Title

Quantification of ONO-2952 occupancy of translocator protein 18kDa in conscious monkey brain using positron emission tomography

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Running title

ONO-2952 brain TSPO occupancy in conscious monkey

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Abbreviations
PET, positron emission tomography; SUV, standardized uptake value; TSPO, translocator protein 18kDa; Vt, total distribution volume.
Abstract

We have previously shown that ONO-2952, a novel translocator protein 18kDa (TSPO) antagonist, inhibits stress-induced neurosteroids accumulation and noradrenaline release in the rat brain and alleviates the subsequent symptomatic responses with a brain TSPO occupancy of 50% or more. In this study, we measured ONO-2952 brain TSPO occupancy in conscious rhesus monkeys using positron emission tomography (PET) with $^{11}$C-PBR28 as ligand for translational research to clinical application. PET scans were performed following single and repeated oral administration of ONO-2952 at several dose levels for each animal with sequential arterial blood sampling. *In vitro* binding studies showed that ONO-2952 potently binds to brain TSPO in monkeys with an affinity equivalent to that in rats. ONO-2952, given orally prior to PET scans, dose-dependently decreased $^{11}$C-PBR28 uptake without marked brain region-specificity. Results of the quantitative analysis using arterial input function revealed that TSPO occupancy following ONO-2952 single and repeated oral administration tended to increase in parallel with its plasma concentration, reaching the highest level of 100%. These findings indicate that ONO-2952 has sufficient brain distribution in primates and that ONO-2952 TSPO occupancy in human can also be determined using PET.
Introduction

The translocator protein 18kDa (TSPO), which has five transmembrane domains, is widely distributed throughout the body and particularly prevalent in steroids producing tissues, including the brain where this protein is predominantly located in glial cells (Marangos et al., 1982; Gavish et al., 1999; Cosenza-Nashat et al., 2009; Rupprecht et al., 2010). Within cells, TSPO is located in the outer mitochondrial membrane and forms a channel-like structure that allows the transport of cholesterol from intracellular sources into mitochondria, a pathway known as the rate-limiting step in steroidogenesis (Anholt et al., 1986; Rupprecht et al., 2010; Rone et al., 2012). Neurosteroids, which are endogenous steroids produced in the central nervous system, have been shown to act as allosteric modulators of excitatory and/or inhibitory neurotransmission, including adrenergic, GABAergic, glutamatergic, and cholinergic neurotransmission (Belelli and Lambert, 2005; Strous et al., 2006). In this regard, an unbalance in the levels of neurosteroids in the central nervous system would have significant pathophysiological consequences. Consistent with this idea, TSPO expression in the brain or peripheral blood cells, as well as neurosteroids concentration in the cerebrospinal fluid or plasma, have been found to be altered in patients with stress-related or neurological disorders, such as depression, generalized anxiety disorder, post-traumatic stress disorder, Alzheimer’s disease, and schizophrenia (Rasmusson et al., 2006; Rupprecht et al., 2010; Da Pozzo et al., 2012). Put together, these findings indicate that drugs that can act on TSPO in the brain would have beneficial effects on stress-related disorders. However, the clinical use of TSPO ligands for treatment of stress-related disorders has not been fully explored.

ONO-2952 is a novel TSPO antagonist that exhibits high affinity for the rat TSPO (Ki = 0.33 nmol/L) with high selectivity over other receptors, transporters, ion channels and enzymes. Pharmacological studies in rats exposed to acute stress have shown that ONO-2952 inhibits stress-induced neurosteroids.
accumulation and noradrenaline release in the brain, resulting in anti-stress effect (Mitsui et al., 2015). In addition, our *ex vivo* binding study in rats showed that ONO-2952 produces its anti-stress effect with brain TSPO occupancy of more than 50%. As we postulate that a TSPO occupancy over 50% would be needed for ONO-2952 to exert its anti-stress effect in human, a relationship between plasma concentration of ONO-2952 and its brain TSPO occupancy in human is necessary to predict this compound clinical effective doses.

Positron emission tomography (PET) is used as a tool to assess the relationship between compound plasma concentration and its target protein occupancy, thereby allowing selection of optimal clinical dosage (Matthews et al., 2012). TSPO has historically been used as a marker for reactive gliosis, which is closely related to neuronal dysfunction associated with a number of CNS diseases. Accordingly, several structurally different TSPO ligands have been synthesized as imaging tools for detection of brain injury (Chauveau et al., 2008; Chen and Guilarte, 2008). Among these ligands, aryloxyanilide-based $^{11}$C-PBR28 has been shown to be a useful TSPO-specific PET radioligand not only in nonhuman primates, but also in humans (Brown et al., 2007; Fujita et al., 2008; Imaizumi et al., 2008; Owen et al., 2014). As a step to establish a relationship between ONO-2952 plasma concentration and its brain TSPO occupancy in human, we measured ONO-2952 brain TSPO occupancy in conscious rhesus monkeys using PET with $^{11}$C-PBR28 as a radioligand. *In vitro* binding studies using $^3$H-PBR28 in membrane fractions from rat and monkey brain were also conducted to confirm the validity of $^{11}$C-PBR28 as a PET probe for determination of ONO-2952 TSPO occupancy.
Materials and Methods

