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In Vivo Evaluation of ^{11}C-labeled Three Radioligands for Glycine Transporter 1 in the Mouse Brain

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Objective: Glycine transporter 1 (GlyT-1) is one of the most attractive therapeutic targets for schizophrenia. There is great interest in developing radioligands for *in vivo* imaging of GlyT-1 in the brain using positron emission tomography. Here, we report the properties of three novel non-sarcosine-based radioligands [^{11}C]CHIBA-3007, [^{11}C]CHIBA-3009, and [^{11}C]CHIBA-3011, for GlyT-1 imaging in the mouse brain *in vivo*.
Methods: The three radioligands were synthesized by *N*-[^{11}C] methylation of the corresponding desmethyl precursor. A pharmacological characterization of these radioligands for *in vivo* imaging of GlyT-1 in the brain was conducted using male ddY mice.
Results: [^{11}C]CHIBA-3009 and [^{11}C]CHIBA-3011 were scarcely incorporated into the brain, whereas [^{11}C]CHIBA-3007 showed slight but considerable brain uptake. Regional brain uptake of [^{11}C]CHIBA-3007 (medulla oblongata>cerebellum>cortex) was similar to the distribution of the GlyT-1 protein. However, pretreatment with CHIBA-3007 (1 mg/kg) or the GlyT-1 selective inhibitor ALX5407 (N-[(3R)-3-([1,1'-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine) (30 mg/kg) did not significantly decrease brain uptake of [^{11}C]CHIBA-3007, suggesting low specific binding to GlyT-1. Pretreatment with cyclosporin A significantly increased brain uptake of [^{11}C]CHIBA-3009 and [^{11}C]CHIBA-3011, suggesting a role for P-glycoprotein in the brain uptake of these ligands. All three radioligands were rapidly degraded intact forms were 3-18% in plasma and 15-74% in the brain at 15 min after injection.
Conclusion: The results suggest that these three radioligands are not suitable for *in vivo* imaging of GlyT-1 in the brain because of low brain uptake and rapid metabolism. Further structural refinement is necessary to enhance brain uptake.

KEY WORDS: Glycine transporter 1; Brain; Positron-emission tomography; CHIBA-3007; CHIBA-3009; CHIBA-3011.

INTRODUCTION

Multiple lines of evidence suggest that a dysfunction in glutamatergic neurotransmission via *N*-methyl-D-aspartate (NMDA) receptors contributes to the pathophysiology of psychiatric diseases, including schizophrenia.[1-7] Furthermore, glycine acts as an obligatory co-agonist strychnine-insensitive glycine site located on the NMDA receptor.[8-11]

Glycine transporters (GlyT), which belong to the Na^+/Cl^--dependent family of neurotransmitter transporters, regulate synaptic levels of glycine during excitatory neurotransmission via the NMDA receptor and inhibitory neurotransmission via the glycine receptor.[12] Two subtypes of glycine transporters, GlyT-1 and GlyT-2, have been identified, and we focused on GlyT-1. GlyT-1 is expressed at glutamatergic synapses throughout the mammalian brain and co-localizes with the NMDA receptor.[13-15] Potentiation of NMDA receptor function by pharmacological manipulation may be a useful approach for treating diseases associated with NMDA receptor hypofunction. One possible strategy is to increase synaptic levels of glycine by blocking GlyT- 1.[16-18] Therefore, numerous efforts have been made to develop suitable drug candidates to inhibit GlyT-1 in the brain.[17,18] Hashimoto[19] reported that levels of the GlyT-1 protein in the hippocampus, but not the frontal cortex, increased significantly after repeated administration of the NMDA receptor antagonist phencyclidine, suggesting that the increased hippocampal GlyT-1 and subsequent decreased extracellular glycine levels may contribute to NMDA receptor hypofunction in this region. However, no reports are available on the levels of GlyT-1

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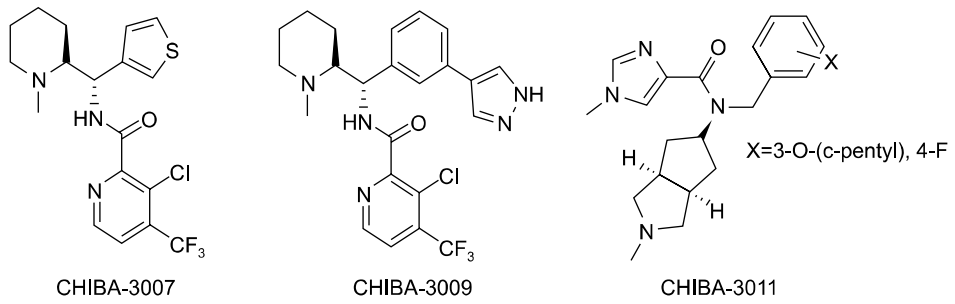

CHIBA-3007 CHIBA-3009 CHIBA-3011

Fig. 1. Chemical structure of Glycine transporter-1 inhibitors used in this study.

in postmortem brain samples from patients with schizophrenia. Therefore, it is of great interest not only to measure the dose-occupancy relationships of potential therapeutic GlyT-1 inhibitors but also to analyze pathological changes in GlyT-1 expression in the intact human brain using positron emission tomography (PET).

