Effect of Dopamine D\textsubscript{2} Receptor Antagonists on [\textsuperscript{18}F]-FEOBV Binding

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ABSTRACT: The interaction of dopaminergic and cholinergic neurotransmission in, e.g., Parkinson’s disease has been well established. Here, D\textsubscript{2} receptor antagonists were used to assess changes in [\textsuperscript{18}F]-FEOBV binding to the vesicular acetylcholine transporter (VACHT) in rodents using positron emission tomography (PET). After pretreatment with either 10 mg/kg haloperidol, 1 mg/kg raclopride, or vehicle, 90 min dynamic PET scans were performed with arterial blood sampling. The net influx rate (K\textsubscript{i}) was obtained from Patlak graphical analysis, using a metabolite-corrected plasma input function and dynamic PET data. [\textsuperscript{18}F]-FEOBV concentration in whole-blood or plasma and the metabolite-corrected plasma input function were not significantly changed by the pretreatments (adjusted p > 0.07, Cohen’s d = 0.28–1.89) while the area-under-the-curve (AUC) of the parent fraction of [\textsuperscript{18}F]-FEOBV was significantly higher after haloperidol treatment (adjusted p = 0.022, Cohen’s d = 2.51) than in controls. Compared to controls, the AUC of [\textsuperscript{18}F]-FEOBV, normalized for injected dose and body weight, was nonsignificantly increased in the striatum after haloperidol (adjusted p = 0.4, Cohen’s d = 1.77) and raclopride (adjusted p = 0.052, Cohen’s d = 1.49) treatment, respectively. No changes in the AUC of [\textsuperscript{18}F]-FEOBV were found in the cerebellum (Cohen’s d = 0.63–0.74). Raclopride treatment nonsignificantly increased K\textsubscript{i} in the striatum 1.3-fold compared to control rats (adjusted p = 0.1, Cohen’s d = 1.1) while it reduced K\textsubscript{i} in the cerebellum by 28% (adjusted p = 0.0004, Cohen’s d = 2.2) compared to control rats. Pretreatment with haloperidol led to a nonsignificant reduction in K\textsubscript{i} in the striatum (10%, adjusted p = 1, Cohen’s d = 0.44) and a 40–50% lower K\textsubscript{i} than controls in all other brain regions (adjusted p < 0.0005, Cohen’s d = 3.3–4.7). The changes in K\textsubscript{i} induced by the selective D\textsubscript{2} receptor antagonist raclopride can in part be quantified using [\textsuperscript{18}F]-FEOBV PET imaging. Haloperidol, a nonselective D\textsubscript{2}/\sigma receptor antagonist, either paradoxically decreased cholinergic activity or blocked off-target [\textsuperscript{18}F]-FEOBV binding to \sigma receptors. Hence, further studies evaluating the binding of [\textsuperscript{18}F]-FEOBV to \sigma receptors using selective \sigma receptor ligands are necessary.

KEYWORDS: animal studies, D\textsubscript{2} receptor, \sigma receptor, vesicular acetylcholine transporter, kinetic modeling, positron emission tomography, Parkinson’s disease

INTRODUCTION

Traditionally, Parkinson’s disease is viewed as a neurodegenerative disorder that is characterized by motor impairments, including slowness of movement, muscle stiffness, and tremor. These motor symptoms result from the degeneration of dopaminergic neurons in the substantia nigra and the consequent deficiency of dopamine in brain regions that receive dopaminergic input from this brain region,\textsuperscript{1} such as the striatum. It is now evident that Parkinson’s disease also involves nonmotor symptoms that can be explained by the degeneration of multiple neurotransmitter systems,\textsuperscript{2,3} including the cholinergic system. Loss of cholinergic neurons has been reported in Parkinson’s disease and has been thought to play a significant role in cognitive decline and motor symptoms such as tremor.\textsuperscript{4,5}

