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PET STUDIES OF THE SEROTONIN TRANSPORTER IN THE HUMAN BRAIN

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Cover image: Summation image based on data from frame 6-20 showing regional radioactivity after intravenous injection of [^{11}C]MADAM, transaxial projection.
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ABSTRACT

The serotonin (5-HT) system attracts considerable attention in research on the pathophysiology and drug treatment of neuropsychiatric disorders. The brain imaging technique Positron Emission Tomography (PET) allows for examination of neurotransmission systems in the human brain *in vivo*. Though suitable radioligands are available for the serotonin 5-HT_{1A} and 5-HT_{2A} receptor subtypes, imaging studies of the 5-HT transporter (5-HTT), a key target for drug treatment of mood and anxiety disorders, has been limited due to lack of optimal radioligands.

In autoradiographic studies on rat and *post mortem* human brain slices the radioligand ^{3}H-labelled MADAM has been shown to bind specifically in regions known to have high 5-HTT density. The first aim of the present thesis was therefore to evaluate ^{11}C-labelled MADAM for *in vivo* quantification of 5-HTT in the human brain using PET. The second aim was to apply this method in control subjects and to address questions relevant for the pathophysiology and drug treatment of major depressive disorder (MDD).

In the first study, a total of four cynomolgus monkeys were examined. The selectivity and reversibility of [^{11}C]MADAM binding was examined. Pretreatment with citalopram resulted in decreased [^{11}C]MADAM uptake in 5-HTT rich regions. Pretreatment with GBR 12909 and maprotiline did not affect [^{11}C]MADAM uptake, thus confirming that [^{11}C]MADAM binds selectively to the 5-HTT in the primate brain.

In the second study [^{11}C]MADAM binding could be described by standard compartment models using an arterial input function. The cerebellum was evaluated as reference region in simplified quantitative approaches showing that the rank order of regional binding potential (BP) values was in good agreement with the rank order reported in binding studies on human brain tissue *post mortem*. The use of simplified quantitative approaches in clinical studies was thus supported.

In the third study the reproducibility of [^{11}C]MADAM binding was studied in a test-retest design tailored to be relevant for future applied studies. Analysis of test-retest data indicate that the reliability of [^{11}C]MADAM binding measurements is good to excellent for averaged regions. The study supports clinical studies also on small regions such as the raphe nuclei, although pooling of data may be required for bilateral regions to improve accuracy.

The fourth study examined expression levels of 5-HTT and 5-HT_{1A} receptors in control subjects, i.e. two proteins suggested in mode of action of antidepressive drugs. A trend towards correlation between [^{11}C]MADAM and [^{11}C]WAY 100635 binding was found in the raphe but not in hippocampus or neocortex. Also, the ratio in the raphe nuclei showed a wide range. This finding suggests that expression of these two proteins may be coregulated in the raphe nuclei. The wide range for binding ratio represents a possible explanation for the clinical variability in response to antidepressant drug treatment.

In the fifth study the use of [^{11}C]MADAM to determine 5-HTT occupancy was examined by comparing 10 mg escitalopram and 20 mg of the racemate citalopram in a two-way cross over, double blind design. Citalopram gave higher occupancy than escitalopram – although the subjects were given equimolar amounts of S-citalopram, the hypothesised active compound in both citalopram and escitalopram. The hypothesis that the lower response rate of citalopram may in part be dependent on R-citalopram interacting with 5-HTT could thus find indirect support.

In summary, [^{11}C]MADAM was shown to be a suitable radioligand for quantitative studies of 5-HTT in the living human brain, and can readily be applied for studies on the pathophysiology and pharmacology of neuropsychiatric disorders.

Vi börjar ana att vår vilsegång
är ännu djupare än först vi trott
att kunskap är en blå naivitet
som ur ett tillmätt mått av tankesyn
fått den idén att Gåtan har struktur.

Aniara, sång 13
Harry Martinson
1956

LIST OF PUBLICATIONS

I. Halldin, C.; Lundberg, J.; Sóvágó, J.; Gulyás, B.; Guilloteau, D.; Vercouillie, J.; Emond, P.; Chalon, S.; Tarkiainen, J.; Hiltunen, J.; Farde, L., [^{11}C]MADAM, a new serotonin transporter radioligand characterized in the monkey brain by PET. *Synapse* **2005,** 58, (3), 173-183.

II. Lundberg, J.; Odano, I.; Olsson, H.; Halldin, C.; Farde, L., Quantification of [^{11}C]MADAM Binding to the Serotonin Transporter in the Human Brain. *J Nucl Med* **2005,** 46, 1505-1515.

III. Lundberg, J.; Halldin, C.; Farde, L., Measurement of serotonin transporter binding with PET and [^{11}C]MADAM: A test-retest reproducibility study. *Synapse* **2006,** 60, (3), 256-263.

IV. Lundberg, J.; Cselenyi, Z.; Borg, J.; Halldin, C.; Farde, L., A PET study on regional coexpression of 5-HT1A receptors and 5-HTT in the human brain**.** *manuscript*.

V. Lundberg, J.; Strøyer Christophersen J, Buchberg Petersen K, Loft H, Halldin C, Farde L., PET measurement of serotonin transporter occupancy: a comparison of escitalopram and citalopram. *submitted.*

CONTENTS

LIST OF ABBREVIATIONS

1TM	One-tissue compartment model
2TM	Two-tissue compartment model
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxy tryptamine, serotonin
5-HTT	serotonin transporter
8-OH-DPAT	8-Hydroxy-2-(di-n-propylamino)tetralin
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BP	binding potential
CNS	central nervous system
CSF	cerebrospinal fluid
DA	dopamine
DALY	disability-adjusted life years
DASB	3-amino-4-(2-dimethylaminomethylphenyltio)benzonitrile
DAT	dopamine transporter
DLPFC	dorsolateral prefrontal cortex
DRN	dorsal raphe nuclei
ECG	electrocardiogram
ECS	electroconvulsive stimulation
FWHM	full with at half maximum
HPLC	High performance liquid chromatography
K_1	Rate constant for influx of ligand over BBB (ml/(ml min))
k_2	Rate constant for efflux of ligand over BBB (1/min)
k_3	Association rate constant (1/min)
k_4	Dissociation rate constant (1/min)
k_5	Association rate constant (1/min)
k_6	Dissociation rate constant (1/min)
K_d	Equilibrium dissociation constant
$K_{i,\,app}$	Apparent equilibrium inhibition constant
LSD	lysergic acid diethylamide
MADAM	N,N-Dimethyl-2-(2-amino-4-methylphenylthio)benzylamine
MAO	monoamine oxidase
McN 5652	(+)-6β-(4-methyltiophenyl)-1,2,3,5,6α,10β-hexahydropyrrolo-[2,1-a]-isoquinoline
MDL 100,907	(R)-(+)-4-[1-hydroxy-1-(2,3-dimethoxyphenyl)methyl]-N-2-(4-fluorophenylethyl)-piperedine
MRI	magnetic resonance imaging
MRN	median raphe nuclei
mRNA	messenger ribonucleic acid
NE	Norepinephrine
NET	Norepinephrine transporter
NEX	Number of excitations
NMSP	N-methylspiperone
PET	positron emission tomography

ROI	region of interest
SPET	single photon emission tomography
SPGR	spoiled gradient recalled
SNRI	selective serotonin/norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
TAC	time activity curve
TCA	tricyclic antidepressant drug
TE	excitation time
TR	relaxation time
WAY 100635	N-[2-(4-(2-metoxyphenyl)-1-piperazinl)ethyl]-N-(2-pyridinyl)-cyclohexanecarboxamide trihydrocloride
WHO	World Health Organisation

1. INTRODUCTION

The development of the serotonin (5-HT) system has been estimated to start some 700-800 million years ago, at the time when vertebrates diverged from invertebrates. This makes the 5-HT system one of the oldest neurotransmission systems in vertebrates.[174] The phylogenesis and its wide distribution within the central nervous system (CNS) are in agreement with the many physiological functions to which the 5-HT system has been associated, many of which are crucial for the existence of man.

In psychiatry, high prevalence, long duration, high risk for recurrence and low response rate to treatment make major depressive disorder (MDD) one of the major causes of disability throughout the world.[152] Several lines of evidence suggest a role for the 5-HT system in the pathophysiology and pharmacology of MDD.

The development of brain imaging techniques such as positron emission tomography (PET) has paved the way for studies of the molecular changes within the central nervous system (CNS) that are associated with MDD and related psychiatric disorders. PET is also the most suitable tool for research on mode of action in relation to pharmacodynamics of CNS drugs in the living human brain.[61] The availability of radioligands for visualisation and quantification of the central 5-HT system are however limited and is a major obstacle for an even more rapid development of the field.

With this background, the aim of the present thesis was first to develop a new promising PET radioligand, [^{11}C]MADAM, for quantification of the serotonin transporter (5-HTT) in the human brain *in vivo* (studies I-III) and, second, to apply [^{11}C]MADAM PET in studies addressing two current hypotheses on the pharmacology of MDD (studies IV-V).

1.1 Positron emission tomography

PET is a non-invasive nuclear medicine imaging technique developed to study organ distribution of tracer molecules, or radioligands, labelled with positron (β^+) emitting isotopes. The method allows for quantification and description of regional brain distribution of the labelled molecule. In a PET measurement, the tracer molecule is typically injected intravenously and distributed throughout the body via the blood stream. After passage of the blood brain barrier (BBB) the radioligand binds to the target molecule. At radioactive decay, the emitted β^+ annihilates with an electron (β^-). The distance travelled by the β^+ before annihilation, one to a few millimetres, is called β^+ range and is dependent on the tissue in which the annihilation occurs and the β^+ energy. The latter varies between isotopes (Table 1).