Some recent reports have indicated that PET GlyT-1 radioligands entered an early clinical study[15,20,21] however, target quantitation was difficult due to the slow kinetics and absence of a GlyT-1 reference region. Therefore, the challenge remains to identify suitable radioligands with the aim of improving imaging properties. Previously, we reported on three carbon-11-labeled non-sarcosine-based GlyT-1 inhibitors, SA1, SA2, and SA3, which possess an *N*-((*S*)-((*S*)-1-methylpiperidin-2-yl)methyl)arylamide structure.[22] Although [^{11}C] SA1 showed a suitable profile for GlyT-1 imaging in the rodent brain, high lipophilicity (calculated Log [cLog] *p*=5.01) of [^{11}C]SA1 led to marginal specific binding in the conscious monkey brain. We selected novel *N*-((*S*)-1-((*S*)-1-methylpiperidine-2-yl)methyl)arylamide derivatives to reduce non-specific SA1 binding, with a pyridine ring replacing the benzene rings.

We have successfully synthesized [^{3}H]3-chloro-*N*-((*S*)-((*R*)-1-methylpiperidin-2-yl)(thiophen-3-yl)methyl)-4-(trifluoromethyl)picolinamide ([^{3}H]CHIBA-3007), a non-sarcosine-based radioligand to evaluate GlyT-1, and determined that it would be a useful new *in vitro* radioligand.[23] These findings promoted further evaluations of carbone-11 labeled CHIBA-3007 as an *in vivo* imaging agent for GlyT-1. We also selected a lipophilic analog of CHIBA-3007, 3-chloro-*N*-((*S*)-(3-(1*H*-pyra z-ol-4-yl)phenyl)((*2S*)-1-methylpiperidin-2-yl)methyl)-4-(trifluoromethyl)piridine-2-carboxamide) (CHIBA-3009), and a low lipophilic GlyT-1 selective inhibitor *N*-(3-(cyclopentyloxy)-4-fluorobenzyl)-1-methyl- *N*-(2-methyloctahydrocyclopenta[c]pyrrol-5-yl)-1*H*-imidazole-4-carboxamide (CHIBA-3011), as new potential structures for GlyT-1 imaging. The chemical structures and cLog *p* values of the GlyT-1 inhibitors are summarized in Supplementary Fig. 1. Here, we report the properties of the three new GlyT-1 non-sarcosine-based radioligands, [^{11}C]CHIBA-3007 (cLog *p*=4.09), and [^{11}C]CHIBA-3009 (cLog *p*=3.94), and [^{11}C] CHIBA- 3011 (cLog *p*=2.84), in the mouse brain *in vivo*.

METHODS

General

CHIBA-3007, CHIBA-3009, CHIBA-3011, and their corresponding desmethyl precursors were synthesized according to methods described previously (Fig. 1).[24-26] ALX5407 (N-[(3R)-3-([1,1'-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine) and cyclosporin A (sandimmun) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Novartis Pharma (Tokyo, Japan), respectively. All other chemical reagents were obtained from commercial sources.

Male ddY mice (8 weeks of age; body weight, 36-39 g) were obtained from Tokyo Laboratory Animals (Tokyo, Japan). Animal studies were approved by the institutional committees for animal experiments at the Tokyo Metropolitan Institute of Gerontology and Chiba University.

Radiosynthesis

Radioligands were synthesized by *N*-[^{11}C]methylation of the corresponding desmethyl precursor with [^{11}C]methyl triflate prepared using an automated synthesis system, as described previously (Supplementary Fig. 2).[27] [^{11}C] Methyl triflate was trapped in acetone (0.25 ml) containing 0.25 mg of each desmethyl precursor. After adding a 1.3 ml mixture of water and mobile phase (1 : 1) as described below, the reaction mixture was applied to a high-performance liquid chromatography (HPLC) system with a reversed-phase column (YMC-Pack-Pro ODS- A, 10 mm i.d. ×250 mm length; YMC, Kyoto, Japan), UV absorbance (260 nm), and a semiconductor radiation detector. The mobile phase was a mixture of CH_3CN, 50 mM CH_3CO_2H, and 50 mM $CH_3CO_2NH_4$ (35/32.5/32.5, v/v/v)

for [^{11}C]CHIBA-3007 or a mixture of CH_3CN, 50 mM CH_3CO_2H, and 50 mM $CH_3CO_2NH_4$ (30/35/35, v/v/v) for [^{11}C]CHIBA-3009 and [^{11}C]CHIBA-3011 at a flow rate of 5 ml/min. The objective fraction (retention time: 8.7 min for [^{11}C]CHIBA-3007, 14.4 min for [^{11}C]CHIBA-3009, and 9.7 min for [^{11}C]CHIBA-3011) was collected in a flask containing 0.2 ml of 100 mg/ml ascorbate and evaporated to dryness. The residue was dissolved in physiological saline and filtered through a 0.22-μm membrane filter (Millipore, Billerica, MA, USA).

The labeled compound was analyzed by HPLC on aTSKgel Super-ODS column (4.6 mm i.d. ×100 mm length; Tosoh, Tokyo, Japan), with a mobile phase of CH_3CN/50 mM CH_3CO_2H/50 mM $CH_3CO_2NH_4$ (30/35/35) for [^{11}C]CHIBA-3007 and [^{11}C]CHIBA-3009 and CH_3CN/0.1% CF_3CO_2H (28/72) for [^{11}C]CHIBA-3011. The flow rate was 1.0 ml/min, and retention times were 4.5 min for [^{11}C]CHIBA-3007, 2.9 min for [^{11}C]CHIBA-3009, and 4.2 min for [^{11}C]CHIBA-3011.