To gain more insight into the relation between dopaminergic and cholinergic neurotransmission in Parkinson’s disease, the noninvasive imaging technique positron emission tomography (PET) can play an important role. [\textsuperscript{18}F]-FEOBV, a benzovesamicol analog, is a PET ligand that binds allosterically to the vesicular acetylcholine transporter (VACHT).\textsuperscript{6–8} VACHT is expressed in presynaptic cholinergic neurons and transports acetylcholine into synaptic vesicles. While VACHT is not a direct marker of acetylcholine synthesis or release, it has...
been shown that the expression of VACHT can be used to study cholinergic neurons.\textsuperscript{5,10} For example, clinical studies using \textsuperscript{[18F]}-FEOBV have revealed changes in the cholinergic system in patients with Parkinson’s disease.\textsuperscript{11,12}

Using microdialysis in rodents, it has been shown that the turnover of acetylcholine and its release into the synaptic cleft is increased after blocking of the dopamine D\textsubscript{2} receptors.\textsuperscript{13−15} Additionally, an increased uptake or binding of benzovesamicol radioligands in the striatum has been reported after the block of D\textsubscript{2} receptors.\textsuperscript{16−18} While the exact mechanisms leading to the increased binding of benzovesamicols following D\textsubscript{2} receptor block are unknown, it has been suggested that changes in the vesamical binding site of the VACHT are dependent on cholinergic activity, e.g. increased acetylcholine release.\textsuperscript{10} Here, we aimed to determine if acute blocking of the dopamine D\textsubscript{2} receptors would lead to increased accumulation of \textsuperscript{[18F]}-FEOBV as a measure of cholinergic activity. For this purpose, we chose the selective dopamine D\textsubscript{2}/D\textsubscript{3} receptor antagonist raclopride and the less selective D\textsubscript{2} receptor antagonist haloperidol which have previously been used in this context and estimated cholinergic activity as measured by the net influx rate \((K_i)\) estimated by Patlak graphical analysis.\textsuperscript{16−19}

**EXPERIMENTAL SECTION**

Experimental Animals. The experiments were approved by the National Committee on Animal Experiments (CCD:AVD1050020151666) and the Institutional Animal Care and Use Committee of the University of Groningen (AVD: 15166-01-003). Male Wistar rats \((n = 18,\) Hsd/Cpb:WU, aged 12.2 ± 2.3 weeks, 364 ± 33 g) were obtained from Envigo (The Netherlands) and housed in groups in humidity and temperature-controlled \((21 ± 2 °C)\) rooms with a 12 h/12 h light/dark cycle \((\text{lights on at 7 a.m.})\). Water and standard laboratory chow were supplied ad libitum. After arrival the rats were acclimatized for at least 7 days and then randomly divided in three groups treated with either vehicle \((\text{control, } n = 6),\) 10 mg/kg haloperidol\textsuperscript{19} \((n = 6),\) or 1 mg/kg raclopride\textsuperscript{20} \((n = 6).\) The PET scans were executed between 1 and 5 p.m. No blinding of the investigators was possible during the experiment, but the data analysis was automated and, hence, independent of the operator.

PET Imaging. The rats were anesthetized with isoflurane \((5\% \text{ for induction, } 1−2.5\% \text{ for maintenance})\) in 95\% oxygen, and eye salve was applied to prevent dehydration of the eyes. Rats were then injected intraperitoneally with 100 \(\mu\)L DMSO \((\text{control}),\) 10 mg/kg haloperidol, or 1 mg/kg raclopride in 100 \(\mu\)L DMSO at 52 ± 10 min \((\text{range 44−80 min})\) before injection of \textsuperscript{[18F]}-FEOBV. One cannula was placed in the tail vein for injection of \textsuperscript{[18F]}-FEOBV, and a second was in the femoral artery for arterial blood sampling. A dedicated small animal PET scanner \((\text{Focus 220 MicroPET, Siemens Healthcare, USA})\) was used, and two rats were scanned simultaneously. For attenuation correction, a transmission scan with \(^{57}\text{Co}\) point source was performed before each emission scan. During the PET scan, the heart rate and oxygen saturation were monitored and heating pads were used to maintain the body temperature of the rats between 37 and 38 °C. \textsuperscript{[18F]}-FEOBV \((28 ± 8 \text{ MBq, molar activity } >300 000 \text{ GBq/mm})\) was injected over 1 min at a rate of 1 mL/min via an infusion pump and a dynamic PET scan of 90 min was started simultaneously with injection. Synthesis of \textsuperscript{[18F]}-FEOBV was performed according to Mulholland et al. with adjustments to local infrastructure.\textsuperscript{22}

