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Pharmacological characterization of the cannabinoid CB_1 receptor PET ligand ortholog, [^{3}H]MePPEP

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ABSTRACT

MePPEP ((3*R*,5*R*)-5-(3-methoxy-phenyl)-3-((R)-1-phenyl-ethylamino)-1-(4-trifluoromethyl-phenyl)-pyrrolidin-2-one) is an inverse agonist shown to be an effective PET ligand for labeling cannabinoid CB_1 receptors in vivo. [^{11}C]MePPEP and structurally related analogs have been reported to specifically and reversibly label cannabinoid CB_1 receptors in rat and non-human primate brains, and [^{11}C]MePPEP has been used in human subjects as a PET tracer. We have generated [^{3}H]MePPEP, an ortholog of [^{11}C]MePPEP, to characterize the molecular pharmacology of the cannabinoid CB_1 receptor across preclinical and clinical species. [^{3}H]MePPEP demonstrates saturable, reversible, and single-site high affinity binding to cannabinoid CB_1 receptors. In cerebellar membranes purified from brains of rat, non-human primate and human, and cells ectopically expressing recombinant human cannabinoid CB_1 receptor, [^{3}H]MePPEP binds cannabinoid CB_1 receptors with similar affinity with K_d values of 0.09 nM, 0.19 nM, 0.14 nM and 0.16 nM, respectively. Both agonist and antagonist cannabinoid ligands compete [^{3}H]MePPEP with predicted rank order potency. No specific binding is present in autoradiographic sections from cannabinoid CB_1 receptor knockout mouse brains, demonstrating that [^{3}H]MePPEP selectively binds cannabinoid CB_1 receptors in native mouse tissue. Furthermore, [^{3}H]MePPEP binding to anatomical sites in mouse and rat brain is comparable to the anatomical profiles of [^{11}C]MePPEP in non-human primate and human brain in vivo, as well as the binding profiles of other previously described cannabinoid CB_1 receptor agonist and antagonist radioligands. Therefore, [^{3}H]MePPEP is a promising tool for translation of preclinical cannabinoid CB_1 receptor pharmacology to clinical PET ligand and cannabinoid CB_1 receptor inverse agonist therapeutic development.

1. Introduction

Marijuana has been used for thousands of years to treat pain, nausea, emesis, and muscle spasms, but is limited by psychotropic side effects (Iverson, 2000). The primary active principle of marijuana, Δ^9-THC, elicits effects centrally through agonism of the cannabinoid CB_1 receptors which are broadly expressed in brain (Pertwee, 2006; Di Marzo et al., 2004; Di Marzo, 2008). Endogenous cannabinoids, such as anandamide and 2-arachidonoylglycerol, activate both cannabinoid CB_1 and CB_2 receptors in vivo. The cannabinoid CB_1 receptor is one of the most abundant G protein-coupled receptors in the central nervous system, localized primarily in cerebral cortex, hippocampus, molecular layer of the cerebellum, striatum, globus pallidus, and substantia nigra, and some peripheral tissues. The cannabinoid CB_2 receptor is localized almost exclusively in peripheral tissues, but recent evidence suggests the presence of low levels in central tissues which increase during pathological states (Benito et al., 2003; Onaivi et al., 2006).

Considerable effort has been extended to develop cannabinoid CB_1 receptor agonists and antagonists, but high lipophilicity has limited most ligands. Recent efforts produced rimonabant (Acomplia®), a cannabinoid CB_1 receptor inverse agonist, for the treatment of obesity (Leite et al., 2009; Bifulco et al., 2007); however, psychiatric adverse events led to withdrawal of this compound. Availability of additional tools will aid study of the potential for inverse agonists and antagonists of this receptor to treat obesity, binge eating, neuropsychiatric conditions and cognitive dysfunctions.

To advance the clinical benefits of cannabinoid-based therapeutics, it is important to accurately select a clinically efficacious dose while maintaining a sufficient margin of safety. Cannabinoid drug development efforts have been facilitated by the recent introduction of effective positron emission tomography (PET) ligands; however, no ligand has been used to translate efforts from initial in vitro screening through animal and human PET studies (Terry et al., 2008; Yasuno et al., 2008; Horti et al., 2006; Burns et al., 2007). [^{11}C] is commonly used for labeling PET ligands due to the high energy

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output required for PET imaging studies, but has a short half life (20 min) and requires special shielding. Therefore, MePPEP was labeled with [^{3}H] to provide safer handling and a longer half life for preclinical use. Ligand affinity, binding kinetics, and cannabinoid CB_1 receptor density were assessed using [^{3}H]MePPEP in membranes of Chinese hamster ovary (CHO) cells stably expressing the human cannabinoid CB_1 receptor (CB_1/CHO) and cerebellum from human, non-human primate, and rat brain. Localization of [^{3}H]MePPEP binding is assessed in tissues from wild-type and cannabinoid CB_1 receptor deficient mouse brains. [^{3}H]MePPEP demonstrates specific, single site, reversible, high affinity binding to cannabinoid CB_1 receptors expressed ectopically and in native tissues with no significant difference in affinity constants across species. Localization of [^{3}H]MePPEP is consistent with previous reports of cannabinoid CB_1 receptor binding and is specific as demonstrated by lack of binding in cannabinoid CB_1 receptor knockout mice tissues. Therefore, [^{3}H]MePPEP shows promise as a pharmacological tool that can translate from in vitro screening efforts to development of clinical compounds targeting inverse agonism or antagonism of the cannabinoid CB_1 receptor.