Measurement of [^{3}H]glycine Uptake Inhibition

The inhibition of [^{3}H]glycine uptake by CHIBA-3007 and CHIBA-3009 was performed as described previously.[22] IC_{50} values due to binding displacement by these drugs were determined using GraphPadPrism (GraphPad Software, San Diego, CA, USA).

Tissue Distribution Study

Tissue distribution studies, including the whole body and regional brain, were conducted in normal male ddY mice to explore the *in vivo* characteristics of the three ^{11}C-labeled compounds.

[^{11}C]CHIBA-3007, [^{11}C]CHIBA-3009, or [^{11}C]CHIBA-3011 was intravenously injected into mice through the tail veil. The injected dose of each radioligand was 2.0-2.9 MBq/17-74 pmol. Mice were killed by cervical dislocation at 1, 5, 15, 30, and 60 min after injection (n=4 in each group). Blood was collected by heart puncture, and the brain, heart, lung, liver, pancreas, spleen, kidney, small intestine, and muscle were removed. The regional brain distribution of each radioligand was determined in the same groups of mice. The brain was divided into the cerebellum, medulla oblongata, cerebral cortex, and residual tissue. Carbon-11 was counted in the samples with an auto-gamma-counter (Compu-gamma 1282CS; LKB Wallac, Turku, Finland), and the tissues were weighed. Tissue uptake of carbon-11 was expressed as a percentage of the injected dose per gram of tissue (% Injected dose [ID]/g).

[^{11}C]CHIBA-3007 Blocking Study

GlyT-1 blocking studies were performed by pretreatment with the intraperitoneal administration of CHIBA-3007 (1 mg/kg, n=7) or sarcosine-based GlyT-1 inhibitor ALX5407 (30 mg/kg, n=6) dissolved in dimethyl sulfoxide (DMSO). The same volume of DMSO was injected into control mice; (n=7). At 30 min after treatment, [^{11}C]CHIBA-3007 (2.0 MBq/37 pmol) was injected intravenously into mice; 15 min later they were killed, and then the blood and brain were removed. The radioactivity levels of the regional brain tissue and blood were measured as %ID/g.

Effect of Cyclosporin A (CysA)

To investigate the effect of P-glycoprotein (P-gp) on brain uptake of [^{11}C]CHIBA-3009 and [^{11}C]CHIBA-3011, three groups of mice were pretreated with an intravenous injection of CysA (50 mg/kg) dissolved in physiological saline 30 min prior to the radioligand injection. Immediately after the treatment, group 1 mice were injected intraperitoneally with unlabeled CHIBA-3009 (1 mg/kg) or CHIBA-3011 (1 mg/kg) carrier, group 2 mice were injected intraperitoneally with ALX5407 (30 mg/kg), and group 3 mice were injected with physiological saline. As the control, a group of mice were given the same volume of physiological saline. Each radioligand was intravenously injected into the four groups of mice (n=4-7 for each group) 15 min later, they were killed, and the tissue uptake of carbon-11 was measured as %ID/g.

Metabolite Study

Each radioligand (160-220 MBq/0.6-1.8 nmol) was intravenously injected into mice (n=3 for each ligand), and 15 or 30 min later they were sacrificed by cervical dislocation. Blood was removed by heart puncture using a heparinized syringe, and the brain was removed. After centrifugation of the blood at 7,000× g for 1 min at 4^{o}C to obtain plasma, 0.2-0.5 ml of the plasma was diluted with water up to 0.5 ml and denatured with 0.5 ml 20% trichloroacetic acid (TCA) in CH_3CN in an ice-water bath. The plasma suspension was centrifuged and divided into soluble and precipitable fractions. The precipitate was re-suspended in 0.5 ml 10% TCA in CH_3CN followed by centrifugation. This procedure was repeated twice. The cerebellum (61-80 mg) was homogenized in 1 ml of 10% TCA in CH_3CN/H_2O (1/1, v/v). The homogenate was treated as described for plasma. Radioactivity in the three soluble fractions and precipitates was measured with an auto-gamma counter. The recovery yields in the soluble

fraction were 91-98% and 86-98% for plasma and brain, respectively. The soluble fractions were combined, diluted with two equivalent volumes of 50 mM sodium acetate buffer (pH 4.5), and centrifuged as described above. A portion of the supernatant was analyzed by HPLC equipped with a radioactivity detector (Radiomatic 150TR; Packard Instruments, Meriden, CT, USA). The reversed-phase column used for [^{11}C]CHIBA-3007 was a NOVA-Pak C_{18} equipped with a radial compression module (RCM) 8×10 module (8 mm i.d. ×100 mm; Waters, Milford, MA, USA) with a mobile phase mixture of CH_3CN and 50 mM sodium acetate buffer (pH 4.5) (40/60) at a flow rate of 4 ml/min. The corresponding columns, mobile phases, and flow rates were: YMC ODS-A (10 mm i.d. ×250 mm, YMC), a mixture of CH_3CN and 50 mM sodium acetate buffer (pH 4.5) (30/70) and 2.5 ml/min for [^{11}C]CHIBA- 3009; a YMC ODS-A (10×150 mm), column, a mixture of CH_3CN and 50 mM sodium acetate buffer (pH 4.5) (35/65), and 4.0 ml/min for [^{11}C]CHIBA-3011. The elution profile was monitored with a radioactivity detector (FLO-ONE 15 0TR, Packard Instruments). The retention times of [^{11}C]CHIBA-3007, [^{11}C]CHIBA-3009, and [^{11}C]CHIBA-3011 were 6.2 min, 7.5 min,and 7.3 min, respectively. Recovery of the injected radioactivity in the eluate was essentially quantitative.

