

# Combination of *In Vivo* [<sup>123</sup>I]FP-CIT SPECT and Microdialysis Reveals an Antipsychotic Drug Haloperidol-induced Synaptic Dopamine Availability in the Rat Midbrain and Striatum

So Hyeon Park<sup>1</sup>, Yoo Sung Song<sup>2</sup>, Byung Seok Moon<sup>3</sup>,  
Byung Chul Lee<sup>2</sup>, Hyun Soo Park<sup>1,2\*</sup> and Sang Eun Kim<sup>1,2,4\*</sup>

<sup>1</sup>Department of Transdisciplinary Studies, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 08826, <sup>2</sup>Department of Nuclear Medicine, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seoul 03080, <sup>3</sup>Department of Nuclear Medicine, Ewha Woman's University Seoul Hospital, Ewha Womans University College of Medicine, Seoul 07804, <sup>4</sup>Advanced Institutes of Convergence Technology, Suwon 16229, Korea

Synaptic dopamine (DA) is mainly regulated by the presynaptic DA transporter (DAT). Single-photon emission computerized tomography (SPECT) with the DAT radiotracer [<sup>123</sup>I]FP-CIT assesses changes in synaptic DA availability when endogenous DA displaces [<sup>123</sup>I]FP-CIT or competes for DAT. Here, we investigated the effects of haloperidol (HAL) and clozapine (CLZ) on [<sup>123</sup>I]FP-CIT binding in the rat striatum and midbrain to assess the utility of [<sup>123</sup>I]FP-CIT SPECT to quantify changes in synaptic DA availability. Rats underwent [<sup>123</sup>I]FP-CIT SPECT after intraperitoneal administration of normal saline (vehicle), HAL (1 and 7 mg/kg), CLZ (10 and 54 mg/kg) and bupropion (BUP, a DAT blocker, 20 and 100 mg/kg). In the striatum and midbrain, percent differences in the nondisplaceable binding potential (BP<sub>ND</sub>) of [<sup>123</sup>I]FP-CIT compared to the vehicle were calculated for the various drugs and doses. In another experiment, changes in endogenous striatal DA concentration were measured by *in vivo* microdialysis under the conditions used in the SPECT study. BUP dose-dependently occupied DAT at considerable levels. Compared to the vehicle, HAL decreased [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> in the striatum (-25.29% and -2.27% for 1 and 7 mg/kg, respectively) and to a greater degree in the midbrain (-58.74% and -49.64% for 1 and 7 mg/kg, respectively), whereas the CLZ-treated group showed a decrease in the midbrain (-38.60% and -40.38% for 10 and 54 mg/kg, respectively) but an increase in the striatum (18.85% and 38.64% for 10 and 54 mg/kg, respectively). Antipsychotic-induced changes in endogenous striatal DA concentrations varied across drugs and doses. The data demonstrate that [<sup>123</sup>I]FP-CIT SPECT may be a useful preclinical technique for detecting increases in synaptic DA availability in the midbrain and striatum in response to HAL, with results comparable to those of *in vivo* microdialysis.

**Key words:** [<sup>123</sup>I]FP-CIT SPECT, Dopamine availability, Haloperidol, Clozapine, *In vivo* microdialysis

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\*To whom correspondence should be addressed.  
Sang Eun Kim, TEL: 82-31-787-7671, FAX: 82-31-787-4018  
e-mail: kse@snu.ac.kr  
Hyun Soo Park, TEL: 82-31-787-2936, FAX: 82-31-787-4018  
e-mail: hyuns@snu.ac.kr

## INTRODUCTION

Synaptic dopamine (DA) availability is implicated in the pathology of various neurological and psychiatric diseases, such as Parkinson's disease [1, 2] and schizophrenia [3, 4]. DA availability in the brain is mainly regulated by the DA transporter (DAT) [5], and DA receptors for its D2 and D3 subtypes expressed on the cell membrane of DA neurons (i.e., autoreceptors) also play a key role in regulating the activity of DAergic neurons and controlling DA

synthesis, release, and reuptake [6]. Under the DA hypothesis, antipsychotic drugs act mainly by regulating the DAergic systems, as evidenced by the significant association between DA receptor antagonism and improved positive and/or negative symptoms. Some of them have been proven to regulate DA release [6-8]. An antipsychotic drug haloperidol (HAL) is known to increase DA synthesis and release in the striatum and related mesolimbic structures [9-12]. The proposed mechanism of action consists of presynaptic terminal blockade by DA autoreceptors [11], which abolishes feedback inhibition, leading to enhanced DA synthesis or release. Clozapine (CLZ), despite acting as a DA antagonist as part of its therapeutic effect against schizophrenia symptoms, also stimulates DA release in the ventral striatum (i.e., nucleus accumbens) of rats [13] and in the hippocampus of schizophrenia patients [14]. Presynaptic modulation induced by antipsychotic drugs affects the extracellular DA concentration by altering autoregulation [15]. An increased extracellular DA concentration can lead to stimulation of DA autoreceptors, which inhibits DA release [6, 16]. DA release and metabolism in the rat striatum *in vivo* were reported to be differentially modulated by typical and atypical antipsychotic drugs, e.g., HAL and CLZ, respectively [17, 18]. HAL and CLZ may have different effects on the release of DA according to their pharmacological characteristics and whether they are typical or atypical antipsychotics.