2. Materials and methods

2.1. Compounds

SR141716A, ((*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride); SR144528, (*N*-(1,3,3-trimethylbicyclo(2.2.1)heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide); MePPEP, ((3*R*,5*R*)-5-(3-methoxy-phenyl)-3-((*R*)-1-phenyl-ethylamino)-1-(4-trifluoromethyl-phenyl)-pyrrolidin-2-one); FEPEP, ((3*R*,5*R*)-5-[3-(2-fluoro-ethoxy)-phenyl]-3-((*R*)-1-phenyl-ethylamino)-1-(4-trifluoromethyl-phenyl)-pyrrolidin-2-one); FMPEP, ((3*R*,5*R*)-5-(3-fluoromethoxy-phenyl)-3-((*R*)-1-phenyl-ethylamino)-1-(4-trifluoromethyl-phenyl)-pyrrolidin-2-one); FMePPEP, ((3*R*,5*R*)-3-[(*R*)-1-(4-fluoro-phenyl)-ethylamino]-5-(3-methoxy-phenyl)-1-(4-trifluoromethyl-phenyl)-pyrrolidin-2-one); and FMPEP-d2, ((3*R*,5*R*)-5-((3-fluoromethoxy-*d2*)phenyl)-3-((*R*)-1-phenyl-ethylamino)-1-(4-trifluoromethyl-phenyl)-pyrrolidin-2-one) were synthesized at Eli Lilly and Company (Indianapolis, IN) (Donohue et al., 2008). CP-55,940, (5-(1,1-dimethylheptyl)-2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-phenol) and R-1 Methanandamide, (*N*-(2-hydroxy-1*R* methylethyl)-5Z, 8Z, 11Z, 14Z-eicosatetraenamide) were purchased from Cayman Chemical Company (Ann Arbor, MI). WIN-55,212-2, (+)-(*R*)-(5-methyl-3-(morpholinomethyl)-2,3-dihydro-[1,4]oxazino[2,3,4-hi]indol-6-yl)(naphthalen-1-yl)methanone mesylate salt was purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]MePPEP was radiolabeled by Amersham Biosciences (Piscataway, NJ). *Other Reagents*: Fatty acid free bovine serum albumin (BSA) pH 7.0 was obtained from Serologicals Corporation (Norcross, GA).

2.2. Synthesis of [^{3}H]MePPEP

Alkylation of (3*R*,5*R*)-5-(3-hydroxy-phenyl)-3-((*R*)-1-phenyl-ethylamino)-1-(4-trifluoromethyl-phenyl)-pyrrolidin-2-one (Donohue et al., 2008) with [^{3}H]iodomethane followed by HPLC purification (Amersham Biosciences, Piscataway, NJ) provided [^{3}H]MePPEP with a specific activity of 83 Ci/mmol (Fig. 1).

2.3. Brain tissues and hCB_1/CHO cells

Human cannabinoid CB_1 receptor expressing Chinese Hamster Ovary (CHO) cell membranes were purchased from Applied Cell Sciences (Rockville, MD). Rat cerebella were purchased from Pel-Freez Biologicals (Rogers, AR). Collaborators at the National Institute of Mental Health (Bethesda, MD) provided non-human primate (*Macaca mulatta*) and human cerebella which were obtained following NIH guidelines for use and obtaining animal and human tissue. Human cerebellar tissue was also obtained from the Indiana Organ Procurement Organization (Indianapolis, IN).

2.4. Membrane preparation

Membranes were prepared from rat, non-human primate, and human whole cerebellum tissues by breaking frozen tissue into small pieces on dry ice and then homogenizing in 5 volumes cold homogenization buffer (10% sucrose in 50 mM Tris, pH 7.4, plus protease inhibitors) using a polytron. Homogenized tissue was diluted to 60 volumes with homogenization buffer and centrifuged for 10 min at 1000×*g* at 4 °C. Supernatant was saved and the pellet rehomogenized as described above. The combined supernatant was centrifuged at 11,000×*g* for 30 min at 4 °C and the supernatant was discarded. The pellet was homogenized in 40 volumes 50 mM Tris (pH 7.4) and centrifuged for 30 min at 27,000×*g* at 4 °C. The pellet was resuspended in an appropriate volume 50 mM Tris (pH 7.4) and membranes were stored at −80 °C until assay. Membrane protein was quantified with bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL).

