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Radiosynthesis and evaluation of 5-methyl-*N*-(4-[^{11}C]methylpyrimidin-2-yl)-4-(1*H*-pyrazol-4-yl)thiazol-2-amine ([^{11}C]ADX88178) as a novel radioligand for imaging of metabotropic glutamate receptor subtype 4 (mGluR4)

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ABSTRACT

ADX88178 (**1**) has been recently developed as a potent positive allosteric modulator for metabotropic glutamate receptor 4 (mGluR4). The aim of this study was to develop [^{11}C]**1** as a novel positron emission tomography ligand and to evaluate its binding ability for mGluR4. Using stannyl precursor **3**, [^{11}C]**1** was efficiently synthesized by introducing an [^{11}C]methyl group into a pyrimidine ring via C–^{11}C coupling and deprotection reactions, in 16 ± 6% radiochemical yield (n = 10). At the end of synthesis, 0.54–1.10 GBq of [^{11}C]**1** was acquired with >98% radiochemical purity and 90–120 GBq/μmol of specific activity. In vitro autoradiography and ex vivo biodistribution study in rat brains showed specific binding of [^{11}C]**1** in the cerebellum, striatum, thalamus, cerebral cortex, and medulla oblongata, which showed dose-dependent decreases by administration with multi-dose of unlabeled **1**.

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors that can activate excitatory neurotransmission via stimulation of secondary messengers in the central nervous system (CNS).[1] MGluRs are classified into three groups including eight subtypes based on sequence homology, intracellular transduction pathways, and pharmacological properties.[2] Of these, mGluR4, mGluR6, mGluR7, and mGluR8 belonging to group III are identified primarily on presynaptic terminals of GABAergic and glutamatergic neurons. These receptors interact with the Gi protein negatively coupled with adenylate cyclase to inhibit the cAMP-dependent signaling pathway, which is responsible for regulation of synaptic transmission via inhibition of voltage-gate calcium flow across the cell membrane within the basal ganglia circuitry of the brain.[3]

Based on their physiological backgrounds, the therapeutic potential of group III mGluRs is rapidly expanding. Of these, mGluR4 has received particular attention because of the potential benefits of mGluR4 activation in several CNS disorders. In particular, several pharmaceuticals for mGluR4 were reported to show neuroprotective activity in models of Parkinson's disease, a degenerative disorder of dopaminergic neurons in the basal ganglia.[4]

The development of radioligands for positron emission tomography (PET) imaging of mGluR4 in brain has become of increasing interest. Kil et al. first developed two PET ligands for mGluR4, such as [^{11}C]ML128 and [^{18}F]KALB001 (Fig. 1).[5] However, the binding of these radioligands did not correspond to the regional distribution of mGluR4 in rat and monkey brains, likely because of low affinity (ML128[5]: EC_{50} = 240 nM; KALB001: EC_{50} = 50 nM) for mGluR4. More recently, 5-methyl-*N*-(4-methylpyrimidin-2-yl)-4-(1*H*-pyrazol-4-yl)thiazol-2-amine (ADX88178, **1**) (Fig. 2) was developed as a potent and selective mGluR4 positive allosteric modulator.[6] Compound **1** potentiated glutamate-mediated activation of human mGluR4 with EC_{50} values of 4 nM without significant effects on other mGluRs (EC_{50} > 30 μM). In addition, compound **1** showed neuroprotection in rodent models of anxiety, obsessive–compulsive disorder, fear, depression, and psychosis.[6]

In present study, we developed [^{11}C]ADX88178 ([^{11}C]**1**, Fig. 2) as a novel PET ligand for mGluR4. Compound **1** contains methyl groups in the pyrimidine and thiazole ring. We labeled the methyl group at the 4-position in pyrimidine to synthesize [^{11}C]**1** by a C–^{11}C coupling reaction with [^{11}C]CH_3I, using stannyl precursor **2** or **3** (Fig. 2). Although introduction of a [^{11}C]methyl group into