The competition between endogenous transmitters and radio-labeled ligands for *in vivo* binding to receptors and transporters may provide a method to measure endogenous neurotransmitter release in the living central nervous system with noninvasive molecular imaging techniques from nuclear medicine. This method is valuable for studying altered neuronal activity in anatomical and chemical systems in several neurologic and psychiatric conditions and for assessing the regulatory effect of drugs on their target systems. [<sup>123</sup>I]N-omega-fluoropropyl-2-beta-carbomethoxy-3-beta-(4-iodophenyl)nortropane ([<sup>123</sup>I]FP-CIT) single-photon emission computerized tomography (SPECT) is used in scientific studies in humans and is also used in preclinical studies to assess the integrity of the nigrostriatal system in the diagnosis of Parkinson's disease [19-21] and schizophrenia [22, 23]. [<sup>123</sup>I]FP-CIT SPECT is increasingly used for diagnostics and drug development for neurological and psychiatric diseases as well as for precision medicine in cases of complicated medication status—for instance, a Parkinson's disease patient receiving antipsychotic drugs. Consequently, there is a need for further experimental evidence supporting the utility of [<sup>123</sup>I]FP-CIT SPECT for quantitation of changes in DA availability. For example, it remains controversial whether changes in synaptic DA availability induced by acute treatment with antipsychotic drugs such as HAL can be quantitatively assessed by

[<sup>123</sup>I]FP-CIT SPECT. In one study, DAT imaging with [<sup>123</sup>I]FP-CIT SPECT revealed acute changes in DAT activity with a 25% baseline reduction in the specific-to-nonspecific binding ratio at equilibrium ( $V_3''$ ) of [<sup>123</sup>I]FP-CIT after acute HAL administration (1 mg/kg *i.p.*), possibly because the increased levels of endogenous DA displaced [<sup>123</sup>I]FP-CIT or competed for presynaptic binding sites [24]. However, based on results reproduced from this experiment, another investigator argued that synaptic changes in DA levels resulting from acute HAL administration were not detectable using storage phosphor imaging with [<sup>123</sup>I]FP-CIT in rats [25] because the dose was too low to alter [<sup>123</sup>I]FP-CIT binding.

We investigated differences in the nondisplaceable binding potential of [<sup>123</sup>I]FP-CIT in the rat striatum and midbrain, which compose the nigrostriatal DA system, after HAL and CLZ compared to vehicle treatment in an attempt to assess the validity of [<sup>123</sup>I]FP-CIT SPECT as a measure of synaptic DA availability induced by HAL and CLZ. The reliability of this noninvasive imaging technique was further examined for the striatum by conducting an *in vivo* microdialysis study that directly measured the changes in DA concentration; this method, having been proposed as a measure of the magnitude of DA release, was selected for the important task of investigating the relationship between antipsychotic drug-induced DA release and [<sup>123</sup>I]FP-CIT displacement. We will discuss the combined results of the [<sup>123</sup>I]FP-CIT SPECT study (differences in [<sup>123</sup>I]FP-CIT binding) and the *in vivo* microdialysis study (changes in DA concentration).

## MATERIALS AND METHODS

### Animals and drugs

This study was approved by the Institutional Animal Care and Use Committee of the Seoul National University Bundang Hospital. Animals were purchased from Orient Bio Inc., Seoul, Korea. A total of 35 and 20 Sprague Dawley (SD) rats (male, 6-week-old, 260~300 g body weight) were used for [<sup>123</sup>I]FP-CIT SPECT and *in vivo* microdialysis studies, respectively. The animals were physiologically acclimated for one week in a clean room after arriving (12 h/12 h light/dark cycle, temperature 20~25°C, relative humidity 40~60%). The rats were fasted for at least 6 h before the [<sup>123</sup>I]FP-CIT SPECT study but were provided water *ad libitum*.

HAL, CLZ and bupropion (BUP) hydrochloride were purchased from Sigma-Aldrich Korea, Yongin, Korea. HAL and CLZ were dissolved in 1% tartaric acid and 1 N HCl. A DAT blocker, BUP, the positive control drug for HAL and CLZ, was used to test whether endogenous DA displaces [<sup>123</sup>I]FP-CIT or BUP competes with [<sup>123</sup>I]FP-CIT for DAT. BUP was dissolved in NS.

In the [<sup>123</sup>I]FP-CIT SPECT study, the animals were divided into

four groups defined by different drugs (vehicle (n=5), HAL (n=10), CLZ (n=10), BUP (n=10) treatment groups). While the vehicle-treated group had no dose conditions, the others had low and high dose conditions for the drug treatment. The low and high doses of HAL, CLZ and BUP were 1 and 7 mg/kg body weight, 10 and 54 mg/kg body weight, and 20 and 100 mg/kg body weight, respectively (n=5 per drug and dose condition). The doses were selected based on previous studies showing that low and high doses induce ~25% increases in synaptic DA availability and greater than 80% DA receptor occupancy by drugs in the striatum, respectively [24-27].

In another experiment, changes in endogenous DA concentration in the striatum were monitored by *in vivo* microdialysis under the same conditions as in the [<sup>123</sup>I]FP-CIT SPECT study (n=5 per drug and dose condition).

In both experiments, drugs were injected intraperitoneally (*i.p.*) while the animals were awake.