Statistical Analysis

Data are expressed as mean±standard deviation. The statistical analysis was performed using a one-way analysis of variance and the *post hoc* Bonferroni/Dunn test. p values < 0.05 were considered statistically significant.

RESULTS

Radiosynthesis

[^{11}C]CHIBA-3007, [^{11}C]CHIBA-3009, and [^{11}C] CHIBA-3011 were radiosynthesized by [^{11}C]methylation of the desmethyl precursor. The radiochemical yields based on [^{11}C]methyl triflate for [^{11}C]CHIBA-3007, [^{11}C] CHIBA-3009, and [^{11}C]CHIBA-3011 were 53.2±12.6%

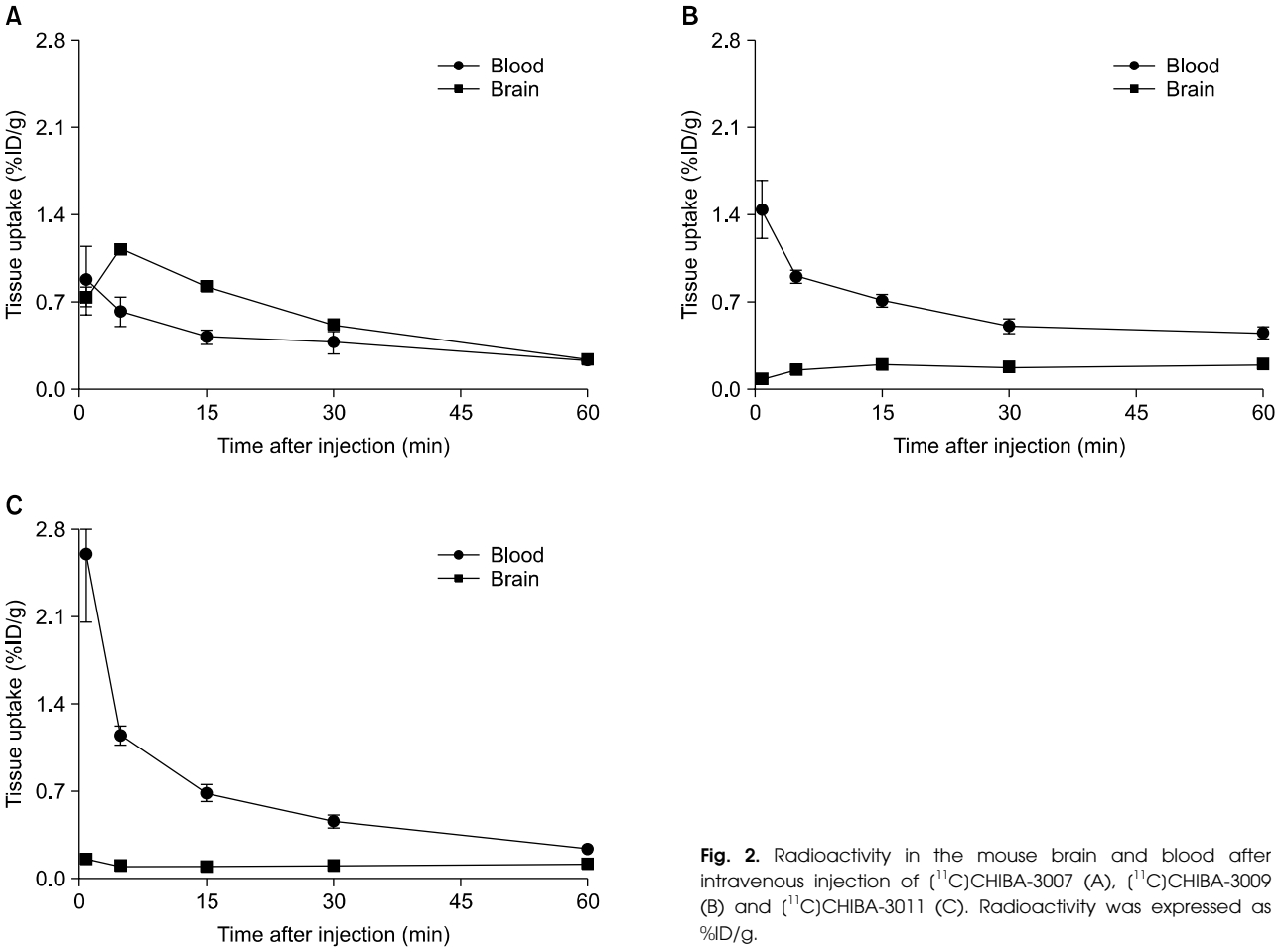

Fig. 2. Radioactivity in the mouse brain and blood after intravenous injection of (^{11}C)CHIBA-3007 (A), (^{11}C)CHIBA-3009 (B) and (^{11}C)CHIBA-3011 (C). Radioactivity was expressed as %ID/g.

(n=4), 35.9±9.6% (n=4), and 60.4±7.9% (n=4), respectively. The total preparation time for these three radioligands, including purification and formulation, was approximately 30 min from the end of irradiation. The radiochemical purities of the radioligands were >95%, and the specific activities at 30 min after the end of irradiation were 184±94 GBq/μmol (n=4), 147±133 GBq/μmol (n=4), and 164±86 GBq/μmol (n=4) for [^{11}C]CHIBA-3007, [^{11}C]CHIBA-3009, and [^{11}C]CHIBA-3011, respectively. In the case of the [^{11}C]CHIBA-3011 synthesis, the precursor was freshly dissolved in acetone because of its instability.

