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Presynaptic Selectivity of a Ligand for Serotonin 1A Receptors Revealed by *In Vivo* PET Assays of Rat Brain

Takeaki Saijo[1,2,3☯], Jun Maeda[1☯], Takashi Okauchi[1], Jun-ichi Maeda[4], Yasunori Morio[6], Yasuhiro Kuwahara[5], Masayuki Suzuki[3], Nobuharu Goto[7], Toshimitsu Fukumura[1], Tetsuya Suhara[1,2], Makoto Higuchi[1,2]*

1 Molecular Imaging Center, National Institute of Radiological Sciences, Chiba, Japan, **2** Department of Molecular Neuroimaging, Tohoku University School of Medicine, Sendai, Japan, **3** DMPK Research Laboratory, Mitsubishi Tanabe Pharma Corporation, Kisarazu, Japan, **4** Research Quality Assurance Department, Mitsubishi Tanabe Pharma Corporation, Yokohama, Japan, **5** Advanced Medical Research Laboratory, Mitsubishi Tanabe Pharma Corporation, Yokohama, Japan, **6** Development Project Management Department, Mitsubishi Tanabe Pharma Corporation, Tokyo, Japan, **7** Early Stage Clinical Research Center, Mitsubishi Tanabe Pharma Corporation, Tokyo, Japan

Abstract

A novel investigational antidepressant with high affinity for the serotonin transporter and the serotonin 1A ($5\text{-}HT_{1A}$) receptor, called Wf-516 (structural formula: (2S)-1-[4-(3,4-dichlorophenyl)piperidin-1-yl]-3-[2-(5-methyl-1,3,4-oxadiazol-2-yl)benzo[b]furan-4-yloxy]propan-2-ol monohydrochloride), has been found to exert a rapid therapeutic effect, although the mechanistic basis for this potential advantage remains undetermined. We comparatively investigated the pharmacokinetics and pharmacodynamics of Wf-516 and pindolol by positron emission tomographic (PET) and autoradiographic assays of rat brains in order to elucidate their molecular interactions with presynaptic and postsynaptic $5\text{-}HT_{1A}$ receptors. In contrast to the full receptor occupancy by pindolol in PET measurements, the binding of Wf-516 to $5\text{-}HT_{1A}$ receptors displayed limited capacity, with relatively high receptor occupancy being achieved in regions predominantly containing presynaptic receptors. This selectivity was further proven by PET scans of neurotoxicant-treated rats deficient in presynaptic $5\text{-}HT_{1A}$ receptors. In addition, [^{35}S]guanosine 5′-O-[γ-thio]triphosphate autoradiography indicated a partial agonistic ability of Wf-516 for $5\text{-}HT_{1A}$ receptors. This finding has lent support to reports that diverse partial agonists for $5\text{-}HT_{1A}$ receptors exert high sensitivity for presynaptic components. Thus, the present PET data suggest a relatively high capacity of presynaptic binding sites for partial agonists. Since our *in vitro* and *ex vivo* autoradiographies failed to illustrate these distinct features of Wf-516, *in vivo* PET imaging is considered to be, thus far, the sole method capable of pharmacokinetically demonstrating the unique actions of Wf-516 and similar new-generation antidepressants.

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* E-mail: mhiguchi@nirs.go.jp

☯ These authors contributed equally to this work.

Introduction

Selective serotonin reuptake inhibitors (SSRIs), such as fluvoxamine, fluoxetine and paroxetine, are currently the most frequently prescribed antidepressants [1–3]. SSRIs induce fewer adverse effects than classical tricyclic agents [4], thereby contributing to improved quality of life. These drugs increase serotonin (5-HT) concentration at synaptic clefts through inhibitory binding to 5-HT transporters (5-HTTs) responsible for 5-HT reuptake, thus enhancing serotonergic neurotransmissions and producing an antidepressant effect [5]. However, this serotonergic reinforcement does not take place immediately after the initiation of treatment, as increased 5-HT stimulates 5-HT 1A ($5\text{-}HT_{1A}$) autoreceptors as negative feedback, inhibiting the release of 5-HT at presynaptic terminals [6,7]. The persistent rise of 5-HT levels following repeated SSRI administration subsequently induces desensitization of $5\text{-}HT_{1A}$ autoreceptors, and the firing frequencies of 5-HT neurons gradually recover [8,9], resulting in the delayed appearance of antidepressant effects. In practice, this delayed therapeutic benefit of SSRIs has been a source of distress for both depressive patients and psychiatrists.

Pindolol, a therapeutic agent used for the treatment of hypertension, antagonistically binds to not only β adrenergic receptors but also to central $5\text{-}HT_{1A}$ receptors [10], and its antagonism for $5\text{-}HT_{1A}$ receptors is assumed to interrupt the autoreceptor-mediated negative feedback. To date, several clinical trials have demonstrated that pindolol accelerates the alleviation of depressive symptoms following initiation of SSRI treatment [11–14]. For this serotonergic modulation, it is necessary for pindolol to preferentially block presynaptic $5\text{-}HT_{1A}$ autoreceptors without profound suppression of postsynaptic receptors, since postsynaptic antagonism could counteract

the indirect agonism by SSRIs [10]. This selective binding property of pindolol for 5-HT_{1A} autoreceptors has been investigated using positron emission tomography (PET) with [^{11}C]N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexane-carboxamide] ([^{11}C]WAY-100635), a specific radioligand suitable for PET imaging of 5-HT_{1A} receptors [15], thus enabling quantification of occupancies of these receptors by therapeutic agents. Several reports have supported the selectivity of pindolol for presynaptic receptors abundantly located in the pontine raphe nucleus [16,17], although such a binding preference has not been confirmed by other studies [18]. In a non-clinical PET study using [^{11}C]WAY-100635, preferential binding of pindolol to 5-HT_{1A} autoreceptors was observed [19], but this was inconsistent with the findings of an *ex vivo* autoradiography (ARG) study that used intravenous administration of [^{3}H]WAY-100635 and identified nonselective binding of pindolol to 5-HT_{1A} receptors in rat brain [20].

Wf-516 (structural formula: (2 S)-1-[4-(3,4-dichlorophenyl)piperidin-1-yl]-3-[2-(5-methyl-1,3,4-oxadiazol-2-yl)benzo[b]furan-4-yloxy]propan-2-ol monohydrochloride), a novel investigational antidepressant with high affinity for 5-HTT and 5-HT_{1A} receptors [21], has been shown to have more rapid antidepressant-like effects than the classical tricyclic antidepressant imipramine in a rat chronic mild stress model of depression [22]. Moreover, a recent *in vivo* electrophysiological study using rats has indicated that Wf-516 at low and medium doses was an antagonist for presynaptic but not postsynaptic 5-HT_{1A} receptors [23]. Although our previous PET study of rats demonstrated *in vivo* binding of Wf-516 to central 5-HTTs in a dose-dependent manner [24], the pharmacological mechanisms involved in the presynaptic/postsynaptic selectivity of binding of Wf-516 to 5-HT_{1A} receptors in living brains had still remained to be clarified using neuroimaging assays.

The present study was conducted in order to determine the properties of the interaction between Wf-516 and presynaptic and postsynaptic 5-HT_{1A} receptors localized predominantly in the raphe nucleus and hippocampus, respectively. Occupancies of these receptors by Wf-516 and pindolol were measured and compared in rat brains using PET with [^{11}C]WAY-100635, and presynaptic selectivity of Wf-516 was further examined by treating rats with a toxin for 5-HT neurons.