An attenuation-weighted 2-dimensional ordered subset expectation-maximization algorithm \((\text{OSEM2D, 4 iterations, 16 subsets})\) following Fourier rebinning was used for iterative reconstruction of the list mode data binned into 24 frames \((6 \times 10, 4 \times 30, 2 \times 60, 1 \times 120, 1 \times 180, 4 \times 300, 6 \times 600 \text{ s}).\) The data were normalized and corrected for scatter, attenuation, and decay. The resulting image matrix was 256 \(\times\) 256 \(\times\) 95 with a slice thickness of 0.796 mm and a pixel width of 0.633 mm.

One rat in the haloperidol treatment group died 54 min after the injection of \textsuperscript{[18F]}-FEOBV and was excluded from the analysis.

Arterial Blood Sampling and Metabolite Analysis. At approximately 10, 20, 30, 40, 50, 60, 90 s and 2, 3, 5, 7.5, 10, 15, 30, 60, and 90 min after \textsuperscript{[18F]}-FEOBV, injection blood samples \((0.10−0.13 \text{ mL each})\) were drawn from the femoral artery. The same volume of heparinized saline was injected after collection of each blood sample to compensate for blood loss. Plasma was obtained by centrifugation of whole-blood for 5 min at 30 000g. An automated well-counter \((\text{Wizard2480, PerkinElmer, USA})\) was used to measure radioactivity \((\text{decay corrected})\) in 25 \(\mu\)L of whole-blood and 25 \(\mu\)L of plasma.

Metabolite analysis was performed for all plasma samples obtained between 1 and 90 min.\textsuperscript{19} After acetonitrile \((50 \text{ \(\mu\)L})\) was added to each sample, the samples were vortexed and centrifuged \((3000g \text{ for 8 min})\). A 1 to 2 \(\mu\)L portion of the supernatant from each sample were pipetted onto a silica gel 60 F\textsubscript{254} plate \((\text{Merck, Germany})\), and the elution was performed with a mixture of hexane/dichloromethane/diethyl ether/triethylamine \((2.3/1/1/0.2)\). A phosphor storage screen \((\text{PerkinElmer, USA})\) was exposed overnight to the eluted silica plates and scanned the next day using a Cyclone \((\text{PerkinElmer, USA})\) phosphorescence imager. OptiQuant software 3 was used to obtain the percentage of intact tracer for correction of the plasma input curve.

PET Data Analysis. PET data analysis was performed using PMOD 3.9 software. Each individual PET image was automatically coregistered to a \textsuperscript{[18F]}-FEOBV template.\textsuperscript{22} A volume of interest \((\text{VOI})\) atlas, containing frontal cortex, remainder of the cortex \((\text{referred to as } \text{“cortex”}), \text{ striatum, thalamus, hypothalamus, hippocampus, brainstem, and cerebellum})\), was then placed on each coregistered PET image. For each individual rat, time−activity curves \((\text{kBq/mL})\) were generated for each VOI. Based on previous analysis from our laboratory, irreversible plasma input models were best suited for \textsuperscript{[18F]}-FEOBV quantification in rats. Irreversible plasma input models estimate the net influx rate \((K_i = K_i k_i/(k_j + k_i))\) which estimates the irreversible binding but also the net influx of the radioligand. In this study, Patlak graphical analysis showed a lower variation of the net influx rate compared to the irreversible two-tissue compartment model \((\text{2TCM}).\) \(K_i\) from Patlak graphical analysis was estimated with a stretch time of 10 min, whole-blood, and metabolite-corrected plasma input.\textsuperscript{23}

Statistical Analysis. Statistical analysis was performed using SPSS 23. The area under the curve \((\text{AUC})\) was determined for the parent fraction, the plasma \((\text{with or without correction for metabolites})\) and whole-blood time−activity curves and the striatal and cerebellar time−activity curves. The time−activity curves for plasma, whole-blood, and brain regions were normalized to standardized uptake values \((\text{SUV} = \text{tissue activity concentration/(injected dose/body weight)})\). Differences in AUC between treatments were assessed using one-way analysis of variance \((\text{ANOVA})\).
Posthoc analysis was performed using Bonferroni correction for multiple comparisons between control and treatments and the adjusted p-values are given.