#### [<sup>123</sup>I]FP-CIT SPECT/CT study

The [<sup>123</sup>I]FP-CIT SPECT/CT study was performed on a dedicated small-animal SPECT/CT system (NanoSPECT/CT, Mediso Inc., Budapest, Hungary). Helical small-animal SPECT scans were performed using a 4-head  $\gamma$ -camera outfitted with multipinhole collimators (1.4-mm-diameter pinholes) designed for rats. [<sup>123</sup>I]FP-CIT was injected at a dose (mean $\pm$ SD) of 39.5 $\pm$ 7.2 MBq 1 h after drug treatment; 2 h later (once [<sup>123</sup>I]FP-CIT had reached equilibrium in the striatum), SPECT/CT data were acquired from the animals for 30 min under 2% isoflurane anesthesia. After the scan, the SPECT data were reconstructed using iterative three-dimensional ordered subset expectation maximization with the single-slice rebinning method. CT-based attenuation correction was performed, as were scatter and random correction. The reconstructed images were 176 $\times$ 176 $\times$ 136 pixels with a voxel size of 0.6 $\times$ 0.6 $\times$ 0.6 mm (x, y, z). PMOD software (PMOD Technologies LLC., Geneva, Switzerland) was used for processing and analysis of SPECT and CT images. Images were spatially normalized to standard stereotaxic space with the predefined magnetic resonance imaging (MRI) rat brain template. The striatum, midbrain and cerebellum were defined using automated anatomical labeling embedded in PMOD software [28]. Synaptic DA availability in the striatum and midbrain was quantitatively assessed in terms of the nondisplaceable binding potential (BP<sub>ND</sub>) of [<sup>123</sup>I]FP-CIT, which is proportional to the density of available binding sites (i.e., DAT). The cerebellum (which is known as a DAT-poor region or nondisplaceable binding site of [<sup>123</sup>I]FP-CIT) was set as the reference region for estimating BP<sub>ND</sub> according to the following equation:  $BP_{ND} = (C_T - C_{ND}) / C_{ND} = C_T / C_{ND} - 1$ , where  $C_T$  and  $C_{ND}$  are the con-

centrations of [<sup>123</sup>I]FP-CIT in the striatum or midbrain and in the cerebellum, respectively.

#### *In vivo* microdialysis study

Extracellular DA concentrations in the striatum of freely moving rats were directly measured by *in vivo* microdialysis following HAL and CLZ treatment. Rats were anesthetized with a mixture of Alfaxan (0.3 ml/g, *i.p.*) and Rompun (0.05 ml/g, *i.p.*). A guide cannula (CMA/12; CMA Microdialysis, Solna, Sweden) was stereotaxically implanted such that it terminated in the center of the striatum (anteroposterior: 1.0 mm; lateral: 3.2 from the bregma; height: 3.0 from the dura) [29] and attached to the skull using skull screws and dental cement. The cannula was then closed with a tight-fitting stainless-steel obturator. Following 3~4 days of recovery, a 4-mm microdialysis probe (CMA/12; CMA Microdialysis) connected via a dual liquid swivel to a syringe pump was inserted into the guide cannula and perfused with artificial cerebrospinal fluid (Harvard Apparatus, Cambridge, UK) at a constant rate of 1.5 ml/min. In order to monitor changes in extracellular DA concentration on the same time scale as the [<sup>123</sup>I]FP-CIT SPECT study, dialysate samples were collected at 20-min intervals for 1 h at baseline and 3 h after drug treatment via an outlet tube connected to a microfraction collector (CMA Microdialysis). The dialysate (injection volume of 15  $\mu$ L) was assayed for DA by high-performance liquid chromatography (HPLC) with an API 4000QTRAP detection system (ABSCIEX, Foster City, CA, USA). The mobile phase consisted of 0.1% formic acid in distilled water and HPLC-grade acetonitrile. A Luna hydrophilic interaction liquid chromatography (HILIC) column (3- $\mu$ m particle size, 2.0 $\times$ 50 mm) was used, and the flow rate of the system was 0.25 ml/min. The DA level in dialysates is expressed as a percentage of the three baseline samples collected immediately before drug treatment. For the analysis, only results derived from healthy rats with correctly positioned dialysis probes were included in the data analysis. The mean DA level across the 3 baseline timepoints was designated as 0% at 0 min.