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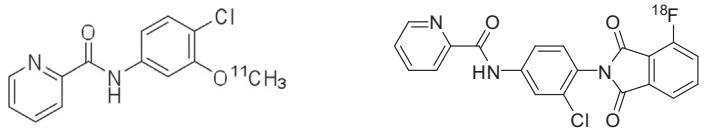
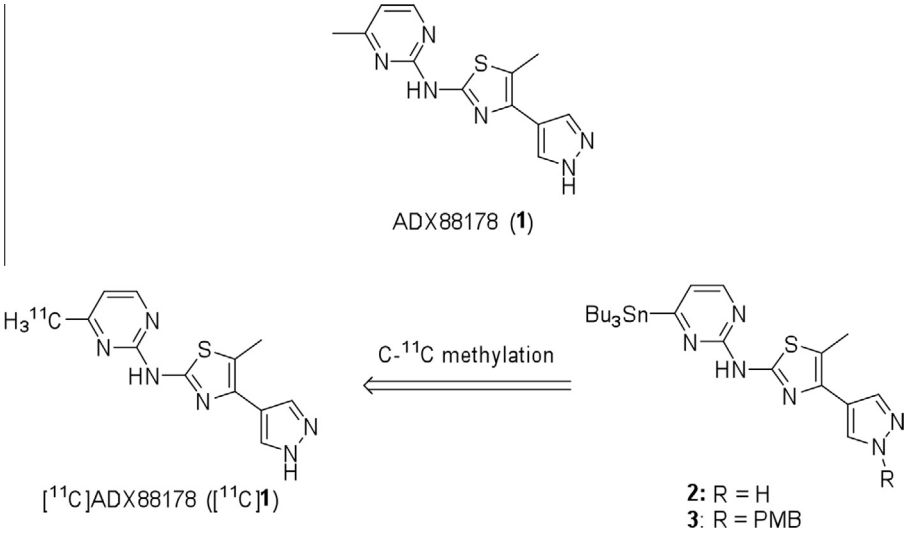


[^{11}C]ML128 [^{18}F]KALB001

Figure 1. Chemical structures of primary two radioligands for mGluR4.



[^{11}C]ADX88178 ([^{11}C]**1**)

Figure 2. Targeted compound and radiolabeling strategy for synthesis of [^{11}C]**1**.

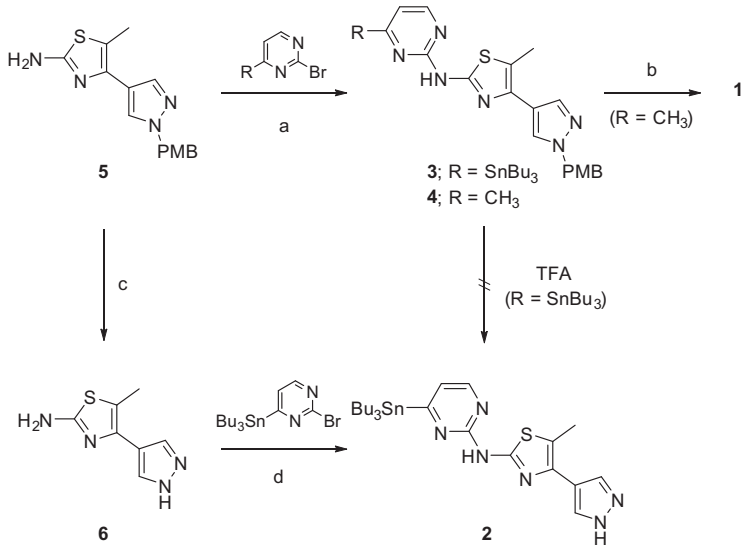
an electron-deficient heteroaromatic ring such as a pyridine or pyrimidine has been reported by Suzuki et al. [7] To our knowledge, no practical PET ligand bearing a [^{11}C]methylated pyrimidine moiety has been reported. Herein, we introduced a [^{11}C]methyl group into pyrimidine ring to synthesize [^{11}C]**1** as a practical PET ligand bearing [^{11}C]methylated pyrimidine moiety for the first time and evaluated the binding ability of [^{11}C]**1** to mGluR4 in brain in vitro and in vivo.