2.5. Receptor binding studies

Saturation, kinetic and competition experiments were performed in assay buffer consisting of 20 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, and 0.5% BSA. Incubations were performed (90 min) at room temperature at a final assay volume of 1 mL. The amount of P2 membranes added to each assay differed for each species due to varying CB_1 expression levels, and was optimized such that the percent of radioligand bound (cpm) compared to the total radioligand added (cpm) did not exceed 10% (hCB_1/CHO: 5 μg/well; human: 60 μg/well; non-human primate: 35 μg/well; rat: 10 μg/well). For each species, the amount of P2 membranes added was equal for saturation, kinetic and competition experiments. In saturation binding assays, 12 concentrations of [^{3}H]MePPEP were

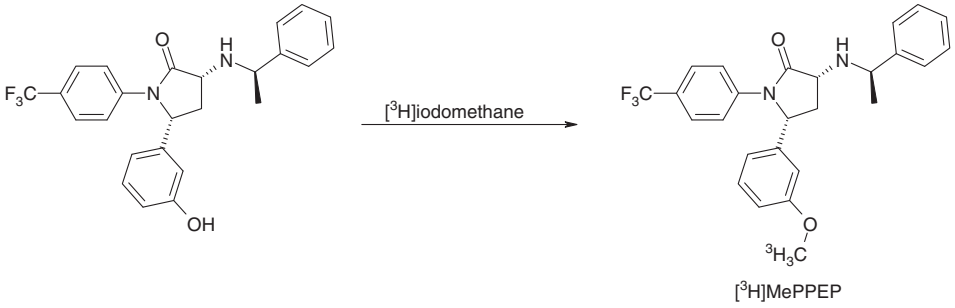

Fig. 1. [^{3}H]MePPEP was generated by alkylation of MePPEP with [^{3}H]iodomethane followed by HPLC purification.

used. In kinetic assays, dissociation rate constants (K_{off}) were determined after addition of 10 μM SR141716A at different times before filtration of membranes pre-incubated with 0.2 nM [^{3}H] MePPEP. Time courses for estimating association rate constants (K_{on}) were measured following the addition of 0.2 nM [^{3}H]MePPEP to membranes at different times before filtration. Eleven point competition curves were used to generate an IC_{50} competing 0.2 nM [^{3}H]MePPEP in all tissues. Nonspecific binding was determined in the presence of 10 μM SR141716A. Bound radioligand was separated from free by rapid filtration through FilterMat type A (Wallac, Turku, Finland) presoaked in 0.3% polyethyleneimine using a Tomtec Harvester (Hamden, CT). The filters were washed three times with cold 50 mM Tris-HCl, pH 7.0. Filters were dried for 90 min at 60 °C, then imbedded with MeltiLex A solid scintillant (Perkin Elmer, Turku, Finland) and counted using a Wallac Microbeta (4 min/well). Data were analyzed using nonlinear regression analysis in GraphPad Prism (San Diego, CA). All saturation binding analyses fit to single site models. pK_i values were calculated for competition experiments by determining the negative logarithm of the inhibition constant (K_i in Molar units) determined in GraphPad Prism using the equation of Cheng and Prusoff (1973). All values represent the mean ± S.E.M. of at least three independent assays ($n = 3$ for kinetic and competition assays; $n = 4$ for saturation experiments where samples from 2 human subjects and 4 non-human primate subjects were each tested as $n = 4$ and data combined).

2.6. Autoradiographic studies

Rat brain sagittal sections, cut from the midline of the brain, (10 μm) were purchased from Zyagen Corp. (San Diego, CA). Mouse brains (wild-type: C57BL6, males, age 8–10 weeks (Taconic, Hudson, NY); cannabinoid CB_1 receptor knockout mice: males, age 8–10 weeks (Taconic Private Breeding Colony)) were removed and rapidly frozen in isopentane. Coronal sections (12 μm) were thaw mounted onto chromium-gelatin coated slides and desiccated at 4 °C, placed at −20 °C overnight, then stored at -80 °C until day of assay. Slides were thawed on a slide warmer (37 °C) and then air dried at room temperature for 20 min. Sections were pre-incubated in assay buffer (50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, and 0.5% BSA) for 25 min at room temperature to remove endogenous ligands, then incubated in assay buffer with 0.14 nM [^{3}H]MePPEP with or without 10 μM SR141716A for 2 h at room temperature. Sections were rinsed 2 × 7 min in ice cold 50 mM Tris–HCl, pH 7.4 with 0.1% BSA. Sections were then briefly immersed in ice cold distilled H_2O, then air dried in a cold stream of air. Sections were apposed to tritium imaging plates (Fujifilm Life Science, Stamford, CT) with [^{3}H] Microscale Standards (Amersham, Piscataway, NJ) for 12 days. Sections were digitized with a BAS-5000 Image Reader (Fujifilm Life Science, Stamford, CT). Images were further processed with MCID Elite imaging software (Cambridge, UK).

