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**THE BRAIN SEROTONIN TRANSPORTER
BINDING IN YOUNG ADULTS;
METHODOLOGICAL CONSIDERATIONS
AND ASSOCIATION WITH BULIMIA
NERVOSA AND ACQUIRED OBESITY**

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ACADEMIC DISSERTATION

**To be publicly discussed with the permission of the Medical Faculty of the
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ABSTRACT

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The brain serotonin transporter binding in young adults; methodological considerations and association with bulimia nervosa and acquired obesity

Serotonin (5-HT) is one of the brain neurotransmitters, and it modulates many functions important for life, including appetite, body temperature, sexual drive and circadian rhythms. It is also involved in controlling the development of the neural system during gestation and infancy, and is likely to play a role in adult neurogenesis. Disturbed 5-HT function is implicated in several psychiatric disorders, including mood, anxiety and eating disorders. Its actions on feeding behavior make it also an interesting target in obesity research. The amount of effective 5-HT in the extracellular space is controlled by the serotonin transporter (SERT), which terminates 5-HT's action by removing it from the extracellular space. Medications acting on SERTs are widely used in treatment of psychiatric disorders, and to some extent also as antiobesity drugs. In vivo investigations of the brain SERTs are possible by using the radionuclide imaging methods single photon emission tomography (SPET) and positron emission tomography (PET).

The aim of this thesis was to investigate methodological aspects of SERT imaging with SPET. This was achieved by comparing different methods for defining target regions, and by investigating the existence of physiological seasonal variation in SERT binding between summer and winter scans. Further aims included investigating the association of SERTs and Bulimia Nervosa, and the association between SERTs and acquired obesity.

The study population consisted of young adults, most of whom were monozygotic (MZ) or dizygotic (DZ) twins recruited from the national FinnTwin16 twin cohort. Two radioligands for SERT imaging, [¹²³I]ADAM and [¹²³I]nor-β-CIT, were used. The first study validated the use of an automated brain template in the analyses of [¹²³I]ADAM images. The second study investigated within-subject variation in SERT binding of [¹²³I]ADAM between scans done in summer and winter, and found no systematic variation in the regions investigated (midbrain and thalamus). The third and fourth studies applied twin study designs. The third study compared SERT binding of [¹²³I]ADAM between BN women, their unaffected co-twin sisters (MZ or DZ), and unrelated healthy twin women. No significant differences were found between the three groups in the midbrain or thalamus areas, and the unaffected co-twins had similar SERT binding as the unrelated healthy control women in both investigated areas. In *post hoc* analyses, a subgroup of purging BN women had significantly higher SERT binding in the midbrain as compared to all healthy women. In the fourth study, MZ twin pairs were divided into twins with higher body mass index (BMI) and co-twins with lower BMI; twins with higher BMI were found to have higher SERT binding of [¹²³I]nor-β-CIT in the hypothalamus/thalamus than their leaner co-twins.

Based on our results, the following conclusions can be made: 1) No systematic seasonal variation exists between SERT binding in summer and winter in the midbrain and thalamus regions. This further suggests that seasonal variation does not need to be considered as significant confounding factor in studies assessing SERT binding in these areas. 2) In a population-based sample, BN does not associate with altered SERT status as such, but in purging BN women such alterations are possible. 2) The higher SERT binding in MZ twins with higher BMIs as compared to their leaner co-twins suggests non-genetic effect of body weight and acquired obesity on the brain SERT binding and the 5-HT system, which may have implications regarding feeding behavior and satiety. These studies add to the existing literature on physiological regulation of SERTs, association between 5-HT function and BN, and 5-HT function and obesity.