Compound **1**,[8] precursors **2**[9] and **3**[10] were synthesized as shown in Scheme 1. 4-(1-(4-methoxybenzyl)-1*H*-pyrazol-4-yl)-5-methylthiazol-2-amine (**5**) was prepared as described previously.[11] Reaction of **5** with 2-bromo-4-methyl or (tributylstannyl)pyrimidine derivatives[12] in the presence of $Pd(OAc)_2$ and Xantphos afforded **3** and **4** in moderate yields of 39% and 52%, respectively. Debenzylation of **4** with TFA gave **1** in 38% yield. However, debenzylation of **3** with TFA did not give the desired compound **2** and protodestannylation was mainly observed on the pyrimidine ring in **3**. Other research groups had also reported such protodestannylation of heteroarylstannanes under acidic conditions.[13] Thus, to avoid protodestannylation of **3** in the acid condition, **5** was treated with TFA and then reacted with 2-bromo-4-(tributylstannyl) pyrimidine to successfully give precursor **2**.

Radiosynthesis[14] conditions of [^{11}C]**1** via C–[^{11}C]methylation and results are summarized in Table 1. [^{11}C]CH_3I was obtained by the reduction of cyclotron-produced [^{11}C]CO_2 with $LiAlH_4$, followed by treatment with 48% HI.[15] The resulting [^{11}C]CH_3I was distilled from the reaction mixture and trapped in a DMF solution containing precursor **2** or **3**, $Pd_2(dba)_3$, P(*o*-tol)$_3$, CuCl, and base. When K_2CO_3 was used as a base, only trace [^{11}C]**1** was produced and unreacted [^{11}C]CH_3I was mainly observed (entry 1). Changing the base from K_2CO_3 to CsF did not improve the reaction efficiency for C–^{11}C coupling (entry 2). Based on these data, we considered that the unprotected pyrazole ring in **2** may hinder the C–^{11}C coupling reaction.

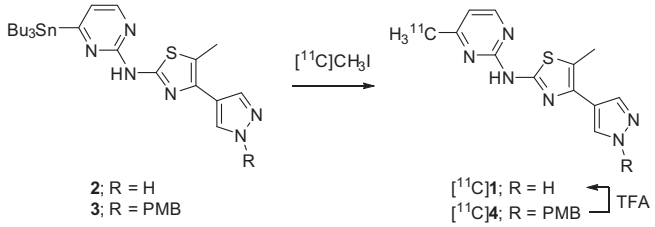
Thus, the precursor for synthesis of [^{11}C]**1** was changed from **2** to **3** in which the pyrazole ring was protected with a *p*-methoxybenzyl (PMB) group (entry 3–6). As expected, use of CsF as a base was effective for C–^{11}C coupling, giving [^{11}C]**4** in a high yield. However, the following deletion of the PMB group in [^{11}C]**4** did not proceed by adding TFA to the reaction mixture in DMF (entry 4). When DMF was partly removed after ^{11}C-methylation, the deprotection with TFA produced a mixture of [^{11}C]**1** and [^{11}C]**4** in an approximately 1:1 ratio (entry 5). As shown in entry 6, to accomplish efficient deprotection of PMB in [^{11}C]**4**, DMF was completely removed under reduced pressure after the C–^{11}C coupling reaction, and TFA was then added to the reaction mixture. Under a neat condition, cleavage of the PMB group in [^{11}C]**4** proceeded efficiently at 100 °C for 5 min.

After optimization of conditions for C–^{11}C coupling and deprotection reactions, radiosynthesis of [^{11}C]**1** was achieved according to entry 6 using a home-made automated synthesis system.[16] Reversed-phase HPLC purification of the reaction mixtures gave [^{11}C]**1** in 16 ± 6% (n = 10) radiochemical yield (n = 10, based on [^{11}C]CO_2, corrected for decay). Starting from 22.2 to 24.0 GBq [^{11}C]CO_2, 0.54–1.10 GBq [^{11}C]**1** was produced within 45 min (n = 10) of averaged synthesis time from the end of bombardment. In the final product solution, identity of [^{11}C]**1** was confirmed by co-injection with unlabeled **1** onto analytic HPLC.[14] The radiochemical purity of [^{11}C]**1** was greater than 98% and the specific activity was 90–120 GBq/μmol at the end of synthesis. No significant peaks corresponding to chemical impurities were observed on HPLC charts for the final product solutions. Moreover, [^{11}C]**1** did not show radiolysis at room



Scheme 1. Chemical syntheses of compound **1**–**3**. Reagents and conditions: (a) $Pd(OAc)_2$, Xantphos, Cs_2CO_3, 1,4-dioxane, 100 °C, 10–17 h, for **3**: 39%, for **4**: 52%; (b) TFA, 90 °C, 3 h, 38%; (c) TFA, 90 °C, 4 h, 88%; (d) $Pd(OAc)_2$, Xantphos, Cs_2CO_3, 1,4-dioxane, 100 °C, 6 h, 22%.