3. Results

3.1. Saturation receptor binding and kinetic analysis of [^{3}H]MePPEP

The affinity of [^{3}H]MePPEP at cannabinoid CB_1 receptors from multiple species was examined. Saturation binding experiments were performed in P2 fraction membranes from human, non-human primate and rat brain, and human cannabinoid CB_1 receptors stably expressed in CHO cells. Affinity constants (K_d) were determined in identical binding conditions for each species. Fig. 2 illustrates the saturation isotherms for each species tested. Nonspecific binding was determined in the presence of saturating levels of SR141716A. Nonlinear regression analysis revealed saturable, single site and high affinity specific binding to cannabinoid CB_1 receptors from cerebella of human, non-human primate, and rat brains and human

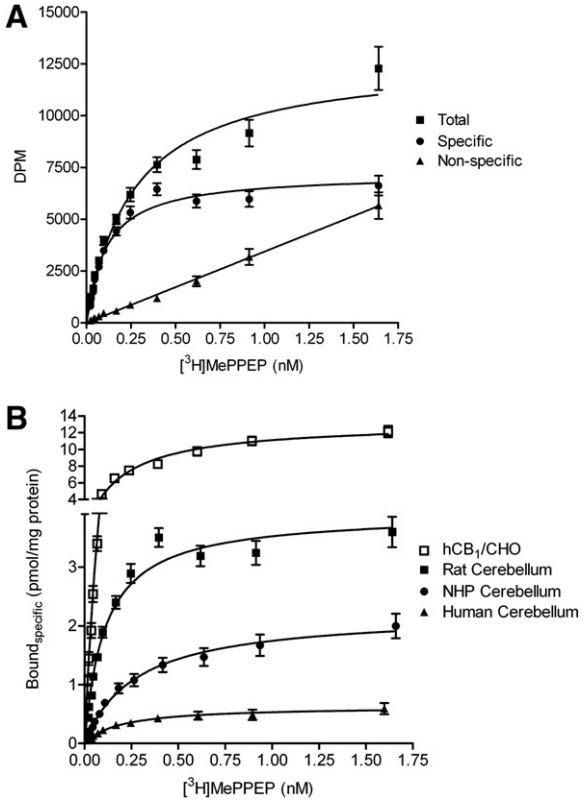

Fig. 2. (A) Example of [^{3}H]MePPEP binding characteristics in rat cerebellum including total, nonspecific, and specific binding (DPM). (B) Saturation binding isotherms of [^{3}H] MePPEP binding to CHO membranes expressing human cannabinoid CB_1 receptor (□); and cerebellar membranes from rat (■), non-human primate (●) and human (▲) brain. Specific binding was defined as the difference in binding in the absence and presence of 10 μM SR141716A. Each data point represents the mean ± S.E.M. of duplicate data points determined from four independent experiments, where for human and non-human primate samples from 2 human subjects and 4 non-human primate subjects were each tested as $n = 4$ and data combined. Affinity constants (K_d) and maximal binding sites (B_{max}) were calculated using nonlinear regression analysis in GraphPad Prism. Binding constants are presented in Table 1. For scatchard plot analysis, see Supplemental data.

CB_1/CHO membranes with no statistical difference in K_d values between species. The maximal number of [^{3}H]MePPEP binding sites was also determined across species (Table 1). The highest levels of [^{3}H]MePPEP binding sites in native tissue were detected in rat cerebellum followed by non-human primate with human cerebellum having the lowest level of binding sites.

Since [^{3}H]MePPEP displayed equal affinity at each species of cannabinoid CB_1 receptor, kinetic analysis was only performed with human CB_1/CHO membranes. Association of specific [^{3}H]MePPEP binding to CB_1/CHO membranes was time dependent, and reached

Table 1
Binding constants for [^{3}H]MePPEP to human cannabinoid CB_1 receptors expressed in CHO membranes, and cerebellar membranes from rat, non-human primate and human brain.

Species	K_d (nM)	B_{max} (fmol/mg protein)
hCB_1/CHO	0.163 ± 0.021	12,900 ± 744
Human cerebellum	0.141 ± 0.032	521 ± 105
NHP cerebellum	0.188 ± 0.022	1890 ± 241
Rat cerebellum	0.093 ± 0.008	3830 ± 238

Binding constants calculated using nonlinear regression analysis. Values represent the mean ± S.E.M. from four separate experiments, where for human and non-human primate samples from 2 human subjects and 4 non-human primate subjects were each tested as $n = 4$ and data combined. ANOVA analysis determined no statistical difference in K_d values between species ($P = 0.097$).

steady state at approximately 70 min. [^{3}H]MePPEP binding to human CB_1/CHO membranes was best fit to a one-phase association model and yielded a K_{on} value of 1.8×10^8 (M^{-1}, min^{-1}) (Fig. 3). The dissociation rate constant (K_{off}) was 0.0156 (min^{-1}) and was best fit to a one-phase dissociation model (Fig. 3). The dissociation constant (K_d) determined from the ratio of K_{off}/K_{on} was 0.09 nM and was not statistically different from the K_d value of 0.16 nM calculated from the saturation binding studies.