Drugs and Chemicals

ONO-2952 (1-[(15)-1-(4-chloro-2-methoxyphenyl)-5-fluoro-1,9-dihydrospiro[β-carboline-4,1'-
cyclopropan]-2(3H)-yl]ethanone, purity: ≥ 95%), PBR28 (N-acetyl-N-(2-methoxybenzyl)-2-phenoxy-5-
pyridinamine) and O-desmethyl PBR28 were synthesized in our laboratories. PK11195 (1-(2-
chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide), a selective TSPO antagonist
(Le Fur et al., 1983), was purchased from Sigma-Aldrich (St. Louis, MO), and 3H-PBR28 (specific
radioactivity: 3.02 TBq/mmol) from Sekisui Medical Co., Ltd. (Tokyo, Japan). All other chemicals
were purchased from Sigma-Aldrich, unless otherwise noted. Compounds used for the binding study
were first dissolved in 0.5% dimethylsulfoxide (DMSO) and then added to the assay buffer. ONO-2952
was suspended in 0.5% (w/v) methylcellulose (MC) solution for oral administration at a dosing volume
of 5 mL/kg.

Animals

Male Wistar rats (Slc:Wistar, Japan SLC, Inc.; 11 weeks old) were used in this study. Rats were housed
in groups of less that of 5 animals/cage in a temperature- and humidity-controlled animal room (temp:
24 ± 2°C, relative humidity: 55 ± 15%) under a 12-h light/dark cycles (light on from 8:00 to 20:00).
Three male rhesus monkeys (Macaca mulatta, Shin Nippon Biomedical Laboratories, Ltd., weight: 4.1
to 5.6 kg) were used for in vitro TSPO-binding study. Six male rhesus monkeys (Macaca mulatta,
Hamri Co., Ltd., weight: 4.5 to 8.0 kg) were used for the PET study. Monkeys were housed in 1
animal/cage in a temperature- and humidity-controlled animal room (temp: 24 ± 4°C, relative humidity:
50 ± 20%) under a 14-h light/10-h dark cycles (light on from 7:00 to 21:00). All experimental
procedures were approved by the institutional animal care and use committee of ONO pharmaceutical
Co., Ltd., and Central Research Laboratory, Hamamatsu Photonics K.K.
In vitro binding assay

ONO-2952 and PK11195 inhibition constants (Ki) for displacement of $^3$H-PBR28 TSPO-binding in mitochondrial membrane fractions prepared from rat whole brain and monkey brain (hippocampus and occipital cortex) were determined as described in our previous report (Mitsui et al., 2015) with minor modifications. Protein concentration in each brain homogenate was adjusted to 1 mg/mL with HEPES buffer, and the homogenate (50 µL) was incubated for 90 min at 4°C with 0.5 nmol/L $^3$H-PBR28 (final concentration), in a final reaction volume of 200 µL. Non-specific binding was defined as binding in the presence of unlabeled PK11195 (20 µmol/L). $^3$H-PBR28 saturation binding assay was also performed for each membrane fraction.

To confirm whether ONO-2952 competitively inhibits PBR28 binding to TSPO in monkey brain tissue, dissociation constants (Kd) and maximum binding (Bmax) of $^3$H-PBR28 were determined with increasing concentrations of non-radioactive test-compound in homogenates derived from the occipital cortex. Competitive inhibition was defined as significant increase in Kd values with no influence on Bmax values (Obata et al., 1992). When the 95% confidence interval of Kd or Bmax did not overlap between assays (i.e; without and with ONO-2952), the change was considered as significant.

All assays were performed in duplicate. GraphPad Prism software (version 5.01; GraphPad Software Inc., La Jolla, CA) was used to calculate Ki values of test-compounds, and Kd and Bmax values of $^3$H-PBR28 in each membrane fraction.

Determination of ONO-2952 TSPO occupancy in the rat brain: Ex vivo experiment

The relationship between ONO-2952 plasma concentration and TSPO occupancy in the brain was evaluated in rats. ONO-2952 or vehicle (0.5% MC) was orally administered once to each rat. Three hours after ONO-2952 administration, approximately 0.5 mL of blood samples were collected via the jugular vein using a heparinized syringe, and the plasma was separated by centrifugation and frozen at -
80°C until use. The rats were then decapitated and the cerebral cortex and hippocampus were dissected. The brain was next weighed and homogenized in 5 times (w/v) ice-cold 50 mmol/L HEPES buffer. Protein concentration in the homogenate was adjusted with HEPES to 10 mg/mL for use in the determination of ONO-2952 TSPO occupancy. TSPO occupancy of ONO-2952 was determined from \(^{3}H\text{-PBR28}\) specific binding to TSPO in the brain homogenate. Each homogenate (50 \(\mu\)L) was incubated for 90 min at 4°C with \(^{3}H\text{-PBR28}\) (final concentration 0.5 nmol/L), in a final reaction volume of 200 \(\mu\)L. Assays were performed in duplicate, and ONO-2952 TSPO occupancy was calculated using the following equation.

\[
\text{TSPO occupancy (\%)} = (1 - X / Y) \times 100
\]

Where,

- \(X\): Specific binding level in each brain homogenate
- \(Y\): Mean specific binding level in the control group treated with the vehicle

Plasma concentration of ONO-2952 was determined by positive turbo ion spray LC/MS/MS with solid-phase extraction. The lowest limit of quantitation was 0.1 ng/mL.