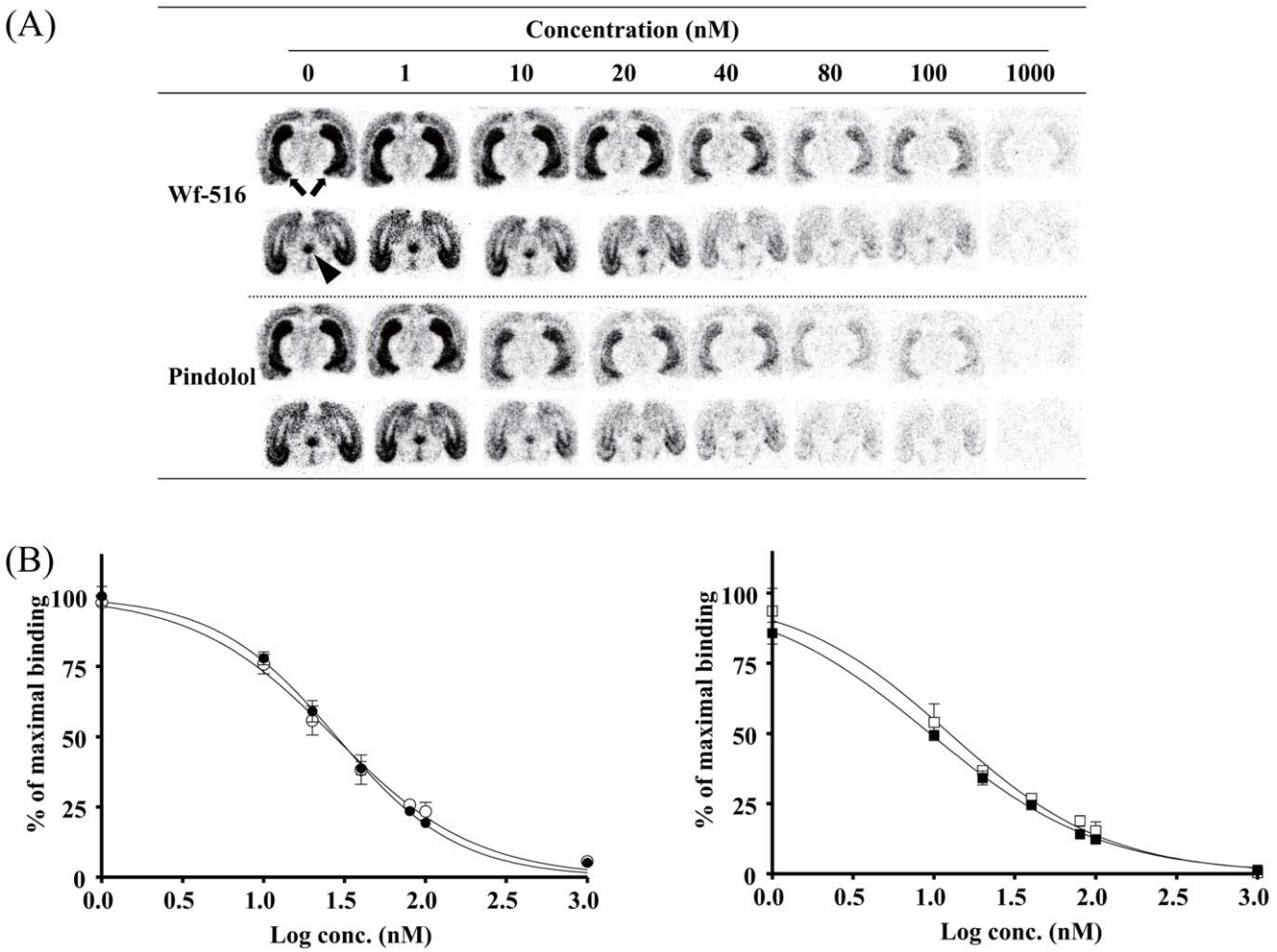

Figure 1. Binding affinities of Wf-516 and pindolol for central 5-HT_{1A} receptors quantified by *in vitro* ARG. (A) Representative autoradiograms showing distribution and intensity of [^{11}C]WAY-100635 radiosignals in rat brain sections containing the hippocampus (arrows) and raphe nucleus (arrowhead) and its attenuation by different concentrations of Wf-516 or pindolol. (B) Inhibition curves of [^{11}C]WAY-100635 binding to the hippocampus (closed symbols) and raphe nucleus (open symbols) by Wf-516 (left) and pindolol (right). Bars indicate S.E. (n = 3).
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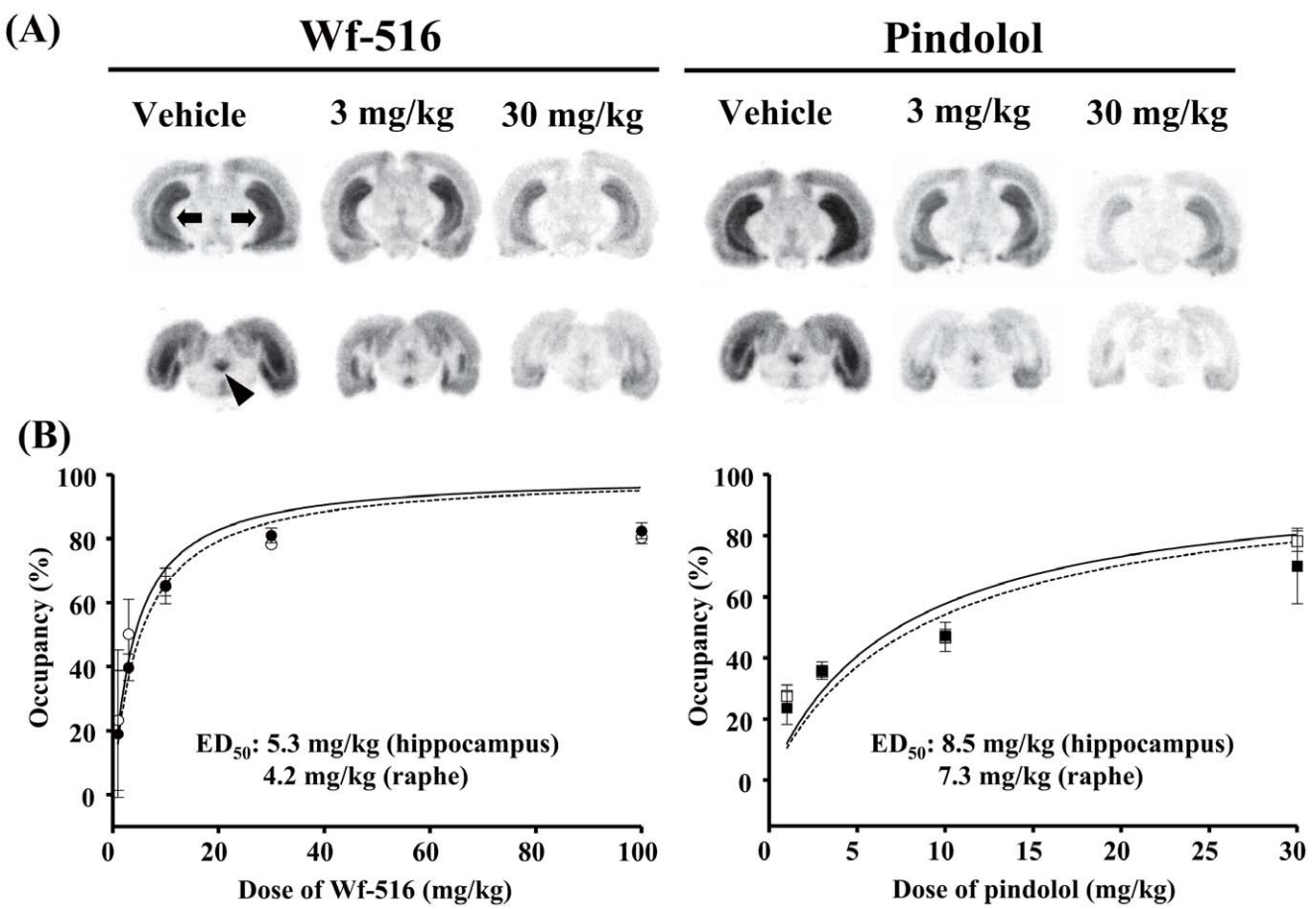

Figure 2. Occupancies of 5-HT$_{1A}$ receptors by Wf-516 and pindolol assessed by *ex vivo* ARG. (A) Representative autoradiograms showing distribution and intensity of [^{11}C]WAY-100635 radiosignals in rat brain sections containing the hippocampus (arrows) and raphe nucleus (arrowhead) after oral administration of Wf-516 and intraperitoneal administration of pindolol. (B) Relationships between dose of Wf-516 (left) or pindolol (right) and 5-HT$_{1A}$ receptor occupancy in the hippocampus (closed symbols) and raphe nucleus (open symbols). Regression curves in the hippocampus and raphe nucleus are denoted by dashed lines and solid lines, respectively, and were generated by the following equation: Occ $=100\times D/(D+ED_{50})$, where Occ is 5-HT$_{1A}$ receptor occupancy and D is the dose of the drug. Bars indicate S.E. (n = 3).
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Methods

Drugs and Chemicals

Wf-516 was synthesized at Mitsubishi Tanabe Pharma Co. (Osaka, Japan). Pindolol in a racemate form, fluvoxamine, 5,7-dihydroxytryptamine (DHT), WAY-100635, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), (±)-Metoprolol, guanosine diphosphate (GDP) and guanosine-5′-O-(3-thio)-triphosphate (GTPγS) were obtained from Sigma-Aldrich (St Louis, MO). Hydroxypropylmethyl cellulose (HPMC), desipramine hydrochloride, pentobarbital and [^{35}S]GTPγS (47.46 TBq/mmol) were purchased from Shin-Etsu Chemical (Tokyo, Japan), Research Biochemicals International (Natick, MA), Dainippon Sumitomo Pharma (Osaka, Japan) and Perkin Elmer (Waltham, MA), respectively. Wf-516 was dissolved or suspended in 0.5% HPMC, and pindolol was dissolved in saline containing 0.1 M citrate when administered to rats. All other chemicals were of analytical grade and commercially available.

Animals

The research protocols in the present study were approved by the Animal Ethics Committee of the National Institute of Radiological Sciences. Male Wistar rats at 8 weeks of age were purchased from CLEA Japan Inc. (Tokyo, Japan). Prior to the PET measurements, neuroanatomical template magnetic resonance images (MRI) of the rat brains were generated as described elsewhere [24].