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindolacetic acid
5-HT	Serotonin, 5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
5-HTTLPR	5-HTT gene-linked polymorphic region
A ₃ ARs	A ₃ adenosine receptors
ACh	Acetylcholine
[¹²³ I] ADAM	[¹²³ I]-2-((2-((dimethylamino)-methyl)phenyl)thio)-5-iodophenylamine
AgRP	Agouti Related Protein
AN	Anorexia Nervosa
ATD	Acute tryptophan depletion
BED	Binge Eating Disorder
BDNF	Brain derived neurotrophic factor
B _{max}	Total concentration of receptors
BMI	Body Mass Index
BN	Bulimia Nervosa
BP	Binding potential (B _{max} / K _d)
cAMP	cyclic adenosine monophosphate
[¹¹ C]5-HTP	5-Hydroxy-L-[β- ¹¹ C]tryptophan
[¹¹ C]-αMtrp	[¹¹ C]-α-methyl-L-tryptophan
CBT	Cognitive behavioral therapy
CNS	Central nervous system
COMT	Catechol- <i>O</i> -methyl transferase
CREB	cAMP response element-binding
CT	Computed tomography
DA	Dopamine
DAG	Diacylglycerol
DAT	Dopamine transporter
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth edition
DV	Distribution volume
DVR	Distribution volume ratio
DZ	Dizygotic
17β-E	17β-estradiol
E	Epinephrine
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
[¹²³ I]β-CIT	[¹²³ I]methyl 3 beta- (4-iodophenyl) tropane-2 beta-carboxylate
[¹²³ I]nor-β-CIT	[¹²³ I]2beta-carbomethoxy-3beta-(4-iodophenyl)
IL-1β	interleukin 1 beta
ICC	Intra-class correlation coefficient

IP ₃	inositol- <i>tris</i> -phosphate
kcts	kilocounts
K _d	Equilibrium dissociation constant
keV	kiloelectronVolt
MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase
MBq	MegaBecquerel
MD	Medical doctor
MDD	Major depressive disorder
MDMA	3,4-methylenedioxy-N-methylamphetamine (extasy)
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
α-MSH	α-Melanocyte stimulating hormone
MZ	Monozygotic
mSv	milliSievert
NE	Norepinephrine
NET	Norepinephrine transporter
OCD	Obsessive compulsive disorder
P	Progesterone
PET	Positron emission tomography
PK	Proteinkinase (PKA, PKC, PKG)
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PP	Protein phosphatase (PP1, PP2)
PTK	Protein tyrosine kinase
PVE	Partial volume effect
ROI	Region of interest
SAD	Seasonal affective disorder
SBR	Specific binding ratio
SERT	Serotonin transporter (SLC6A4, 5-HTT)
SLC6A4	Gene coding for serotonin transporter
SNP	Single nucleotide polymorphism
SPE(C)T	Single photon emission (computed) tomography
SPM	Statistical Parametric Mapping
SRTM	Simplified reference tissue model
SSAGA	Semi-Structured Assessment for the Genetics of Alcoholism
SSRI	Selective serotonin reuptake inhibitor
SUR	Specific uptake ratio (= SBR)
TNF-α	Tumor necrosis factor-alpha
TRP	Tryptophan
VNTR	Variable number tandem repeat
VOI	Volume of interest

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to in the text by the Roman numerals I-IV:

I Kauppinen T., Koskela A., Diemling M., Keski-Rahkonen A., Sihvola E., Ahonen A.: Comparison of manual and automated quantification methods of [¹²³I]ADAM. *Nuklearmedizin* 2005; 44:205-212.

II Koskela A., Kauppinen T., Keski-Rahkonen A., Sihvola E., Kaprio J., Rissanen A. and Ahonen A.: The brain serotonin transporter binding of [¹²³I]ADAM: within-subject variation between summer and winter data. *Chronobiology Int* (in press).

III Koskela A. K., Keski-Rahkonen A., Sihvola E., Kauppinen T., Kaprio J., Rissanen A., Ahonen A.: Serotonin transporter binding of [¹²³I]ADAM in bulimic women, their sisters and healthy women: A SPET study. *BMC Psychiatry* 2007;7:19.

IV Koskela A. K., Kaurijoki S., Pietiläinen K.P., Karhunen L., Kuikka J., Kaprio J. and Rissanen A.: Serotonin Transporter Binding and Acquired Obesity – an Imaging Study of Monozygotic Twin Pairs. *Physiol Behav* 2008;93:724-32.

1. INTRODUCTION

Serotonin (5-HT) is one of the brain monoamine neurotransmitters, and it modulates the homeostasis of several systems that are important for life, e.g., body temperature, appetite, sexual drive, and circadian rhythms. Its actions are also implicated in modulation of emotions, cognition, motor function and pain (1). Furthermore, recent studies have shown that it affects the development of the neural system during gestation and infancy (2) as well as neurogenesis in adults (3). Disturbances of 5-HT function are believed to play a role in the pathophysiology of many psychiatric disorders, e.g. anxiety (4), mood disorders (5), eating disorders (6), obsessive compulsive disease (OCD) (7) and impulsivity and aggression (8,9). Medications acting on 5-HT system are used in treatment of these disorders (10), as well as anti-obesity drugs (11).