**Evaluation of ONO-2952 TSPO occupancy in conscious monkey brain: PET study**

The relationship between plasma concentration of ONO-2952 and its TSPO occupancy in the brain of conscious rhesus monkeys was determined using \(^{11}\text{C-PBR28}\) as a PET ligand. The PET study was conducted by Central Research Laboratory, Hamamatsu Photonics K.K.

**Synthesis of \(^{11}\text{C-PBR28}\):** \(^{11}\text{C-PBR28}\) was synthesized as reported previously (Brown et al., 2007). Positron-emitting carbon-11 was produced by \(^{14}\text{N(p,\alpha)}^{11}\text{C}\) nuclear reaction using a cyclotron (HM-18, Sumitomo Heavy Industries, Ltd., Tokyo, Japan). \(^{11}\text{C-PBR28}\) was radiolabelled by methylation of its
desmethyl precursor using $^{11}$C-methyl triflate, and purified by high-performance liquid chromatography (HPLC, Megapak SIL C18-10 7.6 × 250 mm, JASCO Corporation, Tokyo, Japan) with a mobile phase of 500/500 acetonitrile/30 mmol/L ammonium acetate at a flow rate of 6 mL/min. After a fraction corresponding to $^{11}$C-PBR28 was collected and the eluate evaporated, the residue was dissolved in physiological saline to obtain the final $^{11}$C-PBR28 product. Radioactivity of the final product was measured using a curiemeter (IGC-3, Aloka, Tokyo, Japan), and a portion was analyzed by HPLC (Finepack C18-S 4.6 × 150 mm, JASCO Corporation, Tokyo, Japan) using 500/500/2 acetonitrile/30 mmol/L ammonium acetate/acetic acid at a flow rate of 2 mL/min (wavelength: 254 nm). The product radiochemical purity was more than 99%, and its specific radioactivity was more than 150 GBq/μmol.

**Imaging method:** PET scans were performed as reported previously (Tsukada et al., 2000; Noda et al., 2003). At least 1 month before the start of chair training, an acrylic plate, by which the test animal was fixed to a monkey chair, was attached to the top of monkey’s head under pentobarbital anesthesia. The monkeys were then trained to sit on the monkey chair twice a week for more than 3 months to acclimate and relieve stress of head fixation during PET measurement. After being fasted overnight, the test animals were anesthetized with 2.5% sevoflurane, and a cannula was placed in the cephalic vein or saphenous vein for intravenous administration of test compounds. Another cannula was placed in the femoral artery or posterior tibial artery for arterial blood sampling. The animal was then fixed onto the monkey chair. After confirmation of full recovery from anesthesia, the animal was transferred to a high-resolution animal PET scanner (SHR-7700, Hamamatsu Photonics K.K., Hamamatsu, Japan) and its head was fixed into a stereotactic apparatus (SFCT-RB-PR-2, Hamamatsu Photonics K.K., Hamamatsu, Japan). A transmission scan was then performed for 30 min using a $^{68}$Ge/$^{68}$Ga calibration source. Next, an emission scan (121 min in total) was started simultaneously with the start of $^{11}$C-PBR28 injection.
For quantitative analysis of $^{11}$C-PBR28, PET scans were performed during arterial blood sampling. Approximately 0.5 mL of arterial blood was collected at 20 time points, i.e. 8, 16, 24, 32, 40, 48, 56, 64, 90, and 150 s, and 4, 6, 10, 20, 30, 45, 60, 75, 90, and 120 min after $^{11}$C-PBR28 injection. The blood samples were centrifuged to separate the plasma, and the radioactivity was measured. To the plasma (100 μL) separated from blood samples collected at 16, 40, and 64 s, and 6, 10, 30, 45, 60, 75, 90, and 120 min after $^{11}$C-PBR28 injection, ethanol (200 μL) was added, and the mixture was centrifuged for metabolites analysis. The resulting supernatant was developed on a thin-layer chromatography plate (AL SIL G/UV, Whatman, Kent, Buckinghamshire, United Kingdom) with a mobile phase of 45/5/1 chloroform/methanol/triethylamine. The ratio of metabolites to the parent $^{11}$C-PBR28 was determined using an imaging plate (BAS-IIIIs, Fujifilm Corporation, Tokyo, Japan).

Arterial input function was defined as time-course change in radioactivity concentration of the parent $^{11}$C-PBR28 in arterial plasma.

For ONO-2952 experiment, PET scans were performed at 2 and/or 24 h after single or repeated (twice daily for 3 days and once on Day 4) oral administration of ONO-2952 at several doses ranging from 0.3 to 30 mg/kg. The vehicle (0.5% MC) was administered orally once 2 h before PET scans. Venous blood was collected for measurement of ONO-2952 plasma levels immediately before injection of $^{11}$C-PBR28. ONO-2952 plasma concentrations were determined by the method described above. For PK11195 experiment, PET scans were performed 1 min after intravenous administration of PK11195 (0.1mg/kg) or vehicle (5% ethanol-0.5% Tween80 saline solution).

Images analysis and quantification: PET images were reconstructed with a Filtered Back Projection method using a Hanning filter of 4.5 mm in SHR Control II program (Hamamatsu Photonics K.K., Hamamatsu, Japan). PMOD program (PMOD Technologies Ltd., Zurich, Switzerland) was used to calculate time-activity curves (TACs) of $^{11}$C-PBR28 in the brain. The regions of interest (ROIs)
(cerebellum, hippocampus, corpus striatum, thalamus, occipital cortex, temporal cortex, frontal cortex, and parietal cortex) were drawn on PET reconstructed images of individual animals with reference to brain MRI images previously obtained for each animal using a 3T MRI scanner (Allegra; Siemens, Erlangen, Germany) to determine the TAC of $^{11}$C-PBR28 in each ROI.