Transporter Affinity and Specificity

CHIBA-3007 and CHIBA-3009 inhibited [^{3}H]glycine uptake in mouse brain homogenates with IC_{50} values of 2.7 nM and 5.0 nM (n=3), respectively. Furthermore, CHIBA-3007 (1 μM) has been demonstrated to be devoid of activity (inhibition<50%) in a 28-standard receptor-binding profile.[23)]

Tissue Distribution Study

Whole Body

Tissue distribution studies of the three radioligands in the entire body and the regional brain of mice are summarized in Tables 1-3. The three radioligands exhibited a relatively fast clearance rate of radioactivity from the blood within 60 min. The half-lives for blood clearance were 15 min, 15 min, and 5 min for [^{11}C]CHIBA-3007, [^{11}C]CHIBA-3009, and [^{11}C]CHIBA-3011, respectively (Fig. 2). The highest initial uptake (%ID/g) of [^{11}C]CHIBA-3007 occurred in the lung followed by the kidney, heart, pancreas, small intestine, spleen, muscle, liver, blood, and brain sequentially (Table 1). Additionally, [^{11}C]CHIBA-3007 displayed increased radioactivity in the small intestine, which might reflect hepatobiliary clearance of the parent tracers and/or their radioactive metabolites. The highest initial uptake of [^{11}C] CHIBA-3009 was found in the lung followed by the kidney, liver, heart, pancreas, small intestine, spleen, muscle, blood, and brain (Table 2). The highest initial uptake of [11C]CHIBA-3011 was found in the kidney, followed by the spleen, lung, heart, pancreas, small intestine, liver,

Table 1. Tissue distribution of radioactivity after intravenous injection of ([^{11}C)CHIBA-3007 into mice

	% Injected dose/g tissue (mean±standard deviation n=4)				
	1 min	5 min	15 min	30 min	60 min
Blood	0.88±0.08	0.62±0.03	0.42±0.04	0.37±0.01	0.23±0.04
Heart	8.08±2.94	3.82±0.16	1.75±0.19	1.15±0.26	0.56±0.12
Lung	36.81±10.00	24.00±4.79	10.70±2.81	8.38±2.81	3.05±1.25
Liver	2.19±0.67	6.05±0.88	10.74±2.05	10.25±0.21	6.14±1.24
Pancreas	5.38±0.99	10.14±1.41	9.01±0.79	7.49±1.36	3.29±0.64
Spleen	3.22±1.31	6.07±1.26	6.76±0.94	4.88±0.55	1.80±0.45
Kidney	13.81±3.53	13.04±0.59	7.77±1.71	4.28±0.81	2.72±1.30
S. intestine	4.01±1.18	7.20±0.67	13.65±3.88	12.30±2.78	12.45±3.66
Muscle	3.07±0.63	2.49±0.44	1.65±0.26	1.03±0.11	0.54±0.16
Brain	0.74±0.27	1.11±0.12	0.82±0.06	0.51±0.09	0.24±0.03

Table 2. Tissue distribution of radioactivity after intravenous injection of ([^{11}C)CHIBA-3009 into mice

	% Injected dose/g tissue (mean±standard deviation n=4)				
	1 min	5 min	15 min	30 min	60 min
Blood	1.44±0.23	0.90±0.05	0.71±0.05	0.51±0.06	0.45±0.05
Heart	7.93±0.94	5.64±0.37	3.04±0.52	1.55±0.26	0.85±0.19
Lung	17.22±1.47	12.50±0.94	8.61±0.39	6.54±0.69	5.13±0.63
Liver	8.57±0.98	10.05±0.77	8.33±1.01	8.06±1.24	6.43±0.73
Pancreas	4.74±0.77	7.48±0.91	6.98±0.95	6.84±1.01	6.29±0.75
Spleen	3.09±0.44	4.87±0.51	4.29±0.38	3.20±0.68	2.37±0.30
Kidney	16.84±2.58	14.08±1.14	11.97±1.61	6.38±1.27	4.53±1.30
S. intestine	3.12±0.38	3.32±0.22	3.28±0.90	2.87±0.24	2.90±0.31
Muscle	1.48±0.11	1.82±0.40	1.57±0.23	0.91±0.15	0.61±0.05
Brain	0.08±0.00	0.15±0.02	0.19±0.03	0.17±0.05	0.19±0.01

Table 3. Tissue distribution of radioactivity after intravenous injection of (^{11}C)CHIBA-3011 into mice

	% Injected dose/g tissue (mean±standard deviation, n=4)				
	1 min	5 min	15 min	30 min	60 min
Blood	2.60±0.55	1.15±0.07	0.69±0.07	0.46±0.05	0.24±0.02
Heart	7.20±0.33	2.33±0.51	0.89±0.11	0.52±0.08	0.29±0.03
Lung	12.16±2.28	9.04±1.59	5.52±1.01	2.95±0.34	1.13±0.06
Liver	4.41±1.69	3.36±0.55	1.85±0.17	0.93±0.19	0.50±0.06
Pancreas	6.66±0.75	6.09±0.84	3.36±0.43	2.22±0.39	1.79±0.30
Spleen	14.24±2.58	20.30±4.46	15.34±1.59	11.42±2.38	5.38±0.45
Kidney	20.05±3.48	12.19±3.53	4.78±0.34	2.66±0.60	1.16±0.22
S. intestine	6.31±0.63	11.84±2.82	11.06±3.43	8.39±1.88	6.72±2.77
Muscle	2.08±0.04	1.29±0.08	0.89±0.11	0.48±0.10	0.34±0.13
Brain	0.15±0.01	0.10±0.01	0.10±0.01	0.11±0.01	0.11±0.01