3.2. Pharmacology of [^{3}H]MePPEP at cannabinoid CB_1 receptors

The pharmacology of [^{3}H]MePPEP was examined by homologous competition of MePPEP and heterologous competition with known cannabinoid agonists and antagonists, including other known cannabinoid CB_1 receptor PET ligands and a cannabinoid CB_2 receptor antagonist. Fig. 4 displays representative [^{3}H]MePPEP competition curves at human CB_1/CHO membranes. Inhibition constants (K_i) of various cannabinoid CB_1 receptor antagonists and agonists across all species are displayed in Table 2. Structures for MePPEP and its analogs have been published (Donohue et al., 2008) or can be found in supplemental data. Competition binding studies were performed under identical binding conditions as the saturation and kinetic studies; nonspecific binding was determined in the presence of 10 μM SR141716A. The pharmacological selectivity of [^{3}H]MePPEP against cannabinoid CB_2 receptors was examined with SR144528, a selective cannabinoid CB_2 receptor antagonist. Depending on species, MePPEP was 630- to 1260-fold more selective than SR144528 at competing with [^{3}H]MePPEP binding at cannabinoid CB_1 receptors. To evaluate MePPEP against other clinically relevant compounds, highly potent and selective analogs of MePPEP were examined, as well as the known, nonradiolabeled and structurally distinct, cannabinoid CB_1 receptor PET ligands, JHU-75528 and MK-9470 (Donohue et al., 2008; Horti et al., 2006; Burns et al., 2007). The in vitro potencies of MePPEP analogs were similar to MePPEP in all species tested. The unlabelled cannabinoid CB_1 receptor PET ligand MK-9470 (an analog of taranabant) displayed similar affinities to MePPEP, whereas JHU-75528, an SR141716A analog, displayed approximately 10-fold lower affinities than MePPEP across species. The cannabinoid CB_1 receptor inverse agonist SR141716A displayed similar affinities to JHU-77528. Several structurally diverse cannabinoid CB_1 receptor agonists were also evaluated. The bicyclic compound CP-55,940 was effective at competing with [^{3}H]MePPEP; however, WIN-55,212, an aminoalkylindole, and methanandamide, the stable analog of the endocannabinoid anandamide, were significantly less efficacious at competing [^{3}H]MePPEP from cannabinoid CB_1 receptors. Each active cannabinoid compound was able to compete [^{3}H]MePPEP with similar potency across cannabinoid CB_1 receptors in the three species tested (Table 2).

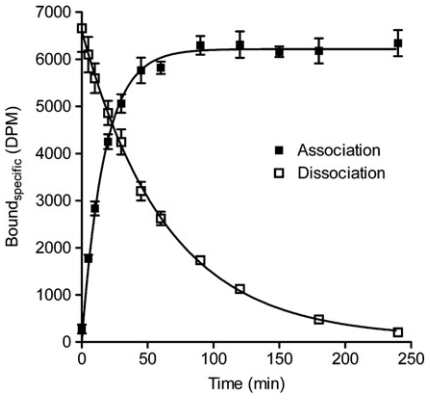

Fig. 3. Time course for the association (■), and dissociation (□) of [^{3}H]MePPEP (0.2 nM) at human cannabinoid CB_1 receptors expressed in CHO membranes. Association was initiated by the addition of [^{3}H]MePPEP to membranes at different times before filtration. Dissociation studies allowed binding of [^{3}H]MePPEP to reach equilibrium before an addition of 10 μM SR141716A at different times before filtration. Kinetic constants were determined with nonlinear regression analysis using GraphPad Prism. $T_{1/2}$ association = 11.8 ± 0.61 min; $T_{1/2}$ dissociation = 44.4 ± 1.54 min. Data were expressed as mean ± S.E.M. from 3 separate experiments.

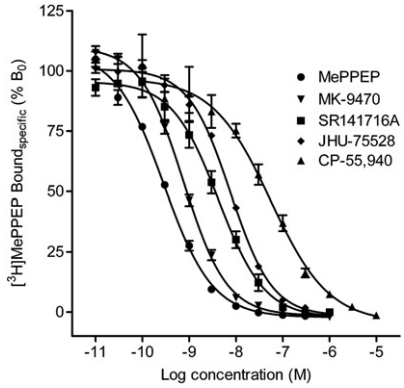

Fig. 4. The pharmacology of [^{3}H]MePPEP binding to CHO membranes expressing recombinant human cannabinoid CB_1 receptor. Competition curves for MePPEP (●), MK-9470 (▼), SR141716A (■), JHU-75528 (◆) and CP55,940 (▲) were generated against 0.2 nM [^{3}H]MePPEP by varying the concentration of unlabeled test compound. The K_i was calculated from the resulting IC_{50} values using the equation of Cheng and Prusoff (1973) $K_i = IC_{50}/(1 + R/K_d)$ where R equals the radioligand concentration and K_d equals the binding affinity of the radioligand. Data represent mean ± S.E.M. of three independent experiments.

3.3. [^{3}H]MePPEP receptor autoradiography in mouse and rat brain

To evaluate the selectivity and anatomical localization of [^{3}H]MePPEP binding sites, receptor autoradiography was employed using coronal brain sections from cannabinoid CB_1 receptor knockout and wild-type mouse controls. Binding to brain sections was performed in a Tris–HCl based buffer which differs slightly from the buffer used in the in vitro binding assays. Nonspecific binding was determined in the presence of 10 μM SR141716A. All autoradiographic binding studies were performed with a concentration of [^{3}H]MePPEP approximate to the determined K_d value (0.14 nM) since higher concentrations resulted in increased nonspecific binding (data not shown). Fig. 5 illustrates the representative specific and nonspecific [^{3}H]MePPEP binding profiles at three anatomical regions of wild-type and cannabinoid CB_1 receptor knockout mouse brains. Specific [^{3}H]MePPEP binding was detected throughout the brain, but highest levels of binding were localized to cerebral cortex, hippocampus, caudate putamen, and the cerebellum. No specific binding was detected in

Table 2
Pharmacology of [^{3}H]MePPEP binding in native brain tissue and in membranes expressing recombinant human cannabinoid CB_1 receptors.