TABLE 1. Main features of frequently used radionuclides[81]

		^{11}C	^{13}N	^{15}O	^{18}F
Half life	(min)	20	10	2	110
Mode of decay		β^+ (100%)	β^+ (100%)	β^+ (100%)	β^+ (97%)
Maximum specific radioactivity	(Ci/mmol)	$9.2x10^6$	$1.9x10^7$	$9.1x10^7$	$1.7x10^6$
Maximal energy	(MeV)	0.97	1.2	1.74	0.64
Penetration distance	(mm)	4	5	8	2

The annihilation results in two 511keV γ-particles (photons). These travel at approximately $180^0 \pm 1^0$ and the coincidences are detected by the PET system outside the subject. The divergence from 180^0 depends on the momentum of β^+ and e^- at the time of annihilation and together with the β^+ range it sets the lower limit to the spatial

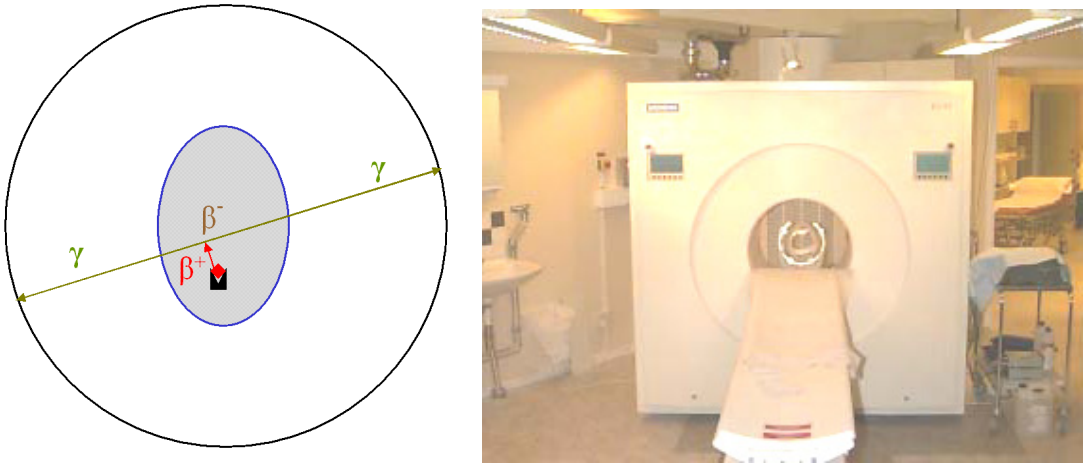


FIGURE 1. Left: The PET principle. A radioligand (red diamond) is bound to a protein (black) within the brain (blue ellipse), e.g. a receptor. After β^+ decay annihilation with an electron (β^-) occurs within a few millimetres, resulting in two γ particles that travel in approximately opposite directions. The coincidence is detected by the PET system (black circle). Right: The PET system at Karolinska Institutet used for the present thesis: ECAT EXACT HR 47.

resolution of PET systems to about 2 mm (Figure 1).[58, 159] Accuracy in PET image data is mainly determined by the sensitivity and spatial resolution of the PET system. The spatial resolution is defined as the degree to which the representation of an object is blurred in the image, commonly expressed in terms of its full with at half maximum (FWHM). FWHM is defined in the Gaussian representation of a perfect point source, as the distance where the intensity in the image is half of the maximal value (Figure 2). The PET system used in the present thesis, ECAT EXACT HR 47, has a in-plane resolution of about 3.8 mm, FWHM.[218]

PET data are most commonly reconstructed by filtered back-projection.[58] In this way images showing the distribution of radioactivity in tissue are computed. In molecular imaging studies the regional activity is corrected for decay and plotted versus time to generate time-activity curves (TAC). Different mathematical models are then applied to the TACs to calculate biological parameters describing ligand-protein binding *in vivo*.

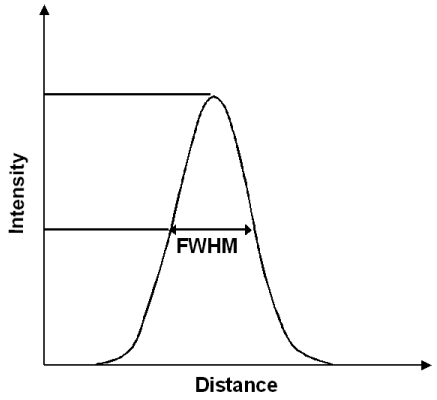


FIGURE 2. Illustration of the concept Full Width at Half Maximum (FWHM), used to estimate resolution of an imaging system.

More complex models such as compartment analyses may require a metabolite corrected arterial input function describing the time curve for unchanged radioligand in plasma.

By consequence of the point-spread function, quantitative PET measurements of objects smaller than 2-3 times the FWHM will result in an underestimation of the signal (Figure 2).[91, 106] Also, activity from surrounding tissue will influence the signal measured in a volume element (spill-over effect). These phenomena are summarized as partial volume effects (PVE) and have to be taken into consideration when measuring radioactivity in small regions.[106]

1.2 The serotonin system

Serotonin was first identified, isolated and characterized as a vasoconstrictive substance in bovine serum 60 years ago.[183] Twarog and Page reported in 1953 that 5-HT is present in the mammalian brain[210] and its role as a chemical mediator was suggested three years later.[28, 32]

Phylogenetic comparisons between the three major classes of 5-HT receptors suggest that the primordial 5-HT receptor was developed some 700-800 million years ago, at the time when vertebrates diverged from invertebrates. This was probably before the development of muscarinic, dopaminergic and adrenergic receptor systems.[174]

The phylogenetic importance of the 5-HT system as well as its wide distribution in CNS is in agreement with the many physiological functions to witch it has been associated, many of which are crucial for the existence of man. Examples of such functions are thermoregulation[49], pain control[185], feeding behavior and body weight[120], sleep[212], mood, social interaction[224] and cognition.[138]

The activity of the 5-HT system is mediated by binding to several distinct receptor proteins. The extracellular concentration of 5-HT is regulated by a specific 5-HT transporter protein (5-HTT), as well as degrading enzymes such as Monoamine

Oxidase (MAO). Several of the 5-HT receptor subtypes have been suggested to be involved in the pathophysiology of psychiatric disorders such as MDD, anxiety disorders [145, 226] and schizophrenia.[137]

1.2.1 Serotonergic pathways

In 1964 Dahlström and Fuxe published the first anatomical description of 5-HT pathways in CNS.[51] This was achieved by applying newly developed histochemical fluorescence methods on rodent brain tissue. It was demonstrated that 5-HT cell bodies are concentrated to the raphe nuclei near the midline of the brainstem. The axonal terminals of these cell clusters innervate virtually all regions of the CNS. The 5-HT neurons in the caudal raphe nuclei project mainly to the grey matter of the spinal cord. The 5-HT neurons in the rostral raphe nuclei, which primarily consists of the dorsal raphe nuclei (DRN) and the median raphe nuclei (MRN), project rostrally and innervate structures such as the cerebellar cortex, thalamus, striatum and the cerebral cortex.[198]

The neuroanatomy of the 5-HT system in the primate brain has been described in some detail. Studies on the *Macaca Fascicularis* has revealed evident differences when compared to subprimate species in terms of nuclear organization, projection pathways, axon morphology and termination pattern.[14] Still, the basic infrastructure in the expansive primate CNS 5-HT system is similar to that in subprimate species. For instance, also in man the cell bodies of ascending 5-HT neurons are confined mainly to the DRN and MRN close to midline in the mesencephalon and rostral pons. The human DRN is a well defined heterogeneous group of neurons that extends for some 20 mm and contains cell bodies of about 250 000 neurons.[16, 17, 157]

Axons of these neurons project to forebrain structures throughout two major pathways: the lateral cerebral cortex is mainly innervated via a lateral pathway through the capsula interna. Medial cortical regions including the hippocampal complex, hypothalamus, basal forebrain and amygdala are all innervated via a medial pathway through the medial forebrain (Figure 3).[14, 207]

1.2.2 The Serotonin transporter

Neuronal uptake of catecholamines was described in the early sixties.[88, 153] Based on measurements of [^{3}H]5-HT uptake in brain slices at different pharmacological conditions a similar specific membrane transport mechanism for uptake of 5-HT was first described in 1967.[186] Five years later it was shown in rodents that this mechanism is associated to 5-HT neurons.[111] More recently, this mechanism has been associated to a selective Na^{+}/Cl^{-}-dependent transport protein.[161] The single gene encoding the human 5-HTT has been mapped to chromosome 17q11.1-q12.[182]

The distribution of 5-HTT in the human brain has been extensively mapped *post mortem*.[15, 48, 118, 179] An overview of these studies is given in table 2. The rank order of 5-HTT density findings has been confirmed in the living human brain using PET and the radioligands [^{11}C]DASB, [^{11}C]MADAM and [^{11}C]McN 5652.[70, 74, 96, 107, 129, 167, 204] Immunohistochemical studies of the subcellular localisation of plasma membrane 5-

HTT have shown the highest densities in association with axons, dendrites and neuronal perikarya.[90, 202, 225]

1.2.3 Serotonin receptor subtypes

In 1957 Gaddum and Picarelli published a work on guinea-pig ileum suggesting the presence of two classes of 5-HT receptors. Their functions were selectively blocked by dibenzyline and morphine, respectively, and they were thus referred to as type D and type M.[72] The current nomenclature is based on a classification made possible by pharmacological characterisation and radioligand binding techniques developed in the 1970s. For instance, measuring binding of the radioligands [^{3}H]5-HT, [^{3}H]lysergic acid

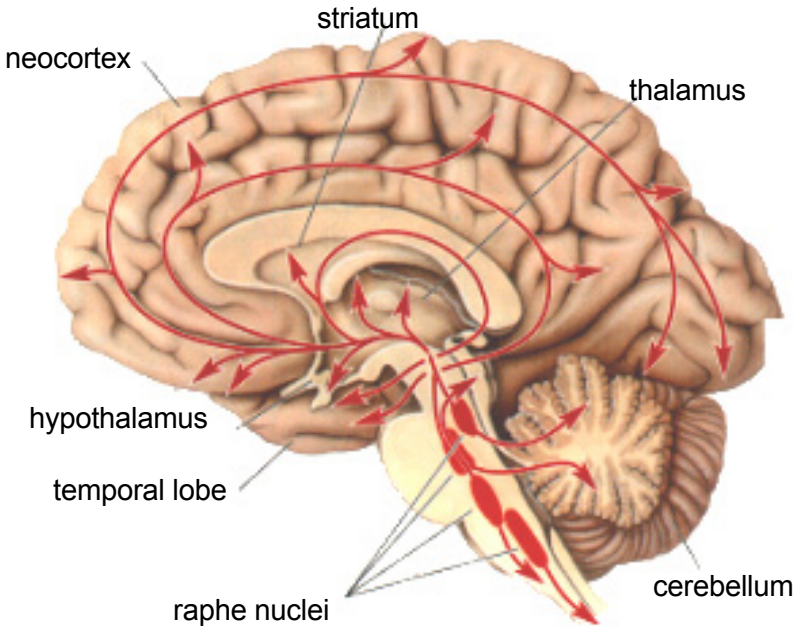


FIGURE 3. Mediosagittal view showing distribution of 5-HT containing cell bodies within the raphe nuclei and major ascending projections.