For the $K_i$ obtained from PET imaging, the differences between the three groups were assessed using generalized estimating equations (GEE) with treatment as a between-group factor, brain region as a within-subject factor, and treatment $\times$ brain region as the interaction. Posthoc tests for differences between treatments were performed for each brain region and corrected for multiple comparisons using the Bonferroni method. GEE was used to assess differences between the three groups while accounting for intraindividual variation.

Figure 1. Uptake of $[^{18}\text{F}]-\text{FEOBV}$ in the brain of control rats and rats pretreated with 10 mg/kg haloperidol or 1 mg/kg raclopride. The net influx rate ($K_i$) images of representative rats from each group are shown (A) as well as the time-activity curves of the striatum and cerebellum for all rats in each group (mean + SD) (B).

Figure 2. Time-activity curves of $[^{18}\text{F}]-\text{FEOBV}$ uptake in whole-blood (A) and plasma (B) and the parent fraction of $[^{18}\text{F}]-\text{FEOBV}$ (C) for control rats and rats pretreated with 10 mg/kg haloperidol or 1 mg/kg raclopride (mean + SD).
differences and repeated measures, i.e. the dependence of brain regions in each rat (within-subject). Cohen’s $d$ was calculated for each treatment group using the means and pooled standard deviation of the control and treatment group.

Differences were deemed statistically significant with $p < 0.05$ (adjusted for multiple comparisons using Bonferroni correction if necessary). Effect sizes with Cohen’s $d > 0.5, 0.8, 1.2, and 2.0$ were considered medium, large, very large, and huge, respectively.

One rat of the control group showed an exceptionally large value for $K_i$ as obtained by Patlak graphical analysis in all brain regions (>1.5× interquartile range) and was excluded from the analysis.

## RESULTS

### Tracer kinetics and Metabolism

The time-activity curves of $^{[18F]}$-FEOBV were analyzed for two brain regions representing the highest (striatum) and lowest (cerebellum) uptake of $^{[18F]}$-FEOBV. $^{[18F]}$-FEOBV showed an initial high uptake in both regions, with the highest $^{[18F]}$-FEOBV uptake in the striatum at approximately 3 to 4 min after injection in control and haloperidol treated rats and at 6 to 8 min in rats treated with raclopride (Figure 1). The area-under-the-curve (AUC) of the striatal time-activity curve tended to be higher in haloperidol (Cohen’s $d = 1.77$) and raclopride treated rats (Cohen’s $d = 1.49$) when compared to control rats, but these differences were not statistically significant ($F(2, 15) = 3.7, p = 0.053$). In the cerebellum, the highest initial uptake of $^{[18F]}$-FEOBV was found between 1 and 3 min, with a 9% and 14% higher uptake in haloperidol and raclopride treated rats, respectively, than in control rats. On average the AUC of the cerebellar time–activity curve tended to be increased in raclopride (Cohen’s $d = 0.74$) and decreased in haloperidol treated rats (Cohen’s $d = 0.63$), but this was not statistically significant ($F(2, 15) = 2, p = 0.2$).

The AUC of the plasma and whole-blood time-activity curve (Figure 2) was not significantly changed by pretreatment with haloperidol (plasma Cohen’s $d = 0.63$, whole-blood Cohen’s $d = 1.10$) or raclopride (plasma Cohen’s $d = 0.39$, whole-blood Cohen’s $d = 0.41$) compared to control rats (plasma, $F(2, 15) = 0.6, p = 0.5$; whole-blood, $F(2, 15) = 2.3, p = 0.1$). A statistically significant difference in the AUC of the parent fraction between the three groups was found ($F(2, 11) = 6, p = 0.022$). Posthoc tests revealed a statistically significantly higher AUC of the parent fraction in haloperidol treated rats when compared to controls (adjusted $p = 0.022$, Cohen’s $d = 2.51$), while no statistically significant difference was found between the AUC of the parent fraction of the control and raclopride treatment groups (adjusted $p = 0.6$, Cohen’s $d = 1.16$). Despite a statistically significant difference in the AUC of the parent fraction between the haloperidol and control rats, the difference in the AUC of the metabolite-corrected plasma curve was not significant ($F(2, 15) = 3.3, p = 0.07$; haloperidol, Cohen’s $d = 1.89$; raclopride, Cohen’s $d = 0.28$).