Radioligand Synthesis

[^{11}C]WAY-100635 was prepared by ^{11}C-acylation of WAY-100634 with ^{11}C-cyclohexanecarbonyl chloride as previously described [25]. Radiochemical purity of the radioligand was more than 95%, and specific radioactivity at the end of radiosynthesis was 183±67 GBq/μmol.

Measurement of Ki Values of Wf-516 and Pindolol for 5-HT$_{1A}$ Receptors by *In vitro* ARG

Rats were sacrificed by decapitation. Twenty-micrometer-thick frozen brain sections were preincubated for 30 min in 50 mM Tris-HCl (pH 7.4) containing 1 mM $MnCl_2$. The samples were then incubated at room temperature for 60 min in the same buffer containing 0.9 nM [^{11}C]WAY-100635 and either Wf-516 or pindolol at eight different concentrations (0–1000 nM). Nonspecific binding of the radioligand was estimated using 10 μM nonradioactive WAY-100635. Subsequently, the sections were rinsed twice with ice-cold Tris-HCl buffer for

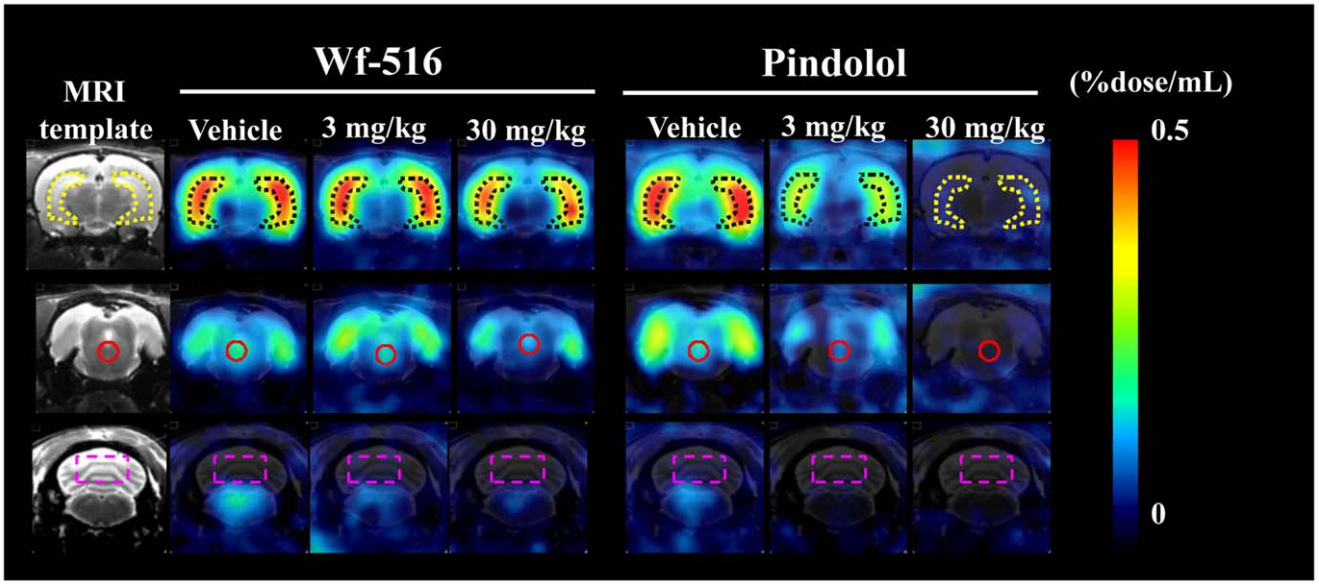

Figure 3. Representative PET images showing distribution of [^{11}C]WAY-100635 in rat brains after oral administration of Wf-516 and intraperitoneal administration of pindolol. Each pretreatment drug was repeatedly administered to the same rat at different doses. PET images were generated by summation of dynamic data 60–90 min after intravenous injection of [^{11}C]WAY-100635, and were overlaid on the MRI template displayed in the far left column. Coronal brain sections shown here were obtained at −5.2 mm (top row), −7.8 mm (middle row) and −12.5 mm (bottom row) from the bregma. ROIs were placed on the hippocampus (dotted lines), raphe nucleus (solid lines) and cerebellum (dashed lines).
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5 min and then desalted with ice-cold distilled water for 10 sec. These samples were warmly blow-dried and placed in contact with an imaging plate (Fuji Film, Tokyo, Japan) for 60 min. Signals on the imaging plate were detected by BAS5000 system (Fuji Film). The inhibition constants of Wf-516 and pindolol for [^{11}C]WAY-100635 (Ki values) were calculated using the following equation:

$$Ki = IC501 + LKd$$

where IC_{50}, L and Kd are median inhibitory concentration, radioligand concentration (0.9 nM) and dissociation constant of WAY-100635 (0.37 nM) [26], respectively.

Measurement of 5-HT_{1A} Receptor Occupancy by Drugs Using *Ex vivo* ARG

Wf-516 (vehicle only, 1, 3, 10, 30 and 100 mg/kg) and pindolol (vehicle only, 1, 3, 10 and 30 mg/kg) were orally and intraperitoneally administered to rats, respectively. Animals were then killed by decapitation 5 h and 30 min after their administration, respectively. Autoradiographic analysis was performed using procedures similar to *in vitro* ARG except for incubation temperature (37°C) and times for preincubation (30 sec), incubation (5 min) and rinsing (1 min). Occupancy of 5-HT_{1A} receptors by Wf-516 and pindolol was calculated as the drug-induced reduction of [^{11}C]WAY-100635 binding relative to vehicle-treated controls in each region of interest (ROI). The doses of these drugs at half-maximal effect (ED_{50}) were determined according to the following relationship:

$$Occupancy = 100 \times DD + ED50$$

where D is the dose of the drug.

Measurement of 5-HT_{1A} Receptor Occupancy by Drugs Using [^{11}C]WAY-100635-PET

A series of 6 and 5 dynamic PET scans was performed for each rat approximately 5 h and 30 min after oral and intraperitoneal pretreatments with graded doses of Wf-516 (vehicle only, 1, 3, 10, 30 and 100 mg/kg) and pindolol (vehicle only, 1, 3, 10 and 30 mg/kg), respectively. Scans for the same individual rat receiving Wf-516 (n = 4) and pindolol (n = 3) were conducted more than 2 weeks and 1 week apart, respectively. PET imaging was also carried out for rats receiving oral administration of 30 mg/kg fluvoxamine dissolved in 0.5%HPMC 30 min before pindolol treatment in order to investigate the effects of fluvoxamine-induced increase of endogenous 5-HTs on the measurements of 5-HT_{1A} receptor occupancies.

All PET scans were carried out using a microPET Focus 220 scanner (Siemens Medical Solutions USA, Knoxville, TN) [27]. The rats were anesthetized with 1.5–2% isoflurane in air (2 L/min flow rate). Emission scans were acquired for 90 min in 3D list mode immediately after intravenous injection of [^{11}C]WAY-100635 (at a dose of 106±10 MBq and specific radioactivity of 132±51 GBq/μmol at the time of injection). The injected mass of WAY-100635 ranged from 0.373 to 3.4 nmol, averaging 0.945±0.501 (S.D.) nmol. There were no marked differences in the injected mass of the tracer between the Wf-516 (0.801±0.482 nmol) and pindolol (1.184±0.649 nmol) studies. In the pindolol study, blood samples were collected from the tail vein upon initiation of the scan, and plasma was frozen at −80°C pending assays. All list-mode data were stored into 3D sinograms, which were then Fourier-rebinned into 2D sinograms (26 frames: 4×1, 8×2, and 14×5 min). Images were reconstructed using 2D-filtered back-projection with a 0.5-mm Hanning filter. All PET images were manually coregistered to the MRI template by spatial translation and rotation of original PET images without geometric expansion and contraction. ROIs were placed on the hippocampus and raphe nucleus using PMOD® image analysis software