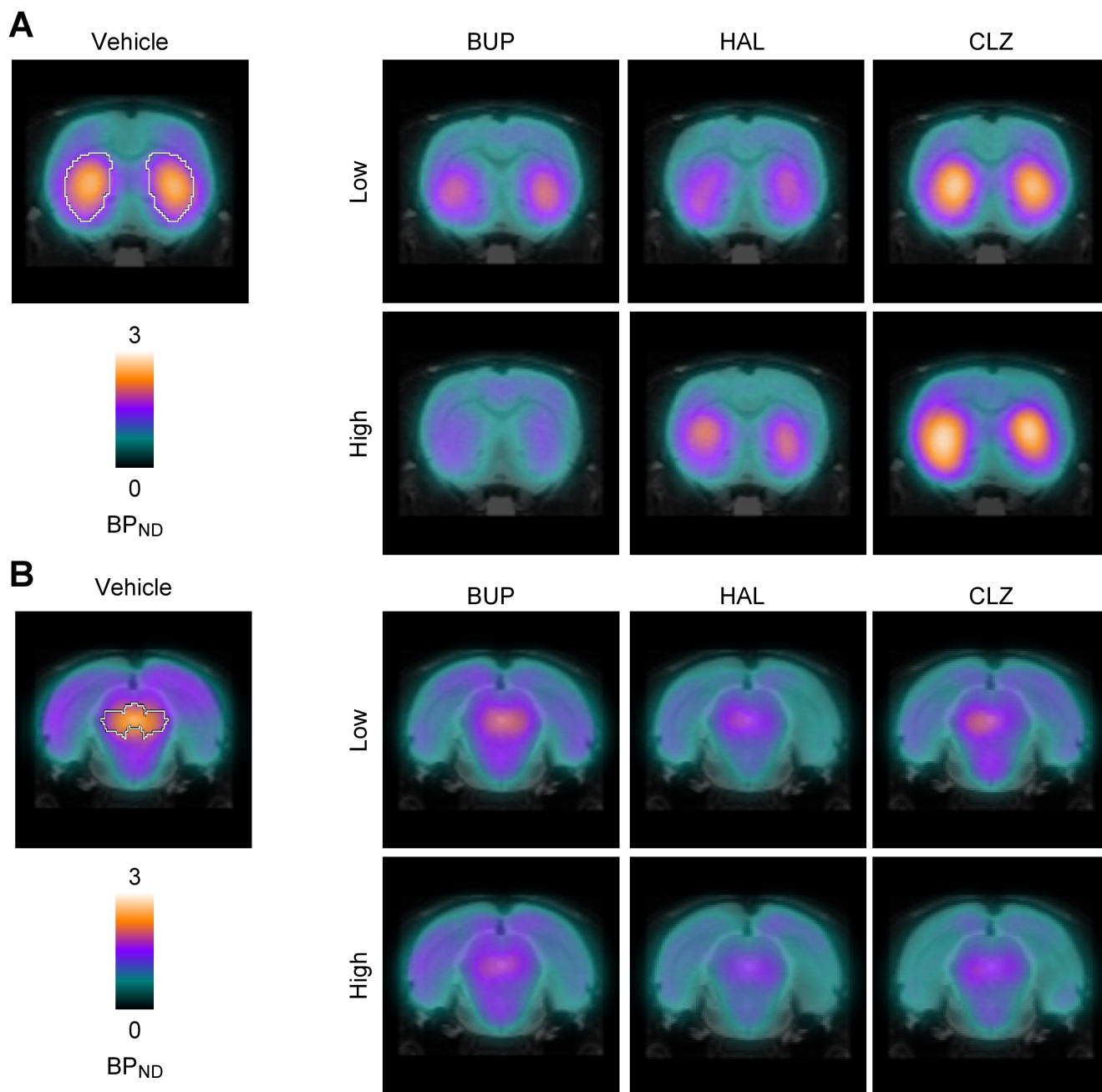
#### Statistical analysis

Data were analyzed using GraphPad Prism (version 7.0, GraphPad Software Inc., La Jolla, CA, USA). The statistical significance of differences in mean BP<sub>ND</sub> between drug and dose conditions was tested by two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test between dose conditions for each drug. *In vivo* microdialysis data were statistically tested with repeated-measures ANOVA, followed by Dunnett's multiple comparison test for the factor of time.  $p < 0.05$  was considered statistically significant. Values are represented as the mean $\pm$ SEM.

RESULTS

In the vehicle-treated group, the mean [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> was 1.64±0.13 and 1.69±0.32 in the striatum and midbrain, respectively; these values are comparable to the results of previous studies [24, 30]. BUP dose-dependently occupied DAT to a considerable

degree, as evidenced by decreases in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> of -16.50% (20 mg/kg) and -56.29% (100 mg/kg) in the striatum and -31.57% (20 mg/kg) and -53.08% (100 mg/kg) in the midbrain, implying that [<sup>123</sup>I]FP-CIT SPECT is a reliable and sensitive technique for measuring drug-induced changes in DAT activity and can conceptually allow the assessment of changes in synaptic DA availability



**Fig. 1.** Group mean (n=5 per drug and dose condition) BP<sub>ND</sub> parametric images comparing changes in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> in the striatum (A) and midbrain (B) after treatment with BUP, HAL and CLZ. These changes are also summarized in graphs (C). VEH, BUP, HAL and CLZ represent the vehicle-, bupropion-, haloperidol- and clozapine-treated groups. The low and high doses of HAL, CLZ and BUP were 1 and 7 mg/kg body weight, 10 and 54 mg/kg body weight, and 20 and 100 mg/kg body weight, respectively. Values are the mean±SEM. White lines overlaid on the SPECT images of the striatum (A) and midbrain (B) for the vehicle-treated group show the margin of each region.

