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Journal
The Journal of Comparative Neurology, 524(7)

ISSN
1550-7149

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Publication Date
2016-05-01

DOI
10.1002/cne.23919

Peer reviewed

HHS Public Access

Author manuscript

J Comp Neurol. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

J Comp Neurol. 2016 May 1; 524(7): 1457–1471. doi:10.1002/cne.23919.

Comparative assessment of ^{18}F-Mefway as a serotonin 5-HT$_{1A}$ receptor PET imaging agent across species-rodents, nonhuman primates, and humans¶

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Abstract

We have developed ^{18}F-*trans*-Mefway (^{18}F-Mefway) for PET imaging studies of serotonin 5-HT$_{1A}$ receptors which are implicated in various brain functions. Translation of imaging the 5-HT$_{1A}$ receptor in animal models to humans will facilitate an understanding of the role of the receptor in human brain disorders. We report comparative brain distribution of ^{18}F-Mefway in normal mice, rats, monkeys and healthy human volunteers. Mefway was found to be very selective with subnanomolar affinity for the serotonin 5-HT$_{1A}$ receptor. Affinities of >55 nM were found for all other human-cloned receptor subtypes tested. Mefway was found to be a poor substrate (>30 μM) for the multidrug resistance 1 protein, suggesting low likelihood of brain uptake being affected by P-glycoprotein. Cerebellum was used as a reference region in all imaging studies across all species due to the low levels of ^{18}F-Mefway binding. Consistent binding of ^{18}F-Mefway in cortical regions, hippocampus and raphe was observed across all species. ^{18}F-Mefway in the human brain regions correlated with the known postmortem distribution of 5-HT$_{1A}$ receptors. Quantitation of raphe was affected by the resolution of the PET scanners in the rodents, while monkeys and humans showed a raphe to cerebellum ratio approximately 3. ^{18}F-Mefway appears to be an effective serotonin 5-HT$_{1A}$ receptor imaging agent in all models including humans. ^{18}F-

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Associate Editor: Dr. Kathleen Rockland

¶Presented in part at the 61th Annual Meeting of Society of Nuclear Medicine, St. Louis, MO, June 7–11, 2014.

Conflict of interest statement.

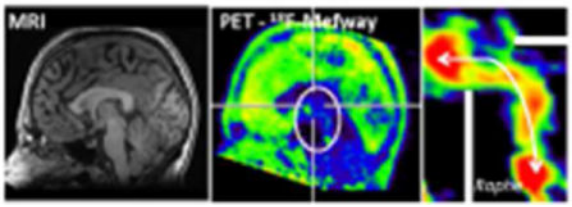
The authors have no conflict of interest in the work reported here.

Role of authors.

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: JM, BTC. Acquisition of data: MLP, NS, SKP, DWW, ATH, AKB. Analysis and interpretation of data: MLP, NS, DWW, ATH, SKP, AKB. Drafting of the manuscript: JM, AKB. Statistical analysis: JM, BTC, DWW, ATH, MLP, AKB. Obtained funding: JM, BTC. Administrative, technical, and material support: AKB Study supervision: JM, BTC.

Mefway therefore may be used to quantify serotonin 5-HT_{1A} receptor distribution in brain regions for the study of various CNS disorders.

Graphical abstract



Keywords

Raphe nucleus; Hippocampus; Translational research; Receptor selectivity; P-Glycoprotein

1. INTRODUCTION

Serotonin 5-HT_{1A} receptor, a G protein coupled receptor (GPCR), has been implicated in several neurological and psychiatric disorders (Popova and Naumenko, 2013) and is therefore targeted for in vivo imaging studies (Hesselgrave and Parsey, 2013; Billard et al., 2014). Translation of the use of radiopharmaceuticals across various species leading up to human studies is an important goal in positron emission tomography (PET) research. Studies of the 5-HT_{1A} receptor in humans have been carried out with several PET agents, most common of which include ^{11}C-WAY 100635, ^{18}F-FCWAY and ^{18}F-MPPF (Paterson et al., 2013). PET has benefitted over the last two decades with the development of high resolution scanners that are being used for imaging studies of various species including mice, rats, rabbits, pigs, monkeys and humans. In parallel, there is a significant effort to develop PET imaging agents for use in the various species. This enables research in animal models, findings of which can then be applied in human research and clinical studies. Several animal and human studies using these agents have pointed to a role of 5-HT_{1A} receptor in depression (Parsey, 2010), epilepsy (Theodore et al., 2012), Alzheimer's disease (Kepe et al., 2006; Truchot et al., 2008) and other brain functions.

Important issues in translational research include the following: (1). Similarity (homology) of the biomarker (serotonin 5-HT_{1A} receptor) across different species. (2). Ability of imaging agent (radiopharmaceutical) to withstand molecular, cellular and physiological differences between species. Distribution of the 5-HT_{1A} receptor in the various brain regions across the different species is very similar (Duncan et al., 1998; Hall et al., 1997). Serotonergic projections to the cortical areas arise from the small mid-brain raphe nuclei, including dorsal raphe (DR) across all the species (Burnet et al., 1997; Khawaja, 1995). Moderate levels of the 5-HT_{1A} receptor are found in the DR and higher levels of the receptor are found in the hippocampus and temporal cortex. Homology of the 5-HT_{1A} receptor across the various species is very high (>84%; Albert et al., 1990; Charest et al., 1993; www.uniprot.org), suggesting a high degree of similarity in receptor-ligand interaction. These are promising attributes of the 5-HT_{1A} receptor for translational research

across different species. The challenge is now on the radiopharmaceutical to provide quantitative noninvasive imaging measures of the 5-HT_{1A} receptor across different species taking into account the imaging capabilities and resolution of scanners for a mouse (~25 g), rat (~250 g), monkey (~10,000 g) and human (~70,000g).

WAY-100635, labeled with ^{11}C-carbon (Fig-1a) has been the mainstay of imaging 5-HT_{1A} receptor (Pike et al., 1996; Stein et al., 2008). For several reasons discussed in Saigal et al., 2006, such as the challenging radiosynthesis of ^{11}C-WAY 100635, cranial uptake of ^{18}F-fluoride of ^{18}F-FCWAY and moderate affinity of ^{18}F-MPPF, efforts to improve the biological properties of currently used 5-HT_{1A} agents for human studies have been made (e.g., Hussainy et al., 2012; Kumar and Mann, 2014). Our goal was to develop Mefway (Saigal et al., 2006), as a fluorinated radiotracer for imaging 5-HT_{1A} receptors that would be relatively more stable to metabolism, easily synthesized, and would retain high affinity and selectivity for the 5-HT_{1A} receptors. Thus, fluorine-18 radiolabeled *trans*-^{18}F-Mefway (Fig-1b; heretofore referred as Mefway) was characterized for imaging 5-HT_{1A} receptors (Wooten et al., 2011a).

One of the major aims of this report is to compare previously reported biological properties of ^{18}F-Mefway reported in our studies of rodents (Saigal et al., 2006, 2013), monkeys (Saigal et al., 2006; Wooten et al., 2011b, 2012) and humans (Hillmer et al., 2014) in order to assess “the similarity of binding of ^{18}F-Mefway across the different species”. To further ascertain the usefulness of ^{18}F-Mefway imaging of 5-HT_{1A} receptors in humans, in vitro receptor selectivity of Mefway was carried out in human cloned receptors, substrate selectivity for the multi-drug resistance-1 (MDR-1) protein was evaluated, toxicology of Mefway was carried out in rats, and in vitro binding in the canine model were assessed and compared to findings in humans. Correlation of in vivo binding parameters will be very valuable in conducting rodent and monkey studies for translating to human studies.