TABLE 2. Estimation of the 5-HTT density in human brain in vitro and in vivo using [^{11}C]MADAM

	Laruelle et al[118] *Saturation study* (pmol/g tissue)	Bäckström et al[15] *Saturation study* (pmol/g tissue)	Plenge et al[179] *Saturation study* (pmol/g tissue)	Cortés et al[47] *Microdensito-metry* (pmol/g tissue)	Lundberg et al[129] *PET* (B_{max}/k_d)
frontal cortex	5	6	5	37	0.6
cingulate cortex	14	11	9	-	0.7
hippocampal complex	14	7	7	42	1.2
putamen	14	15	14	63	1.4
raphe nuclei	-	-	82	200	4.0
cerebellum	2	3	-	18	-

diethylamide (LSD) and [^{3}H]spiroperidol to rat cortical membranes Peroutka and Snyder were able to differentiate between two separate populations of 5-HT receptors denoted 5-HT_1 and 5-HT_2 receptors.[175] Today 14 separate receptor subtypes have been described on the basis of operational, structural and transductional information.[93]

All known 5-HT receptors are G-protein coupled with exception of the 5-HT_3 receptor, which is a ligand gated ion channel.[20, 54] 5-HT_1 receptors are negatively coupled to adenylate cyclase, 5-HT_2 couple to the hydrolysis of inositol phosphates and 5-HT_4, 5-HT_6 and 5-HT_7 receptors are positively coupled to adenylate cyclase. Although the transductional mechanism of 5-HT_5 receptors remains to be understood in more detail they seem to be members of the G-protein coupled superfamily.[20]

1.2.3.1 The 5-HT_{1A} receptor

The human 5-HT_{1A} receptor gene is located on chromosome 5q11.2-q13.[93] The brain distribution pattern has been extensively characterised. In autoradiographic studies on the human brain post mortem high concentrations has been demonstrated in the hippocampal complex, superficial layers of the cortex and in the raphe nuclei.[80, 172] The cellular localization of the 5-HT_{1A} receptor seems to be somatodendritical, as autoradiographic findings largely have been shown to correspond to the regional brain distribution of 5-HT_{1A} receptor mRNA.[35, 148, 180] *In vivo* quantification of 5-HT_{1A} receptor binding in the human brain using PET and [^{11}C]WAY 100635 has confirmed the pattern of distribution and the rank order of receptor density suggested from post mortem studies.[66, 98, 177] Presynaptic 5-HT_{1A} receptors are located in the cell bodies and dendrites of serotonergic neurons in the raphe nuclei.[196] In frontal cortex and hippocampal complex the 5-HT_{1A} receptors are located postsynaptically in pyramidal neurons (Figure 4).

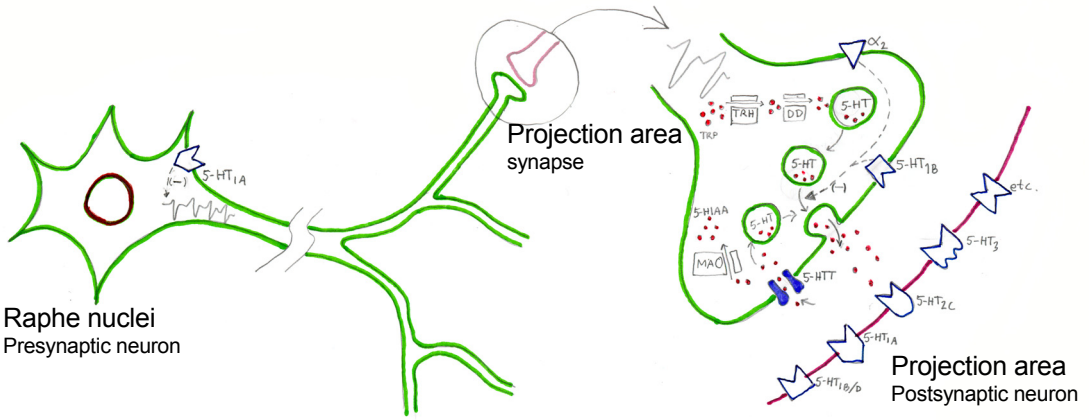


FIGURE 4. Illustration of a serotonergic neuron with cell body in the raphe nuclei (left) and synaptic region with postsynaptic receptors (right, blow up). 5-HT, serotonin; 5-HTT, serotonin transporter; 5-HIAA, 5-hydroxy indole acetic acid; DD, dopa decarboxylase; MAO, monoamine oxidase; TRP, tryptophan; TRH, tryptophan hydroxylase. Note that all 5-HT receptors not are expressed in all post synaptic neurons. After Blier and Montigny, 1999.[26]

Each of the 14 5-HT receptor subtypes has a distinct distribution in the human brain, and may thus be viewed as having distinct functional implications. Of the other 5-HT receptor subtypes, the 5-HT_{2A} receptor is the best characterised with regard to regional brain expression and functional importance.[79, 173, 136, 221]

1.3 Radioligands for the serotonin system

Research on expression of 5-HT proteins has been facilitated markedly by the development of radioligands for binding studies *in vitro* and *in vivo.* Accordingly, *in vitro* research on the 5-HTT has benefited from radioligands such as [^{3}H]imipramine,[48, 179] [^{3}H]paroxetine[15, 48, 118, 179] and [^{3}H]citalopram.[179]

The first selective radioligand used for quantification of the 5-HT_{1A} receptor in the human brain *post mortem* was [^{3}H]8-OH-DPAT (8-Hydroxy-2-(di-n-propylamino) tetralin).[94] However, antagonists without intrinsic activity have for a long time been preferred. The first 5-HT_{1A} receptor antagonist radioligand was [^{3}H]WAY 100635 (N-[2-(4-(2-metoxyphenyl)-1-piperazinil)ethyl]-N-(2-pyridinyl)-cyclo-hexanecarboxamide trihydrocloride).[80]

[^{3}H]spiperone and [^{3}H]LSD were the first radioligands used to label what later was shown to be 5-HT_{2A} receptors.[165, 175] However, these radioligands both suffer from a lack of selectivity to the 5-HT_{2A} receptor which make the more recently developed [^{3}H]MDL 100907 ((R)-(+)-4-[1-hydroxy-1-(2,3-dimethoxyphenyl)methyl]-N-2-(4-fluorophenylethyl)-piperedine) a preferred tool.[127]

At present, three targets specific for the 5-HT system are possible to quantify using PET: the 5-HTT, the 5-HT_{1A} receptor and the 5-HT_{2A} receptor. A need for suitable radioligands for quantification of 5-HTT *in vivo* was identified already in the 80s when PET or single photon emission tomography (SPET) were first applied for molecular imaging. Since then, several compounds of diverse structural families have been synthesised and labelled with β^+ or γ emitters. Most compounds have however proven to be non-optimal for imaging purposes due to poor binding characteristic, unfavourable binding kinetics or lack of selectivity for 5-HTT over the dopamine (DA) transporter (DAT) and the norepinephrine (NE) transporter (NET; for reviews, see Halldin 2001, Laruelle 2002 and Wellsow 2002[82, 117, 215]).

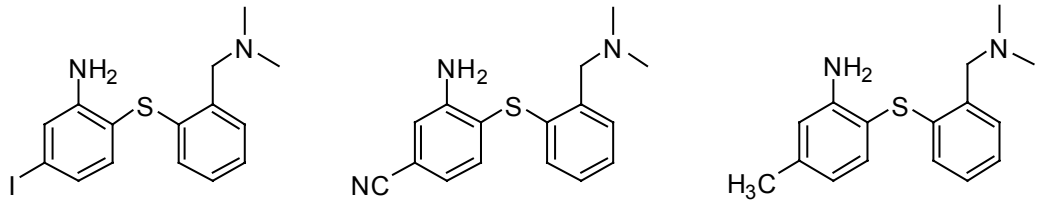
A promising step in the development of radioligands for 5-HTT was the isoquinoline derivative (+)-[^{11}C]McN 5652 (^{11}C-(+)-6β-(4-methyltiophenyl)-1,2,3,5,6α,10β-hexahydropyrrolo-[2,1-a]-isoquinoline) which has been used to image 5-HTT in non-human primates and humans.[200, 203] Major limitations of (+)-[^{11}C]McN 5652 are high non-specific binding and slow binding kinetics.[70]

Cocaine (phenyltropane) congeners, such as nor-[^{11}C]β-CIT represent another structural class of compounds which has been used to image 5-HTT in the living monkey and human brain.[21, 89, 116] These radioligands are however not selective for 5-HTT since they also bind to DAT and NET.[29, 30, 65]

More recently, several derivatives from the diphenyl sulfide family[69] have been proposed as potential candidates for *in vivo* imaging of 5-HTT. Iodine-123 labelled ADAM (Figure 5) is the current SPET tracer of choice for visualisation of 5-HTT.[1, 164, 104] Due to the relatively slow binding kinetics of carbon-11 labelled ADAM, this radioligand is not suitable as a PET radioligand.[84, 216]

The cyano-substituted phenylthio-benzylamine derivative DASB, 3-amino-4-(2-dimethylaminomethylphenyltio)benzonitrile (Figure 5), is a carbon-11 labelled analogue of ADAM. This radioligand has been demonstrated to have suitable characteristics for PET examination of 5-HTT in the human brain.[74, 92, 141]

The radioligand MADAM, N,N-Dimethyl-2-(2-amino-4-methylphenylthio) benzyl amine (Figure 5), contains three methyl substituents in two different located parts of the molecule: in the N,N-dimethyl position and in the 4-methyl position of the phenyl ring. The *in vitro* evaluation of [^{3}H]MADAM[41, 56, 115] and the labelling of MADAM



ADAM K_i = 0.14 nM

DASB K_i = 1.1 nM

MADAM K_i = 1.65 nM

FIGURE 5. The chemical structure and affinity to 5-HTT of ADAM, DASB and MADAM respectively.

with carbon-11 in two different positions using [^{11}C]methyl iodide has been reported earlier.[205] In autoradiographic studies on rat and post-mortem human brain slices [^{3}H]MADAM bound in regions known to have high 5-HTT density and the binding could be inhibited by reference ligands.[41] Characterisation of [^{11}C]MADAM in monkey and man is described in papers I-III of the present thesis.