Compound	hCB$_1$/CHO	Human cerebellum	NHP cerebellum	Rat cerebellum
SR141716A	8.6 ± 0.09	8.4 ± 0.09	8.4 ± 0.07	8.8 ± 0.05
MePPEP	9.7 ± 0.04	9.6 ± 0.10	9.6 ± 0.07	9.9 ± 0.06
MK-9470	9.5 ± 0.02	9.5 ± 0.05	9.5 ± 0.04	9.8 ± 0.01
JHU-75528	8.5 ± 0.05	8.3 ± 0.04	8.3 ± 0.08	9.0 ± 0.07
FEPEP	9.0 ± 0.09	8.9 ± 0.11	8.9 ± 0.03	9.2 ± 0.04
FMPEP	9.6 ± 0.07	9.5 ± 0.15	9.4 ± 0.08	9.7 ± 0.15
FMPEP-d2	9.7 ± 0.06	9.6 ± 0.08	9.6 ± 0.10	9.9 ± 0.08
FMePPEP	9.6 ± 0.03	9.4 ± 0.14	9.4 ± 0.06	9.6 ± 0.16
SR144528	6.9 ± 0.02	6.5 ± 0.11	6.6 ± 0.11	7.0 ± 0.05
CP-55,940	7.6 ± 0.14	7.8 ± 0.02	7.8 ± 0.15	7.8 ± 0.03
Methanandamide	5.5 ± 0.09	5.4 ± 0.08	5.5 ± 0.15	5.6 ± 0.14
WIN-55,212-2	5.8 ± 0.06	6.4 ± 0.01	6.2 ± 0.12	6.8 ± 0.01

Values expressed as pK_i.
Data shown are mean ± S.E.M. from three independent experiments. pK_i values were calculated for competition experiments by determining the negative logarithm of the inhibition constant (K_i in Molar units) determined in GraphPad Prism using the equation of Cheng and Prusoff (1973).

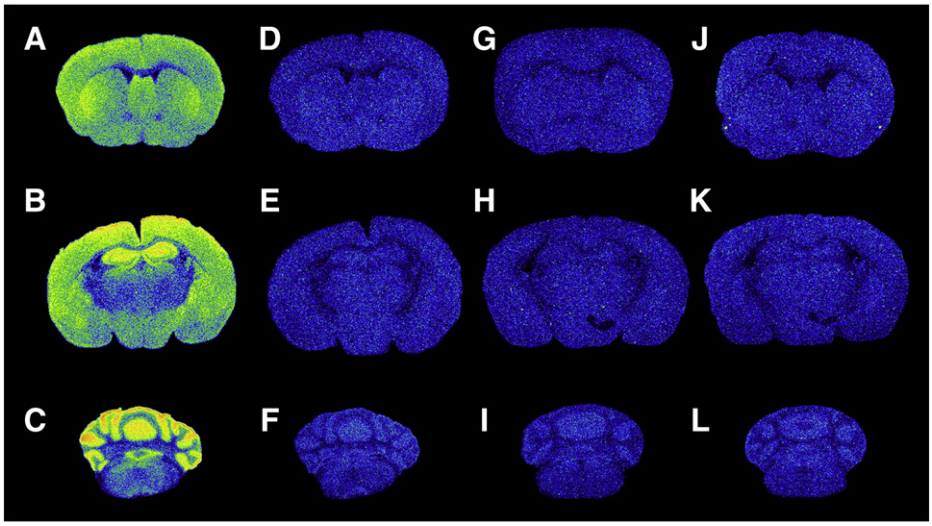

Fig. 5. Representative autoradiograms of [^{3}H]MePPEP total (A, B, C) and nonspecific (D, E, F) binding in wild-type mouse, and total (G, H, I) and nonspecific (J, K, L) binding in cannabinoid CB_1 receptor knockout mouse brains. Nonspecific binding was determined in the presence of 10 μM SR141716A. Areas of high binding appear in green, yellow and red pseudocolor. Images were digitized with MCID Elite software.

brain of cannabinoid CB_1 receptor knockout animals. [^{3}H]MePPEP binding in this report was not quantified. Qualitative observations of binding in knockout brains were similar to the binding in wild-type animals incubated in the presence of a saturating dose of the cannabinoid CB_1 receptor inverse agonist, SR141716A (10 μM).

To complement the rat brain in vitro binding in this study, [^{3}H]MePPEP binding in rat brain sections was also examined. Binding conditions in rat brain were identical to the mouse brain binding. Fig. 6 demonstrates [^{3}H]MePPEP binding to a representative sagittal section of rat brain. Similar to mouse, specific [^{3}H]MePPEP binding was detected throughout the rat brain with highest levels also in cerebral cortex, caudate putamen, hippocampus and cerebellum. Although not visible in the mouse brain coronal sections, the sagittal section of rat brain revealed dense levels of specific [^{3}H]MePPEP binding in the globus pallidus, dentate gyrus and substantia nigra (Fig. 6). No specific [^{3}H]MePPEP binding was detected in sections incubated with 10 μM SR141716A.