5-HT was established as a neurotransmitter in the 1950's, and its role in the development of mental illnesses was first suggested in the 1960's (12). Since then thousands of studies have aimed to investigate its role. The methods for studying the 5-HT system have evolved during this time. While animal models have allowed direct experiments of the brain 5-HT system, their results cannot be directly applied to humans due to species differences. Until recently, central 5-HT system in humans could only be investigated indirectly, by measuring peripheral responses to agents influencing the central 5-HT system; direct investigations were possible only in *post mortem* brains. Since the early 1990's, *in vivo* studies of brain neurotransmitter systems have been performed using imaging methods applying radioactive isotopes, i.e. single photon emission tomography (SPET) and positron emission tomography (PET). Initially, the number of suitable radioligands was small, allowing limited studies on few neurotransmitter systems. During the last few years, the number of available targets and ligands has increased. This development is sure to continue, and we are only beginning to understand all the things that need to be considered when investigating these complicated systems.

Eating disorders Anorexia Nervosa (AN) and Bulimia Nervosa (BN) are important psychiatric disorders affecting predominantly adolescent girls and women (13), while the most common eating disorder, Binge Eating Disorder (BED), affects both men and women (14). Given 5-HT's role in the regulation of appetite (11), anxiety (4) and impulsive behavior (15), 5-HT could theoretically play a role in the pathophysiology of eating disorders. Several studies investigating indirect measures of central 5-HT function have supported its role both in AN and BN (6), and in BN, medications acting on the 5-HT system are known to alleviate symptoms (16). To date, only few imaging studies have investigated the brain 5-HT system in eating disorders, and some uncertainty remains regarding 5-HT's role. Furthermore, if dysfunction of the 5-HT system underlies eating disorders, it is of interest to know whether disturbances are present before the onset of symptoms, representing an eating disorder specific endophenotype, i.e., genetic neurobiological vulnerability for the disorder.

Obesity is one of the main health issues of our time, developing as a consequence of imbalance between ingested and expended energy. The rapid increase in its prevalence suggests a major involvement of environmental factors, of which excessive supply of food together with western sedentary lifestyle is considered as most important (17). However, the complicated mechanisms behind appetite and feeding behavior, involving interplay of peripheral and central control mechanisms (18), are still not fully understood. In brain, more than forty signalling molecules affecting feeding behavior are known (19), and 5-HT is one of them, having an anorexigenic effect. Given the rapid spread of obesity and its role as a major risk factor for several chronic diseases and associated mortality, understanding all the causative mechanisms is of paramount importance. Despite this, only few neuroimaging studies on obesity and neurotransmitters have been published to date, and they have concentrated on the dopamine system (20-23).

This thesis focuses on the brain serotonin transporters (SERTs), which are proteins responsible for reuptake of 5-HT from the extracellular space and thus control the amount of effective 5-HT. The work presented concentrates on methodological aspects as well as SERTs' association to BN and acquired obesity in young adults.

2. REVIEW OF THE LITERATURE

2.1 SEROTONIN (5-HT)

2.1.1. Overview

Serotonin (5-hydroxytryptamine, 5-HT), is one of the brain neurotransmitters. It belongs to the group of biogenic amine neurotransmitters, including also the catecholamines dopamine (DA), epinephrine (E) and norepinephrine (NE). However, 5-HT also exists and has functions outside the central nervous system (CNS), e.g., in the gastrointestinal system, platelets and mast cells. The greatest concentration of 5-HT (approximately 90 %) is found in the gastrointestinal system, and only 1-2 % of the 5-HT is found from the CNS (24). It was discovered in the early 1930's from the rabbit gastric mucosa and named enteramine, and later on isolated and thereafter named as serotonin in 1948. The name serotonin originates from its initial discovery as a vasoconstrictor substance in blood serum. It was established as a neurotransmitter in the early 1950's, and its role in the brain development and mental illnesses was suggested in the 1950's and 1960's (12).