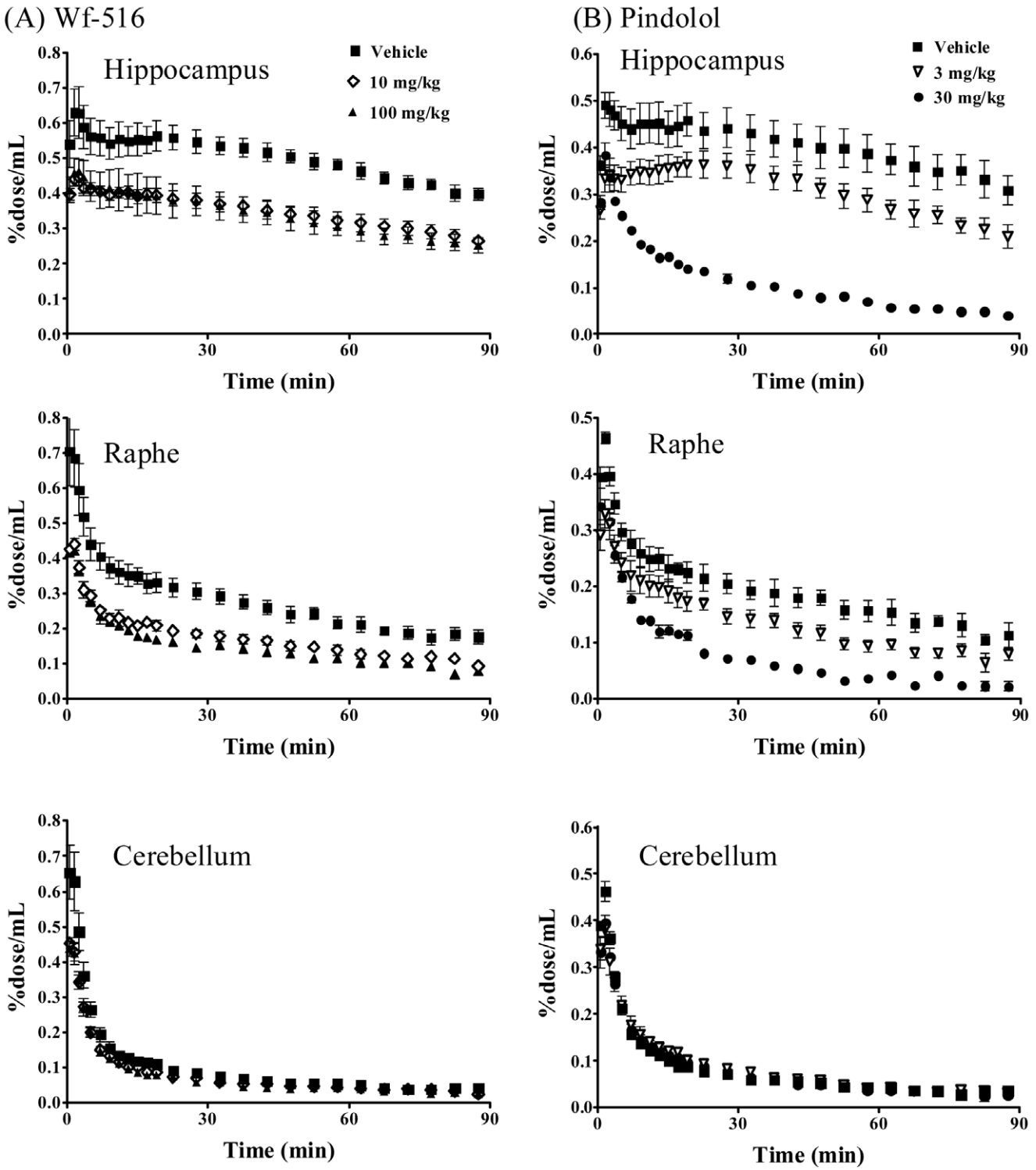

Figure 4. Time-radioactivity curves for [^{11}C]WAY-100635 in the hippocampus (top panels), raphe nucleus (middle panels) and cerebellum (bottom panels) after pretreatments with Wf-516 (A) and pindolol (B) at representative doses. Data were generated by placing ROIs on different brain structures on the PET images illustrated in Figure 3. Radiotracer uptake into each region was expressed as a percentage of the injected dose per unit tissue volume (%dose/mL). Bars indicate S.E. (n = 4 and 3 in Wf-516 and pindolol treatment groups, respectively).
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(PMOD Group, Zurich, Switzerland) with reference to the MRI template. Fine-tuning of the location of ROIs was then performed based on PET images at an early phase (0–30 min) of the scan in order to compensate for small mismatches between coregistered PET and MR images. The cerebellum was used as reference tissue because of its negligible density of 5-HT$_{1A}$ receptors [28]. Binding potential based on specific binding compared to nondisplaceable uptake (BP_{ND}) for [^{11}C]WAY-100635 was quantified by simplified

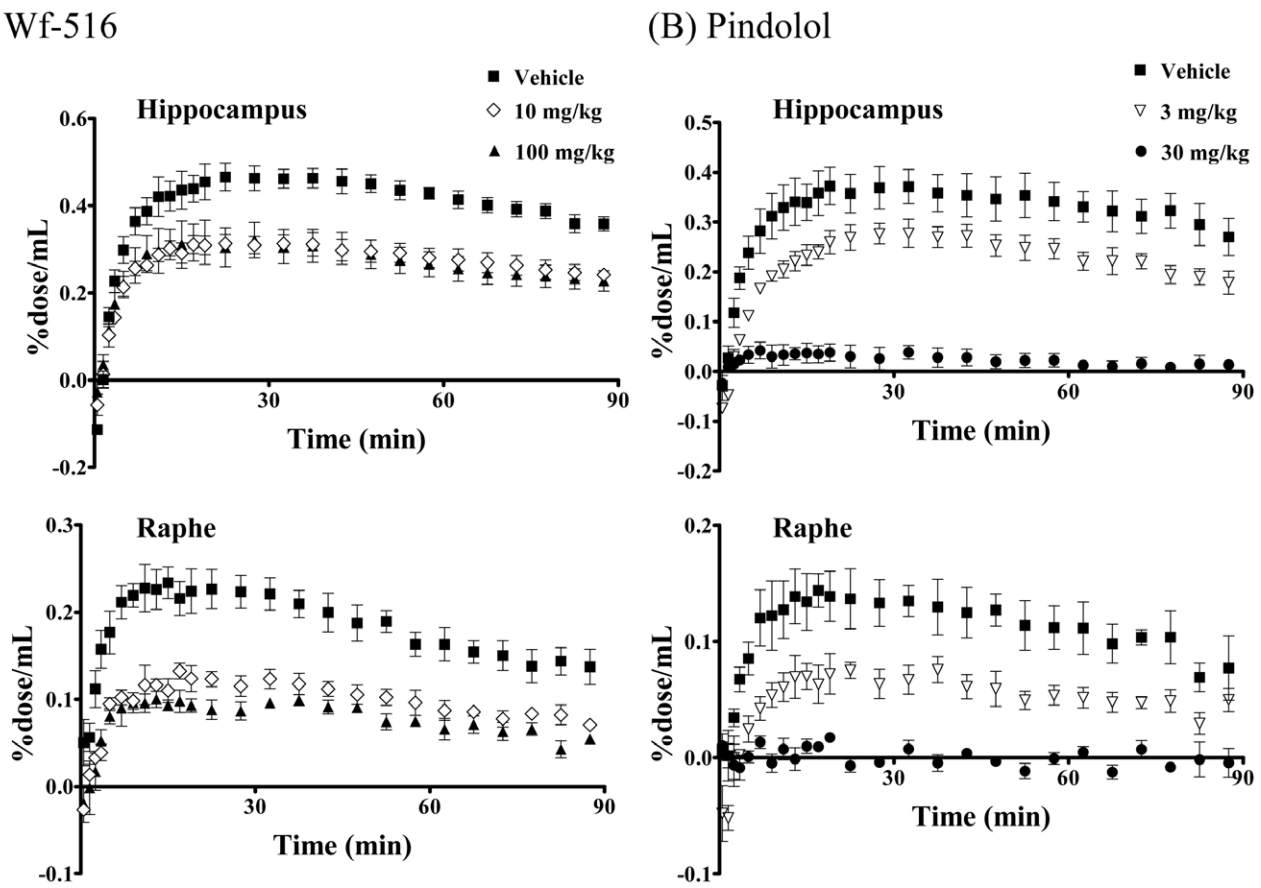

Figure 5. Time course of specific [^{11}C]WAY-100635 binding to 5-HT$_{1A}$ receptors in the hippocampus (top panels) and raphe nucleus (bottom panels) after pretreatments with Wf-516 (A) and pindolol (B) at representative doses. Data were generated by placing ROIs on different brain structures on the PET images illustrated in Figure 3. Binding was estimated as the difference in radiosignals between target and cerebellar regions, and expressed as the percentage of the injected dose per unit tissue volume (%dose/mL). Bars indicate S.E. (n = 4 and 3 in Wf-516 and pindolol treatment groups, respectively).
doi:10.1371/journal.pone.0042589.g005

reference tissue model (SRTM), as in a previous clinical PET study using the same radioligand [29]. Occupancies of 5-HT$_{1A}$ receptors by Wf-516 and pindolol were calculated using the following equation:

$$\text{Occupancy} = \text{BPNDvehicleA}|\text{BPNDdrug} \times 100\text{BPNDvehicle}$$

where $BP_{NDvehicle}$ and BP_{NDdrug} are BP_{ND} values in PET analyses after pretreatment with vehicle only and test drug, respectively. The plasma concentration of the test drug needed for 50% occupancy (EC_{50}) was determined according to the following relationship:

$$Occupancy = 100 \times CC + EC50$$

where C is the plasma concentration of pindolol. The ED_{50} value of pindolol was determined as described above.