Table 4. Effects of pretreatment with carrier and ALX5407 on brain uptake of (^{11}C)CHIBA-3007 in mice

	% Injected dose/g tissue (mean±standard deviation)		
	Control (n=7)	Carrier* (n=7)	ALX5407* (n=6)
Blood	0.60±0.04	0.91±0.29	0.48±0.05
Whole brain	1.28±0.22	1.32±0.18	1.02±0.09
Medulla oblongata	1.79±0.44	1.43±0.14	1.53±0.26
Cerebellum	1.28±0.22	1.04±0.12	1.06±0.09
Cerebral cortex	1.00±0.16	1.33±0.22	0.78±0.06

*Unlabeled carrier CHIBA-3007 or GlyT-1 inhibitor ALX5407 was intraperitoneally injected with a dose of 1 mg/kg and 30 mg/kg, respectively, into mice 30 min before injection of (^{11}C)CHIBA-3007. Brain uptake was measured as % Injected dose/g after 15 min of (^{11}C)CHIBA-3007 injection.

blood, muscle, and brain (Table 3).

Brain

Whole brain uptake of [^{11}C]CHIBA-3007 was low, with a maximum value of 1.11 %ID/g at 5 min after administration followed by a slow decrease over 60 min (Fig. 2). The order of %ID/g for [^{11}C]CHIBA-3007 was as follows: medulla oblongata > cerebellum > cerebral cortex (Table 4). In contrast, [^{11}C]CHIBA-3009 and [^{11}C]CHIBA-3011 were scarcely incorporated into the brain, and the maximum values were 0.19 %ID/g for [^{11}C]CHIBA-3009 and 0.15 %ID/g for [^{11}C]CHIBA-3011 at 15 and 1 min, respectively. A slight increase and/or relatively slow clearance of radioactivity was observed for both radioligands (Fig. 2).

[^{11}C]CHIBA-3007 Blocking Study

The blocking effects of [^{11}C]CHIBA-3007 are shown in Table 4. Pretreatment with unlabeled carrier CHIBA-3007 or ALX5407 did not significantly decrease brain uptake of [^{11}C]CHIBA-3007 in the whole brain or in the three regional areas.

Effect of CysA

After pretreatment with CysA, brain uptake of both [^{11}C]CHIBA-3009 and [^{11}C]CHIBA-3011 increased significantly compared with that in the control (CysA/cControl ratios 4.0 and 11.7, respectively) ($p < 0.05$); however, the radioactivity entering the brain was almost homogeneous (Table 5). In the CysA-treated mice, the increased uptake of [^{11}C]CHIBA-3011 was blocked (60-65%) by ALX5407 in the three regions ($p < 0.05$), but similar blocking did not occur with [^{11}C]CHIBA-3009. A tendency for decreased uptake of [^{11}C]CHIBA-3011 was found in the carrier group, but the difference was not significant.

Metabolite Study

The results of HPLC analyses for radiolabeled metabolites in plasma and brain are summarized in Table 6. A few radioactive peaks other than the unchanged form were detected for the three radioligands in both plasma and brain. Among the three ligands, [^{11}C]CHIBA-3009 was most rapidly degraded, whereas [^{11}C]CHIBA-3007 was relatively stable. A large amount of metabolites existed in the brain.

DISCUSSION

We successfully labeled three non-sarcosine-based GlyT-1 inhibitors with carbon-11 and investigated their potential for *in vivo* GlyT-1 imaging in mice.

The *in vivo* distribution study in mice showed that [^{11}C]CHIBA-3007 was the only radioligand that could cross the blood-brain barrier (BBB) among all three radioligands, although its brain permeability was low. The affinity (IC_{50}=2.7 nM) of CHIBA-3007 for [^{3}H]glycine uptake sites was high. Additionally, the regional brain distribution of [^{11}C]CHIBA-3007 radioactivity in the mouse brain was similar to previous findings on the localization

Table 5. Effects of cyclosporin A (CysA) treatment and co-injected carriers and ALX5407 on brain uptake of (^{11}C)CHIBA-3009 and (^{11}C)CHIBA-3011 in mice

	% Injected dose/g tissue of (^{11}C)CHIBA-3009*				% Injected dose/g tissue of (^{11}C)CHIBA-3011*			
	Control (n=7)	CysA†			Control (n=4)	CysA†		
		Control (n=5)	Carrier‡ (n=4)	ALX5407‡ (n=6)		Control (n=4)	Carrier‡ (n=4)	ALX5407‡ (n=4)
Blood	1.21±0.10	1.24±0.24	1.17±0.12	1.06±0.32	1.00±0.08	1.96±0.43§	1.81±0.33	1.96±0.35
Whole brain Medulla	0.42±0.08	1.69±0.50§	1.48±0.31	1.44±0.52	0.15±0.02	1.76±0.19§	1.47±0.18	0.68±0.41¶
oblongata	0.48±0.12	1.82±0.56§	1.60±0.36	1.55±0.60	0.21±0.03	1.86±0.28§	1.35±0.58	0.70±0.39¶
Cerebellum	0.42±0.08	1.84±0.57§	1.61±0.39	1.19±0.51	0.17±0.03	1.92±0.29§	1.32±0.60	0.68±0.41¶
Cerebral cortex	0.35±0.10	1.64±0.49§	1.49±0.32	1.47±0.56	0.10±0.02	1.76±0.21§	1.22±0.58	0.71±0.45¶