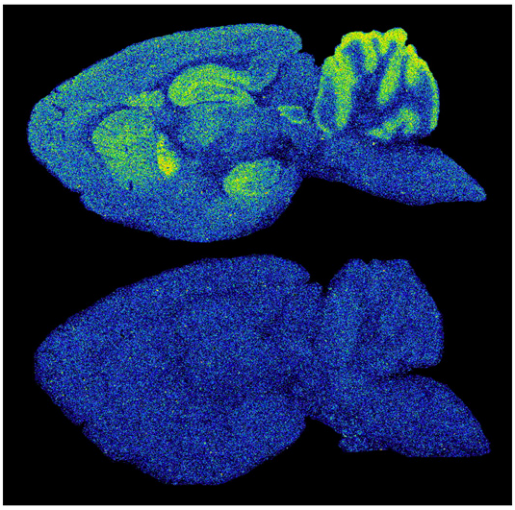

Fig. 6. Representative autoradiograms of total (top) and nonspecific (bottom) binding of [^{3}H]MePPEP to sagittal sections of rat brain. Nonspecific binding was determined in the presence of 10 μM SR141716A and was equal to film background (data not shown). Areas of high binding appear in green, yellow and red pseudocolor. Images were digitized with MCID Elite software.

4. Discussion

This report provides pharmacological characterization of the tritium-labeled radioligand, [^{3}H]MePPEP, which is a selective inverse agonist for the cannabinoid CB_1 receptor. Binding was performed with brain membrane samples derived from three species: rodent, non-human primate, and humans. Terry et al. (2008) demonstrated that [^{11}C]MePPEP selectively binds cannabinoid CB_1 receptors in mouse brain and is not a substrate for P-glycoprotein. Yasuno et al. (2008) reported that [^{11}C]MePPEP reversibly and selectively binds cannabinoid CB_1 receptors in brains of non-human primate, justifying use of this PET ligand in human subjects (Terry et al., 2009). In this study, the tritiated ortholog of [^{11}C]MePPEP, [^{3}H]MePPEP, demonstrates saturable and reversible binding to cannabinoid CB_1 receptors. [^{3}H]MePPEP binds a high affinity binding site with equal affinity at cannabinoid CB_1 receptors from rat, non-human primate and human brain, and at ectopically expressed human cannabinoid CB_1 receptors. [^{3}H]MePPEP binding is saturable, reversible and characterized as a single site. Cannabinoid agonists and antagonists compete [^{3}H]MePPEP with predictable rank order potency consistent with previously published reports. Despite its high lipophilicity, [^{3}H]MePPEP displays no specific binding in autoradiographic sections from cannabinoid CB_1 receptor knockout mice demonstrating this radioligand binds selectively to cannabinoid CB_1 receptors. [^{3}H]MePPEP binds similar anatomical structures in mouse and rat brain compared to previously published reports using other cannabinoid CB_1 receptor agonist and antagonist radioligands (Herkenham et al., 1990, 1991; and Rinaldi-Carmona et al., 1996). Consequently, [^{3}H]MePPEP binding in mouse and rat brain autoradiographic sections was similar to coronal views of rat, non-human primate and human brain labeled with the PET radioligand [^{11}C]MePPEP.

For rat, non-human primate and human studies, we used cerebellum as a source for cannabinoid CB_1 receptors. This tissue has a high density of cannabinoid CB_1 receptors, thus making it practical for study of cannabinoid CB_1 receptor pharmacology. MePPEP has been demonstrated to be an inverse agonist (Yasuno et al., 2008), thus capable of binding cannabinoid CB_1 receptors in active (R^*) and inactive (R) conformations, similar to [^{3}H]SR141716A (Kearn et al., 1999). The effect of sodium ions or guanine nucleotides on [^{3}H]MePPEP binding was not examined; however, the binding of another cannabinoid CB_1 receptor inverse agonist radioligand, [^{3}H]SR141716A, was unaffected by inclusion of these agents in the

binding buffers (Rinaldi-Carmona et al., 1996). In rat brain cerebella, the published number of cannabinoid CB_1 receptor binding sites labeled by [^{3}H]SR141716A compares favorably to the number of sites labeled by [^{3}H]MePPEP in this report (Petitet et al., 1996; Govaerts et al., 2004). [^{3}H]MePPEP binding constants were not significantly different at rat, non-human primate, human cannabinoid CB_1 receptors, and human cannabinoid CB_1 receptors ectopically expressed in CHO cells. However, the Bmax values were significantly different between species. It is possible that this observed difference is an artifact of tissue procurement and handling, rather than a true species difference, as the post-mortem interval, agonal score and other sample handling details are not known. The uniformity of binding constants across species and its relatively high affinity make [^{3}H]MePPEP an ideal tool for studying cannabinoid CB_1 receptor pharmacology. Moreover, the favorable pharmacology of radiolabeled MePPEP, both in vitro and in vivo will facilitate translational studies of the cannabinoid CB_1 receptor.