### Net Influx Rate of $^{[18F]}$-FEOBV

Patlak graphical analysis revealed average $K_i$ values ranging from 0.031 to 0.167 mL/cm$^3$ min for different treatment groups and brain regions. The highest $K_i$ was found in the striatum, and the lowest in the cerebellum, for all three groups (Table 1). A statistically significant effect of treatment ($\chi^2(1, 2) = 40, p < 0.0001$) and brain region ($\chi^2(1, 7) = 69.1, p < 0.0001$) on the $K_i$ of $^{[18F]}$-FEOBV was found, as well as a statistically significant interaction between treatment and brain region ($\chi^2(1, 13) = 3475, p < 0.0001$). Posthoc analysis revealed a statistically significant difference in $K_i$ between control and haloperidol treated rats and between haloperidol and raclopride treated rats for all brain regions together (adjusted $p < 0.0001$), but no statistically significant difference was found between control and raclopride treated rats (adjusted $p = 1$). Comparison of the treatments in each individual brain region (Figure 3) revealed a statistically significant difference between haloperidol treated and control rats in brainstem, cerebellum, cortex, frontal cortex, hippocampus, hypothalamus, and thalamus (adjusted $p$...
< 0.0005, Cohen’s d 3.29−4.66), but not in striatum (adjusted $p = 1$, Cohen’s d 0.44) most likely due to the large variation in $K_i$ (coefficient of variation 23%) in this brain region. In all other brain regions, the $K_i$ was statistically significantly decreased by an average of 45 ± 3%, with the largest decrease of 49−50% in the cerebellum and hippocampus and the lowest of 40% in the cortex. Contrarily, the only individual brain region with a significant difference between control and raclopride treated rats was the cerebellum (adjusted $p = 0.0004$, Cohen’s d 2.15) with a decrease in $K_i$ of 28%. In the striatum, an increase in $K_i$ of 30% was found. Despite a large effect size, this difference did not reach the threshold for statistical significance (adjusted $p = 0.1$, Cohen’s d 1.09). In the other brain regions, $K_i$ was found to be decreased by 8 ± 5%, but this effect was not statistically significant (Cohen’s d 0.06−0.97). Significant differences between haloperidol and raclopride treatment were found in all individual brain regions (adjusted $p < 0.05$) with an average difference in $K_i$ of 60 ± 13% (42−81%).

**Discussion**

In the current study, we have found changes in the net influx rate of $[^{18}F]$-FEOBV after acute treatment with D$_2$ receptor antagonists, using PET imaging. The specific D$_2$ receptor antagonist raclopride led to a statistically significant decrease in the net influx rate of $[^{18}F]$-FEOBV in the cerebellum and nonstatistically significant increase of the net influx rate in the striatum. In contrast, the less specific D$_2$ receptor antagonist haloperidol led to a decreased net influx rate in all brain regions except the striatum. Although haloperidol significantly decreased the metabolism of $[^{18}F]$-FEOBV, treatment with either raclopride or haloperidol did not affect the whole-blood, plasma, and the metabolite-corrected plasma input, showing that changes found in the net influx rate $K_i$ were not due to hemodynamic differences.