Labelling of WAY 100635 and its metabolite desmethyl-WAY 100635 with carbon-11 has resulted in two radioligands, [carbonyl-^{11}C]WAY 100635 (or simply [^{11}C]WAY 100635), and [carbonyl-^{11}C]desmethyl-WAY 100635. They have both successfully evaluated for quantification of the 5-HT$_{1A}$ receptor in the living human brain using PET.[5, 63, 66, 77, 178]

[^{11}C]N-methylspiperone ([^{11}C]NMSP) was the first radioligand used in PET on human subjects when Wagner and co-workers in 1983 published a work describing [^{11}C]NMSP binding to dopamine D2 receptors in the human brain *in vivo.*[213] However, as [^{11}C]NMSP has similar affinity for the D2 and 5-HT$_{2A}$ receptor it was also the first radioligand used for the quantification of the 5-HT$_{2A}$ receptor using PET.[6, 7, 76, 155] The low specific-to-nonspecific binding ratio and the affinity to several receptors make

[^{11}C]NMSP a less optimal radio ligand. This is the background for the development of MDL 100,907 as a PET radioligand for quantification of the 5-HT_{2A} receptor.[100]

1.4 Serotonin hypotheses

1.4.1 The serotonin hypothesis for schizophrenia

The 5-HT hypotheses of the pathophysiology of psychiatric disorders have largely been driven by pharmacological observations. Already in 1954 Woolley and Shaw suggested the 5-HT system a target for the treatment of schizophrenia. This suggestion was mainly based on two observations: First, several compounds structurally similar to 5-HT, ergot alkaloids such as LSD, yohimbine and harmala alkaloids, have all been shown to antagonize the contraction of artery walls induced by 5-HT. Second, these substances all cause mental disturbances: some of them similar to those seen in schizophrenia.[223] It was later shown that for 14 drugs with hallucinogenic potency, the mechanism of action involved 5-HT_2 receptor related events.[75] Today, several antipsychotic drugs have 5-HT_{2A} receptor antagonist properties.[136]

1.4.2 The serotonin hypothesis for major depressive disorder

The 5-HT hypothesis for depression is somewhat younger than that for schizophrenia. In 1958 imipramine as the first drug in the class of tricyclic antidepressants (TCA) was shown to relieve depressive symptoms.[112] It was later shown that the TCA drugs imipramine, amitryptiline and clomipramine all inhibited reuptake of 5-HT, and of NE.[38, 39] It was also shown that by inhibiting 5-HT synthesis, the antidepressant effect of imipramine could be blocked.[194] Among other things, these findings motivated the development of drugs that selectively inhibits the 5-HTT.[37, 227] The first SSRI developed, zimelidine, was indeed found to have antidepressive properties.[149] Zimelidine was taken off market after being associated with development of Guillain-Barré syndrome, but has been followed by other SSRIs such as citalopram, fluoxetine, fluvoxamine, paroxetine and sertraline.

In 1962 imipramine was also shown to be effective in what today would be classified as panic disorder.[108] The hypothesis of a specific role in anxiety disorders for the 5-HT system finds support in the fact that 5-HT_{1A} receptor agonists such as *m*-chlorophenyl piperazine is anxiogenic, that 5-HTT inhibitors such as imipramine and clomipramine are effective anxiolytics but that the NET inhibitor maprotiline is not. These findings are all from the late eighties and early nineties.[217]

1.5 Major depressive disorder

MDD is characterised by symptoms such as low mood, anergia, anxiety, feelings of guilt or shame, hopelessness, anhedonia, loss of appetite, insomnia, impairment of cognitive functions, suicidal thoughts and ideation.[8] It is associated with a substantial morbidity, disability and also mortality, reflected in the fact that it has been determined to be the fourth most disabling (in terms of disability-adjusted life years (DALY))

condition globally, preceded only by lower respiratory tract infections, diarrhoeal diseases and perinatal conditions. Furthermore, projections by the world health organisation (WHO) suggest that by the year 2020, unipolar major depression will be second only to ischemic heart disease in terms of DALY.[152] In a developed country such as Sweden, this scenario is already true.[123]

1.5.1 Epidemiology

In a recent study on some 20 000 inhabitants in six European countries, the 12-month and lifetime prevalence of MDD was reported to be approximately 4% and 14%, respectively.[4] These results are in good agreement with recently published data for the USA.[105] The lifetime risk was estimated to be approximately double for women, compared to that for men.[4, 105]

1.5.2 Pathophysiology in relation to the serotonin system

Several lines of evidence suggest 5-HT to have a role in the pathophysiology of depression. Analyses of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) in CSF of MDD patients have revealed decreased concentrations.[2, 12] A relation between this specific biological finding and suicide risk has been described.[3, 13, 208] Studies looking at the endochrinological response (e.g. cortisol, growth hormone or prolactine) to increased 5-HT concentrations (e.g. by fenfluramine or tryptophan stimulation) have reported a difference both between MDD patients and controls, and between MDD patients with active disease and patients in remission.[44, 86, 158, 195, 211]

Expression levels of the 5-HTT in MDD have been examined by comparing MDD patients and controls, together with other paradigms. Post mortem studies show inconsistent results. Decreased[119, 176, 197], unchanged[27, 162, 206] and increased[143] 5-HTT concentrations have been found in MDD subjects compared to age-matched control subjects. This inconsistency may to some extent be explained by the use of less selective ligands such as [^{3}H]imipramine in some studies, that not the same brain regions have been examined in all studies, that most studies have included suicide victims to a variable degree, that some of the subjects were treated with drugs affecting the serotonin system, but also that there is a large interindividual variability in 5-HTT concentration in the human brain *in vivo*[128] and that small samples thus may lead to contradictory results.

The development of radioligands for quantification of the 5-HTT in the human brain *in vivo* using SPET and PET has allowed for studies with better validity with regards to molecular differences in the human brain between MDD patients and control subjects. The most common finding is decreased 5-HTT density in the MDD group, although conflicting as well as non-significant results also exists (Table 3).[95, 113, 132, 140, 154, 166, 220] The brain regions described are not identical in all reports, but the midbrain is included in six studies comparing MDD patients with controls. In three of these, lower 5-HTT densities were detected in the MDD population, and in the other three no significant difference was noted. In these studies a number of radioligands were used. The lack of coherence in the results is at least in part an expression for the need of selective radioligands with good signal to noise ratio.

TABLE 3. Measurement of 5-HTT binding in MDD patients using PET or SPET

Study	sample size	antidepressant treatment	method/ radioligand	regions examined	comparison/main results
Malison et al 1998[132]	MDD=15 C=15	3w to 7yrs wash-out	SPET [^{123}I]β-CIT	brainstem*	MDD<C
Willeit et al 2000[220]	SAD=11 C=11	>6months wash out	SPET [^{123}I]β-CIT	thalamus/hypothalamus midbrain/pons*	SAD<C SAD vs C; ns
Ichimiya et al 2002[95]	MDD=7	>2weeks wash out	PET	thalamus	(MDD+BP-D)>C
	BP-D=6 C=21		[^{11}C](+)McN5652	midbrain	(MDD+BP-D) vs C; ns
Laasonen-Balk et al 2004[113]	MDD_R=7 MDD_{NR}=11		SPET [^{123}I]β-CIT	midbrain*	$\Delta MDD_R > \Delta MDD_{NR}$
Meyer et al 2004[140]	MDD=20 C=20	>3 months wash out	PET [^{11}C]DASB	prefrontal cortex anterior cingulate bilateral caudate bilateral thalamus midbrain	MDD vs C; ns MDD vs C; ns MDD vs C; ns MDD vs C; ns MDD vs C; ns
Newberg et al 2005[154]	MDD=7		SPET	medial temporal lobe	MDD vs C; ns
	C=6		[^{123}I]ADAM	basal ganglia midbrain	MDD vs C; ns MDD<C
Parsey et al 2006[166]	MDD=26 C=43	>2weeks wash out	PET [^{11}C](+)McN5652	anterior cingulate hippocampus thalamus amygdala putamen midbrain	MDD vs C MDD vs C MDD vs C MDD<C, post hoc MDD vs C MDD<C, post hoc

MDD = major depressive disorder, ongoing

SAD = seasonal affective disorder

BP-D = bipolar disorder, current episode depression

MDD_R = MDD patients responding to antidepressant treatment

MDD_{NR} = MDD patients responding to antidepressant treatment

ΔMDD_R = specific binding at baseline-specific binding after 6 months of antidepressant treatment, group of responders

ΔMDD_{NR} = specific binding at baseline-specific binding after 6 months of antidepressant treatment, group of non-responders

*[^{123}I]β-CIT has similar affinity for 5-HTT and the dopamine transporter (DAT) (Laruelle et al 1994, carroll et al 1995). It is thus expected that midbrain binding reflect both 5-HTT in the raphe nuclei and DAT in the substantia nigra

C = control group

Together with the 5-HTT, the 5-HT_{1A} receptor is the most examined marker of the 5-HT system studied in relation to MDD. Possible differences in 5-HT_{1A} receptor binding between patients suffering from MDD and controls has been studied post mortem. For cortical regions decreased 5-HT_{1A} receptor binding in MDD patients compared to control subjects has been reported.[9, 31] Results from studies of the DRN are inconclusive.[9, 199] Results from the few studies of 5-HT_{1A} receptor mRNA levels reported do show differences between MDD subjects and controls, both decreased[125, 126] and increased[59] levels.

PET studies of patients suffering from MDD have come to somewhat inconclusive results (Table 4). The sample sizes in these studies are sometimes small. Four papers have reported significantly decreased [^{11}C]WAY 100635 binding in medication free MDD subjects compared to controls.[24, 55, 135, 188] One paper shows no significant difference between controls and MDD patients, despite a reasonable sample size and sound methodology.[169] More studies on large samples are warranted.

Also 5-HT_{2A} receptor density in the brain of MDD patients has been compared to controls in a number of studies. The results of the PET and SPET studies so far published are however inconsistent.[23, 139, 147, 190, 193]

1.5.3 Pharmacological treatment

The mechanism of action for most antidepressant drugs involves an inhibition of the 5-HTT.[38, 39, 149] Alternative mechanisms involve inhibition of monoamine oxidase (MAO)[71] or blockade of 5-HT receptors[131, 189] A recent development of 5-HTT inhibitors is the synthesis of pure enatiomeres of SSRI compounds, such as escitalopram.[33]

Two major limitations of current pharmacological treatment approaches warrant continued research of the pathophysiology and pharmacology of MDD: First, the response rate is only 60-70%. Second, the time to onset of antidepressant effect is two to three weeks.[19, 103]

Apart from the 5-HTT, several of the 5-HT receptors have been suggested as targets for antidepressant treatment. Many of these targets are only evaluated in animal models. One of the most studied receptors in this context is the 5-HT_{1A} receptor.

Addition of a 5-HT_{1A} receptor agonist such as buspirone to SSRI treatment has been suggested to be an augmentation strategy in the treatment of MDD, possibly by means of direct activation of postsynaptic 5-HT_{1A} receptors.[101, 184, 209] Also, 5-HT_{1A} receptor antagonists such as pindolol have been suggested to enhance and/or accelerate the effect of SSRI in treatment of depression.[93, 189] Several clinical trials and one meta-analysis of nine randomised controlled trials evaluating the addition of pindolol to SSRI treatment of MDD have been published.[18] The meta-analysis suggests that addition of pindolol increase the response rate the first two weeks of treatment significantly (number needed to treat = 6).