The importance of 5-HT as a neurotransmitter is highlighted by the fact that it has the highest number, altogether 14, of receptors of any of the neurotransmitters (25). 5-HT regulates the homeostasis of many systems important for life, e.g., body temperature, appetite, sexual drive, sleep and circadian rhythms. Furthermore, 5-HT is known to modulate emotion, cognition, motor function and pain sensitivity. In addition to its role as a neurotransmitter, 5-HT has an important role in the development of neural system. Pharmacological and molecular genetic studies have shown that during gestation and infancy 5-HT can modulate a number of developmental processes, including neurogenesis, axon branching, dendritogenesis and apoptosis (2). 5-HT also has demonstrable effects on synaptic plasticity and adult neurogenesis (3,26,27). Furthermore, disturbance of 5-HT function has been implicated in many psychiatric disorders including e.g., major depressive disorder (MDD) (5), anxiety (4), Anorexia and Bulimia Nervosa (6), obsessive compulsive disease (7), autism (28), and impulsivity and aggression (8,9).

2.1.2. 5-HT synthesis and degradation

5-HT consists of a five member ring containing nitrogen joined to a benzene ring. It is synthesized from the essential amino acid tryptophan (TRP), derived primarily from the diet. Both TRP and 5-HT belong to a group of aromatic compounds called the indoles (24).

5-HT does not cross the blood brain barrier, hence it must be synthesized in the CNS. Both TRP and its next derivative before 5-HT, 5-hydroxytryptophan (5-HTP),

can cross this barrier. TRP is transported across the blood brain barrier by an active uptake process performed by the large neutral amino acid carrier. Not only TRP uses this transport mechanism, but also other large amino acids, e.g., tyrosine, phenylalanine, leucine, isoleucine and valine, compete for the same transport process. Therefore, not only the plasma concentration of TRP, but also its ratio to other competing large amino acids, affects the amount of TRP in brain. Nevertheless, the carbohydrate and protein content of the diet affect the plasma TRP concentration and have an effect on the 5-HT synthesis in the brain. The plasma TRP level has circadian rhythmic variation, which probably leads to some circadian variation also in the 5-HT synthesis. (24).

Two enzymatic steps are needed for the synthesis of 5-HT from TRP. Firstly, TRP is oxidized to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase. Secondly, 5-HTP is decarboxylated by the enzyme 5-hydroxytryptophan decarboxylase to yield 5-HT (24). The oxidation of TRP by TRP-hydroxylase is the rate limiting step of 5-HT synthesis. The activity of TRP-hydroxylase is affected by the amount of its substrate TRP as well as by the amount of available oxygen, as TRP-hydroxylase requires oxygen to function. The end product of the enzymatic step, 5-HTP, does not affect the activity of TRP-hydroxylase, neither does the amount of 5-HT nor the metabolic end product of 5-HT's catabolism, 5-hydroxyindolacetic acid (5-HIAA). On the other hand, the activity of 5-HT neurons can affect the functional capacity of TRP-hydroxylase, serving as an autoregulative factor (24). Pharmacologic inhibition of TRP-hydroxylase (e.g., by p-chlorophenylalanine) reduces the brain 5-HT content by 80 %.

The catabolism of 5-HT also requires two steps and enzymes. After being reuptaken from the extracellular space into the presynaptic terminal by the serotonin transporter (SERT), 5-HT can be deaminated by the enzyme monoamine oxidase (MAO), yielding 5-hydroxyindoleacetaldehyde. This can be further oxidized by the enzyme acetaldehyde hydrogenase to 5-HIAA or reduced to 5-hydroxytryptophol (24).