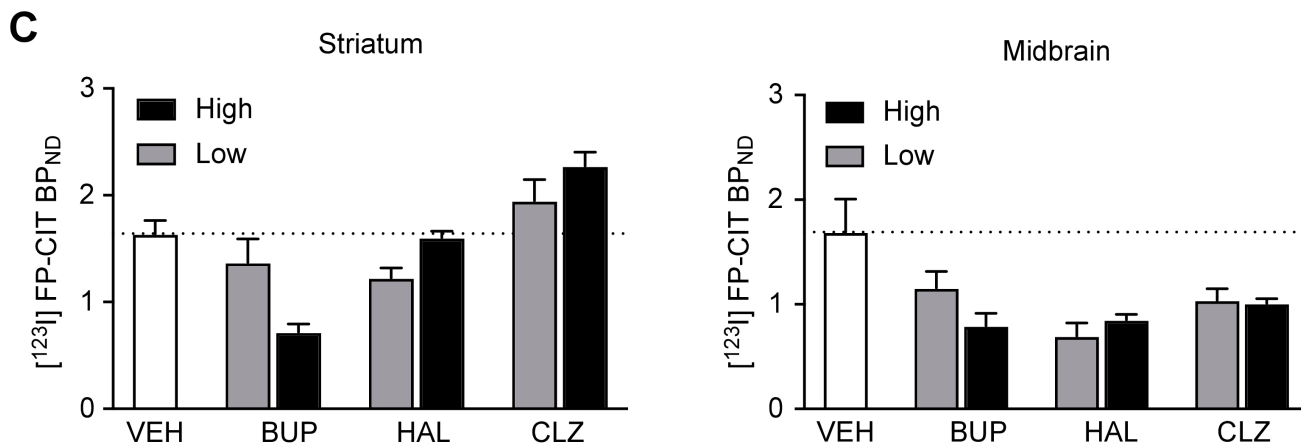


Fig. 1. Continued.

Table 1. Differences in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub>

Drug	Dose (mg/kg)	Striatum			Midbrain		
		BP <sub>ND</sub>	% Difference	p	BP <sub>ND</sub>	% Difference	p
VEH	-	1.64±0.13	-	-	1.69±0.32	-	-
BUP	20	1.37±0.22	-16.50±13.41	0.3954	1.16±0.16	-31.57±9.26	0.2165
	100	0.72±0.08	-56.29±4.59	0.0006	0.79±0.12	-53.08±7.21	0.0376
HAL	1	1.22±0.09	-25.29±5.60	0.0749	0.70±0.12	-58.74±7.38	0.0041
	7	1.60±0.06	-2.27±3.65	0.9797	0.85±0.05	-49.64±3.13	0.0215
CLZ	10	1.95±0.20	18.85±12.00	0.2257	1.04±0.11	-38.60±6.53	0.0668
	54	2.27±0.13	38.64±7.87	0.0061	1.01±0.05	-40.38±2.76	0.0676

Values are the mean±SEM. VEH, vehicle; BUP, bupropion; HAL, haloperidol; CLZ, clozapine.

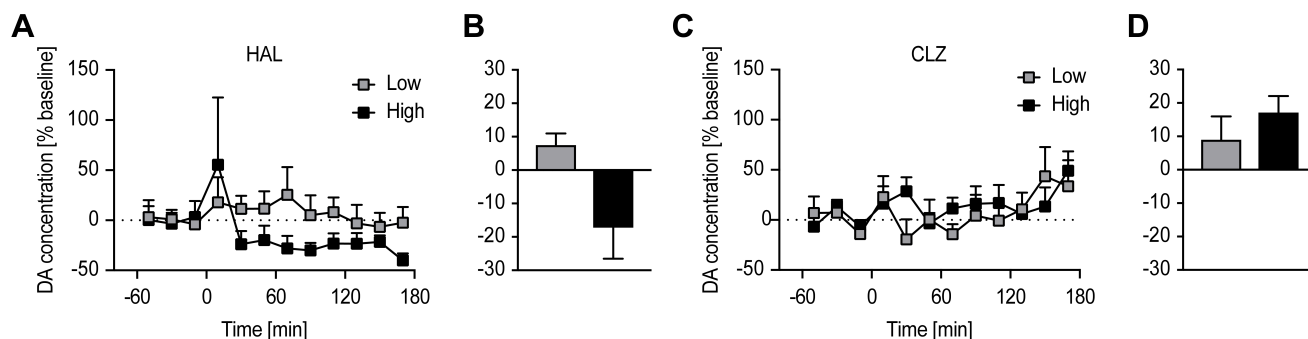
*in vivo* when endogenous DA displaces [<sup>123</sup>I]FP-CIT or competes for DAT.

Treatment with HAL and CLZ markedly altered synaptic DA availability compared to the vehicle, as evidenced by changes in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> in both the striatum and the midbrain. Intriguingly, the level of changes in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> treatment varied across drugs, doses, and regions (Fig. 1 and Table 1). Compared to the vehicle, HAL decreased [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> in the striatum (-25.29% and -2.27% for 1 and 7 mg/kg, respectively) and to a greater degree in the midbrain (-58.74% and -49.64% for 1 and 7 mg/kg, respectively), whereas the CLZ-treated group showed an increase in the striatum (18.85% and 38.64% for 10 and 54 mg/kg, respectively) but a decrease in the midbrain (-38.60% and -40.38% for 10 and 54 mg/kg, respectively).