[^{11}C]WAY-100635-PET Scans of Rats Treated with a Toxicant for 5-HT Neurons

Prior to disruption of 5-HT neurons, rats were scanned using [^{11}C]WAY-100635-PET. Central 5-HT neurons were lethally injured by 5,7-DHT as previously described [28]. Briefly, four rats were intraperitoneally pretreated with desipramine (20 mg/kg) to protect noradrenergic neurons, and with pentobarbital (60 mg/kg) 45 min and 10 min before 5,7-DHT treatment, respectively. 5,7-DHT (150 µg as free base in 10 µL of saline containing 0.1% ascorbic acid) was unilaterally infused into the right lateral ventricle (stereotactic coordinates: 0.8 mm anterior to the bregma, 1.2 mm lateral to the midline, and 3.5 mm below the dura mater) for 5 min. More than 2 weeks after 5,7-DHT treatment, a second PET scan was performed to evaluate the extent of the loss of presynaptic 5-HT$_1$A receptors. Then, more than 1 week later, a third PET scan was carried out approximately 5 h after oral administration of Wf-516 (30 mg/kg) to the rats. BP_{ND} for 5-HT$_{1A}$ receptors in each PET scan was calculated as described above.

Measurement of Plasma Pindolol Concentration

(±)-Metoprolol was used as internal standard. Pindolol in the plasma sample was extracted using a solid phase extraction cartridge (OASIS® MCX; Waters, Milford, MA). The eluate was injected into a liquid chromatography-tandem mass spectrometry system equipped with a high performance liquid chromatograph (CLASS-VP HPLC system; Shimadzu, Kyoto, Japan) and a tandem mass spectrometer (TSQ-7000, Thermo Fisher Scientific, San Jose, CA). HPLC analysis was performed on a Xbridge C_{18}

Table 1. BP_{ND} of [^{11}C]WAY-100635 and occupancy of 5-HT_{1A} receptors by Wf-516 and pindolol.

			BP_{ND}		Occupancy (%)	
	PET ligand	Dose (mg/kg)	Hippocampus	Raphe	Hippocampus	Raphe
Wf-516	[^{11}C]WAY-100635	0	6.037±0.338	2.348±0.101	–	–
		1	5.612±0.356	2.128±0.109	7.0±2.7	8.5±7.6
		3	5.589±0.410	2.024±0.097	6.8±7.1	13.2±6.1
		10	4.937±0.550	1.620±0.126	18.8±6.0	30.6±6.4
		30	4.836±0.441	1.271±0.194	20.3±4.1	46.3±6.9
		100	4.962±0.400	1.212±0.073	17.8±4.9	48.2±3.3
	[^{18}F]MPPF	0	1.433±0.051	0.418±0.051	–	–
		30	1.100±0.015	0.227±0.027	23.3±1.5	45.7±6.5
Pindolol	[^{11}C]WAY-100635	0	5.709±0.724	1.798±0.316	–	–
		1	4.787±0.676	1.129±0.164	15.0±11.8	33.7±14.5
		3	3.517±0.119	0.752±0.093	37.0±5.6	57.3±2.8
		10	2.190±0.757	0.459±0.153	58.1±18.4	69.6±14.6
		30	0.441±0.173	0.029±0.029	91.8±3.4	98.3±1.7
		3+ FLV*	3.670±0.315	0.783±0.178	33.0±18.8	53.1±15.1
		30+FLV*	0.343±0.065	0	91.6±1.2	100.0

Data represent mean ± S.E. of 3–4 rats.
*Thirty mg/kg of fluvoxamine (FLV) was orally administered 30 min before pindolol treatment.
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column (3.5 μm, 2.1×50 mm; Waters, Milford, MA) at 40°C. The eluted pindolol and (±)-metoprolol were ionized using an electrospray interface and detected by selected reaction monitoring of the transitions of m/z 249.0 to 116.0 and 268.0 to 133.0, respectively.

Autoradiographic Evaluation of Agonistic Properties of Drugs

Autoradiographic procedures were performed as described previously [30], with some modifications. The sections were preincubated for 15 min in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4) containing 10 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM ethylene glycol tetraacetic acid. A second preincubation using the same buffer containing 2 mM GDP was performed for 15 min. The sections were subsequently incubated in the same buffer containing 2 mM GDP, 0.2 mM dithiothreitol and 0.04 nM [^{35}S]GTPγS for 120 min. Basal [^{35}S]GTPγS binding was defined in the absence of test drugs. One μM 8-OHDPAT was used as the agonist control, and test drugs included Wf-516 (10^{-7}–10^{-5} M), pindolol (10^{-7}–10^{-5} M) and WAY-100635 (10^{-8}–10^{-6} M). Nonspecific binding was estimated using 10 μM nonradioactive GTPγS. The incubation was terminated by ice-cold 50 mM HEPES (pH 7.0). The sections were desalted with ice-cold distilled water, dried, and placed in contact with an imaging plate for 2 days. Signal detection and image analysis were performed as described above.

Results

In vitro ARG revealed that the inhibition of [^{11}C]WAY-100635 binding to 5-HT_{1A} receptors in the hippocampus and raphe nucleus of rats in a manner dependent upon the concentrations of Wf-516 and pindolol in the reaction buffer (Figure 1). Ki values for Wf-516 in the hippocampus and raphe nucleus were 8.1 nM and 7.9 nM, respectively; these values were nearly equivalent to the *in vitro* estimate (7.4 nM) in our previous assay using rat hippocampal homogenates [21]. Pindolol exhibited a slightly higher affinity than Wf-516, as its Ki values in the hippocampus and raphe nucleus were 2.7 nM and 3.6 nM, respectively, also in reasonable agreement with previous *in vitro* ARG measures (7.9 nM and 6.5 nM in the hippocampus and raphe nucleus, respectively) [31]. Excess amounts of Wf-516 and pindolol induced a near-complete blockage of 5-HT_{1A} receptors, and there were no differences in their affinities between the hippocampus and raphe nucleus [$p>0.05$ by two-way repeated-measures analysis of variance (ANOVA)]. The lack of regional selectivity of Wf-516 and pindolol was also demonstrated by *ex vivo* ARG analyses (Figure 2), in which Wf-516 and pindolol bound to 5-HT_{1A} receptors in a dose-dependent manner up to approximately 80% and 70%, respectively, irrespective of the region. Oral ED_{50} values for Wf-516 in the hippocampus and raphe nucleus were 5.3 mg/kg and 4.2 mg/kg, respectively, and intraperitoneal ED_{50} values for pindolol in these regions were 8.5 mg/kg and 7.3 mg/kg, respectively. There was no significant difference in drug occupancies between the hippocampus and raphe nucleus ($p>0.05$ by two-way repeated-measures ANOVA).

In contrast to *in vitro* and *ex vivo* autoradiographic imaging, the *in vivo* PET assays indicated a limited capacity of 5-HT_{1A} receptors to be accessible to Wf-516, with a marked regional difference. The partial and full inhibitions of [^{11}C]WAY-100635 binding by pretreated Wf-516 and pindolol, respectively, were visually demonstrated in representative PET images showing dose-dependent changes in the same individual rats (Figure 3). Effects of Wf-516 and pindolol on the radioligand kinetics in the brain were then quantitatively assessed by defining ROIs on dynamic PET data. Time-radioactivity curves after administration of [^{11}C]WAY-100635 demonstrated that the reduction of radioligand retention with increasing doses of pretreated Wf-516 was more prominent in the raphe nucleus than in the hippocampus (Figure 4). However, time-radioactivity curves in these regions showed a substantial difference from that in the cerebellum even with an excessive dose of this drug. Meanwhile, [^{11}C]WAY-