TABLE 4. Measurement of 5-HT$_{1A}$ binding in MDD patients using PET and [^{11}C]WAY 100635

Study	sample size	antidepressant treatment	regions examined	comparison/main results
Drevets et al, 1999[55]	MDD=12 (recurrent MDD=8, BP-D=4) C=8	>2weeks wash out	Raphe mediotemporal cx occipital cx (post hoc) parietal cx (post hoc)	MDD<C MDD<C MDD<C MDD<C
Sargent et al, 2000[188]	$MDD_{unmedicated}$=15 $MDD_{medicated}$=20 C=18	>11 weeks wash out	11 regions	$MDD_{unmedicated}$<C $MDD_{medicated}$<C
Bhagwagar et al, 2004[24]	MDD_{R_PET}=14 C=18	wash-out 8-60 months	Raphe Post synaptic regions (mean BP of 20 ROIs)	MDD_{R_PET} vs C; ns MDD_{R_PET}<C
Meltzer et al, 2004[135]	MDD=17 C=17	wash-out >2weeks	Raphe 6 post synaptic regions	MDD<C MDD vs C; ns
Parsey et al, 2006[169]	MDD=28(MDD_{AN}=13, MDD_{AE}=15) C=43	wash-out >2weeks	Raphe+ 12 post synaptic regions	MDD vs C; ns MDD_{AN} vs MDD_{AE} vs C C<MDD_{AN}; post hoc MDD_{AE}<MDD_{AN}; post hoc
Parsey et al, 2006[168]	MDD=22 (MDD_{R}=9, MDD_{NR}=13) C=43	wash-out >2weeks	Raphe+ 12 post synaptic regions	MDD_{NR_FU}>MDD_{R_FU}

MDD = major depressive disorder

BP-D = bipolar disorder, current episode depression

MDD_{R_PET} = subjects with previous MDD who are in remission at time of PET measurement

MDD_{AE} = subjects with MDD that previously have been exposed to antidepressants

MDD_{AN} = subjects with MDD that are antidepressant naïve

MDD_{R_FU} = subjects who had reached remission at follow-up one year after PET examination

MDD_{NR_FU} = subjects who had not reached remission at follow-up one year after PET examination

C = control group

The mechanism by which this effect may be achieved has been studied. The antidepressant effect of SSRI treatment has been ascribed to enhanced 5-HT transmission.[53] The acute effect of antidepressant exposure is however associated with depressed firing rates in DRN neurons and with decreased 5-HT concentration in frontal cortex.[97, 192] This is due to stimulation by 5-HT of somatodendritic 5-HT$_{1A}$ autoreceptors in the DRN (Figure 4).[42, 73] Electrophysiological studies suggest that chronic SSRI treatment for two to three weeks result in a desensitisation of 5-HT$_{1A}$ autoreceptors and an increase in 5-HT transmission. This time frame corresponds to the

time of onset for SSRI treatment.[25] Thus, blockade of the 5-HT_{1A} autoreceptor might fasten the response to SSRI and TCA treatment of depression.

For the other 5-HT receptors data are more limited. The putative 5-HT_{1B}-receptor antagonist anpirtoline has shown antidepressant like properties in rodents.[191] The selective 5-HT_{2A} receptor antagonist EMD 281014 has been associated with decreased immobility in the forced swim test in rats with congenital learned helplessness.[170] 5-HT_{2C} receptor agonists such as WAY 161503 and WAY 163909 show antidepressant like effects in multiple animal models of depression such as the rat forced swim test.[189] The 5-HT_6 receptor agonist LY586713 has been shown to upregulate expression of brain derived neurotrophic factor (BDNF) messenger ribonucleic acid (mRNA) in the hippocampus. The 5-HT_6 receptor is thus a candidate mediator of the up-regulation of BDNF gene expression associated with SSRI treatment.[189]

1.5.3.1 The concept of occupancy

By comparing PET BP data for a target molecule before and during treatment the fraction of target molecules occupied by the drug can be calculated. This can be related to e.g. the serum concentration of the drug (Study V), to clinical effect or to side effects. The approach is frequently applied in drug development to establish a suitable dosing interval in phase II trials.[61] It is thus of value to explore a possible relation between occupancy and clinical effect or side effect in a patient population. This relation is well studied for antipsychotics.[67, 68, 156] For antidepressants a 5-HTT occupancy of about 80% for many SSRIs and SNRIs has been suggested as being important for therapeutic effect.[87, 110, 142, 201] The number of studies where antidepressant occupancy of the 5-HTT has been studied in an MDD population is however quite small and the field would benefit from more research.

2. AIMS

The overall objective of the present thesis project was to develop and apply a new tool for quantification of 5-HTT in the living human brain. This aim was further specified as follows:

1. To evaluate [^{11}C]MADAM as a PET radioligand for quantification of 5-HTT with regard to selectivity, quantitative modelling properties and reproducibility.

2. To examine interindividual variability and regional co-regulation of the 5-HT$_{1A}$ receptor and 5-HTT , two important biomarkers in current hypotheses on the pathophysiology and treatment of MDD and anxiety disorders.

3. To examine the feasibility of using [^{11}C]MADAM to determine 5-HTT occupancy. R,S-citalopram and S-citalopram occupancy were compared in a single dose, double-blind, two-way cross over study.

3. MATERIALS AND METHODS

3.1 Subjects

3.1.1 Cynomolgus monkeys

One male and three female cynomolgus monkeys weighing between 3.4 and 6.7 kg participated in eight PET measurements (study I). They were supplied by the Swedish Institute for Infectious Disease Control, Solna. Study I was covered by animal ethical committee approvals for pharmacological PET studies on animals.

3.1.2 Human subjects

All studies involving human subjects (study II-V) were approved by the Ethics and Radiation Safety Committees of the Karolinska Hospital, and all subjects participated after giving informed consent. Altogether 21 male subjects, 22-55 years old, participated in a total of 54 PET experiments. They were all healthy according to history, psychiatric interview, physical examination, blood and urine analysis, magnetic resonance imaging (MRI) of the brain (study II-V) and Electro cardiogram (ECG; study V).

3.2 Chemistry

In study I-V the syntheses of *N*-demethyl-MADAM precursor (N-methyl-2-(2-amino-4-methylphenylthio)benzylamine) for the labeling of [^{11}C]MADAM as well as the synthesis of authentic MADAM were carried out as previously described.[164, 205, 216] In study IV, [^{11}C]WAY 100635 was prepared from ^{11}C-acylation of WAY 100634 with carbonyl-^{11}C-cyclohexanecarbonyl chloride as described previously.[80] Other chemicals were obtained from commercial sources and were of analytical grade wherever possible.

The specific radioactivity of [^{11}C]MADAM at the time of injection in study I varied between 34 and 652 GBq/mmol at the time of injection, corresponding to a total mass injected of 0.02-0.46 μg. In study II-V the specific radioactivity of [^{11}C]MADAM varied between 5.4-399 GBq/mmol, corresponding to a total mass injected of 0.2-11.9 μg. In study IV the specific radioactivity of [^{11}C]WAY 100635 varied between 7.4-49.5 GBq/mmol, corresponding to a total mass injected of 2.6-15.2 μg.

3.3 Positron emission tomography and magnetic resonance imaging

The PET system used was ECAT EXACT HR 47 (Siemens, Berlin and Munich, Germany), which was run in the three-dimensional mode.[218] The in-plane and axial resolution is about 3.8 and 4.0 mm respectively, FWHM. A Hanning filter with a cut-off frequency 0.5 of maximum was used, providing an in-plane resolution of 5.5 mm FWHM. Scatter correction was performed as described in the literature.[218] Attenuation correction was done using transmission scan data obtained for each subject. The

reconstructed volume was displayed as 47 sections with a center-to-center distance of 3.125 mm.

All human subjects (study II-V) were examined with MRI. The MRI system used was Signa, 1.5 T (GE Medical Systems, Milwaukee, Wisconsin). Two examinations are made in one session during 15 minutes, one T2-weighted for clinical evaluation regarding pathology and one T1-weighted for delineation of anatomical brain regions. The T2 sequence is a 2-D fast spin echo protocol with the following settings: relaxation time (TR) 5000 ms, echo time (TE) 68 ms, axial field of view (FOV) 260 mm, 260 x 260 matrix, 44 x 3.0 mm slices, 1 number of excitations (NEX) 4 min. The slice gap is set to 0.125 mm in order to make the center to center distance 3.125 mm witch is that of the PET images. The T1 sequence is a 3-D spoiled gradient recalled (SPGR) protocol with the following settings: TR 23 ms, TE 4 ms, flip angle 50 , FOV 260x180x156, matrix 256x192x156, 156 x 1.0 mm slices, 1 NEX 8 min 45 sec. The sequences are optimised for trade off between a minimum of scanning time and a maximum of spatial resolution.

To allow the same head positioning in the two imaging modalities, a head fixation system with an individual plaster helmet was used both in the PET and MRI measurements.[22] In study III-V a coregistration procedure was applied: for each subject, the MR-image was adjusted to position the anterior–posterior commissural (AC-PC) line in the horizontal plane, and the interhemispheric plane orthogonal to the AC-PC plane. It was resampled and cropped to generate a 256 x 256 x 141 matrix with $1mm^2$ pixels before it was used for manual definition of ROIs. The PET images were resampled to a 2 mm^2 pixel size and coregistered to a corresponding MRI half-resolution dummy. Coregistration was done using SPM2.[130]

3.4 Examination procedure

In study I, anesthesia in the cynomolgus monkeys was induced and maintained by repeated i.m. injection of a mixture (50%-50%) of ketamine (Ketalar, 5-10 mg/kg/hour) and xylazine hydrochloride (Rompun, 2-4 mg/kg/hour). A head fixation system was used to secure a fixed position of the monkey head during the PET measurements.[102] The monkey head was positioned so that the imaging planes were parallel to the canto-meatal line. Body temperature was controlled by a heating pad with thermostat. Breath rate, heart rate and body temperature was monitored regularly. A venous cannula was inserted and fixed in a sural vein and used for radioligand administration.

In study II-V the subject was placed recumbent with his head in the PET system. A sterile physiological phosphate buffer (pH=7.4) solution containing the radioligand was injected as a bolus during 2 seconds into a cannula inserted into the right antecubital vein. The cannula was then immediately flushed with 10 ml saline.

Brain radioactivity was measured in a series of consecutive time frames. After injection with [^{11}C]MADAM (study I-V), the examination lasted for 93 minutes and consisted of 20 frames (3 * 1’; 4 * 3’; 13 * 6’). After injection with [^{11}C]WAY100635 (study IV) the examination lasted for 69 minutes and consisted of 16 frames (3 * 1’; 4 * 3’; 9 * 6’).