2. METHODS

2.1. General Methods

All chemicals and solvents were of analytical or HPLC grade from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Hanover Park, IL). ^{18}F-fluoride ion was produced in the MC-17 cyclotron or a RDS 112 cyclotron or a GE PET Trace cyclotron using oxygen-18 enriched water (^{18}O to ^{18}F using p, n reaction). The Mefway tosylate precursor (>95% purity; *trans*-*N*-{2-[4-(2′-methoxyphenyl)piperazinyl]ethyl}-*N*-(2-pyridyl)-*N*(4′-toluenesulfonyloxymethylcyclohexane)-carboxamide) was prepared in house using reported methods (Saigal et al., 2006; Thio et al., 2015) or purchased from Isoflex, San Francisco, CA. ^{18}F-Fluoride radioactivity was counted in a Capintec CRC-15R dose calibrator. Brain slices were prepared using a Leica 1850 cryotome. The apposed phosphor screens were read and analyzed by OptiQuant acquisition and analysis program of the Cyclone Storage Phosphor System (Packard Instruments Co., Boston, MA). Beagle dog brain unfixed tissue (age 4.5 to 15.4 yrs, brains immediately frozen and stored at −80 °C after euthanasia) and postmortem human brain unfixed tissue (postmortem interval between 3.8 to 5.8hr, brain tissue stored at −80 °C) were obtained from University of California Irvine Alzheimer’s Disease Research Center (UCI ADRC) and stored at −80 °C. Postmortem human brain

studies were approved by the Institutional Biosafety Committee of University of California, Irvine. All rodent studies were approved by the Institutional Animal Care and Use Committee (IACUC) of University of California, Irvine. Monkey studies were approved by IACUC of Wright State University, Dayton, Ohio and by the IACUC of University of Wisconsin, Madison. Human studies were carried out at University of Wisconsin, Madison under an approved Food and Drug Administration Investigational New Drug application for ^{18}F-Mefway and with Institutional Review Board approval from University of Wisconsin, Madison.

2.2. Radiopharmaceutical

The radiosynthesis of ^{18}F-Mefway was performed using nucleophilic displacement of the tosylate precursor by ^{18}F-fluoride in an automated CPCU using previously described procedures for the various species (Saigal et al., 2006; 2013; Wooten et al., 2011b; Hillmer et al., 2014). The final formulation of ^{18}F-Mefway was carried out using sterile saline (0.9% NaCl injection, United States Pharmacopeia) followed by sterile filtration through a membrane filter (0.22 μm) into a sterile dose vial for use in the PET studies. Radiochemical purity of ^{18}F-Mefway was >98% and chemical purity was found to be >95% with a measured specific activity >74 GBq/μmol at the end of synthesis.

2.3. Receptor Binding Assays

In vitro affinity studies of Mefway to the serotonin 5-HT$_{1A}$ receptors in rat brain slices (Saigal et al., 2006) and rat brain homogenate assays (Saigal et al., 2013) were previously reported. Binding affinity measurements of Mefway was carried out by the National Institutes of Mental Health (NIMH) psychoactive drug screening program (PDSP) for the following targets: serotonin receptor subtypes: 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_{3}$, 5-HT$_{5A}$, 5-HT$_{6}$, 5-HT$_{7}$; dopamine receptor subtypes: D$_1$, D$_2$, D$_3$, D$_4$, D$_5$; adrenergic receptor subtypes: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, β_3; histaminergic receptor subtypes: H$_1$, H$_2$, H$_3$, H$_4$; muscarinic receptor subtypes: M$_1$, M$_2$, M$_3$, M$_4$, M$_5$; sigma receptors subtypes: σ_1, σ_2; monoamine transporters: serotonin (SERT), dopamine (DAT), norepinephrine (NET); other targets included: opiate receptors (delta, kappa, and mu), GABA-A, and peripheral benzodiazepine receptor. Data represent mean % inhibition (N = 4 determinations) for compound tested at receptor subtypes. Significant inhibition was considered > 50% at a 10 μM Mefway concentration at the different receptor sites. More details of the methods for the individual assays are available at the PDSP website (www.pdsp.med.unc.edu). Mefway was also tested in the multidrug resistance (MDR1) assay in order to make an assessment if it is a potential substrate for P-glycoprotein.

2.4 Autoradiographic Studies

Wild type mice (male BALB\c, 25 g) and male Sprague Dawley rats (250–300g; Charles River, Wilmington, MA; RRID:RGD_737891), were anesthetized and decapitated; the brain was rapidly removed and frozen in isopentane at −20 °C. Horizontal slices were cut at 10 μm thickness. Slices contained regions known to have 5-HT$_{1A}$ receptors which included the hippocampus, frontal cortex and dorsal raphe and cerebellum. Hemi coronal slices of beagle dog brains were cut at 10 μm thickness. Dog brain slices contained the hippocampus and cortex. Human postmortem brain tissue from the hippocampus was cut in 7 μm thick

sections. Brain slices were preincubated in 50 mM Tris-HCl buffer (pH 7.4) for 10 min at room temperature. The slices were then incubated with 37–185 kBq/mL of ^{18}F-Mefway at 37 °C for 1hr. Nonspecific binding was measured in the presence of 10 μM of WAY-100635. After incubation, slides were washed twice (each wash lasting one minute) with ice-cold buffer. Slides were then quickly dipped in cold deionized water, air dried, and exposed to a phosphor screen multisensitive, medium MS (Perkin Elmer, Waltham, MA) for 24hr. The autoradiographs were generated using the Phosphor Cyclone Imager. The amount of binding was evaluated in digital light units (DLU/mm^2) using OptiQuant acquisition and analysis program (Packard Instruments Co.) described previously (Saigal et al., 2013).

2.5 Toxicity Studies

The expanded acute toxicity study of Mefway was carried out by Stanford Research Laboratories (SRI International, Menlo Park, CA). Sprague-Dawley rats, 40 males and 40 females, age 8–9 weeks (243–283 g males; 175–202 g females). Intravenous injections of Mefway were administered on Days 1, 3 and 6 at 4, 20 and 40 μg/kg/day (27.8, 139 and 278 μg/m^2/day). Clinical observations included altered clinical signs including motor and behavioral activities, changes in body weight, food consumption, ophthalmologic examination, clinical pathology on blood samples, hematology parameters, serum chemistry and urinalysis. After euthanasia, histopathologic examination of organ tissues were processed and evaluated.

2.6 PET studies

Whole body mouse (BALB\c 25–30 g, n=6) ^{18}F-Mefway PET/CT scans (Constantinescu et al., 2012) and rat (Sprague-Dawley 250–400 g, n=6) upper body (Saigal et al., 2013) were acquired on the preclinical Inveon PET/CT scanner (Siemens Medical Solutions, Knoxville, TN) with a transaxial full width at half maximum (FWHM) of 1.46 mm, and axial FWHM of 1.15 mm (Constantinescu et al., 2009). Mice and rats were anesthetized with 4% isoflurane and positioned in the PET/CT scanner and maintained on isoflurane (2% for mice and 2.5% for rats and placed on a warm water circulating heating pad set at 35 °C) for the duration of the PET scan. PET data acquisition was followed by a CT scan for attenuation correction and anatomical delineation of mouse and rat PET images. Dynamic PET data was acquired with a bolus injection of ^{18}F-Mefway (approximately 9.25 MBq for mice and 24 MBq for rats, specific activity > 74 TBq/mmol) for up to 120 minutes. Images were analyzed using ASIPro VM (Concorde Microsystems Inc., Knoxville, TN) and Pixelwise Modeling Software (PMOD 3.0; PMOD Technologies Ltd, Zurich, Switzerland).