Interestingly, we found a statistically significant decrease in the net influx rate of $[^{18}F]$-FEOBV in the cerebellum of rats treated with raclopride. Contrarily, a study by Efange et al. using the VACH$T$ radioligand $(+)$-$[^{125}I]$-MIBT in rats found no change in the cerebellar uptake of $(+)$-$[^{125}I]$-MIBT after treatment with the D$_2$ receptor antagonist S-$(−)$-etico mipide.26 In the same study, however, a significant increase in the uptake of the less specific $(−)$-$[^{125}I]$-MIBT in the cerebellum after spiperone (D$_2$ receptor antagonist) treatment was observed. The discrepancy between our and the study of Efange et al. could be related to the different D$_2$ receptor antagonists used and their varying binding affinities to the D$_2$ receptor and potential off-target binding.27 Additionally, Efange et al. performed ex vivo biodistribution after 3 h, while we determined the net influx rate of $[^{18}F]$-FEOBV using Patlak graphical analysis using a 90 min PET acquisition. The difference in time, as well as the more specific quantification via PET imaging with pharmacokinetic modeling compared to the ex vivo distribution, could explain the differences between our study and the study of Efange et al. Previous studies showed only negligible binding of the D$_2$ receptor antagonist raclopride in the cerebellum of rats.28,29 Additionally, it was also determined that raclopride binding in the striatum was saturated at $3.5 \text{ pmol/g}$ which could suggest that the higher dose of approximately $3 \text{ nmol/g (1 mg/kg)}$ of raclopride used in our study could have led to increased off-target binding of raclopride to serotonergic or adrenergic receptors and thereby facilitated the decrease in the net influx rate of $[^{18}F]$-FEOBV via interaction with other neurotransmitters.28

While we found a decrease in $[^{18}F]$-FEOBV net influx rate in the cerebellum, the net influx rate and area-under-the-curve of $[^{18}F]$-FEOBV in the striatum tended to be increased after raclopride treatment as evidenced by the large effect size found between control and raclopride treated rats. Ingvar et al. showed increased uptake of the VACH$T$ radioligand $[^{18}F]$-NEFA in the striatum of primates after raclopride or haloperidol treatment.16 Similarly, Efange et al. showed increased striatal uptake of $(+)$-$[^{125}I]$-MIBT in rats pretreated with spiperone and S-$(−)$-etico mipide at 3 h after injection of $(+)$-$[^{125}I]$-MIBT.17 In addition, after depletion of dopaminergic neurons in the rat striatum with 6-OHDA, a smaller spiperone-induced increase in $(+)$-$[^{125}I]$-MIBT uptake was found in the ipsilateral striatum, when compared to the contralateral side.26 In a more recent study, Jin et al. showed a 1.5-fold increase in binding of the VACH$T$ ligand $[^{11}C]$-T26S9 in striatum after treatment with S-$(−)$-etico mipide.18 These findings indicate that D$_2$ receptor antagonism results in an increased binding of benzovesamol radioligands in the striatum, most likely due to increased acetylcholine turnover facilitated by the antagonized D$_2$ receptor15−15,30 and suggest that with lower variability of the net influx rate in the striatum a significant difference might have been found in our study. Hence, our study suggests that $[^{18}F]$-FEOBV could be capable of measuring increased cholinergic activity in rats after treatment with the D$_2$ receptor antagonist raclopride.

Raclopride tended to increase the net influx rate of $[^{18}F]$-FEOBV in the striatum, whereas, haloperidol, the other D$_2$ receptor antagonist used in this study, nonsignificantly decreased the net influx rate by 10%. In fact, haloperidol significantly reduced the net influx rate by up to 50% in all other brain regions. There are several possible explanations for this result. First, the overall reduction in the net influx rate after haloperidol pretreatment could suggest reduced cholinergic activity. Nevertheless, microdialysis experiments in rats treated with haloperidol indicate that the opposite is the case, as an increase in acetylcholine turnover was observed after haloperidol treatment.13 Hence, it is unlikely that the results of our study can be explained by a decreased cholinergic activity due to haloperidol treatment.

Second, hemodynamic changes after haloperidol treatment could have reduced the uptake of $[^{18}F]$-FEOBV in the brain. Similar to a study by Mulholland et al., we used a high dose (10 mg/kg) of haloperidol. Mulholland found a reduction of $[^{18}F]$-FEOBV brain uptake and blood activity of up to 30 and 50%, respectively, when compared to control mice.19 They suggested that hemodynamic changes after the high-dose haloperidol treatment were responsible for the reduced brain uptake.19 We found the area-under-the-curve of the $[^{18}F]$-FEOBV parent fraction to be significantly higher compared to control rats, indicating a reduced metabolism of $[^{18}F]$-FEOBV after haloperidol pretreatment. However, no significant differences in the area-under-the-curve of the whole-blood or plasma activity curve of $[^{18}F]$-FEOBV (parent and metabolite), as well as no significant changes in the metabolite-corrected plasma input function (parent only) between the control and haloperidol group, were found although the determined effect sizes were large. Furthermore, we found no statistically significant differences in the influx rate constant $K_i$ obtained from the irreversible 2TCM between control and haloperidol or raclopride treated rats in any brain region (Supplemental...
This suggests that hemodynamic changes did not change $^{[18F]}$-FEOBV delivery to the brain after haloperidol pretreatment in our study.