The standardized uptake value (SUV), which represents mean brain radioactivity accumulation between 91 and 121 min post dose normalized for the injected dose of radioactivity and for animal body weight, was calculated according to the following equation.

$$\text{SUV} = \frac{[\text{PET-measured value (Bq/mL)}]}{[\text{amount of }^{11}\text{C-PBR28 injected (Bq)} / \text{body weight (kg)}]}$$

Using metabolite-corrected arterial input function, the total distribution volume ($V_t$) of $^{11}$C-PBR28 was determined from TACs of each ROI by Two-Tissue Compartment (2TC) model (Innis et al., 2007; Fujita et al., 2008), using a calculation tool for PMOD. In addition, $V_t$ images were generated from the reconstructed PET images blurred by 3D Gauss filter of 4 mm FWHM and input function by 2TC analysis using PMOD.

TSPO occupancy in the whole brain was defined as the slope of linear regression of the $V_{t_{\text{base}}}$ vs. ($V_{t_{\text{base}}} - V_{t_{\text{drug}}}$) in each brain ROI, where $V_{t_{\text{base}}}$ and $V_{t_{\text{drug}}}$ were the regional distribution volumes following vehicle and drug administration, respectively, termed the Lassen plot method (Cunningham et al., 2010; Owen et al., 2014).

**Statistical analysis**

Except where noted, data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism software (version 5.01; GraphPad Software Inc., La Jolla, CA). Nonlinear regression analysis was performed with a least squares method.
Results

*In vitro* binding of ONO-2952 to rat and monkey TSPO

As shown in Table 1, Kd value of $[^3]H$-PBR28 in mitochondrial membrane fractions prepared from rat brain was equivalent to that in the fractions prepared from monkey hippocampus or occipital cortex. However, Bmax value in the rat fractions was lower than that in the monkey fractions. ONO-2952 bound to rat TSPO with a Ki value comparable to, or slightly lower than that for binding to monkey TSPO. There were no significant differences in Ki values between ONO-2952 and PK11195 binding to rat TSPO. As for the affinity of these two test-compounds for monkey TSPO, PK11195 showed higher Ki value than ONO-2952 in each brain region.

Effect of ONO-2952 on Kd and Bmax values for $[^3]H$-PBR28 binding to monkey brain TSPO


Relationship between ONO-2952 plasma concentration and its *ex vivo* TSPO occupancy in rat brain

ONO-2952 plasma concentration and TSPO occupancy in the rat brain 3 hours after oral administration are shown in Table 3. ONO-2952 occupancy of TSPO in the rat cerebral cortex and hippocampus increased with increasing plasma concentration, reaching a maximum of approximately 90% in the
cerebral cortex at the dose of 3 mg/kg. The estimated IC$_{50}$ value of ONO-2952 in the cerebral cortex and hippocampus were 9.78 and 18.6 ng/mL, respectively.

**PET study in conscious monkeys**

**Effect of PK11195 on $^{11}$C-PBR28 TAC in the brain of monkey**

$^{11}$C-PBR28 uptake peaked at 25 to 35 min after vehicle administration in all brain regions with an SUV of 1.5 or more, except in the cerebellum (Fig 1). The highest SUV was observed in the occipital cortex, and the lowest in the cerebellum (Fig. 1A). As shown in Fig. 1B, pretreatment with PK11195 shifted peak $^{11}$C-PBR28 uptake to within 5 min after injection, followed by rapid decrease to lower levels in all brain regions at times later than 20 min after injection, compared to the corresponding levels after vehicle administration.

**TSPO occupancy by PK11195 in the brain**

Quantitative PET images demonstrated that PK11195 (0.1 mg/kg, i.v.) decreased $^{11}$C-PBR28 Vt across broad brain regions (Fig. 2 and Table 4). The averaged TSPO occupancy of PK11195 as determined by using the slope of the Lassen plots was 49.2% (Fig. 2C and Table 4).

**Effect of ONO-2952 on $^{11}$C-PBR28 TAC in the brain of monkey**

As shown in Fig 3, pre-treatment with ONO-2952 (0.1 mg/kg) resulted in a slight increase in the uptake of $^{11}$C-PBR28 in the brain compared with pretreatment with the vehicle (Fig. 3A). Oral administration of ONO-2952, at doses of 1 and 10 mg/kg, dose-dependently shifted the peak of $^{11}$C-PBR28 uptake to
approximately 15 and 8 min after injection, respectively (Fig. 3B and 3C). TACs in ONO-2952-treated monkey decreased thereafter in a dose-dependent manner compared to those in the vehicle-treated monkey. Similar effects on $^{11}$C-PBR28 TACs were observed 2 h or 24 h after repeated administration of ONO-2952 (Fig. 4A).