Male rhesus monkey (Macaca mulatta 8–10 kg, n=6) PET studies were carried out either on Siemens ECAT EXACT HR+ PET scanner (Saigal et al., 2006) or in the Concorde P4 MicroPET scanner (Wooten et al., 2011b) with in-plane FWHM of 4.6 mm for EXACT HR + and in plane spatial resolution of 1.8 mm for the P4 scanner. The monkeys were anesthetized using either ketamine (10 mg/kg, IM) and xylazine (0.5 mg/kg, IM) or ketamine (10 mg/kg, IM) and atropine sulfate (0.27 mg, IM) and were then maintained on isoflurane (1–2%) for the duration of the PET scan. PET data acquisition was initiated with a bolus injection of approximately 48 to 111 MBq of ^{18}F-Mefway, and data were acquired for at least 120 minutes. Areas showing maximal radioligand binding in the frontal cortex,

temporal cortex, dorsal raphe and other brain regions were delineated in the images. The PET images were coregistered with an MRI image template of the rhesus brain as reported previously (Wooten et al., 2011b).

^{18}F-Mefway human PET data were acquired on a Siemens ECAT EXACT HR+ PET scanner using 3-D mode in-plane FWHM of 4.6 mm (Hillmer et al., 2014). Dynamic PET data acquisition was initiated with a bolus injection of approximately 185 MBq ^{18}F-Mefway, and data were acquired for 120 minutes. There were no adverse or clinically detectable pharmacologic effects, including no significant changes to vital signs or laboratory results, in any of the 6 subjects. MRI data were acquired on a GE 3.0 T MR750 (Waukesha, WI) for co registration with PET (Hillmer et al., 2014).

2.7 Image analysis

For rodents, using PMOD software package, PET images of rats were normalized to the standard space described by the stereotaxic coordinates (Paxinos and Watson, 2006) via co registration to an MRI rat template (Schweinhardt et al., 2003). Mouse PET images, summed over 20–60 min interval, were co-registered to a MRI mouse brain template (Ma et al., 2005) of size 192 × 96 × 256 voxels with a voxel size of 2 mm, which was preliminarily scaled by a factor of 20. The placing of the volume of interests (VOIs) was guided by examination of the Paxinos and Watson rat atlas. All VOIs were copied to the PET images and time activity curves (TACs) were extracted for each VOI from the dynamic PET data. No additional partial volume correction was applied. Kinetic analysis of rat and mouse *in vivo* PET studies was performed using kinetic analysis toolbox in PMOD. Distribution volume ratio (DVR) in each selected brain region was calculated for using Logan non-invasive method (Logan et al., 1996). Nondisplaceable binding potential (BP_{ND}; Innis et al., 2007) was calculated as “DVR-1” (further details described in Saigal et al., 2013).

For monkeys, raw list mode data from all scans were summed into frames of 4×1 minute, 3×2 minutes, and either 16×5 minutes) with corrections applied for scanner dead time and random coincidence events. Sinograms of the emission scan were reconstructed using filtered back-projection (0.5 cm^{-1} ramp filter) with corrections to account for attenuation, scatter, radioactive decay, and scanner normalization to a final matrix size of 128 × 128 × 63 and voxel dimensions of 1.90 × 1.90 × 1.21 mm^3. The time series were realigned into common space for each subject using the FSL software to obtain the rigid body transformation matrix obtained from the co registration of the summed data over the entire study. Circular regions of interest (ROIs) were drawn in various regions of the brain to extract time-activity curves of the radiotracer in the tissue, which included the brain regions of the cerebellum, raphe nuclei, hippocampus and mesial temporal lobe (MTL) and BP_{ND} were obtained (further details described in Wooten et al., 2011b).

For humans, PET data were histogrammed into frames of 8 × 0.5 minutes, 3 × 2 minutes, 10 × 5 minutes, and 6 × 10 minutes. Sinogram data were then reconstructed with a filtered back projection algorithm (Direct Inverse Fourier Transformation; DIFT) using a 4 mm gaussian filter and included corrections for random events, dead time, signal attenuation, and scanner normalization. Regions of interest were defined with FreeSurfer 5.3 software (http://surfer.nmr.mgh.harvard.edu). The raphe nuclei region was manually drawn for each PET

scan since this region's structure could not be accurately determined based on the MRI data. Time activity curves were extracted from all regions for subsequent analysis. To quantify specific ^{18}F-Mefway binding, binding potential non-displaceable (BP_{ND}) was measured (Further details described in Hillmer et al., 2014).

To compare measured cerebral time activity curves in the four species, standardized uptake value (SUV) was calculated as SUV = PET/[ID/weight], where PET is the measured PET concentration (kBq/cc), ID is the injected dose (MBq), and weight is subject weight (kg). For all imaging studies, cerebellum served as an appropriate reference region since it is largely devoid of 5-HT_{1A} receptors.

3. RESULTS

3.1 ^{18}F-Mefway Design

Mefway was designed to be structurally similar to WAY-100635, containing only an additional fluoromethyl group on the cyclohexyl ring (Fig-1B) so that a more stable fluorine-18 may be incorporated. Inclusion of this fluoromethyl group resulted in the formation of two-isomers (*cis*- and *trans*-) at a given position. The *cis*-isomer of Mefway exhibited lower binding compared to *trans*-isomer (Wooten et al., 2011a). This was similar to previous observations for other WAY-analogs (Lang et al., 2006; Wilson et al., 1999). Additionally, the possibility of repositioning the fluoromethyl group at different carbons was explored in new series of Mefway analogs (Thio et al., 2015). The 3-Mefway analog exhibited lower in vivo binding properties compared to the 4-Mefway reported here (Wooten et al., 2014). The 2-Mefway analogs exhibited higher affinities, similar to 4-Mefway and may be promising in vivo (Thio et al., 2015). Thus, of the various Mefway analogs prepared thus far, the *trans*-4-Mefway reported here appears to be most promising. The radiosynthesis of ^{18}F-Mefway was a single step reaction of the tosylate in acetonitrile with ^{18}F-fluoride and automated.

3.2 In Vitro Binding Selectivity

Figure-2 shows binding affinity values of *trans*-Mefway for human serotonin receptor subtypes 5-HT_{1A} (0.9 nM compared to 0.64 nM for reference 8-OH-DPAT; Fig-2A), 5-HT_7 (297 nM compared to 40 nM for reference clozapine; Fig-2B) and 5-HT_{2B} (260 nM compared to 34 nM for reference SB206553; Fig-2C). Other serotonin receptor subtypes did not show significant affinities for Mefway.

For adrenergic receptor subtypes, Mefway exhibited affinities for adrenergic α_{1A} (70 nM compared to 0.44 nM for reference prazosin; Fig-2D) and α_{1D} (57 nM compared to 0.34 nM for reference prazosin; Fig-2E). Other adrenergic receptor subtypes exhibited poor affinities for Mefway.

Mefway exhibited very poor affinities for D_1-like receptors (D_1 and D_5 subtypes). In the case of D_2-like receptors, for D_3 subtype it had an affinity of 139 nM compared to 6 nM for reference chlorpromazine and for D_2 subtype, Mefway had an affinity of 188 nM compared to 3.2 nM for reference haloperidol. For dopamine receptor subtype D_4 subtype, Mefway had an affinity of 126 nM compared to 12 nM for reference chlorpromazine (Fig-2F).

For all other receptor subtypes tested in section 2.3, Mefway did not exhibit significant affinity at 10 μM concentration.