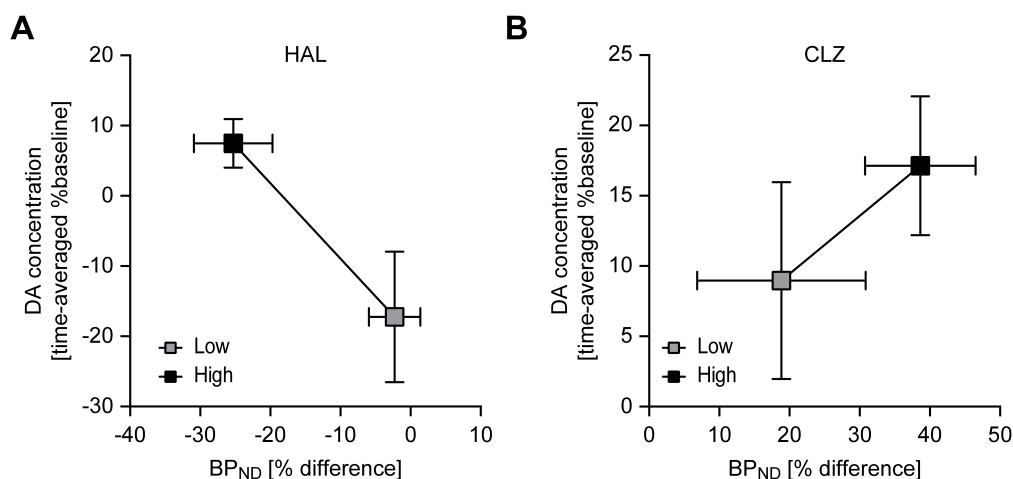
The changes in extracellular striatal DA concentrations by HAL (1 and 7 mg/kg) and CLZ (10 and 54 mg/kg) treatment were evaluated by *in vivo* microdialysis (Fig. 2). Despite the lack of statistical significance in the entire dataset by repeated-measures ANOVA, immediately after the treatment, a low dose of HAL (1 mg/kg) induced a 17.96%±24.90% increase in DA concentration

compared to the baseline, after which DA returned to the baseline level during the study; in contrast, a high dose of HAL (7 mg/kg) induced a decrease in DA concentration that was preceded by a maximal 55.36%±67.22% baseline increase immediately after the treatment (Fig. 2A). We smoothed the temporal changes by using the time-averaged % changes. The time-averaged % change, which also has unit of % baseline, was calculated by averaging the % baseline values of individual dialysate samples along the axis of time, from the time of drug treatment to the end of the study. Overall % baseline values were summed and divided by the number of samples regardless of the time of measurement. The time-averaged percentages of change from baseline for the low and high doses of HAL were 7.48±3.48 and -17.24±9.29% baseline, respectively, corresponding to changes in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> (Fig. 2B). Both doses of CLZ resulted in increased DA concentrations, but the time lag was longer than that of HAL, as the maximal changes appeared late in the measurement period, with 43.53±29.21% and 49.10±19.25% increases from baseline, respectively, whereas immediately after the treatment, the changes from baseline were 22.82±10.70% and 16.26±27.52% for 10 and 54 mg/kg CLZ, respectively (Fig. 2C).





**Fig. 2.** Time course of the effects of HAL (A) and CLZ (C) treatment on DA release in the rat striatum (n=5 per drug and dose condition). Time-averaged (from the time of drug treatment to the end of the study) percentage changes from baseline for each dose of HAL (B) and CLZ (D). HAL and CLZ represent the haloperidol- and clozapine-treated groups. The low and high doses of HAL and CLZ were 1 and 7 mg/kg body weight and 10 and 54 mg/kg body weight, respectively. The value is designated as 0% at time 0, and drugs were treated at time=0 min. Values are the mean±SEM.



**Fig. 3.** Relationship between percentage differences in [<sup>123</sup>I] FP-CIT BP<sub>ND</sub> (x-axis) and time-averaged percentage changes from baseline extracellular DA concentration (y-axis) after treatment with varying doses of HAL and CLZ. HAL and CLZ represent the haloperidol- and clozapine-treated groups. The low and high doses of HAL and CLZ were 1 and 7 mg/kg body weight and 10 and 54 mg/kg body weight, respectively. Values are the mean±SEM.

The time-averaged percentage changes from baseline for 10 and 54 mg/kg CLZ were 8.98±7.00% and 17.13±4.94%, respectively (Fig 2D).

The relationships between percentage differences in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> and time-averaged percentage changes from baseline extracellular DA concentration after treatment with varying doses of HAL and CLZ are depicted in Fig. 3 (A and B for HAL and CLZ, respectively). An inverse relationship between percentage differences in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> and time-averaged % percentage change from baseline extracellular DA concentration (lower [<sup>123</sup>I]FP-CIT BP<sub>ND</sub>, greater synaptic DA availability) appeared only in the HAL-treated group.

**DISCUSSION**

Under the investigational assumption that if endogenous DA displaces radioligands or competes with them for presynaptic binding sites, radioligand binding to the DAT could be affected, we

investigated differences in the [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> in the rat striatum and midbrain, which compose the nigrostriatal DA system, after HAL and CLZ treatments that increased synaptic DA availability. The reliability of this noninvasive imaging technique was further examined for the striatum by using an *in vivo* microdialysis study that directly measured the changes in DA concentration induced by HAL and CLZ.