3.5 Arterial blood sampling

In study II, an automated blood sampling system was used during the first five minutes of each PET measurement.[57] After the first five minutes, arterial blood samples (2 ml) were taken manually at the midpoint of each frame until the end of the measurement.[62]

3.6 Determination of radioactive metabolites in plasma

The fractions of plasma radioactivity corresponding to unchanged [^{11}C]MADAM and metabolites were determined in study I-II as has been described previously for other radioligands.[83] In brief, arterial (study II) or venous (study I) samples (2ml taken at 4, 10, 20, 30, 40 and 50 minutes (study II) or 4, 15, 30 and 45 minutes (study I)) were deproteinized with acetonitrile and analysed by gradient High performance liquid chromatography (HPLC) on a reverse-phase column (Waters μ-Bondapak C18; 7.8 x 300 mm, 10 μm) eluted at 6ml/min over 10 min with acetonitrile 0.01 M phosphoric acid, using a gradient of 10% acetonitrile from 0 to 5.0 min, 10-60% acetonitrile from 5.0 to 6.5 min and 60-10% acetonitrile from 6.5 to 10 min. Unlabelled MADAM was used as reference to provide retention times for possible labelled metabolites in plasma.

3.7 Regions of interest

In study I, regions of interest (ROIs) were defined on reconstructed and summated PET-images according to anatomical boundaries for whole brain, thalamus, mesencephalon, lower brainstem (pons and medulla oblongata), striatum, frontal-, parietal- and temporal lobes, and cerebellum. The anatomical delineation of ROIs was guided by an atlas of a cynomolgus monkey brain cryosectioned *in situ*[102] supplemented by the monkey brain atlas of Paxinos et al.[171]

In studies II-III and V, ROIs according to anatomical boundaries for frontal cortex, cingulate cortex, hippocampal complex, putamen, cerebellum (and in studies III and V also temporal cortex and insula) were defined on the MR images. In study IV ROIs were defined for neocortex, the hippocampal complex and cerebellum on the MR images. On MR images the raphe nuclei cannot be differentiated from surrounding tissue. Therefore, in study II-V these ROIs were delineated directly on the PET images.

To obtain the radioactivity concentration for the whole volume of interest, data for each ROI were pooled. Regional radioactivity was calculated for each frame, corrected for decay and plotted versus time, thus providing regional TACs.

3.8 Quantitative analysis

3.8.1 Kinetic modelling

In study II radioligand binding to 5-HTT was analysed using arterial plasma input function and compartment models. The general configuration is a conventional three tissue compartment model. The input function correspond to the radioactivity

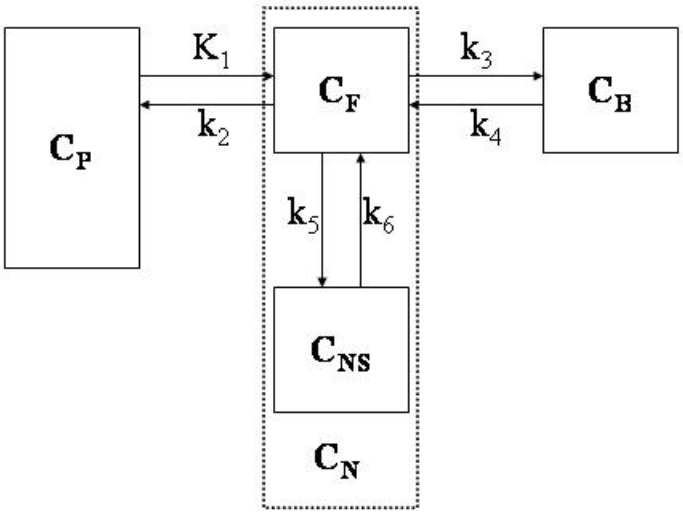


FIGURE 6. The two- (C_N and C_B) and three-tissue (C_F, C_{NS} and C_B) compartment models for radioligand uptake and binding in brain.

concentration of unchanged radioligand in plasma C_P and the three tissue compartments correspond to unbound radioligand in brain C_F, non-specifically bound radioligand in brain C_{NS}, radioligand specifically bound to receptors C_B and to six first-order rate constants (K_1, k_2, k_3, k_4, k_5, k_6; Figure 6). K_1 (ml/(ml min) and k_2 (1/min) correspond to the influx and efflux rates for radioligand diffusion through the blood-brain barrier, respectively. The rate constants k_3 (1/min) and k_4 (1/min) correspond to the rates for radioligand transfer between the compartments for non-displaceable and specific radioligand binding to receptors, respectively. Radioligand transfer between C_F and C_{NS} is described by k_5 and k_6 (both with the dimension 1/min). All compartments were assumed homogenous in concentration and all concentrations have the dimension nCi/ml.

A common assumption is that the two compartments C_F and C_{NS} equilibrate rapidly, thus forming one effective compartment[222] which corresponds to non-displaceable radioligand in brain C_N. Assuming that C_F and C_{NS} equilibrate rapidly, the model can be simplified into two tissue compartments and four first-order rate constants, K_1, k_2, k_3, k_4 (Figure 6). This model (2TM) was used to describe the TACs for [^{11}C]MADAM. The radioactivity concentration in plasma was not corrected for plasma protein binding. Based on this model, the following differential equations can be expressed:

$$dC_N(t)/dt = K_1 C_P(t) - (k_2 + k_3)C_N(t) + k_4 C_B(t) \quad (1)$$

$$dC_B(t)/dt = k_3 C_N(t) - k_4 C_B(t) \quad (2)$$

$$C_T(t) = C_N(t) + C_B(t) \quad (3)$$

where C_T is the radioactivity concentration in brain, corrected for CBV.

The 1TM is a simplification of the 2TM based on the assumption that all the compartments, C_F, C_{NS} and C_B, equilibrate rapidly to form one effective compartment, C_T. The 1TM was used as an alternative approach to describe the TACs for [^{11}C]MADAM.[109] Here K_1 corresponds to the influx rate of radioligand diffusion through the blood-brain barrier. The rate constant k_2' corresponds to the efflux rate, and its relation to k_2, k_3 and k_4 of the three-compartment model is given by equation 4:

$$k_2' = k_2/(1 + k_3/k_4) \quad (4)$$

In the 2TM analysis the four rate constants were determined by curve fitting in a non-linear least squares minimization procedure using the Simplex algorithm[36] with constraints for K_1 being restricted between 0 and 99.9, and for k_2, k_3 and k_4 between 0 and 9.9. The initial value for K_1, k_2, k_3 and k_4 was 0.5, 0.5, 2.0 and 0.5, respectively, and the local minimum of the sum of the squared residuals was determined by an iterative procedure.

Classical receptor binding parameters, such as receptor density B_{max} and affinity K_d cannot be differentiated on the basis of one PET measurement with high specific radioactivity.[146] The ratio B_{max}/K_d is often referred to as the binding potential (*BP*). It corresponds to the ratio k_3/k_4 in the kinetic analysis. In the present study *BP*s calculated directly from k_3 and k_4 of the three-compartment analysis are referred to as $BP_{Direct(1)}$.

A variant of the two tissue compartment analysis, 2TM(2), was applied with the assumption that C_N is identical among all ROIs. In this approach, the K_1/k_2 ratio was fixed to the ratio for the reference region obtained with the 1TM. The *BP*s calculated from the thereby acquired k_3 and k_4 values are referred to as $BP_{Direct(2)}$.

3.8.2 The Volume of Distribution: $BP_{Indirect}$

In study II [^{11}C]MADAM binding was also expressed using the concept of the total volume of distribution, which for the two-tissue compartment model is defined as

$$V_t = (K_1/k_2)(1 + k_3/k_4)\,. \quad (5)$$

The ratio of V_t in the ROI (V_t^{ROI}) and the V_t in the reference region (V_t^{REF}) was entered into the equation

$$BP_{Indirect} = (V_t^{ROI}/V_t^{REF}) - 1 \quad (6)$$

to calculate the $BP_{Indirect}$.

3.8.3 The Simplified Reference Tissue Model: BP_{SRTM}

According to the Simplified Reference Tissue Model (SRTM) which was applied in study II-V, the TAC for a reference region devoid of receptors can be used as an indirect approximation of the plasma input function, C_P. Two assumptions are made; the non-displaceable and specific compartments coalesce, because of high exchange rates between the two, and, the level of non-displaceable binding in the reference and target regions is similar. The expression includes the *BP* (referred to as BP_{SRTM}) and is solved in a convolution manner and fitted to the data in a least squares sense.[114]

3.8.4 The Linear Graphical Analysis: BP_{Logan}

In study II the linear graphic analysis for reversible ligand binding to receptors developed by Logan et al[124] was also used for analysis of [^{11}C]MADAM binding. The slope of the linear phase of the obtained plot corresponds to the total volume of distribution, V_t, of the ligand plus the plasma volume. The regional distribution volume (*DV*) was determined from the slope and the binding potential was calculated as follows:

$$BP = (DV^{ROI} / DV^{REF}) - 1. \quad (7)$$

3.8.5 The Transient Equilibrium Approach: BP_{EqA} and BP_{EqB}

The ratio C_B/C_N is equal to the *BP* when $dC_B/dt=0$. In this moment, i.e. the time of transient equilibrium, the number of molecules associating to the receptors is equal to the number dissociating.[62, 99, 160] In study II the radioactivity concentration in the cerebellum was used as an estimate for $C_N(t)$. $C_B(t)$ is defined as $C_T(t)$-$C_N(t)$, and the *BP* is referred to as BP_{EqA}. A flat shape of $C_B(t)$ may make it difficult to define time of peak equilibrium with high reliability. As an alternative approach, the area under the TAC for $C_B(t)$ and $C_N(t)$ during the time interval 57 to 93 minutes was used to calculate BP_{EqB}.

3.8.6 Receptor occupancy and drug plasma concentration

In study V the relationship between receptor occupancy and plasma concentration of the experimental compounds was examined. The relationship between receptor binding and the concentration of a radioligand or a drug at equilibrium can be described by the hyperbolic function

$$B = \frac{B_{\max} F}{K_{i,app} + F} \quad (8)$$

where *B* is the concentration of ligand bound to receptor, B_{max} the number of available receptors, *F* the concentration of unbound ligand and $K_{i,\ app}$, the apparent inhibition constant. The affinity is called apparent when serum concentration is used as an estimate for free fraction in blood (f_1), and when the concentration of endogenous ligand, i.e. 5-HT, and non-specific binding in brain (f_2) is not corrected for. In drug occupancy studies, equation 8 may be rewritten as

$$occupancy = \frac{occ_{\max} C_s}{K_{i,app} + C_s} \quad (9)$$

where occ_{max} is the maximal occupancy induced by the drug and C_s is the serum concentration of the drug. This is done under the assumption that there is a linear relationship between drug concentration in brain and serum so that F may be substituted with C_s. In the analysis, occ_{max} was fixed to 100%. The calculated 5-HTT occupancy was related to the mean serum concentration of the examined drugs at 6-8 h post dose, i.e. during the PET measurement, and $K_{i,\ app}$ was determined by means of a least square minimization procedure.