*Data represent the mean±standard deviation (n=4-7). †CysA (50 mg/kg) was intravenously injected into mice, 30 min prior to of (^{11}C)CHIBA-3009 or (^{11}C)CHIBA-3011 injection. ‡Unlabeled carrier (CHIBA-3009/CHIBA-3011) or glycine transporter-1 inhibitor ALX5407 was intraperitoneally with a dose of 1 mg/kg or 30 mg/kg, respectively, into mice 30 min before radioligand injection. In control mice, the same amount of dimethyl sulfoxide was injected. Brain uptake was measured as % Injected dose/g after 15 min of each ligand injection. §$p<0.05$: compared with control, ¶$p<0.05$: compared with CysA-treated control.

Table 6. Percentages of radiolabeled metabolites in plasma and brain after intravenous injection of ^{11}C-labeled glycine transporter-1 ligands into mice

		Percentage of metabolites*			
(^{11}C)CHIBA-3007		Metabolite-1 (2.2 min)	Metabolite-2 (3.5 min)		Unchanged form (6.2 min)
Plasma	15 min	8.2±1.0	2.6±1.3		17.7±1.0
	30 min	78.1±1.6	7.8±2.1		11.7±2.6
Brain	15 min	21.6±10.7	3.2±2.9		74.1±13.9
	30 min	42.9±9.4	5.9±4.5		50.7±9.7
(^{11}C)CHIBA-3009		Metabolite-1 (4.2 min)	Metabolite-2 (9.5 min)		Unchanged form (7.5 min)
Plasma	15 min	90.7±0.3	4.7±3.4		3.4±1.2
Brain	15 min	78.3±7.5	5.3±2.4		15.2±4.0
(^{11}C)CHIBA-3011		Metabolite-1 (2.9 min)	Metabolite-2 (3.6 min)	Metabolite-3 (4.5 min)	Unchanged form (7.3 min)
Plasma	15 min	40.2±5.2	44.4±4.4	4.4±0.1	9.4±0.9
Brain	15 min	22.7±3.1	20.0±0.9	7.8±1.4	45.9±5.6

Data show the mean±standard deviation (n=3).
*Metabolites 1-3 with retention times of 2.2-9.5 min may not be single component.

of GlyT-1 mRNA using *in situ* hybridization and the GlyT-1 protein using immunohistochemistry[28,29] in our previous *in vitro* membrane-binding study with [^{3}H] CHIBA-3007 using rat brain homogenate[23] and on *in vivo* distribution studies with PET radioligands such as [^{11}C]GSK931145, [^{18}F]MK-6577, [^{11}C]CMPyPB, and [^{11}C]SA1 in animals and humans.[15,20-22] These findings suggest that [^{11}C]CHIBA-3007 binding sites may be associated with GlyT-1 in the mouse brain. However, [^{11}C] CHIBA-3007 showed low brain uptake (1.28±0.22 %ID/g) and rapid washout from the brain. Furthermore, pretreatment with carrier and the sarcosine-based GlyT-1 inhibitor ALX5407 did not decrease regional brain uptake of [^{11}C]CHIBA-3007 (Table 4). Low brain uptake reduced the statistically significant specific binding signals. Furthermore, a large amount of brain metabolites masked [^{11}C]CHIBA-3007 specific binding.

Radioligand incorporation into the brain is a key prerequisite for its effective use for brain imaging. Previous studies have shown that lipophilicity is one of the most important factors affecting brain uptake of a molecule from blood circulation and indicate that many lipophilic compounds are often P-gp substrates found in the BBB, which promptly transports these compounds back into the blood.[30] The modulation of several radioligands by P-gp indicates limited radioligand incorporation into the brain of rodents.[31-34] Thus, we evaluated whether brain uptake of [^{11}C]CHIBA-3009 and [^{11}C]CHIBA-3011 was affected by pretreatment with CysA.

As shown in Table 5, CysA pretreatment produced four-fold and 12-fold increases in brain uptake of [^{11}C] CHIBA-3009 and [^{11}C]CHIBA-3011, respectively. These results demonstrate that P-gp modulation of both radioligands resulted in very low brain uptake. However, blood levels of radioactivity in mice treated with CysA were higher than those of controls after administering [^{11}C]

CHIBA-3011, although the reasons for the increase in CysA blood levels are unknown. Therefore, it seems that increased blood levels in mice treated with CysA may partly contribute to the increase in brain radioactivity after the administration of [^{11}C]CHIBA-3011.

Regional differences in the uptake of both [^{11}C]CHIBA-3009 and [^{11}C]CHIBA-3011 were unclear in the brains of mice treated with CysA; however, ALX5407 treatment significantly decreased brain uptake of [^{11}C]CHIBA-3011 to 60-65% of the CysA-treated control. These findings suggest that [^{11}C]CHIBA-3011 may bind to GlyT-1 in the brain to some degree. Decreased brain uptake of [^{11}C]CHIBA-3009 was not significant, suggesting low specific binding. The reasons for these results could be that 1) the concentration of the unlabeled carriers (1 mg/kg each for both CHIBA-3009 and CHIBA-3011) in the brain may have been much lower than that of ALX5407 (30 mg/kg) because both compounds were excluded from the brain by P-gp; or 2) the rapid degradation of all radioligands and the presence of radiolabeled metabolites in the brain may have led to a lack of GlyT-1 specific binding.