3.3 In Vitro Autoradiographic studies

3.3.1 Hippocampus—Hippocampus showed the highest amount of binding across the different species. Using cerebellum as the reference region, a ratio of hippocampus to cerebellum was 25 in mice (Fig-3A) and in the rats the ratio was 30 (Fig-3B). All ratios were computed in brain slices incubated for one hr.. In the case of the dog brains, hippocampus exhibited the highest binding consistent with the rodents (Fig-3D). A ratio of hippocampus to white matter within the same brain slice (Fig-3C) in the dog brain was found to be approximately >50, while in the human hippocampus sections the ratio versus white matter was 13, which was similar to the rodents (Fig-3F). Thus all species, including human hippocampus sections exhibited distinct ^{18}F-Mefway binding at comparable levels.

3.3.2 *Raphe*—Mice and rat brain horizontal sections allowed visualization of the raphe. Using cerebellum as the reference region, a ratio of ^{18}F-Mefway in the raphe to cerebellum was 22 in mice (Fig-3A) and in the rats the ratio was 23 (Fig-3B). The level of ^{18}F-Mefway binding in this region was high compared to that found in the hippocampus.

3.3.3 *Cortex*—Cortical regions known to be less densely packed with 5-HT$_{1A}$ receptors, gave ^{18}F-Mefway ratios of >10 in mice and rats (Fig-3). Binding of ^{18}F-Mefway in the various cortical layers were observed and were in agreement with reported 5-HT$_{1A}$ distribution (Eickhoff et al., 2007). Figure-3G and I shows the cortical layers in the dog brain with layers I and II (100%) showing the highest levels of ^{18}F-Mefway binding, followed by layers V and VI (36%) and at and layers III and IV at 31% similar to that found in humans (Burnett et al., 1997). Similar cortical layer distribution of ^{18}F-Mefway was seen in the rat cortex (Saigal et al., 2013). Binding of ^{18}F-Mefway was displaced (>95% in hippocampus and cortex) in the presence of 10 μM of WAY-100635 in all the species, suggesting binding of ^{18}F-Mefway to areas rich in the 5-HT$_{1A}$ receptor.

3.4. Acute Toxicity

There were no morbidity or mortality observed in any animals treated with the vehicle control or Mefway, and all animals survived to their scheduled necropsy with minimal clinical observations. The absence of findings in in-life evaluations of body weight, ophthalmology, clinical pathology, and organ weight parameters and histopathology do not support a toxicological consequence. Microscopic evaluation of tissues presented no findings associated with Mefway treatment. Intravenous injections of Mefway administered on Days 1, 3 and 6 at 4, 20 and 40 μg/kg/day (27.8, 139 and 278 μg/m^2/day) were well tolerated in Sprague-Dawley male and female rats. Histopathology evaluation on Days 7 and 20 presented no dose-related findings from treatment of Mefway. Based on these finding the maximum tolerated dose (MTD) and the no observed adverse effect level (NOAEL) are estimated to exceed 40 μg/kg/day (278 μg/m^2/day), the maximum dose level tested in this study. Specific activities of ^{18}F-Mefway have exceeded 74 GBq/μmol, which for a 185 MBq human dose injection amounts to a Mefway mass of less than 1.14 μg and well below the MTD and NOAEL for Mefway. The mass injected in our human studies with ^{18}F-Mefway

was ~0.14 μg (Hillmer et al., 2014) while it was ~1.07 μg in another recently reported ^{18}F-Mefway human study (Choi et al., 2015).

3.5. PET Studies

3.5.1. Hippocampus—^{18}F-Mefway showed rapid uptake in all brain regions including the hippocampus in all the species (Saigal et al., 2013; Wooten et al., 2011a, b; Hillmer et al., 2014). Faster clearance from cerebellum was observed and this was used as a reference region in all studies. Figure-4 illustrates time-activity curves of ^{18}F-Mefway binding in the hippocampus and cerebellum of the four species.

Distribution of ^{18}F-Mefway in the rodent hippocampus was consistent with the observations in the in vitro brain slices (Fig-5B, D). As seen in Fig. 5D, ^{18}F-Mefway binding in the mouse and rat MicroPET images reflected some extracranial uptake in rats possibly due to faster metabolism. The cerebellar time-activity curves of both mice and rats (Fig-4) may reflect some “spill-over” of radioactivity during later time points due to the upward trend in the curves. In the case of monkeys, hippocampus was visualized both in the HR+ scanner (Fig-5G; resolution of 4.6 mm) and in the P4 scanner (Fig-5F; resolution of 1.8 mm). No cranial uptake was observed in the monkey PET scans with ^{18}F-Mefway, suggesting little defluorination. Human hippocampus exhibited high binding of ^{18}F-Mefway with some retention occurring in the cranium (Fig-5I). Ratio of hippocampus to cerebellum at 90 mins post-injection was as follows: mouse = 3; rat = 10; monkeys = 10 and humans = 4.

3.5.2. *Raphe*—Uptake of ^{18}F-Mefway in the raphe was clearly evident in the humans and monkeys. Using the EXACT HR+ PET scanner, the raphe exhibited ratios with respect to cerebellum of approximately 2 in humans (Fig-5E) and monkeys (Fig-5C) (Hillmer et al., 2014; Saigal et al., 2006). However, with the higher resolution P4 scanner, the monkey raphe showed higher ratios as seen in Fig-5G (greater than 3, Wooten et al., 2011b). Delineation of raphe in the mouse brain in vivo was difficult due to its small size (Fig-5B), whereas the rat raphe was somewhat better visualized (Fig-5D). Of the four species, the monkey raphe provided the highest ratios with respect to the cerebellum, with $BP_{ND}>3$ (Wooten et al., 2011b).

3.5.3. *Cortex*—Significant ^{18}F-Mefway binding was measured in temporal cortex, cingulate gyrus, frontal cortex and occipital cortex in the various species. Entorhinal cortex exhibited a high degree of ^{18}F-Mefway binding across the species. Similar high binding was observed in the anterior cingulate gyrus. The small size of the rodent brains and the cranial uptake seen in Fig-5B, D made regional cortical analysis difficult (Saigal et al., 2013). Ratios between 4 and 7 were found for temporal cortex, frontal cortex, occipital cortex and anterior cingulate in the monkeys. The monkey cortical regions showed the highest BP_{ND} values for ^{18}F-Mefway.

4. DISCUSSION

Advances of PET imaging to clinical practice require well-characterized PET radiotracers, easily available, selective targeting, lack of toxicity, suitable dosimetry, easily quantifiable

and available for large multi-center trials (Coenen et al., 2010; Parsey, 2010). Across the various species, the promise of ^{18}F-Mefway to meet these requirements is very high.

Overall selectivity of Mefway was found to be superior to WAY-100635 for the human receptor clones as summarized in Table-1. Mefway had subnanomolar affinity for rat and human 5-HT$_{1A}$ subtype. It exhibited weak affinity for two other serotonin receptor subytpes, 5-HT$_7$ and 5-HT$_{2B}$. WAY-100635 on the other hand exhibited a 10-fold higher affinity for 5-HT$_{2B}$, compared to Mefway. For the adrenergic receptors Mefway had weak affinities for the various subytpes which was an improvement compared to WAY-100635 which had a 10-fold higher affinity for the α_{1D} subtype compared to Mefway. Mefway exhibited weak affinity for the dopamine D$_4$ receptor subtype compared to WAY-100635. Thus the small change in structure by inclusion of a fluoromethyl group in WAY-100635 has resulted in higher selectivity for the 5-HT$_{1A}$ subtype. This suggests that in vivo imaging using ^{18}F-Mefway in humans is most likely reflective of 5-HT$_{1A}$ receptor subtype in the different brain regions.