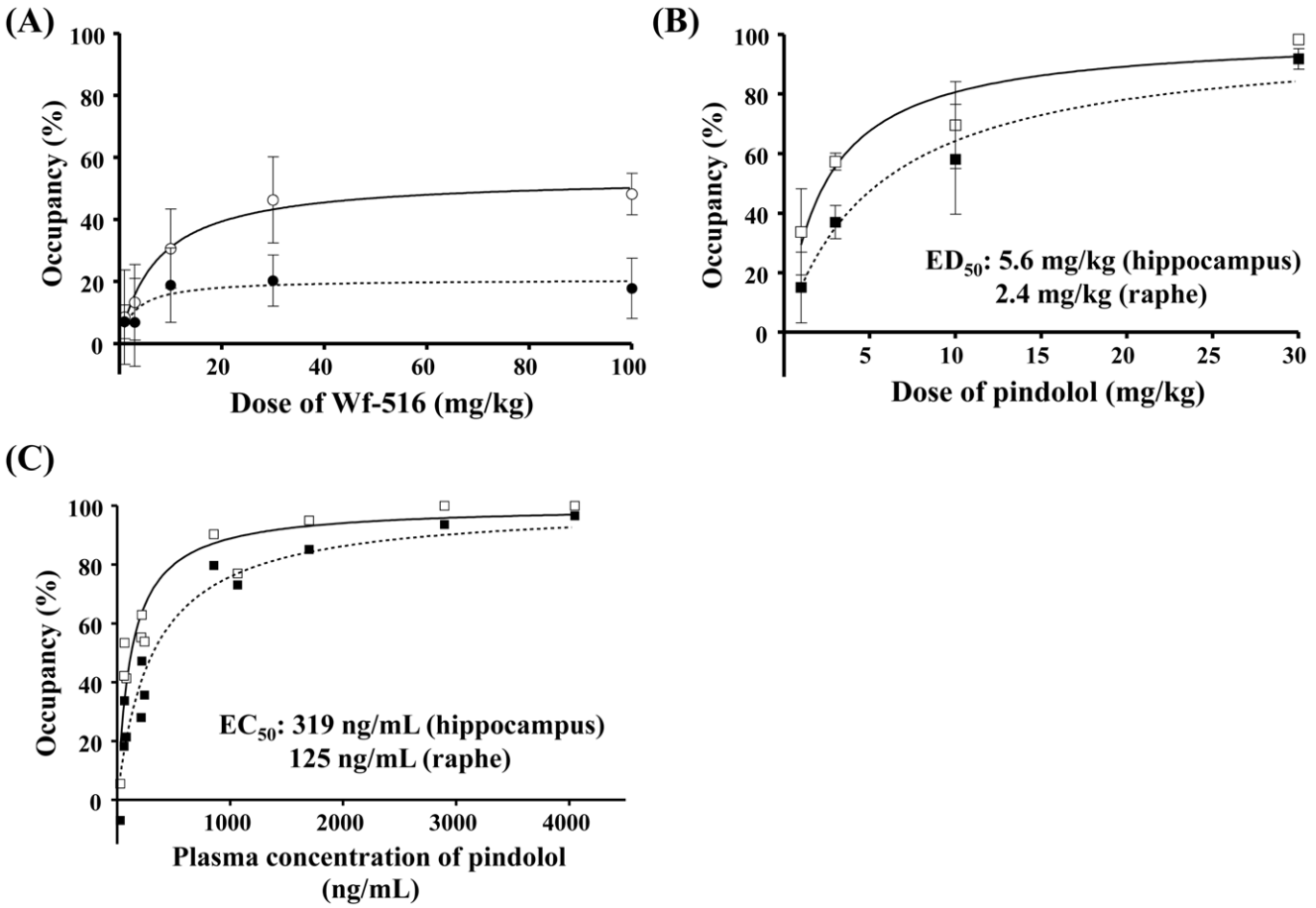

Figure 6. Relationships between dose or plasma concentration of test drugs and 5-HT$_{1A}$ receptor occupancies analyzed with [^{11}C]WAY-100635-PET data. (A) Receptor occupancies in the hippocampus (closed circles) and raphe nucleus (open circles) plotted against oral dose of Wf-516. Regression curves were generated by the following equation: Occ $= Occ_{max} \times D/(D+ED_{50})$, where Occ, Occ_{max}, D and ED_{50} are 5-HT$_{1A}$ receptor occupancy, maximal occupancy, dose of Wf-516, and dose of Wf-516 required for 50% of maximal occupancy, respectively. Bars indicate S.E. (n = 4). (B) Receptor occupancies in the hippocampus (closed squares) and raphe nucleus (open squares) plotted against intraperitoneal dose of pindolol. Regression curves were generated by the following equation: Occ $= 100 \times D/(D+ED_{50})$. Bars indicate S.E. (n = 3). (C) Receptor occupancies in the hippocampus (closed squares) and raphe nucleus (open squares) plotted against plasma concentration of pindolol. Regression curves were generated by the following equation: Occ $= 100 \times C/(C+EC_{50})$, where C is plasma concentration of pindolol. Dashed and solid lines represent regressions in the hippocampus and raphe nucleus, respectively.
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100635 radiosignals in the hippocampus and raphe nucleus were attenuated to a level close to cerebellar values as a function of the dose of pretreated pindolol (Figure 4). These distinct properties in the mode of receptor occupancies by Wf-516 and pindolol were more clearly presented by plotting the specific binding of [^{11}C]WAY-100635 calculated as the difference in radioactivity between target and reference regions (Figure 5). Again, an incomplete but regionally selective suppression of radioligand binding following Wf-516 pretreatment was demonstrated, while these binding components were fully displaceable by pindolol.

We then quantified BP_{ND} values for radioligand binding and occupancies of 5-HT$_{1A}$ receptors by Wf-516 and pindolol at each dose (Table 1 and Figure 6). Both drugs induced attenuation of BP_{ND} in a dose-dependent fashion. Notably, Wf-516 preferentially bound to 5-HT$_{1A}$ receptors in the raphe nucleus compared to the hippocampus. This regional selectivity was particularly striking at a high dose, and a statistically significant interaction between Wf-516 dose and region was observed ($p<0.01$, $F(4,12) = 7.53$ by two-way repeated-measures ANOVA). Saturation occupancy by Wf-516 was far below 100%, but there was a more than 2.5-fold difference in maximal occupancy as calculated by the regression curve between the hippocampus (20.7%) and raphe nucleus (53.8%). In contrast, the dose-dependent binding of pindolol to 5-HT$_{1A}$ receptors reached nearly full occupancy in these regions. A significant main effect of region on receptor occupancies by pindolol was found ($p<0.01$, $F(1,2) = 157.6$ by two-way repeated-measures ANOVA), indicating that this drug also binds to 5-HT$_{1A}$ receptors in a somewhat regionally selective manner, although there was no marked interaction between dose and region ($p>0.05$ by two-way repeated-measures ANOVA). Curve fitting in scatterplots of 5-HT$_{1A}$ receptor occupancy against dose and plasma concentration of pindolol yielded the ED_{50} and EC_{50} values for this drug, respectively (Figure 6). ED_{50} estimates in the hippocampus and raphe nucleus were 5.6 mg/kg and 2.4 mg/kg, respectively, and EC_{50} measures in these regions were 319 ng/mL and 125 ng/mL, respectively. The occupancy by 3 mg/kg Wf-516, corresponding to ED_{50} for its blockade of 5-HTTs as reported in our previous study [24], differed by approximately 100% between the hippocampus and raphe nucleus, while 3 mg/kg pindolol, close to its ED_{50} value in the raphe nucleus, resulted

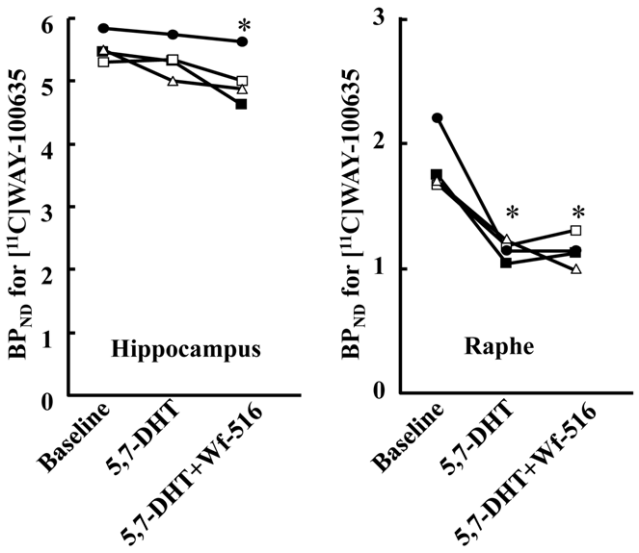

Figure 7. Alteration of BP_{ND} for [^{11}C]WAY-100635 in rats treated with a toxicant for 5-HT neurons, 5,7-DHT. Four rats each underwent three [^{11}C]WAY-100635-PET scans, at baseline, after 5,7-DHT treatment, and after oral administration of 30 mg/kg Wf-516 (5,7-DHT + Wf-516), in the indicated chronological order. Each symbol represents individual BP_{ND} in the hippocampus (left) and raphe nucleus (right). These changes of BP_{ND} in each region were statistically examined by one-way repeated-measures ANOVA followed by least significant difference test. *$p<0.05$ compared with each baseline.
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in an approximately 50% difference in the occupancy between these two regions, suggesting a prominent regional selectivity of Wf-516 in comparison with pindolol. Additionally, administration of 30 mg/kg fluvoxamine prior to pindolol treatment, which is capable of inhibiting more than 80% of 5-HTTs according to our previous investigation [24], had no effect on 5-HT_{1A} receptor occupancies by pindolol (Table 1). This result implies that binding of 5-HT_{1A} receptor ligands was not overtly affected by a concurrent blockade of 5-HTT and consequent increase of synaptic 5-HT, supporting the view that the characteristics of the interaction between Wf-516 and 5-HT_{1A} receptors do not stem from its dual action on 5-HT_{1A} receptor and 5-HTT.

As the preferential binding of Wf-516 to the raphe nucleus could be due to its selectivity for presynaptic 5-HT_{1A} autoreceptors enriched in this region, we assessed the occupancy of 5-HT_{1A} receptors by Wf-516 in rats treated with 5,7-DHT, which abolished 5-HT neurons expressing presynaptic 5-HT_{1A} receptors. Consistent with our previous *ex vivo* study [28], BP_{ND} for [^{11}C]WAY-100635 in the hippocampus was almost unchanged by treatment with 5,7-DHT, while that in the raphe nucleus was profoundly decreased, indicating a selective disruption of 5-HT neurons bearing presynaptic 5-HT_{1A} receptors (Figure 7). The residual radioligand binding in the raphe nucleus after treatment with 5,7-DHT was presumed to primarily reflect the presence of binding sites other than presynaptic 5-HT_{1A} autoreceptors, as documented in a previous study [32]. Oral administration of 30 mg/kg Wf-516 to these 5,7-DHT-treated rats induced a significant decrease of BP_{ND} in the hippocampus as compared with baseline, but no additional reduction of BP_{ND} was observed in the raphe nucleus.