4. RESULTS AND COMMENTS

4.1 Study I: [^{11}C]MADAM, a New Serotonin transporter Radioligand Characterised in the Monkey Brain by PET

To explore the potential of the new selective 5-HTT inhibitor MADAM as a PET radioligand for examination of 5-HTT in the non-human primate brain MADAM binding was characterized by PET in four cynomolgus monkeys. The highest uptake of radioactivity was observed in striatum, thalamus, mesencephalon, thalamus, and the lower brainstem. Lower binding was detected in neocortex and the lowest radioactive uptake was found in the cerebellum. This distribution is in accordance with the known expression of 5-HTT *post mortem*. The fraction of the total radioactivity in monkey plasma representing unchanged [^{11}C]MADAM was 20% at 45 minutes after injection, as measured by gradient HPLC. Pretreatment measurements using unlabelled citalopram, a selective 5-HTT inhibitor, GBR 12909, a selective DAT inhibitor and maprotiline, a selective NET inhibitor, as well as a displacement measurement using unlabelled MADAM confirmed that [^{11}C]MADAM binds selectively and reversibly to 5-HTT, and support the use of the cerebellum as reference region.

The characterization of binding in the monkey brain suggested that [^{11}C]MADAM is a potential PET radioligand for quantitative studies of 5-HTT binding in the human brain.

4.2 Study II: Quantification of [^{11}C]MADAM Binding to the Serotonin Transporter in the Human Brain

The purpose of this study was to examine the radioligand [^{11}C]MADAM and its potential for quantitative PET studies of 5-HTT in applied clinical studies. PET examination was performed in each of nine male subjects after intravenous injection of [^{11}C]MADAM with high specific activity. A metabolite-corrected arterial input function was used in kinetic one- and two- tissue compartment analyses. Cerebellum was used as reference region in an examination of six reference tissue approaches.

The highest radioactivity concentration was detected in the raphe nuclei, followed consecutively by the striatum, the hippocampal complex, cingulate cortex, neocortex and the cerebellum. The time-curve for the fraction of unchanged [^{11}C]MADAM in plasma was best described by a sigmoid function. After 50 minutes, the fraction was 40%. The labelled metabolites were more polar than the mother compound. The two compartment model approaches converged, and could describe the time-activity curves in all regions (for example of regions, see figure 7). The total volume of distribution V_t was similar to the regional distribution volumes obtained by the linear graphic analysis. The BPs for six different approaches yielded similar values in all regions but the raphe nuclei, where the two equilibrium methods provided lower values.

The regional binding distributions of this study are consistent with *post mortem* data acquired with [^{3}H]MADAM[41] as well as with other reference ligands *in vitro*.[15, 48, 118, 179] The TACs were well described by current major quantitative approaches and the

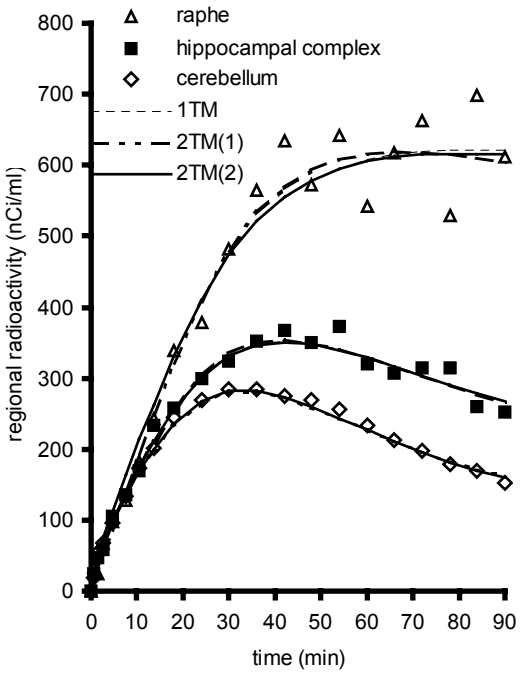


FIGURE 7. Experimental values of regional activity and corresponding fitted curves obtained by the 1TM, the unconstrained 2TM (2TM(1)) and the 2TM with the K_1/k_2 ratio given by that of cerebellum (2TM(2)).

suitability of the cerebellum as a reference region could be confirmed. It was concluded that simplified methods such as the SRTM may be advantageous for applied clinical studies

4.3 Study III: Measurement of Serotonin Transporter Binding With PET and [^{11}C]MADAM: A Test-Retest Reproducibility Study

The purpose of this study was to examine the test-retest reproducibility of [^{11}C]MADAM using a design tailored for future applied studies. Nine healthy male subjects were examined with PET and [^{11}C]MADAM at baseline conditions on two occasions four to eight weeks apart. The subjects participated in a Phase 1 trial (study V) to which the present study was an addendum. Test-retest data were calculated from BPs, and included BP quotient, BP difference and the intraclass correlation coefficient. The quotient was about one in all regions and the mean difference varied between 0 and 11 percent. The intraclass correlation coefficient varied between 0.96 and 0.51 in the raphe nuclei and averaged bilateral regions (Table 5). [^{11}C]MADAM was shown to have good to excellent reliability in measurements of 5-HTT binding in brain regions of interest in research on psychiatric disorders

4.4 Study IV: Indication of co-regulation: a PET examination of 5-HT$_{1A}$ receptors and 5-HTT in the human brain

The 5-HT$_{1A}$ receptor and 5-HTT are of central interest in psychopharmacology. The 5-HTT is the target for SSRI which have a well documented effect in the treatment of

TABLE 5. Test-retest characteristics of [^{11}C]MADAM binding to the serotonin transporter

	BP PET 1		BP PET 2		quotient PET2/PET1		difference (%)		ICC
	mean±SD	CV(%)	mean±SD	CV(%)	mean±SD	range	mean±SD	range	
Raphe nuclei	2.53±0.49	19	2.51±0.65	26	1.00±0.20	0.64-1.30	0±20	-30-36	0.51
Putamen	0.96±0.25	26	0.90±0.22	24	0.97±0.23	0.63-1.35	3±23	-35-37	0.51
dx	0.96±0.24	25	0.89±0.19	21	1.10±0.32	0.70-1.83	3±24	-43-45	0.39
sin	0.99±0.26	27	0.91±0.27	30	1.12±0.29	0.77-1.55	5±25	-30-35	0.50
Insula	0.47±0.08	16	0.46±0.06	13	0.98±0.15	0.77-1.25	2±15	-25-23	0.51
dx	0.47±0.09	19	0.46±0.08	18	1.06±0.25	0.62-1.52	1±26	-61-34	0.06
sin	0.50±0.08	17	0.44±0.08	18	1.17±0.24	0.89-1.57	11±17	-12-36	0.17
Anterior cingulum	0.41±0.15	37	0.37±0.11	30	0.96±0.26	0.71-1.51	4±26	-51-29	0.79
dx	0.38±0.14	37	0.37±0.15	42	1.35±1.16	0.51-4.34	-3±47	-97-77	0.68
sin	0.44±0.18	40	0.38±0.15	38	1.23±0.47	0.76-2.18	9±30	-31-54	0.68
Hippocampus	0.33±0.09	26	0.35±0.10	27	1.07±0.16	0.85-1.26	-7±16	-26-15	0.82
dx	0.36±0.16	44	0.38±0.12	32	0.94±0.26	0.60-1.44	-14±33	-67-31	0.67
sin	0.37±0.17	44	0.34-0.16	47	1.26±0.82	0.57-3.22	-2±48	-77-69	0.12
Temporal cortex	0.20±0.08	41	0.19±0.09	50	0.93±0.10	0.77-1.08	7±10	-8-23	0.96
dx	0.18±0.07	42	0.16±0.05	33	1.18±0.50	0.59-2.24	3±37	-69-55	0.56
sin	0.23±0.08	33	0.20±0.05	26	1.19±0.28	0.83-1.57	11±21	-21-36	0.52
Frontal cortex	0.18±0.13	70	0.17±0.10	56	1.04±0.21	0.82-1.46	-4±21	-46-17	0.96
dx	0.16±0.13	79	0.12±0.06	48	1.45±0.93	0.41-3.69	6±62	-146-73	0.33
sin	0.20±0.11	55	0.19±0.07	40	1.13±0.41	0.75-1.89	2±29	-34-47	0.59

depression and anxiety disorders.[50, 214] The 5-HT$_{1A}$ receptor antagonist pindolol has been suggested to accelerate the onset of the antidepressant effect of SSRIs [11, 18, 78].

The short (S) allele of the frequent 5-HTT gene (SLC6A4) polymorphism, 5-HTTLPR (5-HTT gene-linked polymorphic region) has been associated with restricted transcriptional activity *in vitro*. [45] Interestingly, carriers of the S allele have also been shown to have an increased frequency of anxiety and mood disorders.[40, 85, 122, 134] In addition, it has been reported that carriers of the S allele have a lower 5-HT$_{1A}$ receptor density.[52] This finding suggests a dependency between the expression levels of these two markers for serotonergic neurotransmission.

The possible relationship between 5-HT$_{1A}$ and 5-HTT gene expression levels has to some extent been approached experimentally. In 5-HTT knock out (KO) mice, both 5-HT$_{1A}$ receptor proteins and mRNA has been shown to be decreased in the DRN, increased in the hippocampus and unchanged in other forebrain areas.[60] A pharmacological examination of the 5-HT$_{1A}$ receptor mediated cell activity in 5-HTT

KO mice showed correspondingly a desensitisation in the DRN but no alteration in the hippocampus.[133] In an autoradiography study on prefrontal cortex of suicide victims and control subjects a negative correlation was found between the two markers, suggesting common regulatory factors.[10]

The aim of this work was a direct comparison of regional expression levels for these two serotonergic markers in the human brain *in vivo*. Eight male control subjects were examined with PET twice on the same day, using the radioligands [^{11}C]WAY 100635 and [^{11}C]MADAM for quantification of the 5-HT1A receptor and the 5-HTT respectively. BP was calculated for raphe nuclei, hippocampus and neocortex. In all regions the BP for both radioligands as well as the quotient between the two varied severalfold between subjects. There was a trend towards a positive correlation of the binding potentials in raphe ($r_{xy} = 0.69$, $p = 0.06$) but not in hippocampus ($r_{xy} = 0.62$, $p = 0.10$) or neocortex ($r_{xy} = 0.028$, $p = 0.95$; Figure 8). The results support a correlation in expression levels of the 5-HT$_{1A}$-receptor and the 5-HTT in the raphe nuclei but not in serotonergic projection areas. The interindividual variability in 5-HT$_{1A}$-receptor/5-HTT quotients may be further explored in relation to the individual response to SSRI treatment.