Our results showed that [<sup>123</sup>I]FP-CIT SPECT allows the detection of apparent increases in synaptic DA concentration induced by low (1 mg/kg *i.p.*) and high doses (7 mg/kg *i.p.*) of HAL in both the striatum and midbrain in terms of reduced [<sup>123</sup>I]FP-CIT BP<sub>ND</sub>. The [<sup>123</sup>I]FP-CIT SPECT results for the striatum were compatible with those obtained in the *in vivo* microdialysis study. For instance, elevated extracellular DA concentration corresponded to decreased or increased [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> in the striatum induced by 1 or 7 mg/kg HAL, respectively (Fig. 3A). Although the high dose (7 mg/kg) of HAL unexpectedly induced an 18% decrease from baseline in striatal DA concentration determined by *in vivo* microdialysis,

this change corresponded to an elevation in [ $^{123}$ I]FP-CIT BP<sub>ND</sub>. The decreased DA concentration after the high-dose (7 mg/kg) HAL treatment may be attributable to desensitization of autoreceptors for the DA D2 and DA D3 subtypes and the consequent loss of feedback excitation by DA or to activation of monoamine oxidase B (MAO-B) to maintain the homeostasis of DAergic neurotransmission. MAO plays a major role in the inactivation of multiple catecholamines, including DA, and synaptic DA is mainly metabolized by MAO-B in the brain [31]. The 7 mg/kg HAL was high enough to abnormally increase synaptic DA concentration in rats and may cause MAO activation. One study demonstrated that HAL inhibited DA release in the striatum and in the nucleus accumbens using *in vivo* electrochemical techniques and provided evidence for the induction of a depolarization block of DA cell firing as a possible mechanism underlying this effect [32, 33].

The [ $^{123}$ I]FP-CIT SPECT and *in vivo* microdialysis techniques detected changes in striatal synaptic DA availability and extracellular DA concentrations induced by CLZ treatment. However, CLZ reduced [ $^{123}$ I]FP-CIT BP<sub>ND</sub> in the midbrain while increasing the value in the striatum. Moreover, direct measurements of elevated striatal DA concentrations (8.98±7.00% and 17.13±4.94% increases from baseline induced by low and high doses of CLZ, respectively) did not affect [ $^{123}$ I]FP-CIT BP<sub>ND</sub>. The discrepancy shown here as increases in both [ $^{123}$ I]FP-CIT BP<sub>ND</sub> and DA concentration after CLZ treatment was unexpected (Fig. 3B), although we expected that the degree of change in [ $^{123}$ I]FP-CIT BP<sub>ND</sub> in the brain would differ between HAL and CLZ since their binding targets and temporal behavior with respect to the target were distinct. This may be due to differences in the pharmacodynamics profiles of CLZ, time course of drug action at the target site, and experimental protocol (time window) in the [ $^{123}$ I]FP-CIT SPECT scan. The results from the *in vivo* microdialysis study showed that the increases in extracellular DA concentration induced by CLZ follow distinct time courses from the increases induced by HAL. The maximum striatal DA concentration was reached between 60 and 80 min after treatment with HAL, whereas CLZ showed a more prolonged effect on releasing DA. In the present study, HAL and CLZ were administered 30 min before [ $^{123}$ I]FP-CIT application. Thus, the radioligand was administered before attaining the maximum striatal DA concentration induced by HAL but after reaching that induced by CLZ. Since [ $^{123}$ I]FP-CIT—which requires 2 h to reach a state of equilibrium [34]—was injected 30 min after antipsychotic drug treatment, the time course of the drug action may have been missed. Thus, it is possible that the time window for [ $^{123}$ I]FP-CIT detection did not cover the maximal DA concentration induced by CLZ treatment, leading to an error in the measurement of [ $^{123}$ I]FP-CIT BP<sub>ND</sub>. The elevation in [ $^{123}$ I]FP-CIT BP<sub>ND</sub>

following CLZ treatment could instead represent a decrease in DA availability in the recovery phase of drug action. Although our data did not provide evidence supporting previous explanations (i.e., the DA concentration during the late phase was increased rather than decreased), this outcome could be due to variations between datasets acquired from different animals.

Although it is possible that there were experimental errors and unknown contamination factors that could have led to these results, the magnitude of error in the measured values (BP<sub>ND</sub> and DA concentration) was quite acceptable. The reliability of increased striatal [ $^{123}$ I]FP-CIT BP<sub>ND</sub> could be validated from decreases in the midbrain of the same subject on CLZ treatment. In the midbrain, non-dose-dependent decreases in [ $^{123}$ I]FP-CIT binding after treatment were consistently shown among drugs (although the DAT blocker BUP occupied DAT dose-dependently). Regardless of dose, the %difference was greater for HAL (-49.64~58.74% difference) than CLZ (-38.60 to -40.38% difference) in accordingly with pharmacologic characteristics in DA regulation of HAL and CLZ, implying their own typicality. That CLZ affects increasing binding affinity to DAT is unlikely, but it is not impossible, as some ligands occasionally act in this way. For example, an antiepileptic drug, tiagabine, which binds to the central benzodiazepine receptor, affects the increased binding affinity of radiolabeled ligand ([ $^{18}$ F] flumazenil) to the central benzodiazepine receptor, known as the “GABA shift” [35]. On the other hand, the interaction between CLZ and [ $^{123}$ I]FP-CIT could also be considered. The potential effect of CLZ on the group of antipsychotic drugs was examined; for instance, only CLZ induced a decrease in the protein kinase C level [36], but the effect of decreased protein kinase C level and interaction between [ $^{123}$ I]FP-CIT and DAT is unexpected.