We subsequently investigated the agonistic and antagonistic properties of Wf-516, in consideration of the fact that several drugs acting as partial agonists for 5-HT_{1A} receptor display a high selectivity for presynaptic binding sites relative to postsynaptic components [33–35]. *In vitro* ARG binding of [^{35}S]GTPγS was enhanced by the addition of a 5-HT_{1A} receptor agonist, 8-OH-DPAT, in the hippocampus and raphe nucleus (Figure 8). In the raphe nucleus, a significant interaction between [^{35}S]GTPγS binding and three different concentrations of each of Wf-516, pindolol and WAY-100635 was observed ($p<0.05$, $F(4,20) = 3.58$ by two-way repeated-measures ANOVA). Furthermore, 1 μM 8-OH-DPAT and 10 μM Wf-516 significantly increased [^{35}S]GTPγS binding in the raphe nucleus as compared with 1 μM WAY-100635 ($p<0.01$ by one-way repeated-measures ANOVA followed by least significant difference test), with the increment by Wf-516 being smaller than that by 8-OH-DPAT. The increased binding of [^{35}S]GTPγS by Wf-516 was abolished in the presence of 1 μM 8-OH-DPAT (data not shown). In the hippocampus, test compounds other than 8-OH-DPAT did not induce a significant increase in [^{35}S]GTPγS binding ($p>0.05$ by one-way repeated-measures ANOVA followed by least significant difference test), although there was a tendency for enhancement of radioprobe binding by Wf-516 at higher concentrations. Pindolol at high concentrations also produced a slight increase in [^{35}S]GTPγS binding in the hippocampus and raphe nucleus, but this change was not statistically significant ($p>0.05$ by one-way repeated-measures ANOVA followed by least significant difference test). These experimental data suggest a partial agonist action of Wf-516 on 5-HT_{1A} receptors, which may be relevant to its binding selectivity for presynaptic elements.

Discussion

In this study, we demonstrated preferential binding of Wf-516 to presynaptic 5-HT_{1A} autoreceptors by means of *in vivo* PET imaging of rat brains. This investigational drug dually acting on 5-HTTs and 5-HT_{1A} receptors yielded approximately 20% and 50% occupancies at maximum in the hippocampus and raphe nucleus of living rats, respectively. PET experiments following toxic injuries of 5-HT neurons by 5,7-DHT indicated a selective affinity of Wf-516 for presynaptic receptors accounting for this regionality. The limited availability of 5-HT_{1A} receptors to Wf-516 and its regional difference was in sharp contrast with an established 5-HT_{1A} antagonist, pindolol, which fully occupied these receptors at a high dose irrespective of region. Since one of the major differences in pharmacological features between Wf-516 and pindolol is the blockage of 5-HTTs, it was initially presumed that synaptic 5-HT, intensified by the inhibitory effects of Wf-516 on 5-HTTs, could in turn compete with Wf-516 for 5-HT_{1A} receptors, resulting in limited receptor occupancy by Wf-516. However, this possibility was ruled out by our PET observation that the receptor occupancies by pindolol were apparently not influenced by co-treatment with a typical SSRI, fluvoxamine, at a dose of 30 mg/kg, supposedly blocking 80% of 5-HTTs [24]. Furthermore, these observations are not likely to result from partial volume effects in the raphe ROI, which could lead to underestimation of BP_{ND} but not overestimation of the receptor occupancy by Wf-516. Indeed, we defined a relatively large brainstem ROI including the raphe and surrounding structures for increasing the signal-to-noise ratio, and therefore the receptor occupancy might be somewhat underestimated due to the presence of nonspecific signals from non-raphe areas, which were not displaceable by Wf-516. Despite this possible effect, the occupancy by Wf-516 in the raphe nucleus was higher than that in the hippocampus. Another possibility for the regional difference in the receptor

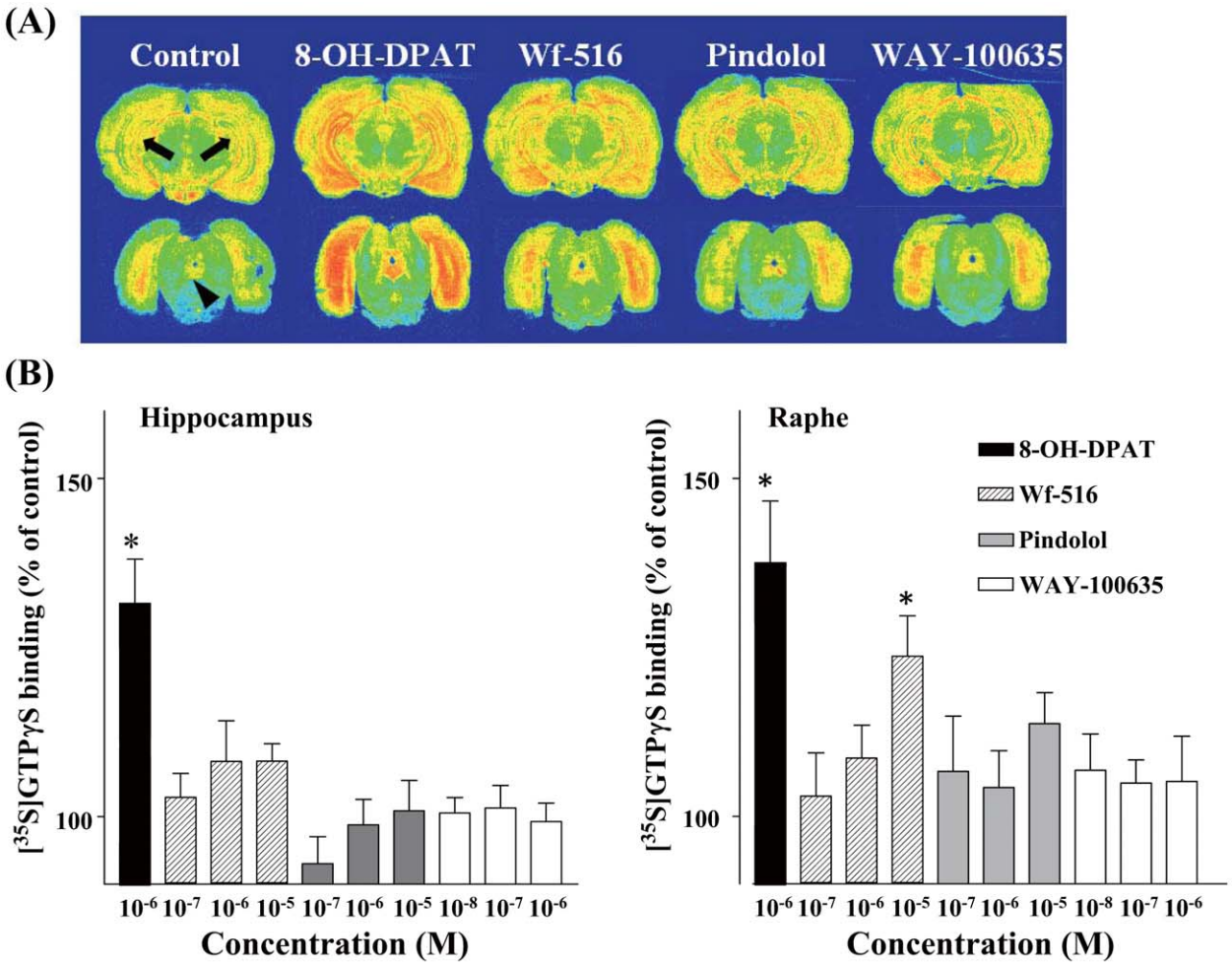

Figure 8. Effects of Wf-516 and pindolol on autoradiographic [^{35}S]GTPγS binding in rat brains. (A) Representative autoradiograms showing radiolabeling with [^{35}S]GTPγS in the hippocampus and raphe nucleus at baseline (control) or in the presence of 1 μM 8-OH-DPAT (full agonist for 5-HT$_{1A}$ receptors), 10 μM Wf-516, 10 μM pindolol and 1 μM WAY-100635 (full antagonist for 5-HT$_{1A}$ receptors). (B) Ratio of [^{35}S]GTPgγS binding to the control level in the hippocampus (left) and raphe nucleus (right). Changes in radiotracer binding were statistically examined using one-way repeated-measures ANOVA followed by least significant difference test. *$p<0.01$ compared with 1 μM WAY-100635. In the raphe nucleus, a significant interaction between [^{35}S]GTPγS binding and three concentrations each of Wf-516, pindolol and WAY-100635 was demonstrated by two-way repeated-measures ANOVA ($p<0.05$, $F(4,20) = 3.58$).
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occupancy by Wf-516 is the variability of its uptake among brain areas attributed to locally differential effects of efflux transporters. However, this is also unlikely in light of: 1) efficient transfer of Wf-516 to the brain with the brain-to-plasma ratio approximating 2.0 at the time of reaching maximal plasma concentration (unpublished data); and 2) our previous observation that the occupancy of 5-HTT by Wf-516 was homogenous among regions [24].