Table 1
Effect of the reaction conditions[a] for synthesis of [^{11}C]**1**



Entry	Precursor	Base	TFA (μL)	RCY (%) of [^{11}C] **4**[d]	RCY (%) of [^{11}C] **1**[d]
1	**2**	K_2CO_3	—	—	Trace
2	**2**	CsF	—	—	Trace
3	**3**	K_2CO_3	—	35	—
4	**3**	CsF	300	91	0
5	**3**	CsF	500[b]	45	52
6	**3**	CsF	500[c]	Trace	97

[a] Reagents and conditions: from **2**; $Pd_2(dba)_3$, CuCl, P(*o*-tol)$_3$, base, DMF, 80 °C, 5 min. from **3**; (i) $Pd_2(dba)_3$, CuCl, P(*o*-tol)$_3$, base, DMF, 80 °C, 5 min, (ii) TFA, 100 °C, 5 min.
[b] Before addition of TFA, DMF was partly removed.
[c] Before addition of TFA, DMF was completely removed under reduced pressure.
[d] The radiochemical yield was determined by analyzing the reaction mixture using radio-HPLC.

temperature for 90 min after formulation. The analytical results were in compliance with our in-house quality control/assurance specifications of radiopharmaceuticals.

To determine the binding ability of [^{11}C]**1** for mGluR4, we performed in vitro autoradiography using rat brain sections.[17] Figure 3 shows representative in vitro autoradiograms with [^{11}C]**1** containing vehicle (control, A) or 10 μM unlabeled **1** (blockade, B). In the control section, strong radioactivity signals were detected in the cerebellum, striatum, thalamus, midbrain, cerebral cortex, and medulla oblongata, while low radioactivity signals were found in the frontal cortex, hypothalamus, and pons. The radioactivity was significantly decreased by treatment with unlabeled **1**; a decrease of 12.3% for the cerebellum, 13.8% for the thalamus, 12.5% for the medulla oblongata, and 15.8% for the striatum.

The distribution pattern of radioactive accumulation in the control section was similar to the reported biological distribution of mGluR4 in the rat brain.[18] Because of high selectivity of **1** for mGluR4 over other mGluRs, we assumed that [^{11}C]**1** could have some in vitro specific binding with mGluR4 in the rat brain. However, radioactive accumulation was identified in some negligible regions, such as the cerebral cortex and midbrain, suggesting that specific binding of [^{11}C]**1** may contain binding to other receptors.

We further verified specific binding of [^{11}C]**1** in vivo by biodistribution studies using dose–response assessments with unlabeled **1**.[19] Figure 4A shows time–activity curves of [^{11}C]**1** in the blood and brain regions (cerebellum, cerebral cortex, basal ganglia containing striatum and thalamus, and whole brain). All radioactivity

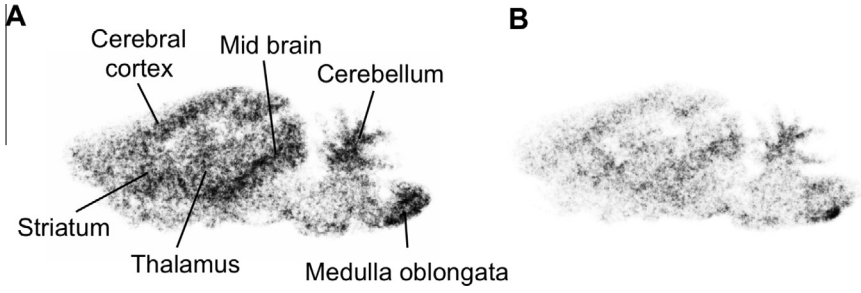


Figure 3. Representative in vitro autoradiograms of rat sagittal brain sections with [^{11}C]**1** in presence of vehicle (A) or 10 μM unlabeled **1** (B).