**TSPO occupancy by ONO-2952 in the brain**

Pre-treatment with ONO-2952 (1 and 10 mg/kg) 2-h before PET scans dose-dependently decreased $^{11}$C-PBR28 Vt in the whole brain (Fig. 4B-a, b, c and Table 5). This trend continued even 24-h after dosing with no inter-regional differences (Fig. 4B-a, d, e and Table 5). The regression lines in each brain ROI of a representative monkey are shown in Fig. 4C. Overall, there was no marked brain region-specificity for ONO-2952 TSPO occupancy at any dose. In addition, ONO-2952 TSPO occupancy was dependent on the plasma concentration of this compound irrespective of the time from the final dosing (Table 5).

**The relationship between ONO-2952 plasma concentration and its brain TSPO occupancy in monkey**

TSPO occupancy of ONO-2952 in the whole brain determined by PET generally increased with increasing plasma concentration, reaching a maximal occupancy as high as 100.2% (Fig. 5). Analysis of brain TSPO occupancy plotted as function of plasma concentration revealed that the two parameters correlated well. In the fit with maximal occupancy fixed at 100%, the estimated IC$_{50}$ value of ONO-2952 in the PET study was 19.6 ng/mL.
Discussion

Using conscious monkeys, we confirmed in this PET study that ONO-2952, a novel TSPO antagonist, has sufficient brain distribution with a clear relationship between its plasma concentration and TSPO occupancy in the brain.

*In vitro* binding experiment using $^3$H-PBR28 as a radioactive ligand showed that ONO-2952 exhibits high affinity for monkey TSPO compared to PK11195, a well characterized TSPO ligand (Table 1). These findings are in agreement with those showing that PK11195 exhibits interspecies variability in its affinity for TSPO with Ki values of 4.35 nmol/L and 0.73 nmol/L for monkey and rat TSPOs, respectively (Briard et al., 2008). Although Ki values of ONO-2952 and PBR28 showed a small interspecies variability, the binding affinity of these compounds to TSPO is considered to be similar across species. As shown in Table 2, Kd value of $^3$H-PBR28 increased with increasing concentration of ONO-2952 although Bmax value did not change. These findings indicate that these two compounds mutually compete for TSPO binding sites in monkey brain. In addition, PBR28 reportedly has good selectivity for TSPO against 36 off-targets (Imaizumi et al., 2008), and $^{11}$C-PBR28 exhibits sufficient signal and appropriate kinetics for imaging TSPO in nonhuman primate brain (Briard et al., 2008; Imaizumi et al., 2008). Based on these findings, we decided to evaluate TSPO occupancy of ONO-2952 in the brain using $^{11}$C-PBR28 as a radiolabeled ligand for the PET study.

Our *ex vivo* experiment, aimed at measuring TSPO occupancy of ONO-2952 in the rat brain, revealed that TSPO occupancy of this compound increases with increasing plasma concentration. This finding is in agreement with our previous work using $^3$H-PK11195 as a radioactive ligand and showing that TSPO occupancy in the rat cerebral cortex and hippocampus treated with 0.3 mg/kg of ONO-2952 reaches about 50% at plasma concentration of 6.30 ng/mL (Mitsui et al., 2015). However, a dose-independent relationship was found between 0.03 and 0.1 mg/kg in the hippocampus in the current
study. This might have been due to variability associated with administration of very low dose of ONO-2952. Our PET study in the monkey, on the other hand, demonstrates the need for a relatively higher plasma concentration than in the rat to achieve target occupancy levels (Table 3 and Fig. 5). In our hands, *in vitro* protein binding levels of ONO-2952 (100 ng/mL) in the rat and monkey sera were 98.7% and 99.7%, respectively (unpublished data). Considering the small interspecies difference in the affinity of ONO-2952 for TSPO in rat and monkey, the difference in protein binding of two species may result in higher requirement of blood exposure in monkey study.

Combining the results obtained from the *in vitro* and *ex vivo* binding studies, $^{11}$C-PBR28 was considered to be a useful ligand for determination of TSPO occupancy of ONO-2952 in the monkey PET study. Consistent with the reported brain kinetic of $^{11}$C-PBR28 in anesthetized monkeys (Imaizumi et al., 2008), this ligand showed wide and sufficient distribution in our PET study using conscious monkeys. In addition, pre-treatment with PK11195 shifted peak $^{11}$C-PBR28 uptake in all brain regions, and then rapidly decreased this uptake to levels lower than those detected in the vehicle-treated monkeys (Fig. 1). It is interesting to note that similar effects on $^{11}$C-PBR28 TACs were observed after oral administration of ONO-2952 in a dose-dependent manner (Fig. 3). These findings indicate that $^{11}$C-PBR28 recognizes and specifically binds to biomolecules, including TSPO, that are inhibited by both PK11195 and ONO-2952. TSPO has been found to be expressed not only in the central nervous system, but also in peripheral organs and peripheral blood cells (Gavish et al., 1999; Kreisl et al., 2010). A blocking study has shown that plasma levels of unmetabolized $^{11}$C-PBR28 increase following pre-treatment with non-radioactive TSPO ligand (Imaizumi et al., 2008). Therefore, the high $^{11}$C-PBR28 uptake observed in the PK11195-treated monkey (Fig. 1) as well as in the ONO-2952-treated monkey (Fig. 3 and Fig. 4) may be explained by increase in unbound $^{11}$C-PBR28 resulting from increased binding of cold ligand to peripheral TSPO, which in turn penetrates the brain. Due to
these peripheral effects on $^{11}$C-PBR28 kinetic, and since no suitable reference brain region free of specific binding sites for $^{11}$C-PBR28 was found, we used metabolite-corrected arterial input function to determine $V_t$ values and TSPO occupancy of ONO-2952. As shown in Fig. 2 and Table 4, a significant reduction in $V_t$ after PK11195 dosing was observed in all ROIs in the monkey brain with TSPO occupancy of about 50%. ONO-2952 also decreased $V_t$ of $^{11}$C-PBR28 in a dose-dependent manner (Fig. 4). The current PET occupancy study shows that ONO-2952 bound to TSPO in all brain ROIs, including the amygdala and hippocampus, both of which are key structures associated with physiological responses to stress (Guan et al., 2003; Myers and Greenwood-Van Meerveld, 2009; Belujon and Grace, 2011). These findings support our hypothesis that ONO-2952 may modify stress response in humans through its binding to TSPO in the brain. Similar inhibitory effects on $V_t$ images characterize brain distribution of ONO-2952 following oral administration 2 h or 24 h before PET scans. Although TSPO occupancy of ONO-2952, as determined by quantitative PET assessment 24 h after administration, was slightly less than that 2 h after dosing, plasma concentration of ONO-2952 and its TSPO occupancy in the whole brain roughly correlated. This suggests that ONO-2952 reversibly binds to TSPO with no excessive accumulation in the brain. As shown in Fig. 5, a good correlation between ONO-2952 pharmacokinetic (PK) and its TSPO occupancy was observed irrespective of the dosing time, which further supports the reversible binding property of this compound.