We then postulated that Wf-516 might exert agonistic effects at 5-HT$_{1A}$ receptors, in view of previous clinical studies documenting that occupancies of 5-HT$_{1A}$ receptors by agonistic agents at regular doses without adverse effects were barely detectable by [^{11}C]WAY-100635-PET [36–38]. This notion was supported by the present ARG measurements of [^{35}S]GTPγS, which indicated the partial agonistic potency of Wf-516, particularly in the raphe nucleus. Since it was reported that 5-HT$_{1A}$ receptors were configured in high- and low-affinity states for agonistic ligands [39], the incomplete occupancy of these receptors by Wf-516 can be explained by its selectivity for the high-affinity state binding components. Agonists are supposed to interact with G protein-coupled and uncoupled 5-HT$_{1A}$ receptors with high and low affinities, respectively, and these two modes of the receptors may coexist in the same synaptic terminal. Previous *in vitro* ligand-receptor binding assays also indicated two such distinct functional modes in other serotonin receptor subtypes, including 5-HT$_{1B}$ [40], 5-HT$_{2A}$ [41,42] and 5-HT$_{2C}$ [42] receptors, and G protein-coupled neurorecptors in non-serotonergic systems [43]. The proportion of the high-affinity sites against total binding components might differ between presynaptic and postsynaptic terminals, which might be associated with the previous observation that 5-HT$_{1A}$ receptor reserve for agonists in the hippocampus was lower than that in the dorsal raphe nucleus [44,45]. An *in vivo* electrophysiological study of rats demonstrated that Wf-516 intravenously administered at low and medium doses inhibited the actions of 5-HT$_{1A}$ autoreceptor agonists on firing of 5-HT neurons, but enhanced these agonistic effects at high cumulative doses [23]. This supports the contention that Wf-516 preferentially binds to agonist 5-HT$_{1A}$ receptor sites, acts antagonistically at therapeutic doses, and exerts an agonistic property at excessive doses. The previous finding that Wf-516 at low and medium doses

is devoid of activity in hippocampal postsynaptic 5-HT_{1A} receptors [23] is also consistent with the fact that postsynaptic receptors are relatively insensitive to partial agonists [33–35]. Despite these indications, it is still necessary to assess differences in the density of agonist binding sites in the raphe nucleus and hippocampus of living brains using PET and agonistic radioligands for 5-HT_{1A} receptors. Such imaging agents have recently been developed [46,47] and would serve this purpose.

Unlike Wf-516, pindolol at a high dose showed full occupancy of 5-HT_{1A} receptors in the hippocampus and raphe nucleus, while binding of this compound at low and medium doses appeared somewhat regionally selective. This modest regionality of receptor occupancies by pindolol may account for inconsistent observations in clinical PET investigations of its binding selectivity [16–18]. Accordingly, the present ARG using [^{35}S]GTPγS suggested marginal, insignificant agonistic activity of pindolol at 5-HT_{1A} receptors. This conflicts slightly with a previous study that showed unremarkable modulations of ARG [^{35}S]GTPγS binding by pindolol [30], but is basically in agreement with reports demonstrating the *in vivo* electrophysiological effects of pindolol as an agonist [48] and its mixed antagonistic/agonistic profile in physiological experiments [49]. Considering these observations together with the inconsistency of previous reports on the selectivity of pindolol for presynaptic sites [16–20], we postulate that pindolol does not strongly preferentially interact with high-affinity binding sites for agonists and therefore may not be as suitable as Wf-516 for selectively blocking and/or downregulating presynaptic 5-HT_{1A} autoreceptors.

As illustrated in Figure 6C, EC_{50} estimates for receptor occupancies by pindolol in the raphe nucleus and hippocampus were 125 and 319 ng/mL, respectively, which were not equivalent to the clinical data (27 and 80 ng/mL in the raphe nucleus and postsynaptic receptor-rich region, respectively) of one previous PET study [16], but they were acceptably close to one another to support the predictability of clinical doses based on animal measures. Because the proportions of protein-bound pindolol in plasma or serum have been reported to be 52% in rats [50] and 57% in humans [51], and because no metabolites of this drug active at 5-HT_{1A} receptors have been identified, we assumed that there were no species differences in the relationships between plasma pindolol concentration and 5-HT_{1A} receptor occupancy. In clinical assays, data on the plasma concentration of pindolol to induce full occupancy of receptors are lacking due to concerns about side effects at such high doses, and this may hamper accurate determination of EC_{50} in humans, conceivably resulting in a slight difference in EC_{50} values between non-clinical and clinical studies. Indeed, pindolol was required to fully block 5-HT_{1A} autoreceptors in order to provide benefits for the therapeutic effects of SSRIs, but it was found to occupy only 60% of these receptors at the maximal safe dose [16]. As illustrated by our PET measurements, the high-dose administration of pindolol sufficient for the full occupancy of 5-HT_{1A} autoreceptors in the raphe nucleus simultaneously causes a complete blockade of postsynaptic receptors in the hippocampus, a crucial drawback for the antidepressive effects of SSRIs mediated directly by reinforcement of serotonergic neurotransmissions and indirectly by the resultant enhancement of neurogenesis [52,53]. This could be in line with the results of clinical studies [54,55] that failed to confirm the adjunctive therapeutic effects of pindolol reported in earlier works [11].

Besides the utilization of common methodologies and biological parameters among species to facilitate the translation of findings from non-clinical to clinical studies, *in vivo* PET imaging has the advantage of being able to clarify the status of bioactive molecules in living brains, which might not be revealed by *in vitro* or *ex vivo* techniques. In fact, the pharmacokinetic characteristics of Wf-516, including limited ranges of 5-HT_{1A} receptor occupancy and preference of presynaptic receptors, were demonstrated by PET but not by other assaying modalities. Although the reasons for these discrepant observations among analytical methods remain unclear, one could speculate that alterations in 5-HT_{1A} receptors – including their subcellular localizations, biochemical modifications (e.g., phosphorylation, glycosylation), coupling to G proteins, and proportion of high-affinity sites, in addition to redistribution of the drug – could occur at perimortem and postmortem periods in the preparations of *in vitro* and *ex vivo* samples. Indeed, *ex vivo* receptor occupancy measurements in the present work were conducted by reacting radioligands with brain samples collected from Wf-516- or pindolol-treated rats, and thus could be influenced by perimortem and postmortem alterations of the receptor statuses, unlike *ex vivo* autoradiographic labeling of the receptors with radioligands systemically administered to living animals. This discrepancy between *in vivo* and postmortem assays may be relevant to a previous observation that binding of an agonistic radioligand for 5-HT_{1A} receptors in the cat hippocampus was abundant in autoradiograms but was nearly absent in PET images [47]. One speculative notion would be that recoupling of low-affinity-state receptors to G proteins to form a high-affinity state occurs at a higher rate in perimortem and postmortem conditions than in living brains, and this could facilitate interaction between agonists and receptors in *ex vivo* and *in vitro* autoradiographic assays. Transition between high- and low-affinity states was also implied in dopamine D_2 receptors [56], although the molecular mechanisms by which neuroreceptors are coupled to and uncoupled from G proteins have yet to be clarified.

The present data also provide mechanistic implications for other in-development drugs simultaneously targeting 5-HTT and 5-HT_{1A} receptors, such as vilazodone (also known as EMD68843) [57]. This drug has been developed as an SRI as well as a partial agonist for 5-HT_{1A} receptors, but *in vivo* biochemical assays in animals have supported its activity as an antagonist for these receptors [58]. This is quite similar to observations in a previous electrophysiological experiment using Wf-516 at low and medium doses [23]. Moreover, a clinical PET study with [^{11}C]WAY-100635 implied that occupancies of 5-HT_{1A} receptors by vilazodone exhibited selectivity for presynaptic components and did not reach a complete blockade of these receptors, despite its small sample size [38]. The properties resembling those of Wf-516 can be more precisely assessed by small animal PET measurements, and each subject in such non-clinical studies can be repeatedly scanned after administration of drugs at different doses broadly ranging from minimal to excessive amounts, an approach not possible for human subjects. As vilazodone has been proven to be effective in treating patients with major depression in a phase III clinical trial [59] and has lately been approved by the US Food and Drug Administration [60], our results have brought to light evidence of promising clinical potencies of Wf-516.

In conclusion, the present results, in conjunction with our previous data [24], have proven the pharmacological concept of an emerging antidepressant therapy dually targeting the 5-HTT and presynaptic 5-HT_{1A} receptors. This combination enables prompt serotonergic enhancement without autoreceptor-mediated negative feedback. The partial agonistic properties of Wf-516 could be linked to the presynaptic dominance of the drug action, and would thus be suitable for inhibitory blockade of presynaptic signaling suppressive of 5-HT release.

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Author Contributions

Conceived and designed the experiments: T. Saijo JM T. Suhara MH. Performed the experiments: T. Saijo JM TO MH. Analyzed the data: T. Saijo JM TO. Contributed reagents/materials/analysis tools: J-iM YM YK MS NG TF.Wrote the paper: T. Saijo JM MH.

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