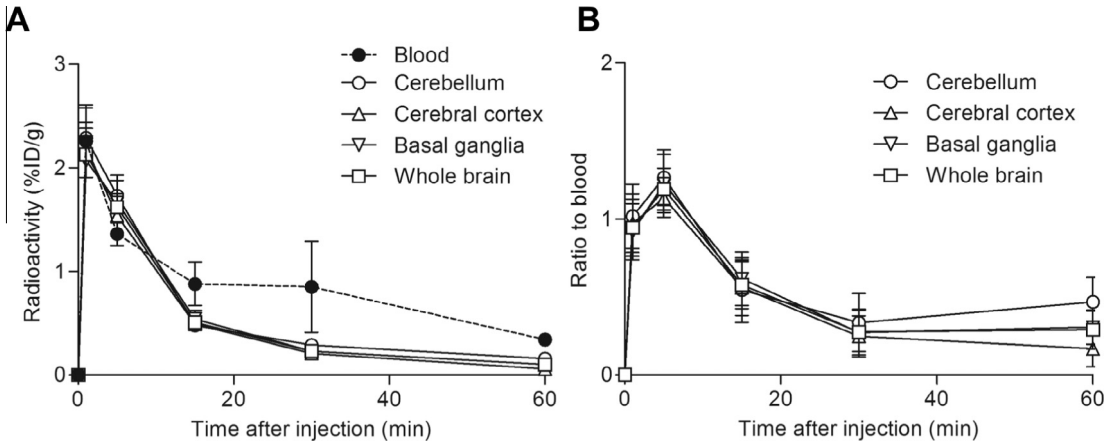


Figure 4. Ex vivo distribution study of [^{11}C]**1** in the brain (n = 3, in each time point). (A) Time-course of radioactivity (%ID/g) in the blood, cerebellum, cerebral cortex, basal ganglia, and whole brain. (B) Time-course of tissue-to-blood ratio in the cerebellum, cerebral cortex, basal ganglia, and whole brain.

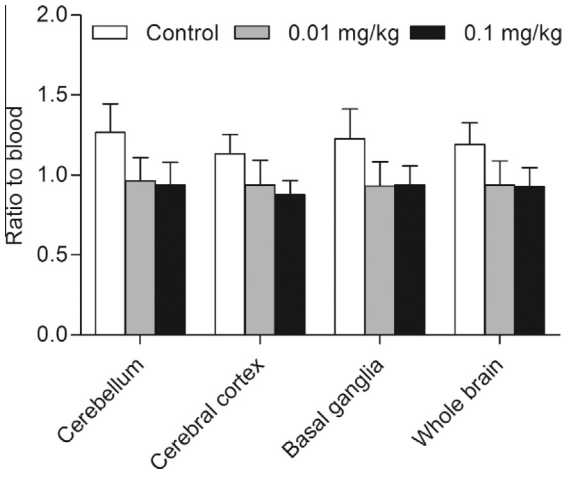


Figure 5. Dose–response of unlabeled **1** (0, 0.01, and 0.1 mg/kg) in the brain uptake of [^{11}C]**1**. Specific radioactive uptakes in the tissue were estimated using ratio to blood under the equilibrium state (5 min after the injection).

(% of injection dose per gram tissue, %ID/g) in brain regions peaked at 1 min after the injection of [^{11}C]**1** and then showed a quick decrease. To estimate equilibrium state for binding of [^{11}C]**1**, the ratio of tissue to blood in brain regions was calculated. Figure 4B shows time-course of tissue-to-blood ratio in brain regions. The estimated equilibrium state for binding of [^{11}C]**1** was heterogeneously determined at 5 min after the injection of [^{11}C]**1**, for example, 1.27 for the cerebellum, 1.13 for the cerebral cortex, 1.23 for the basal ganglia, and 1.19 for the whole brain, respectively. By administration with multi-doses of unlabeled **1**, these ratios decreased with dose-dependency (Fig. 5). Compared with the baseline rat, percentages of decreased radioactivity in the brain regions of rats administrated with 0.1 mg/kg unlabeled **1** were 22–26%. Previously, in vivo blocking study in PET with [^{18}F]KALB001, the most promising PET ligand for mGluR4 until now, showed 10–20% decline compared with baseline rats.[5] Hence, the present study suggested that in vivo specific binding of [^{11}C]**1** for mGluR4 might be higher than that of [^{18}F]KALB001.