Competition studies with [^{3}H]MePPEP demonstrate that this radioligand can be competed with cannabinoid agonists and antagonists, and the affinities (K_i) for each compound were relatively similar between species. [^{3}H]MePPEP was selective for cannabinoid CB_1 receptors, as the cannabinoid CB_2 receptor antagonist, SR144528, had a greatly reduced potency to compete [^{3}H]MePPEP in receptor binding studies, and to reduce functional γ-^{35}SGTP binding at cannabinoid CB_2 receptors (Yasuno et al., 2008). The synthetic cannabinoid agonist, CP-55,940, was competitive and compares favorably with some previously published results in rat brain membranes with [^{3}H]SR141716A (Thomas et al., 1998; Govaerts et al., 2004), but differs from other reports using similar conditions (Rinaldi-Carmona et al., 1996; Griffin et al., 1999). The lower potency of CP-55,940 at MePPEP binding sites was also observed in [^{11}C]MePPEP PET studies using rodent brain (Terry et al., 2008). One plausible explanation is that CP-55,940 and MePPEP may not have overlapping binding sites at cannabinoid CB_1 receptors, consistent with other reports comparing inverse agonists and agonists (McAllister et al., 2003; Murphy and Kendall, 2003). Similar explanations can be offered for methanandamide and WIN-55,212-2, which are ineffective at competing for MePPEP binding sites both in vitro and in vivo (Terry et al., 2008).

The K_i of SR141716A for [^{3}H]MePPEP competition at cannabinoid CB_1 receptor binding sites was similar to the affinity of [^{3}H]SR141716A at cannabinoid CB_1 receptor binding sites in rat brain membranes (Thomas et al., 1998, 2005; Rinaldi-Carmona et al., 1996; Petitet et al., 1996), and human cannabinoid CB_1 receptor-transfected cells (Thomas et al., 2005). SR141716A readily competes with [^{11}C]MePPEP in rodent brain providing evidence that these two cannabinoid ligands bind cannabinoid CB_1 receptors competitively, albeit with different affinities. Competition for [^{3}H]MePPEP binding sites with MePPEP yielded affinities consistent with [^{3}H]MePPEP binding constants indicating the radiolabeled ligand has identical binding profiles as the unlabeled molecule.

The anatomical distribution of cannabinoid CB_1 receptors in rodent, non-human primate and human brain has been well documented, using radiolabeled cannabinoid CB_1 receptor agonists and antagonists (Herkenham et al., 1990, 1991; Rinaldi-Carmona et al., 1996; Burns et al., 2007). Our autoradiographic studies were not designed to provide a complete map of [^{3}H]MePPEP binding sites in mouse brain, but to test the selectivity of [^{3}H]MePPEP binding at cannabinoid CB_1 receptors. Coronal sections representing regions known to display high densities of cannabinoid CB_1 receptor binding sites were employed to evaluate [^{3}H]MePPEP binding efficiency and selectivity. As expected, [^{3}H]MePPEP efficiently bound cannabinoid CB_1 receptor-dense regions in wild-type mouse brain; however, [^{3}H]MePPEP binding to the same regions in cannabinoid CB_1 receptor deficient knockout mice was equivalent to wild-type mouse sections co-incubated with saturating concentrations of SR141716A, demonstrating that this ligand selectively labels cannabinoid CB_1 receptors in native tissue. These results are in agreement with a report by Terry et al. (2008) and support previously reported in vitro data for MePPEP indicating a lack of significant off target interactions in a broad screen of common cell surface targets and a lack of activity against rat fatty acid amide hydrolase (Yasuno et al., 2008; data not shown).

[^{3}H]MePPEP binding in sagittal sections of rat brain demonstrates that this ligand selectively labels cannabinoid CB_1 receptors similarly to [^{3}H]CP-55,940 and [^{3}H]SR141716A. Moreover, the anatomical binding profiles of [^{3}H]MePPEP in the rodent brain were similar to the imaging results achieved with the PET ligand [^{11}C]MePPEP in rat, non-human primate and human brain (Terry et al., 2008, 2009; Yasuno et al., 2008).

In conclusion, we have developed a tritium-labeled version of the cannabinoid CB_1 receptor PET ligand, MePPEP. We have demonstrated that this radioligand selectively and specifically binds cannabinoid CB_1 receptors in native tissue from rat, non-human primate and human, and ectopically expressed human cannabinoid CB_1 receptors. [^{3}H]MePPEP binding was saturable, reversible, selective, high affinity and characterized as a single cannabinoid CB_1 receptor binding site. The affinity constants across the species evaluated were similar, thus this radioligand is suitable for defining cannabinoid CB_1 receptor pharmacology in preclinical and clinical subjects. The binding to cannabinoid CB_1 receptors is competed with cannabinoid receptor agonists and antagonists with predictive rank order affinities. Despite its moderate lipophilicity, [^{3}H]MePPEP selectively binds cannabinoid CB_1 receptors in autoradiographic brain sections with low nonspecific binding. Therefore, [^{3}H]MePPEP is a useful tool to bridge preclinical and clinical studies focused on the cannabinoid CB_1 receptor pharmacology, distribution, and therapeutic drug discovery.

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