We have previously shown that ONO-2952 exerts anti-stress effects with brain TSPO occupancy of 50% or more in rats exposed to acute stress (Mitsui et al., 2015). In addition, the relationship between plasma concentration of ONO-2952 and its brain occupancy in rats is not changed by 1-hour stress exposure (unpublished data). Based on these findings, we assume the target occupancy of this protein for a similar effect in human would be $\geq 50\%$. Although, the contribution of peripheral TSPO to the beneficial effect of ONO-2952 in rats has not been examined, pharmacological profile points at central
TSPO as the principal target involved in the mechanism of action of this compound (Mitsui et al., 2015). Nevertheless, it is important to note that there is a marked difference in PK-occupancy relationship between the two species as discussed above. Taken together, minimal therapeutic dose of ONO-2952 as well as its optimal dose level for clinical use could be estimated using human PET study by targeting a brain occupancy of 50%, rather than using this compound PK profile. More recently, we have published a study showing that ONO-2952 exhibits clinical efficacy in patients with irritable bowel syndrome at whole-brain occupancy level of 77.4% (Whitehead et al., 2017). As we did not investigate here the relationship between anti-stress effect of ONO-2952 and its TSPO occupancy in monkey’s brain, the reason why higher whole-brain occupancy is needed in this POC study compared with rats studies remains unclear. To clarify this remaining question, a determination of expression levels of TSPO in the brain of patients with stress related disorders would be necessary.

In conclusion, this study shows that ONO-2952 sufficiently penetrates conscious monkey’s brain with a clear relationship between its plasma concentration and TSPO occupancy.
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**Authorship Contributions**

Participated in research design: Mitsui, Tsukada, and Katsumata.

Conducted experiments: Mitsui, Morimoto, Niwa, Yamaura, Ohba, Tsukada, and Katsumata.

Performed data analysis: Mitsui, Morimoto, Niwa, Yamaura, Ohba, Tsukada, and Katsumata.

Wrote or contributed to the writing of the manuscript: Mitsui, Morimoto, Tsukada, and Katsumata.
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Footnotes

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**Figure Legends**

**Fig. 1.** Typical $^{11}$C-PBR28 TACs in each ROI of conscious monkey brain after (A) vehicle and (B) PK11195 dosing. PET scans were performed at 1 min after intravenous administration of PK11195 (0.1 mg/kg) or vehicle (5% ethanol-0.5% Tween80 saline solution). The uptake values in each ROI were normalized for the injected dose of radioactivity and for animal body weight. Cere: cerebellum, Hippo: hippocampus, Str: corpus striatum, Thal: thalamus, OccCtx: occipital cortex, TmpCtx: temporal cortex, FrtCtx: frontal cortex, ParCtx: parietal cortex

**Fig. 2.** Typical MR and PET images of $^{11}$C-PBR28 in conscious monkey after (A) vehicle and (B) PK11195 dosing. PET scans were performed at 1 min after intravenous administration of PK11195 (0.1 mg/kg) or vehicle (5% ethanol-0.5% Tween80 saline solution) with sequential arterial blood sampling. Color scale indicates total distribution volume ($V_t$). (C) Linear regression of the $V_t_{base}$ vs. ($V_t_{base}$ – $V_t_{drug}$) in each brain region of interest (Lassen plot).