The PET agents commonly used in human studies for the 5-HT$_{1A}$ receptor system are ^{11}C-WAY-100635, ^{18}F-FCWAY and ^{18}F-MPPF. The PDSP data base indicates WAY-100635 has safely been used up to 700 μg/kg daily dose of WAY-100635 in human and a 14-Day Toxicity Study of FCWAY in rabbits with intravenous administration of FCWAY at doses of 0.15 and 0.3 mg/kg/day for 14 consecutive days did not result in treatment-related histologic lesions. The no-effect level for the study was 0.3 mg of FCWAY/kg body weight/day (corresponding to human equivalent dose (HED) = 97 μg/kg). The calculated Mefway HED based on our rat toxicity results, HED (μg/kg) = 7.6 μg/Kg [40 μg/kg (Animal dose) × (6.95 (Animal K*m*)/37 (Human K*m*)]. Based on the findings of WAY 100635 and FCWAY, the HED for Mefway NOAEL is expected to be significantly higher.

^{18}F-Mefway binding to brain tissue of various species was similar and in agreement with the inter-species homology of the 5-HT$_{1A}$ receptors (Table-2). The binding affinity of Mefway was similar in rat and human (Table-1). Hippocampus across the various species exhibited a high level of binding of ^{18}F-Mefway. In vitro studies were able to decipher the different concentrations of ^{18}F-Mefway in the cortical layers. This was consistent with previous autoradiographic studies of this receptor using ^{3}H-WAY-100635 and ^{3}H-8-OH-DPAT.

Uptake of ^{18}F-Mefway was observed in brain regions across all species. Ratio of brain regions versus cerebellum reached a plateau approximately 90 min post-injection in the various species suggestive of pseudo-equilibrium in vivo. Thus, based on the kinetics of ^{18}F-Mefway, a scan time of 90 minutes may be appropriate across the various species in order to derive reliable quantitative data.

The human cerebellum kinetics of ^{18}F-Mefway was very similar to the kinetics observed for ^{11}C-WAY-100635 (Fig-6C). The cerebellar lobes (Fig-6A, B) had the least amount of activity, although the midline region consisting of the vermis is known to be innervated, similar to our findings in human ^{18}F-fallypride studies (Mukherjee et al., 2002). No uptake of radioactivity was seen in the skull of the monkey suggestive of little defluorination of ^{18}F-Mefway in the PET study (Saigal et al., 2006; Wooten et al., 2011b). A significant

amount of unmetabolized ^{18}F-Mefway was found in the blood in both monkeys and humans at 90 mins post-injection. A free fraction of 15% in monkeys (Wooten et al., 2013) and 5% in humans (Hillmer et al., 2014) was measured (Table-2). The plasma free fraction of ^{18}F-Mefway in humans was similar to that reported for ^{11}C-WAY 100635 (5.8%; Parsey et al., 2000) but lower than ^{18}F-FCWAY (13%; Ryu et al., 2007). The difference in free fraction is most likely due to differences in protein binding across the species and between radiotracers.

One of the metabolites in this class of compounds is the breakdown of the amide bond as seen in Fig-6D, E. Our findings in the rat study with the metabolite ^{18}F-FMCHA showed poor penetration and retention of this metabolite (Saigal et al., 2013). Liver enzyme inhibitors have been used to increase in vivo stability of ^{18}F-FCWAY, resulting in greater uptake of the radiotracer in the brain. In the case of ^{18}F-Mefway using these inhibitors, although increased uptake in the rodent brain was observed, it did not significantly affect the measured BP_{ND} values since uptake in the reference region, cerebellum was similarly affected (Choi et al., 2012; Saigal et al., 2013). However, as can be noted in the time-activity curve of the cerebellum of mouse and rat in Fig-4, the slight upward trend at the end of the scan (absent in the case of the monkeys and humans) is perhaps reflective of some spillover of activity from the skull. In humans, disulfiram decreased plasma clearance of ^{18}F-FCWAY by 47% compared to baseline, while the free fraction of ^{18}F-FCWAY remained at ~13% similar to baseline study without disulfiram (Ryu et al., 2007). In the case of ^{18}F-Mefway in humans, although there appears to be some scalp uptake, defluorination may not be as big an issue (Hillmer et al., 2014) as compared to ^{18}F-FCWAY (Ryu et al., 2007; Choi et al., 2015a).

Efflux of radiotracers from the brain by the multidrug resistance 1 protein has been a concern in PET imaging studies of the 5-HT_{1A} receptor. WAY-100635 and MPPF have been reported as not being suitable substrates for human P-glycoprotein (Tournier et al., 2011). In rodents and nonhuman primates however, tariquidar induced inhibition of P-glycoprotein has been reported to increase brain uptake of ^{18}F-MPPF (La Fougere et al., 2010). Compared to the reference compound cyclosporine A (IC_{50} = 0.88 μM), Mefway was found to be a weaker substrate (IC_{50}>30 μM; Fig-6F). Recent studies of ^{18}F-Mefway in rats pretreated with the P-glycoprotein inhibitor tariquidar and in MDR1 knock-out mice exhibited an increase in brain uptake but binding potentials were not statistically significant compared to control animals (Choi et al., 2015b). Additional studies will be needed to further ascertain the effect of P-glycoprotein on human ^{18}F-Mefway binding in vivo.

^{18}F-Mefway brain uptake went down from 5% ID/cc in mice to 0.002% ID/cc in humans (Table-2). The uptake in humans was similar to that observed for ^{11}C-WAY 100635 (Pike et al., 1996; Farde et al., 1998). Hippocampus to cerebellum ratios and the measured BP_{ND} values in the hippocampus showed some variability across the different species (Table-2). Measurements in mice may be affected due to the small brain size and partial volume effects, causing the underestimation. This partial volume effect may be less in the case of the human brain. Hippocampus in the monkey brains provided more consistent measures (Wooten et al., 2011b; Christian et al., 2013). Small age- and gender effects on the human 5-HT_{1A} receptor have been previously reported (Costes et al., 2005; Stein et al., 2008). Our preliminary study on a small group of male and female monkeys suggest that the females

tend to have a higher BP_{ND} which may be due to lower a lower equilibrium dissociation constant, K_{Dapp} of ^{18}F-Mefway in female monkeys (Wooten et al., 2013). In the case of human ^{18}F-Mefway, a larger study will be required to ascertain such age and gender effects.

It must be noted that the mice, rat and monkey PET studies were carried out under anesthesia, while human studies were without anesthesia. Isoflurane has been shown to significantly lower hippocampal extracellular serotonin levels in mice (<40% of baseline; Whittington and Virag, 2006). A similar isoflurane effect on serotonin release has been reported in the rat frontal cortex (<40% of awake state; Mukaida et al., 2007). Isoflurane causes significant changes in cerebral blood flow in rhesus monkeys (Enlund et al., 1997; Li et al., 2014) although effects on brain serotonin levels are not available. Serotonin exhibited inhibition values of 169.4 ± 5.0 nM (hippocampus) and 218.3 ± 15 nM (frontal cortex) for ^{18}F-Mefway in rat brain slices (Saigal et al., 2006). Because of this competitive inhibition of ^{18}F-Mefway by serotonin, it may be that the higher binding of ^{18}F-Mefway seen in animal models under isoflurane anesthesia (compared to humans without anesthesia) may be attributed to lowered brain serotonin levels. However, this will require further verification because the ability of endogenous serotonin to compete with 5-HT_{1A} receptor radioligands in vivo remains to be firmly established. Several efforts have been made using 5-HT_{1A} radiotracers to measure serotonin-induced effects in vivo but consensus is still lacking (for e.g., Milak et al., 2011; Pinborg et al., 2012). It may be worthwhile to examine serotonin effects on ^{18}F-Mefway in vivo using PET studies.

