CBF tracer generally underestimate true CBF [11].

Neuronal activity relates with metabolism and blood flow of the brain [12]. However, as volatile anesthetics including isoflurane have a dose-dependent direct vasodilating effect, the level of decreased CBF which is due to the indirect extrinsic constricting effect of volatile anesthetics by decreased metabolism is limited, resulting in increased CBF in higher concentration of isoflurane [12, 13]. Previous studies reported that isoflurane increased blood flow of striatum in human and animal [14–16]. Thus, there is a chance that increased CBF of striatum by isoflurane might affect the DAT availability of SD rat striatum. Further study regarding DAT availability and blood flow should be performed. Previously, Ori et al. showed that local cerebral glucose utilization of rat brain was decreased in all cortical regions and increased in CPu with an autoradiography [17], while Lenz et al. observed the decreased glucose utilization in CPu [18]. In addition, Spangler-Bickell et al. reported that isoflurane does not have an effect on striatum of rats [19], consistent with the study by Matsumura et al. that no significant change of glucose metabolism in striatum was visualized after anesthesia with isoflurane [20]. Regarding with DAT, anesthesia with isoflurane either increases [21], or decreases [22] the DAT availability. After anesthesia with isoflurane, internalization of DAT from plasma membrane to cytoplasmic space was observed, while the total amount of DAT was not changed [22, 23]. This inconsistency might be due to either the difference in animals or the concentration of inhaled isoflurane. In post-hoc analysis of this study, striatal SBR of group D (without fasting, without anesthesia) was lower than that of group C (without fasting, with anesthesia), indicating the effect of anesthesia with isoflurane on DAT availability. In addition, although the statistical difference was not significant between group A (with fasting, with anesthesia) and B (with fasting, without anesthesia), mean SBRs of group A showed the trend of higher values than those of group B, consistent with the results of Western blot.

Acute fasting and chronic food restriction is also linked with the dopaminergic system of brain [6]. The microdialysis study reported the decrease of extracellular striatal dopamine after fasting [24]. With ^{125}I-RTI-121, no change of striatal DAT binding was observed after fasting, while DAT mRNA was increased in the ventral tegmental area [25]. On the contrary, feeding induced insulin can act on the insulin receptors to amplify the dopamine uptake by DAT through the PI3 kinase signaling pathway which enhances the surface expression of DAT, and also the release of dopamine in the striatum [26, 27]. However, in post-hoc analysis of this study, striatal SBR of group D (without fasting, without anesthesia) was lower than that of group B (with fasting, without anesthesia), indicating the effect of fasting on DAT availability. As rats in group B were fasted for over 12 h, and those in group D could access the chew before scan (ad libitum feeding), factors other than insulin may contribute to these differences between group B and group D, such as reward system regarding satiety, and taste. Probably, injection of glucose by tail vein other than ad libitum feeding might result in changes of DAT availability differently.

In this study, although SBRs were not completely statistically different between each group according to anesthesia and fasting, the application of anesthesia and fasting showed the different tend of SBRs. Moreover, even though the correlation between DAT/GAPDH and SBR was not significant, the different tend was similar between them. These results might suggest true change of DAT in presynaptic dopaminergic neuron. Further studies are needed to validate these results.

There are several limitations in this study. First, a small number of animals was included in this study. Second, although we cautiously injected radiotracer into the tail vein, injection quality was not always consistent. Third, we analyzed striatal area as a whole rather than each of CPu and NAc. Fourth, we perform the static scans instead of dynamic scans. As semi-quantitative analysis of PET image is known to inferior than binding potential estimation using kinetic analysis with dynamic PET scan [28], this might affect the result. Further studies are needed to investigate the effects of animal handling on DAT availability.

In conclusion, striatal DAT availability can be affected by both fasting and anesthesia with isoflurane in small animal imaging. Therefore, animal studies should be designed with caution.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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