**Fig. 3.** Typical $^{11}$C-PBR28 TACs in each ROI of conscious monkey brain after vehicle and ONO-2952 dosing. PET scans were performed at 2 h after single oral administration of ONO-2952 at (A) 0.1 mg/kg, (B) 1 mg/kg and (C) 10 mg/kg, or vehicle (0.5% MC). Uptake values in each ROI were normalized for the injected dose of radioactivity and for animal body weight. Cere: cerebellum, Hippo: hippocampus, Str: corpus striatum, Thal: thalamus, OccCtx: occipital cortex, TmpCtx: temporal cortex, FrtCtx: frontal cortex, ParCtx: parietal cortex
**Fig. 4.** Typical $^{11}$C-PBR28 (A) TACs in each ROI and (B) PET images in conscious monkey after (a) vehicle and (b-e) ONO-2952 dosing. PET scans were performed at (a, b, c) 2 h and (d, e) 24 h after the final administration of ONO-2952 or vehicle (0.5% MC) with sequential arterial blood sampling. ONO-2952 at doses of (b, d) 1 mg/kg and (c, e) 10 mg/kg was orally administered twice daily for 3 days and once on Day 4. Uptake values in each ROI were normalized for the injected dose of radioactivity and for animal body weight. Cere: cerebellum, Hippo: hippocampus, Str: corpus striatum, Thal: thalamus, OccCtx: occipital cortex, TmpCtx: temporal cortex, FrtCtx: frontal cortex, ParCtx: parietal cortex. Color scale indicates total distribution volume (Vt). (C) Linear regression of the $V_{t\text{base}}$ vs. ($V_{t\text{base}} - V_{t\text{drug}}$) in each brain region of interest (Lassen plot).

**Fig. 5.** Relationship between plasma concentration of ONO-2952 and its TSPO occupancy in the brain of conscious monkeys. PET scans were performed following single and repeated oral administration of ONO-2952 at three or four dose levels for each animal with sequential arterial blood sampling. Plasma concentration was determined from blood withdrawn 2 h or 24 h after oral administration of ONO-2952 at several doses ranging from 0.3 mg/kg to 30 mg/kg, immediately prior to intravenous administration of $^{11}$C-PBR28. TSPO occupancy in the whole brain was defined as the slope of the Lassen plot. Dotted line represents the regression curve of TSPO occupancy-plasma concentration in PET study. IC50: plasma concentration required for 50% of maximal (fixed at 100%) TSPO occupancy in PET study. Single: ONO-2952 was orally administered once; repeated: ONO-2952 was orally administered twice daily for 3 days and once on Day 4. 2h: Assessed 2 h after the final administration; 24 h: Assessed 24 h after the final administration.
## Tables

### TABLE 1
Ki values of ONO-2952 for binding to rat and monkey TSPO

<table>
<thead>
<tr>
<th>Species</th>
<th>Membrane fraction</th>
<th>Kd (nmol/L) (^a)</th>
<th>Bmax (fmol/mg protein) (^a)</th>
<th>Ki (nmol/L) (^b)</th>
<th><strong>ONO-2952</strong></th>
<th><strong>PK11195</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>whole brain</td>
<td>0.230</td>
<td>254</td>
<td>0.164</td>
<td>0.312</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[0.129-0.330]</td>
<td>[231-277]</td>
<td>[0.127-0.211]</td>
<td>[0.249-0.389]</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>hippocampus</td>
<td>0.373</td>
<td>1757</td>
<td>0.681</td>
<td>2.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[0.190-0.555]</td>
<td>[1573-1941]</td>
<td>[0.369-1.30]</td>
<td>[1.03-7.93]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>occipital cortex</td>
<td>0.400</td>
<td>1791</td>
<td>0.558</td>
<td>3.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[0.301-0.500]</td>
<td>[1694-1888]</td>
<td>[0.283-1.10]</td>
<td>[1.52-9.25]</td>
<td></td>
</tr>
</tbody>
</table>

Competitive binding assay was performed using \(^3\)H-PBR28. \(^a\) Dissociation constant (Kd) and maximum binding (Bmax) of \(^3\)H-PBR28 in each membrane fraction. \(^b\) Inhibition constant (Ki) of each test-compound in each membrane fraction. Numerical values in the parentheses are 95% confidence intervals (n=3 in each group).
TABLE 2

Effect of ONO-2952 on Kd and Bmax values for $^3$H-PBR28 binding to monkey brain TSPO

<table>
<thead>
<tr>
<th>ONO-2952 (nmol/L)</th>
<th>Kd (nmol/L)</th>
<th>Bmax (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.405 [0.338-0.472]</td>
<td>1879 [1811-1947]</td>
</tr>
<tr>
<td>0.5</td>
<td>0.620 [0.537-0.703] *</td>
<td>1857 [1799-1915]</td>
</tr>
<tr>
<td>1</td>
<td>0.888 [0.793-0.983] *</td>
<td>1775 [1728-1822]</td>
</tr>
</tbody>
</table>

Saturation binding assay for three separate experiments was performed with membrane fractions prepared from monkey occipital cortex using $^3$H-PBR28. * Significant increase in Kd value with no effect on Bmax value (n=3 in each group).
### TABLE 3

ONO-2952 plasma concentration and TSPO occupancy in the rat brain

<table>
<thead>
<tr>
<th>ONO-2952 concentration (mg/kg)</th>
<th>Plasma concentration (ng/mL)</th>
<th>TSPO occupancy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cerebral cortex</td>
</tr>
<tr>
<td>0.03</td>
<td>0.325 ± 0.018</td>
<td>6.5 ± 13.62</td>
</tr>
<tr>
<td>0.1</td>
<td>1.32 ± 0.09</td>
<td>11.5 ± 4.51</td>
</tr>
<tr>
<td>0.3</td>
<td>6.07 ± 0.18</td>
<td>40.4 ± 4.33</td>
</tr>
<tr>
<td>1</td>
<td>39.6 ± 2.7</td>
<td>77.7 ± 2.13</td>
</tr>
<tr>
<td>3</td>
<td>145 ± 6</td>
<td>90.2 ± 0.66</td>
</tr>
</tbody>
</table>