The raphe nuclei were clearly visualized both in the monkey and humans. An ascending group of nuclei from the raphe to the rest of the brain was visualized with ^{18}F-Mefway (Fig-7 B and C). This might suggest presence of 5-HT_{1A} receptors in other brain stem nuclei (rather than fiber tracts), similar to the reported distribution of the 5-HT_{1A} receptor mRNA in the mouse brainstem (Bonnavion et al., 2010). Further studies may help refine the binding of ^{18}F-Mefway in the human brain stem. There was a significant inter-subject variability of ^{18}F-Mefway BP_{ND} values in the human subjects, similar to previously described studies with ^{11}C-WAY 100635 (e.g., Stein et al., 2008). A larger group of subjects, including blood input function may be valuable in addressing this issue with ^{18}F-Mefway. It is likely that factors affecting BP_{ND} may include variations in endogenous serotonin levels. A correlation of the average BP_{ND} values in the human ^{18}F-Mefway PET scans with the reported 5-HT_{1A} receptor density measured using ^{3}H-WAY100635 (Hall et al., 1997) and ^{11}C-WAY 100635 (Parsey et al., 2011) (Fig-7D), provided some significance to the measures of in vivo ^{18}F-Mefway PET. In the six human subjects, there appears to be a linear correlation of ^{18}F-Mefway binding in the raphe with that measured in the mesial temporal lobe, hippocampus and amygdala, whereas the frontal cortex and temporal cortex were not correlated with raphe (Fig-7E). However, the distribution of ^{18}F-Mefway in the cortex of all the subjects followed the same general pattern (Supplementary Fig-1). A larger study will have to be undertaken to understand the relationship of the raphe 5-HT_{1A} receptors with the rest of the brain. The ventral (or anterior) hippocampus had a significantly greater amount of ^{18}F-Mefway binding compared to the dorsal (or posterior) hippocampus (Supplementary Fig-2). Perhaps this heterogeneous distribution of ^{18}F-Mefway in the human hippocampus may be useful in understanding a potential role of 5-HT_{1A} receptors in the debate over the functional role of ventral (or anterior) hippocampus in emotion and anxiety related behaviors versus

cognitive functions and spatial memory associated with dorsal (posterior) hippocampus (Strange et al., 2014).

5. CONCLUSION

^{18}F-Mefway appears to be an effective serotonin 5-HT_{1A} receptor imaging agent in all models including humans. Our findings with ^{18}F-Mefway reinforce the homology of the 5-HT_{1A} receptor across species and also the similarity in the brain distribution of this receptor across species. ^{18}F-Mefway therefore may be reliably used to quantify serotonin 5-HT_{1A} receptor distribution in brain regions for the study of various CNS disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support for the project was provided by NIH/NIA R21/R33 AG030524 (JM).

Technical assistance of Drs. Robert Coleman, Evegueni Sevrioukov, Cristian Constantinescu, Rama Pichika, Bingzhi Shi and Tanjore K. Narayanan is gratefully acknowledged. We like to thank Dr. Elizabeth Head and the UCI ADRC for the dog and human brain tissue. We like to thank Neerva Rana, Himika Patel and Sharon Kuruvilla for assistance with tissue sectioning and manuscript preparation and Andrew Higgins for preparing the supplementary movie files. Receptor binding profiles was generously provided by the NIMH PDSP, Contract # HHSN-271-2008-025C directed by Dr. Bryan L. Roth at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscol at NIMH, Bethesda MD, USA. We like to thank the reviewers and editors for their suggestions.

Grant Support: NIH/NIA R21/R33 AG030524 (JM).

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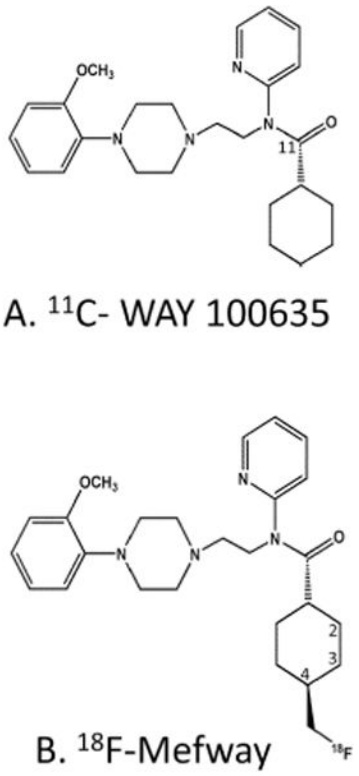

Figure-1.

Chemical structures: (A). Structure of ^{11}C-WAY-100635 currently used in human PET studies. (B). Structure of ^{18}F-*trans*-4-Mefway used in the studies reported here.

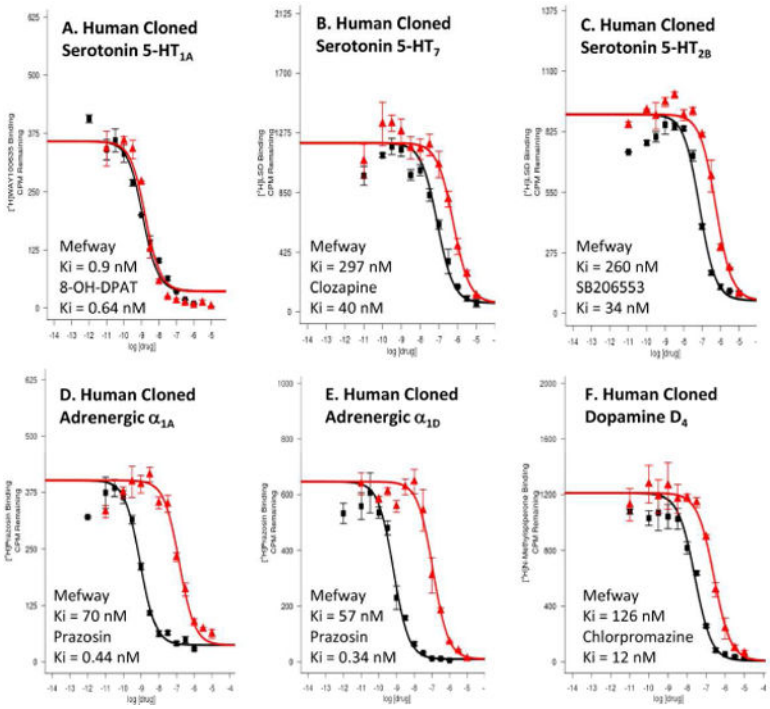

Figure-2.

In vitro receptor binding profiles of Mefway (red curves; PDSP Compound #34292) for human cloned receptors: (A). Serotonin $5HT_{1A}$ (Ki=0.9 nM). (B) Serotonin $5HT_7$ (Ki=297 nM). (C) Serotonin $5HT_{2B}$ (Ki=260 nM). (D). Adrenergic alpha1 (Ki 70=nM). (E). Adrenergic alpha 1D (Ki=57 nM). (F). Dopamine D4 (Ki=126 nM).