Third, it could be possible that due to the high dose of haloperidol used in this study not only $D_2$ receptors were occupied. Haloperidol binds to a variety of neuroreceptors, e.g., serotoninergic, dopaminergic, or adrenergic receptors, which could, in turn, have affected the net influx rate of $^{[18F]}$-FEOBV either directly by changes in cholinergic neurotransmission or indirectly via the dopaminergic or other neurotransmitter systems.

Another possible explanation for the reduced net influx rate of $^{[18F]}$-FEOBV could be the blocking of $\sigma$ receptors by haloperidol pretreatment. Haloperidol has been described as a potent $\sigma_1$ receptor antagonist and $\sigma_2$ receptor agonist. The binding affinity of vesamical derivatives like $^{[18F]}$-FEOBV to $\sigma$ receptors has been a topic of dispute. Some argued that most vesamical-based radioligands, including $^{[18F]}$-FEOBV, should not be considered for VAChT imaging because of their binding affinity for $\sigma$ receptors. The binding affinity of $^{(-)}$-FEOBV has been determined as approximately $200 \mathrm{~nM}$, it was recently shown that its binding to other neuroreceptors by haloperidol used in this study not only $D_2$ receptors were occupied. Haloperidol binds to a variety of neuroreceptors, e.g., serotoninergic, dopaminergic, or adrenergic receptors, which could, in turn, have affected the net influx rate of $^{[18F]}$-FEOBV either directly by changes in cholinergic neurotransmission or indirectly via the dopaminergic or other neurotransmitter systems.

Lastly, it has been shown that isoflurane anesthesia decreases the release of acetylcholine in several brain regions, including striatum and cortex, in a dose-dependent manner. Isoflurane is a widely used anesthetic in PET imaging studies in rodents which, at least for now, cannot be omitted during the procedure. As all rats in this study were treated with approximately the same dose of isoflurane a constant bias between the control and treatment groups can be assumed.

In the present study, we showed that changes in cholinergic activity after treatment with the selective $D_2$ receptor antagonist raclopride and the nonselective $D_2$ receptor antagonist haloperidol can partly be quantified using $^{[18F]}$-FEOBV PET imaging. The seemingly decreased cholinergic activity after treatment with haloperidol appears to be due to off-target binding of $^{[18F]}$-FEOBV to $\sigma$ receptors in rat brain. This hypothesis needs to be confirmed in further studies on the off-target binding of $^{[18F]}$-FEOBV to $\sigma$ receptors in rat brain. Such off-target binding will probably not occur in the human brain due to interspecies differences in the binding affinities of $^{[18F]}$-FEOBV to $\sigma$ receptors.

## CONCLUSION

In the present study, we showed that changes in cholinergic activity after treatment with the selective $D_2$ receptor antagonist raclopride and the nonselective $D_2$ receptor antagonist haloperidol can partly be quantified using $^{[18F]}$-FEOBV PET imaging. The seemingly decreased cholinergic activity after treatment with haloperidol appears to be due to off-target binding of $^{[18F]}$-FEOBV to $\sigma$ receptors in rat brain. This hypothesis needs to be confirmed in further studies on the off-target binding of $^{[18F]}$-FEOBV to $\sigma$ receptors in rat brain. Such off-target binding will probably not occur in the human brain due to interspecies differences in the binding affinities of $^{[18F]}$-FEOBV to $\sigma$ receptors.

## ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.9b001129.

Supplemental Table 1 Influx rate constant ($K_i$, $[\mathrm{min}^{-1}]$, mean $\pm$ SD) of $^{[18F]}$-FEOBV in brain tissue in control rats and rats pretreated with 10 mg/kg haloperidol or 1 mg/kg raclopride. $K_i$ was estimated using the 2-tissue compartmental model (PDF)

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