The results suggest that these three radioligands are not suitable for *in vivo* imaging of GlyT-1 in the brain because of their poor brain permeability and extensive metabolism. Further structural refinement of these tracer agents is necessary to enhance their uptake in the brain.

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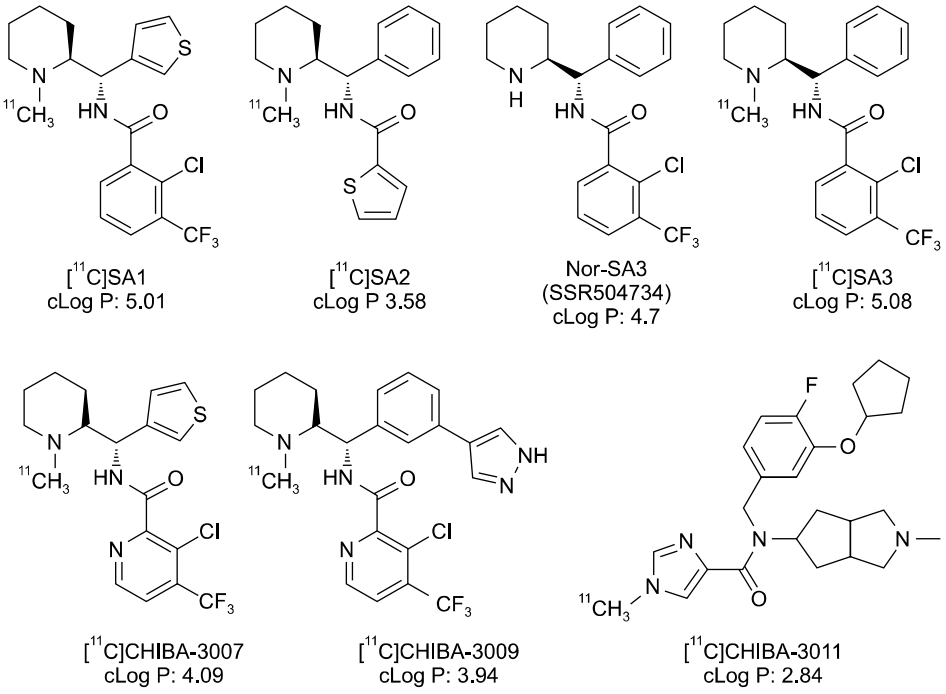
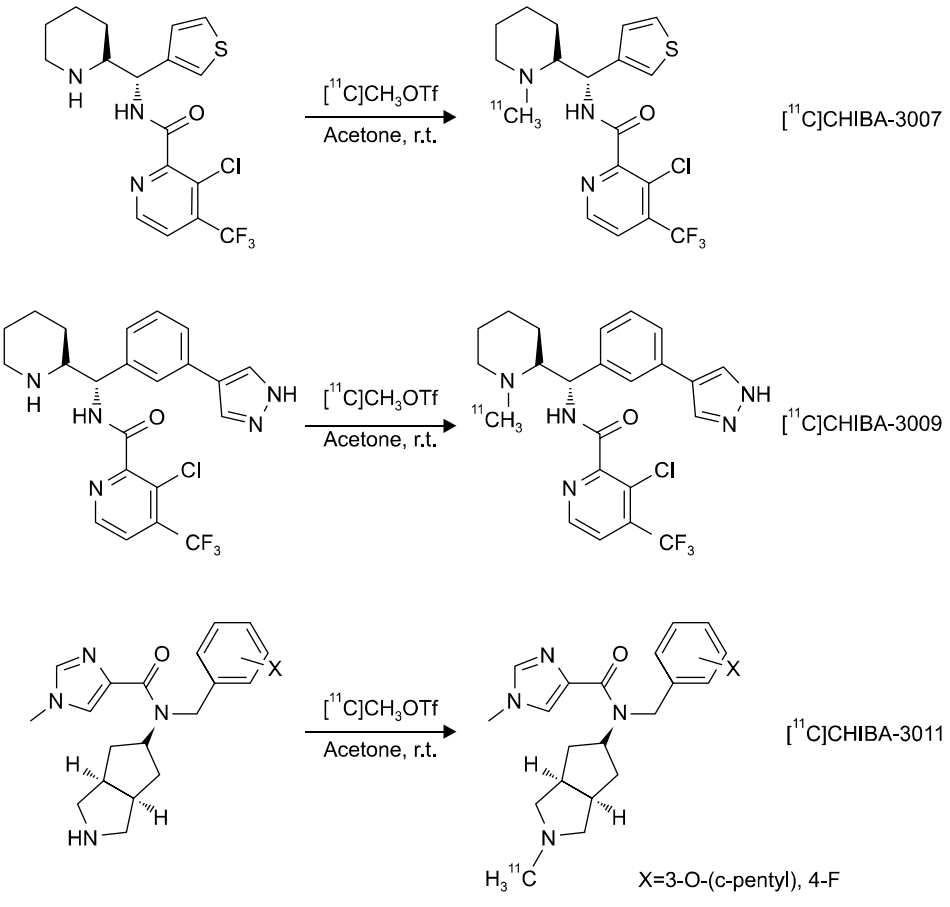
<Appendix>



Supplementary Fig 1. Chemical-structures and calculated Log *p* (cLog) values of the radioligands used in this study.



Supplementary Fig 2. Radiosynthesis of (^{11}C)CHIBA-3007, (^{11}C)CHIBA-3009 and (^{11}C)CHIBA-3011. r.t., room temperature.