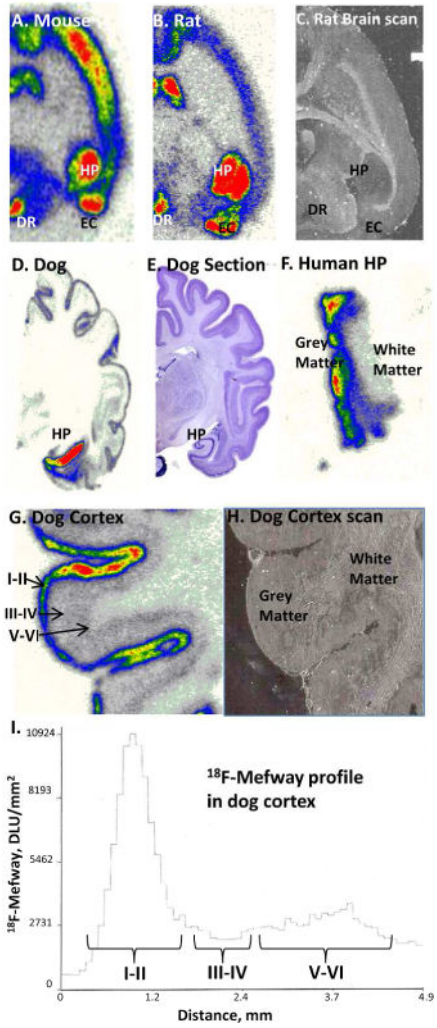

Figure-3.

In vitro autoradiography across species of ^{18}F-Mefway: (A). Horizontal mouse brain slice showing hippocampus (HP), raphe (DR) and entorhinal cortex (EC). (B). Horizontal rat brain slice showing HP, DR and EC (C). Rat brain slice (10 μM thick) scan adjacent to section shown in (B) corresponding approximately to horizontal section Bregma −6.38 mm, interaural 3.62 mm showing HP, DR and EC (Paxinos and Watson, 2006). (D). Coronal dog brain showing hippocampus (HP) and cortex. (E). Stained dog brain coronal section #1360 showing HP and cortex (from brainmuseum.org University of Wisconsin-Madison Brain Collection). (F). ^{18}F-Mefway binding in a section of human hippocampus. (G). Dog brain cortical layers I through VI showing ^{18}F-Mefway binding. (H). Scan of the dog brain slice shown in G. (I). Binding profile of ^{18}F-Mefway in the cortical layers, I–II, III–IV and V–VI of the dog brain slice shown in G. Layers I–II had the highest ^{18}F-Mefway binding in the cortex followed by layers V–VI.

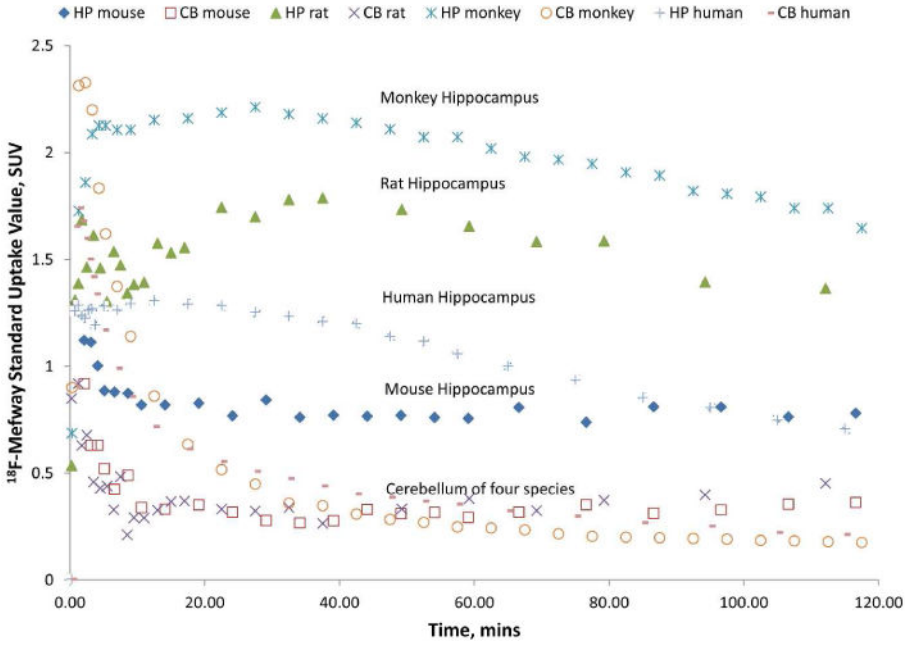

Figure-4.

Time-activity Curves: PET curves across species of ^{18}F-Mefway showing hippocampus (HP) and cerebellum (CB) in mouse brain, rat brain, monkey brain and human brain. Standard uptake value for all species were calculated as SUV= Activity concentration (kBq/cc) in region of interest/[Injected dose (MBq)/Body weight (Kg)]

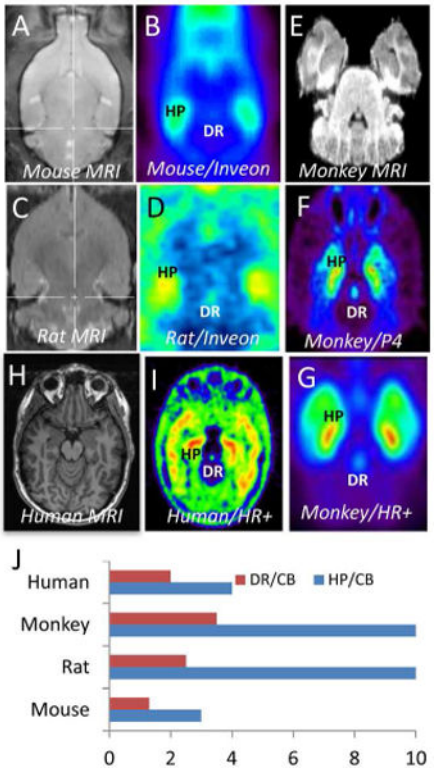

Figure-5.

PET images across species of ^{18}F-Mefway showing hippocampus (HP) and raphe (DR): (A). Mouse brain slice from coregistered MRI template (Saigal et al., 2013). (B). Mouse brain PET slice corresponding to MRI slice in A. (C). Rat brain slice from coregistered MRI template (Saigal et al., 2013). (D). Rat brain PET slice corresponding to MRI slice in C. (E). Monkey brain slice from coregistered MRI template (Wooten et al., 2014). (F) Monkey brain PET slice on P4 Focus scanner corresponding to MRI slice in E. (G). Monkey brain PET slice on ECAT EXACT HR+ scanner. (H). MRI brain slice of human subject shown in I. (I). Human brain PET slice of subject shown in Fig-H on ECAT EXACT HR+ scanner (F) Plot of dorsal raphe (DR) and hippocampus (HP) ratio with respect to cerebellum in the four species from in vivo PET studies. Mouse and rat brain data was from Saigal et al., 2013; monkey data was from Saigal et al., 2006 and Wooten et al., 2011b; human data was from Hillmer et al., 2014. Image slices are from summed dynamic data sets between 0–120 mins for each species.

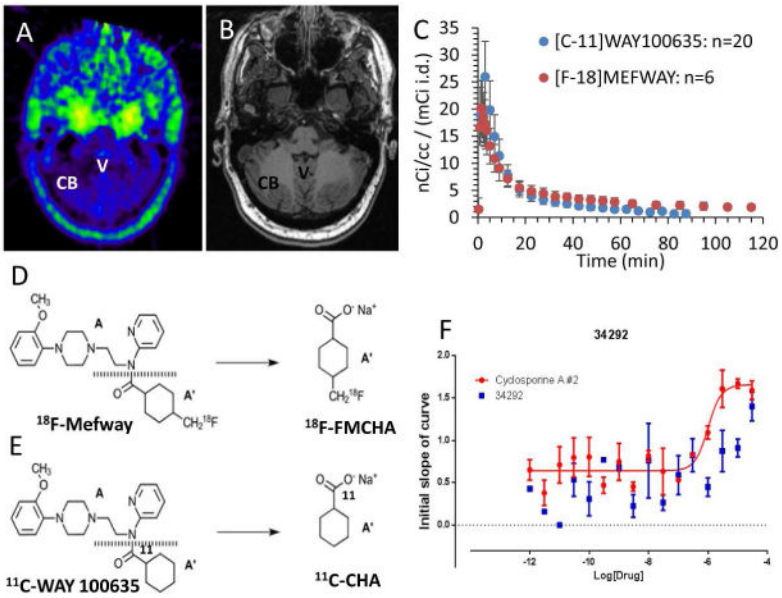

Figure-6.