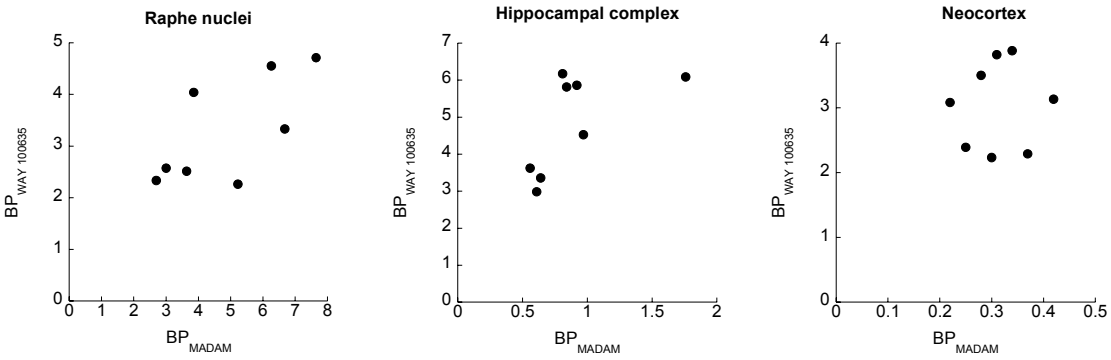


FIGURE 8. Plots showing the relation between binding potentials (BP) for [^{11}C]MADAM and [^{11}C]WAY 100635 for the eight subjects in the raphe nuclei, the hippocampal complex and neocortex.

4.5 Study V: PET Measurement of Serotonin Transporter Occupancy: A Comparison of Escitalopram and Citalopram

The SSRI citalopram (R,S-citalopram) is a racemic compound of two enantiomers. On the basis of *in vitro* studies, inhibition of the human 5-HTT is achieved by the S-enantiomer (S-citalopram or escitalopram).[163] Interestingly, when the amounts of S-citalopram are equal the onset of effect of S-citalopram alone has been shown to be faster compared to that of R,S-citalopram, both in a non-clinical study using a rat behavioural model and in a clinical study on patients with major depressive disorder .[150] This difference in time to response, and in the proportion of responders has been replicated in several clinical studies.[34, 46, 121, 151] The exact molecular mechanism for this effect of R-citalopram on the pharmacodynamics of S-citalopram is not fully understood. One suggested mechanism is that R-escitalopram attenuates the binding of

S-citalopram to the 5-HTT,[187] possibly due to a conformation change resulting from R-citalopram binding to an allosteric site.[43]

The aim of the present PET study was to compare serotonin transporter (5-HTT) occupancy after single equimolar doses (with respect to S-enantiomer) in man *in vivo* of R,S-citalopram (20 mg) and S-citalopram (10 mg) using–PET and the radioligand [^{11}C]MADAM. The design was a single-dose, double-blind, two-way crossover study in eight healthy male subjects. The 5-HTT binding potential at baseline and after single doses of study drugs was used to calculate 5-HTT occupancy in seven brain regions. Serum concentrations of the study drugs were determined in order to calculate the apparent inhibition constant ($K_{i,\ app}$), a secondary parameter of interest for the comparison.

In all brain regions examined, occupancy was numerically higher after treatment with R,S-citalopram (overall comparison: F=14.8, df=1,90, p<0.001, Figure 9)**.** In line with this also the apparent inhibition constant was significantly lower for R,S-citalopram than for S-citalopram (overall comparison: F=6.7, df=1,90, p<0.05). The small but significant difference in occupancy and $K_{i,\ app}$ found between R,S-citalopram and S-citalopram suggests that not only S-citalopram but also R-citalopram to some degree occupies the 5-HTT in the human brain *in vivo*.

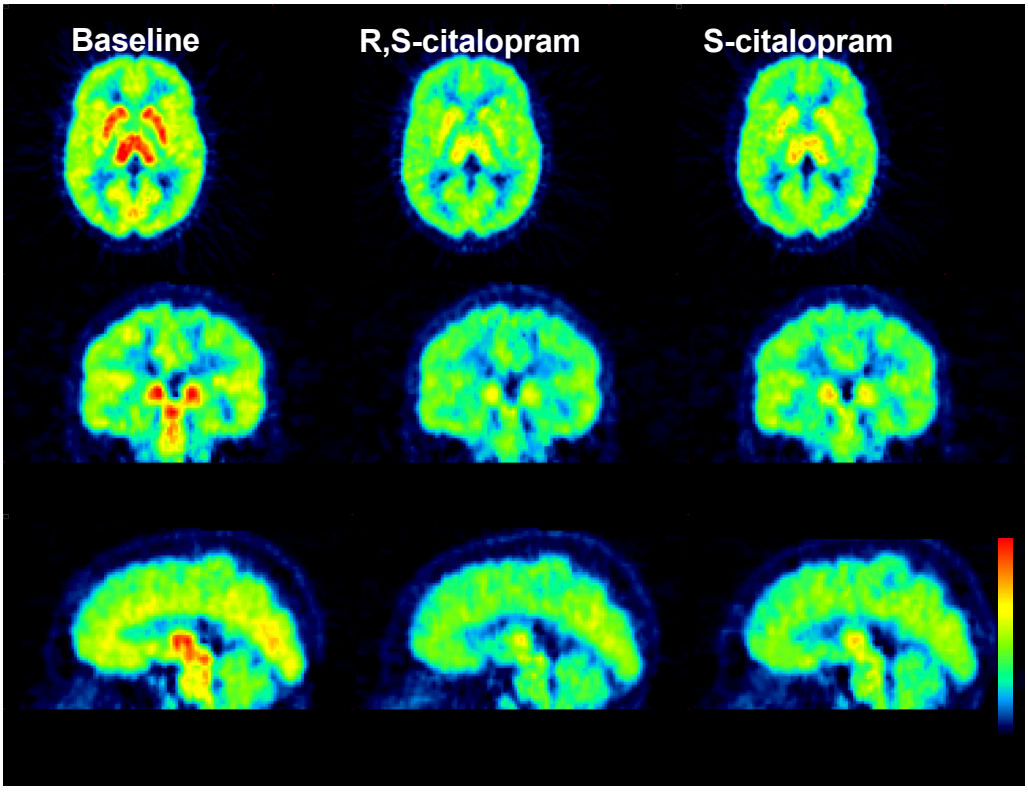


FIGURE 9. Summation images based on data from frame 6 to 20 showing regional radioactivity after intra venous injection with [^{11}C]MADAM at baseline conditions (left), after treatment with 20 mg R,S-citalopram (middle) and after treatment with 10 mg S-citalopram (right). The projections are transaxial (top), coronal (middle) and sagittal (bottom).

5. SUMMARY OF FINDINGS

5.1 On [^{11}C]MADAM

It could be shown that [^{11}C]MADAM binds reversibly and selectively to the 5-HTT in the primate brain.

[^{11}C]MADAM binding in neocortical, limbic and striatal regions as well as in the raphe nuclei could be described by the two tissue compartment model. The rank order of the BP calculated by reference tissue models was in accordance with the rank order reported in binding studies on human brain tissue post mortem.

The validity of C_N in cerebellum as an index for C_N in other regions could be confirmed in pretreatment studies using citalopram in the monkey brain. In the human brain, the 1TM was equally good as the 2TM in 7 out of 9 subjects examined supporting the model assumption of a rapid equilibrium between C_{NS} and C_F in the cerebellum, i.e. a characteristic of a region devoid of specific binding. These findings support *in vitro* data suggesting cerebellum to have negligible 5-HTT density thus making it a favoured reference region in clinical studies where simplified quantitative approaches are preferred.

The reliability of 5-HTT quantification was shown to be good to excellent in most regions. However, the reliability did show regional variability, and in some cases pooling of data for bilateral regions s required to improve accuracy.

5.2 On the expression levels of 5-HTT and 5-HT$_{1A}$ receptor in the human brain

A correlation between 5-HTT and 5-HT$_{1A}$ receptor was noted in the raphe nuclei but not in target regions. As the two markers of the 5-HT system are situated on the same neurons in the raphe nuclei, the finding suggests a common regulatory mechanism.

The quotient between the two markers varied markedly in the raphe nuclei. This suggests that they should be used in parallel in future studies when used as markers for the 5-HT system. Since these markers both are suggested to be involved in the mechanism of action of antidepressant treatment, the finding could in part explain the clinical variability in SSRI treatment response.

5.3 On 5-HTT occupancy of escitalopram and citalopram

Citalopram (R,S-citalopram) was shown to give higher occupancy and to have lower $K_{i,app}$ than escitalopram (S-citalopram). This finding suggests R-citalopram to have higher affinity to 5-HTT *in vivo* than suggested from *in vitro* studies. Whether this binding takes place at an allosteric or similar site as S-citalopram binding could not be addressed in this study.

6. FINAL REMARKS AND FUTURE PERSPECTIVES

The present characterisation of [^{11}C]MADAM suggests it to be a suitable radioligand for PET studies on the 5-HTT in the living human brain. Further studies using [^{11}C]MADAM are thus of great interest.

First, the effect of endogenous ligand on [^{11}C]MADAM binding is not well known. The high k_4 found in study II suggest it to be sensitive to 5-HT concentrations. This hypothesis should be addressed in an experiment where the 5-HT concentrations may be distinctly manipulated pharmacologically. Published data for the structurally similar radioligand [^{11}C]DASB are hitherto inconclusive.[144, 181, 204]

Second, BP reflects the ratio of B_{max} and K_d. In order to translate findings in PET using [^{11}C]MADAM to a biological parameter such as B_{max} repeated experiments with low and high specific activity of [^{11}C]MADAM has to be performed. In this way saturability of radioligand binding to 5-HTT may be demonstrated and the binding parameters can be determined by the Scatchard plot.[64]

Data on small regions such as the raphe nuclei are sensitive to PVE. In the case of [^{11}C]MADAM and [^{11}C]WAY 100635 they both have a strong signal compensating for this to some extent. Also, in both study IV and V, the two sets of raphe data compared did not differ significantly in terms of volume, diminishing the risk of PVE affecting the result of the statistical analysis. Still, any PVE correction was not applied as the raphe nuclei is not possible to define in MR-images. Major improvements in the analysis of small regions such as the raphe nuclei will however be available in the near future with the application of the HRRT, a PET instrument with higher resolution.[219]

The possible relation between clinical effect of antidepressant treatment and occupancy of the 5-HTT should be further examined. On-going work aims at describing this in a wide range of antidepressants including TCAs.

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