In summary, we successfully synthesized [^{11}C]**1** as a novel radioligand for mGluR4 by introducing a [^{11}C]methyl group into the pyrimidine ring. This labeling technique enables synthesis of PET ligands containing electron-deficient [^{11}C]methylpyrimidine. Although [^{11}C]**1** showed low specific binding for mGluR4 in vitro and in vivo, development of new candidates with higher binding affinity to mGluR4 than **1** is required.

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8. *Compound **1** (5-methyl-N-(4-methylpyrimidin-2-yl)-4-(1H-pyrazol-4-yl)thiazol-2-amine, ADX88178):* The title compound was prepared as previously reported.[11] colorless powder; mp: 256–258 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.41 (3H, s), 2.43 (3H, s), 6.87 (1H, d, J = 5.1 Hz), 7.87 (2H, br s), 8.43 (1H, d, J = 4.8 Hz), 11.38 (1H, s), 12.93 (1H, br s). HRMS (FAB) calcd for $C_{12}H_{13}N_6S$, 273.0922; found, 273.0952.
9. *Compound **2** (5-methyl-4-(1H-pyrazol-4-yl)-N-(4-(tributylstannyl)pyrimidin-2-yl)-thiazol-2-amine):* colorless solid; mp: 204–206 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.85 (9H, t, J = 7.3 Hz), 1.06–1.21 (6H, m), 1.25–1.37 (6H, m), 1.49–1.70 (6H, m), 2.40 (3H, s), 7.09 (1H, d, J = 4.8 Hz), 7.80 (1H, br s), 7.94 (1H, br s), 8.34 (1H, d, J = 4.4 Hz), 11.34 (1H, s), 12.94 (1H, s). HRMS (FAB) calcd for $C_{23}H_{37}N_6SSn$, 549.1822; found, 549.1840.
10. *Compound **3** (4-(1-(4-methoxybenzyl)-1H-pyrazol-4-yl)-5-methyl-N-(4-(tributylstannyl)pyrimidin-2-yl)thiazol-2-amine):* yellow solid; mp: 75–77 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.85 (9H, t, J = 7.3 Hz), 1.06–1.20 (6H, m), 1.25–1.37 (6H, m), 1.46–1.67 (6H, m), 2.39 (3H, s), 3.73 (3H, s), 5.29 (2H, s), 6.92 (2H, d, J = 8.1 Hz), 7.09 (1H, d, J = 4.0 Hz), 7.26 (2H, d, J = 8.1 Hz), 7.74 (1H, s), 8.01 (1H, s), 8.34 (1H, d, J = 5.1 Hz), 11.33 (1H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 10.1, 11.7, 13.7, 27.3, 29.0, 55.3, 55.6, 114.1, 117.9, 118.5, 122.9, 127.5, 128.4, 129.3, 137.8, 138.0, 154.2, 155.6, 156.2, 159.4, 186.4. HRMS (FAB) calcd for $C_{31}H_{45}ON_6SSn$, 669.2398; found, 669.2416.
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14. Radiosynthesis of [^{11}C]**1** from precursor **3**: CsF (2.3 mg, 15 μmol), copper(I) chloride (1.5 mg, 15 μmol), and $Pd_2(dba)_3$ (1.3 mg, 1.4 μmol) were added to a minivial (1 mL) equipped with a septum and stirring bar. The vial was purged with N_2 gas, and a solution of P(*o*-tol)$_3$ (1.7 mg, 5.6 μmol) in DMF (0.15 mL) was added to the vial. After the reaction mixture was stirred for 4 min at room temperature, a solution of **3** (2.0 mg, 3 μmol) in DMF (0.1 mL) was added to the reaction mixture. This mixture was purged with N_2 gas and stirred for 1 min. The prepared solution was transferred with a syringe into a reaction vial equipped with the automated synthetic unit. [^{11}C]CH_3I transferred under N_2 gas flow into the prepared solution at −15 to −20 °C. After radioactivity of [^{11}C]CH_3I reached at a plateau, the reaction mixture was heated at 80 °C for 5 min. After removal of DMF under reduced pressure, TFA (0.5 mL) was added to the residue and heated at 100 °C for 5 min. The reaction mixture was neutralized with a solution of 2 mol/L CH_3CO_2Na (0.7 mL) in MeOH (0.3 mL), filtered with a glass fiber prefilter (GF53, 30 mm; Agilent Technologies, Santa Clara, CA), and applied to the HPLC system. HPLC purification was performed using a JASCO HPLC system (JASCO, Tokyo, Japan) under