Human Cerebellum ^{18}F-Mefway PET: (A). Transaxial brain slice of ^{18}F-Mefway in the human cerebellum (CB) and vermis (V). (B). Coregistered MRI of brain slice in (A) showing cerebellum and vermis. (C). Average time-activity curves of ^{11}C-WAY-100635 and ^{18}F-Mefway human subjects showing similarity of kinetics with little binding is seen in the cerebellar lobes which is used as a reference region. (D). Breakdown of ^{18}F-Mefway with ^{18}F-FMCHA as a plausible metabolite in humans. (E). Breakdown of ^{11}C-WAY-100635 with ^{11}C-CHA as a plausible metabolite in humans. (F). Comparison of cyclosporine A with Mefway as a potential substrate of MDR1 (P-glycoprotein; Cyclosporine IC_{50}=0.88 μM; Mefway >30 μM)

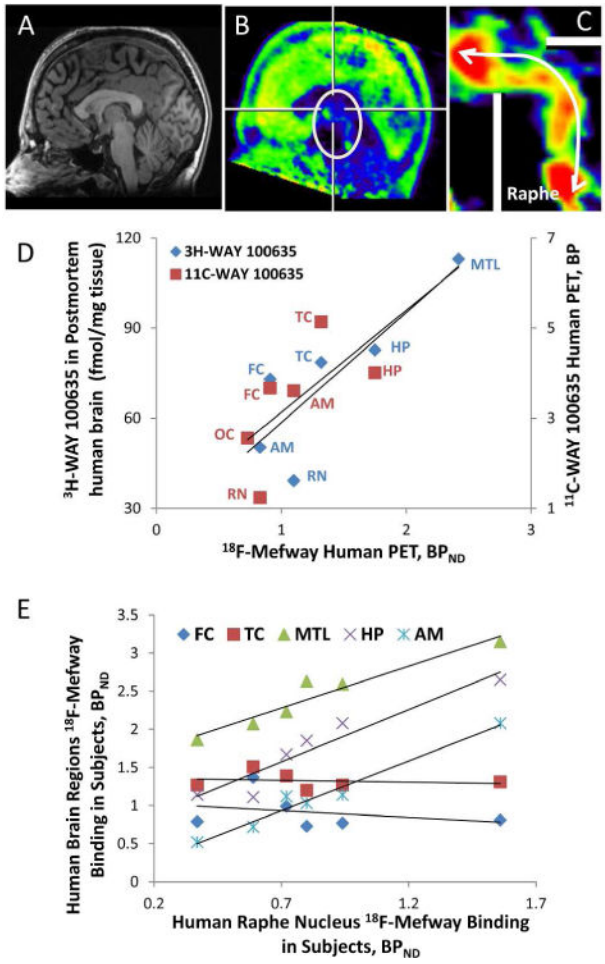

Figure-7.

Human Raphe ^{18}F-Mefway PET: (A). Sagittal MRI brain slice showing cortical regions, mid-brain, cerebellum and brain stem. (B). Coregistered ^{18}F-Mefway sagittal slice of the same subject showing binding of ^{18}F-Mefway to cortical regions and mid-brain regions. (C). Zoom-in view of ^{18}F-Mefway in the raphe (DR) showing several other nuclei ascending to the fornix. (D). Correlation of ^{18}F-Mefway BP_{ND} with postmortem ^{3}H-WAY-100635 in human postmortem brain regions and with ^{11}C-WAY-100635 BP in PET studies. (E). Correlation of ^{18}F-Mefway binding potential in the raphe nucleus with ^{18}F-Mefway binding potential in the other brain regions of 6 subjects previously reported (Hillmer et al., 2014; FC: frontal cortex; TC: temporal cortex; MTL: mesial temporal lobe; HP: hippocampus; AM: amygdala).

Table-1

Binding Affinity and Selectivity for WAY-100635 and Mefway*

Tracer	5-HT$_{1A}$	5-HT$_{7}$	5-HT$_{2B}$	α$_{1A}$	α$_{1B}$	α$_{1D}$	D$_{4}$
WAY-100635, Ki nM	2.2[a], 0.6[b] 1.07[c]	>10000[a]	24[a]	20[a]	322[a]	5[b]	16[a]
% human 5HT$_{1A}$ Selectivity [d,e]	100	0.006 to 0.02	2.5 to 9.16	3 to 11	0.19 to 0.68	12 to 44	3.8 to 14
Mefway, Ki nM	0.9 0.84[c]	297	260	70	505	57	126
% human 5HT$_{1A}$ Selectivity[d,e]	100	0.30	0.35	1.29	0.18	1.58	0.71

*Human cloned receptor affinities obtained from pdsp.unc.edu (also reported by

[a]Chemel et al., 2006;

[b]Hussainy et al., 2011; Lang et al., 1999);

[c]Rat brain homogenates labeled with ^{3}H-WAY 100635 (Saigal et al., 2013).

[d]Percent selectivity for 5HT$_{1A}$ receptor = [5-HT$_{1A}$ affinity/other receptor affinity]×100;

[e]Calculated assuming affinities of 0.6 nM or 2.2 nM for WAY-100635 and 0.9 nM for Mefway for the 5-HT$_{1A}$ receptor.

Table-2

Comparison of Mefway Across Species

Species	$5HT_{1A}$ Receptor amino acid length[a]	In Vitro Binding Affinity, Ki[b]	Brain Uptake, %ID/cc[d]	HP/CB PET ratio[h]	Binding potential HP, BP_{ND}	Free Fraction in plasma
Human	422	0.90 nM	0.002[e]	4[i]	2.7[i]	5%[j]
Monkey	421	NA	0.03[f]	10[f]	7.4[j]	15%[j]
Rat	422	0.84 nM 169 nM[c]	0.8[g]	10[g]	6.4[g]	NA
Mouse	421	NA	5[g]	3[g]	1.2[g]	NA

[a] www.uniprot.org; Receptor homology >84% (Albert et al., 1990; Charest et al., 1993).

[b] Binding affinity for Mefway (from Table-1).

[c] Serotonin competition (IC_{50}) with ^{18}F-mefway in hippocampus of rat brain slices (Saigal et al., 2006).

[d] Initial brain uptake, 1–2 min post-injection of ^{18}F-Mefway.

[e] Human brain uptake of ^{18}F-Mefway (Hillmer et al., 2014) was comparable to ^{11}C-WAY 100635 (0.002–0.004%; Parsey et al., 2000; Pike et al., 1996; Farde et al., 1998).

[f] Saigal et al., 2006, Wooten et al., 2011b

[g] Saigal et al., 2013; mouse ratios and BP_{ND} may be underestimated due to the small size of the brain.

[h] Ratios are at 90 mins post-injection of ^{18}F-Mefway.

[i] Binding potential in human hippocampus for ^{18}F-Mefway ranged from 1.1 to 2.7 (Hillmer et al., 2014).

[j] Wooten et al., 2011b, BP_{ND}, average of 4 monkeys of mesial temporal cortex which includes hippocampus; ^{11}C-WAY100635 BP_{ND}=7.

[j] Free fraction human plasma (Hillmer et al., 2014); Free fraction rhesus monkey (Wooten et al., 2013).

HP=Hippocampus; CB=Cerebellum; NA=Not Available