In addition, this study examined changes in synaptic DA availability after acute administration of HAL and CLZ in an attempt to resolve the previously reported controversy [12, 13] through an analogous approach to the experimental paradigm and to determine whether [ $^{123}$ I]FP-CIT SPECT can be used to assess changes in endogenous DA concentration based on alterations in [ $^{123}$ I]FP-CIT BP<sub>ND</sub> to DAT as it is displaced by endogenous DA. Importantly, we partly overcame the experimental limitations of the earlier studies by performing *in vivo* microdialysis to eliminate technical (imaging vs. direct measurement) and conceptual (changes in DAT radiotracer binding potential vs. DA concentration) differences in our measurements and by including additional experimental variables such as another drug that has a common mode of action in increasing synaptic DA concentrations, varying doses of the drug, and multiple brain regions implicated in DAergic neurotransmission. These observations raised concerns about the validity of these measurements as an indicator of DA release. Both

studies not only used HAL (1 mg/kg, *i.p.*) alone but also lacked a means of directly measuring DA concentration in the striatum. We proposed that changes in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> in the midbrain region should also be assessed to clarify changes in the activity of nigrostriatal DA projections in response to antipsychotic drug treatment.

DAT imaging is increasingly used for diagnostics and drug development for neurological and psychiatric diseases as well as for precision medicine in cases of complicated medication status—for instance, a Parkinson's disease patient receiving antipsychotic drugs. Consequently, there is a need for further experimental evidence supporting the utility of [<sup>123</sup>I]FP-CIT SPECT for quantitation of acute changes in available DA. Recently, there has been growing interest in assessing DAT binding in schizophrenia patients. However, the results obtained to date on striatal DAT binding in schizophrenia subjects have been inconsistent, with reports of elevated [37, 38], reduced [22, 38, 39], or unaltered [23, 40–44] DAT binding. Interestingly, unaltered [43], decreased [22, 39], or increased [37] DAT binding has also been observed in medicated patients. These results are difficult to interpret because factors such as illness duration and phase—which could vary between patients and investigations—are likely to affect the regulation of pre- and postsynaptic binding sites. Moreover, our findings suggest that antipsychotic drugs themselves may confound presynaptic binding data. Schizophrenia patients who are not responding to antipsychotic drug treatment can have a high percentage of occupied D2 receptors without any relief of symptoms [45]. In light of the present findings, it is conceivable that presynaptic autoreceptor or transporter function may be dysregulated in this subgroup of schizophrenia patients. In routine clinical studies as well as scientific studies, patients are frequently on medication and sometimes even take drugs of abuse [46]. Moreover, in preclinical studies, animals are anesthetized for their scans. Prescribed drugs, drugs of abuse, and anesthetics may influence the visual interpretation and/or quantification of [<sup>123</sup>I]FP-CIT SPECT scans.

The present study has several limitations. We used different methods to measure alterations in the DAergic neurotransmission system induced by treatment with the antipsychotic drugs HAL and CLZ. The two techniques employed have pros and cons with respect to their ability to measure alterations in the DAergic neurotransmission system, and they were used under fundamentally different experimental conditions (anesthetized animals vs. awake animals). Nonetheless, we felt that the utility of [<sup>123</sup>I]FP-CIT SPECT compared to the *in vivo* microdialysis technique should be demonstrated due to the lack of alternative *in vivo* imaging techniques other than [<sup>123</sup>I]FP-CIT SPECT. Experimental conditions were maintained as similar as possible between our [<sup>123</sup>I]FP-

CIT SPECT and *in vivo* microdialysis studies. The methodological validity of [<sup>123</sup>I]FP-CIT SPECT for measuring altered DAT activity was partially demonstrated in the present study. We attempted to describe the reliability and sensitivity of [<sup>123</sup>I]FP-CIT SPECT and the DAT blocker BUP for the measurement of DAT activity. The dose-dependent responses in [<sup>123</sup>I]FP-CIT BP<sub>ND</sub> after exposure to low and high doses of BUP demonstrated that BUP displaces [<sup>123</sup>I]FP-CIT or competes for the DAT in both the striatum and midbrain. This result directly indicates that radioligand binding to the DAT could be affected if endogenous DA displaces radioligands or competes with them for presynaptic binding sites. We did not perform an *in vivo* microdialysis study using BUP to validate the significance of the changes in the DA concentration and/or exclude the variation. Instead, we chose to measure changes in the DA concentration using a within-subject design. We felt that this experimental design could reduce experimental error, including variations in DA concentration measurements.

In conclusion, this study demonstrates that [<sup>123</sup>I]FP-CIT SPECT may be a useful preclinical technique for detecting increases in synaptic DA availability induced by HAL treatment in both the midbrain and the striatum, with results comparable to those obtained by *in vivo* microdialysis. Our most compelling hypothesis is that alterations in synaptic DA concentration are reflected as variations in DAT radioligand binding, at least in response to HAL and as detected by [<sup>123</sup>I]FP-CIT SPECT. For [<sup>123</sup>I]FP-CIT SPECT to be established as a standard technique to aid in the diagnosis of neurological and psychiatric diseases or monitor therapeutic responses, the effects of prescribed drugs on patients' imaging results must be carefully investigated first.

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