the following conditions. Column: Capcell Pak C_{18} (Shiseido, Tokyo, Japan), 10 mm × 250 mm; detector: UV 254 nm; eluent: MeOH/H_2O/TFA = 5/5/0.01 (v/v/v); flow rate: 5.0 mL/min. The radioactive fraction corresponding to [^{11}C]**1** [retention time (t_R): 7.9 min] was collected in a sterile flask containing polysorbate (80) (75 μL) and ethanol (150 μL), evaporated to dryness under vacuum, re-dissolved in 3 mL of sterile normal saline, and passed through a 0.22-μm Millipore filter to obtain [^{11}C]**1** for analysis and the animal experiments. HPLC analysis was performed under the following conditions. Column: Capcell Pak C_{18} (Shiseido) 4.6 mm × 250 mm; detector: UV 254 nm; eluent: MeOH/H_2O/TFA = 5/5/0.01 (v/v/v); flow rate: 1.0 mL/min; t_R: 8.3 min. The identity of [^{11}C]**1** with unlabeled **1** was confirmed by analytical HPLC under the same condition.
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17. (a) Animals: Male Sprague-Dawley rats (8 weeks old) and ddY mice (8–9 weeks old) were purchased from Japan SLC (Shizuoka, Japan), kept in a temperature-controlled environment with a 12-h light-dark cycle, and fed a standard diet (MB-1; Funabashi Farm, Chiba, Japan). Animal experiments were performed according to the recommendations of the Committee for the Care and Use of Laboratory Animals, National Institute of Radiological Sciences. (b) In vitro autoradiography: Three rats anesthetized with 1.5% (v/v) isoflurane were sacrificed by the cervical dislocation and their brains were quickly removed and frozen. The sagittal brain sections (20 μm) were prepared from frozen rat brains using a cryostat (HM560, Carl Zeiss, Oberkochen, Germany). Brain sections were pre-incubated for 10 min in Tris-buffer (50 mM Tris–HCl, 1.2 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4) at room temperature. After pre-incubation, these sections were incubated for 30 min at room temperature in fresh buffer containing [^{11}C]**1** (37 MBq/mL in saline) in the absence (vehicle, 0.1% DMSO) or presence of 10 μM unlabeled **1** (dissolved in DMSO, final concentration: 0.1%). After incubation, these sections were washed three times for 5 min each time with cold buffer, dipped in cold distilled water, and dried in air. These sections were placed in contact with an imaging plate (BAS-MS2025; Fujifilm, Tokyo, Japan). Autoradiograms were acquired using a Bio-Imaging Analyzer System (BAS5000; Fujifilm).
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19. Respective mouse (n = 3 in each time point, 34–41 g) was injected a bolus of [^{11}C]**1** (8.6 MBq/0.1 mL, 79 pmol) via its tail vein. Three mice were killed at each experimental time point (1, 5, 15, 30, and 60 min) after the injection of [^{11}C]**1** by cervical dislocation, respectively. For the dose–response assessment, mice (n = 3 in each group, 36–41 g) were administrated with multi-doses (0, 0.01, and 0.1 mg/kg) of unlabeled **1** prior to the injection of [^{11}C]**1** (5.2 MBq/0.1 mL, 49 pmol). Three mice were killed at 5 min after the injection of [^{11}C]**1**. The brain and blood samples were quickly removed. Subsequently, removed brains were separated into the cerebellum, cerebral cortex, basal ganglia, and residue. The radioactivity in these tissues was measured by an autogamma scintillation counter (1480 Wizard, Perkin-Elmer, Waltham, MA) and expressed as the percentage of injected dose per gram of wet tissue (%ID/g). All radioactivity measurements were decay-corrected.