Plasma concentration is expressed as the mean ± standard deviation. TSPO occupancy was determined by *ex vivo* binding using \(^3\)H-PBR28. TSPO occupancy in the cerebral cortex and hippocampus is expressed as the mean ± standard error (n=8 in each group). Plasma concentration required for 50% of maximal (fixed at 100%) TSPO occupancy in cerebral cortex and hippocampus were 9.78 and 18.6 ng/mL, respectively.
# TABLE 4

Effect of PK11195 on $^{11}$C-PBR28 Vt in conscious monkeys

<table>
<thead>
<tr>
<th>ROIs</th>
<th>Vt</th>
<th>Vehicle</th>
<th>PK11195</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum</td>
<td></td>
<td>47.4 ± 14.94</td>
<td>25.1 ± 4.59</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td>75.8 ± 19.78</td>
<td>39.4 ± 7.41</td>
</tr>
<tr>
<td>Corpus striatum</td>
<td></td>
<td>70.1 ± 23.38</td>
<td>32.3 ± 5.64</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
<td>61.6 ± 21.25</td>
<td>26.8 ± 5.51</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td></td>
<td>57.2 ± 18.72</td>
<td>27.2 ± 4.27</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td></td>
<td>73.3 ± 23.82</td>
<td>36.7 ± 5.41</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td></td>
<td>63.3 ± 18.40</td>
<td>32.9 ± 4.07</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td></td>
<td>61.2 ± 15.16</td>
<td>29.7 ± 2.54</td>
</tr>
</tbody>
</table>

PET scans were performed at 1 min after intravenous administration of PK11195 (0.1mg/kg) or vehicle (5% ethanol-0.5% Tween80 saline solution) with sequential arterial blood sampling. Total distribution volume (Vt) in each ROI is expressed as the mean ± standard error (n=3). Averaged TSPO occupancy of three monkeys as determined by using the Lassen plots was 49.2 ± 29.66 %.
### TABLE 5

Effects of ONO-2952 on $^{11}$C-PBR28 Vt and ONO-2952 plasma concentration in conscious monkeys

<table>
<thead>
<tr>
<th>ONO-2952 mg/kg (time *)</th>
<th>Plasma concentration (ng/mL)</th>
<th>Vt</th>
<th>TSPO occupancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cere</td>
<td>Hippo</td>
<td>Str</td>
</tr>
<tr>
<td>Vehicle (-)</td>
<td>74.7</td>
<td>128.3</td>
<td>132.1</td>
</tr>
<tr>
<td>1 (2 h)</td>
<td>24.0</td>
<td>34.6</td>
<td>37.8</td>
</tr>
<tr>
<td>10 (2 h)</td>
<td>29.6</td>
<td>43.4</td>
<td>46.2</td>
</tr>
<tr>
<td>1 (24 h)</td>
<td>10.3</td>
<td>12.2</td>
<td>13.6</td>
</tr>
<tr>
<td>10 (24 h)</td>
<td>16.5</td>
<td>163</td>
<td>10.3</td>
</tr>
</tbody>
</table>

*PET scans were performed at 2 h and 24 h after the final administration of ONO-2952 or vehicle (0.5% MC) with sequential arterial blood sampling. ONO-2952 at doses of 1 and 10 mg/kg was orally administered twice daily for 3 days and once on Day 4. TSPO occupancy in the whole brain was defined as the slope of the Lassen plot. All data were obtained from the experiment shown in Fig. 4. Cere: cerebellum, Hippo: hippocampus, Str: corpus striatum, Thal: thalamus, OccCtx: occipital cortex, TmpCtx: temporal cortex, FrtCtx: frontal cortex, ParCtx: parietal cortex.*
Figure 1
Figure 2

A. Vehicle

B. PK11195 (0.1 mg/kg, i.v.)

C. Lassen plot

\[ y = 0.7075x - 10.12 \]

\[ R^2 = 0.6976 \]
Figure 3

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A. $^{11}$C-PBR28 TACs in each ROI

a) Vehicle (2 h after administration)

- SUV as a function of time after injection of $^{11}$C-PBR28.
- Time range: 0 to 120 min.
- ROIs: Cere, Hippo, Str, Thal, OccCtx, TmpCtx, FrtCtx, ParCtx.

ONO-2952 (2 h after administration)

b) 1 mg/kg
c) 10 mg/kg

d) ONO-2952 (24 h after administration)

d) 1 mg/kg
e) 10 mg/kg

B. MR and PET images of $^{11}$C-PBR28 MRI

a) Vehicle (2 h after administration)
b) ONO-2952 (1 mg/kg, 2 h after administration)
c) ONO-2952 (10 mg/kg, 2 h after administration)
d) ONO-2952 (1 mg/kg, 24 h after administration)
e) ONO-2952 (10 mg/kg, 24 h after administration)

C. Lassen plot

ONO-2952 (2 h after administration)
b) 1 mg/kg  c) 10 mg/kg

d) ONO-2952 (24 h after administration)
d) 1 mg/kg  e) 10 mg/kg

Figure 4
IC$_{50}$=19.6 ng/mL

Figure 5