2.1.3. 5-HT neurons

A simplified illustration of the serotonergic pathways is depicted in **Figure 1**. The nuclei of the serotonergic neurons are mainly located along the midline of the brainstem from the midbrain to the medulla. The shared name, raphe, for the nuclei, is derived from this midline location (raphe, French for seam). Altogether nine raphe nuclei, numbered B1-9, have been described. The nuclei in the midbrain and pons (B4-9), including the dorsal, median and pontine raphe nuclei, project to the upper brainstem, hypothalamus, thalamus, and cerebral cortex. The nuclei in the medulla (B1-3), corresponding to the raphe magnus, raphe pallidus and raphe obscurus, project to the lower brainstem and the spinal cord. The rostral nuclei participate in regulation of the sleep-wake cycles, affective behavior, food intake, thermoregulation, and sexual behavior. The neurons in the lower pons and medulla participate in regulating the perception of pain and the tone in motor systems (1). Some serotonergic cell bodies can be found also outside the raphe nuclei, and not all cell bodies in the raphe nuclei are

serotonergic (29). For the forebrain functions, the dorsal (B6-B7) and median raphe (B8-B9) nuclei in the midbrain are most important. There are functional and morphologic differences between the serotonergic and non-serotonergic neurons of these two nuclei groups (29), and afferent connections exist between them. In the dorsal raphe, there are dendro-dendritic synaptic contacts between the serotonergic neurons, suggesting local autoregulatory interaction between the neurons. The dorsal and median raphe nuclei also get afferents from other cell body groups in the brainstem, such as the substantia nigra and ventral tegmental area (dopamine), superior vestibular nucleus (acetylcholine), locus coeruleus (norepinephrine), nucleus prepositus hypoglossi and nucleus of the solitary tract (epinephrine). Other afferents include neurons from the hypothalamus, cortex and the limbic forebrain structures, e.g., amygdala (29).

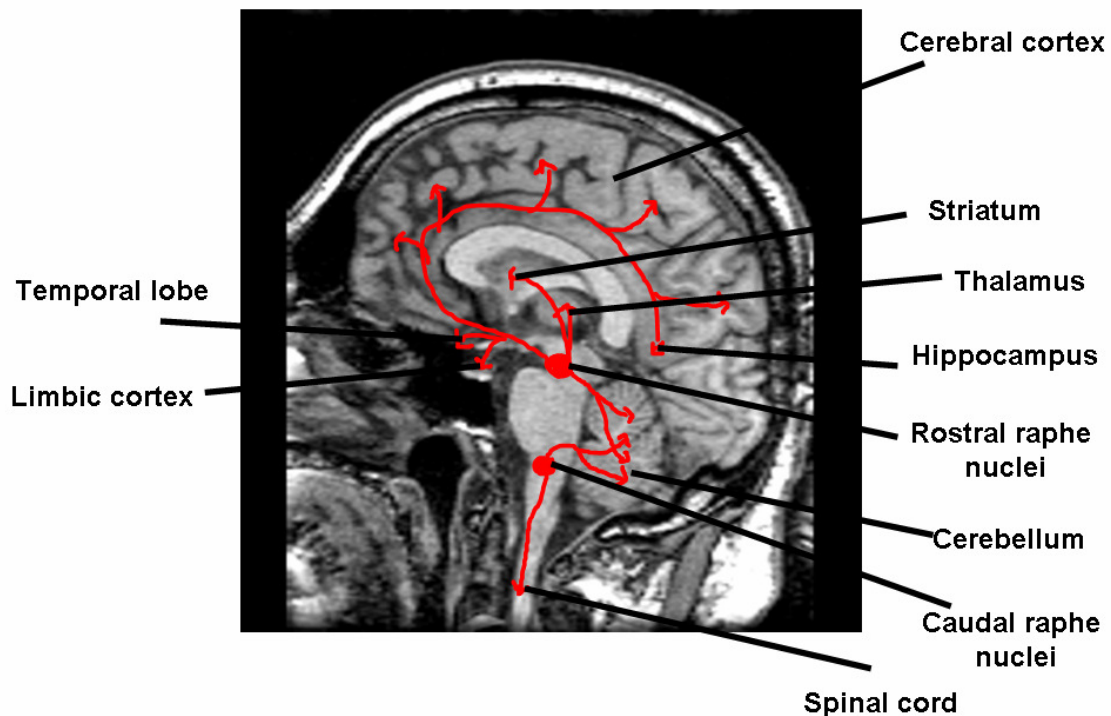


Figure 1. The serotonergic pathways

2.1.4. 5-HT receptors

There are at least 14 different 5-HT receptors in seven receptor subclasses (**Table 1**). While all receptor subtypes are found postsynaptically, 5-HT_{1A} and 5-HT_{1B} are also found on presynaptic cell bodies and dendrites, where they function as autoreceptors (30).

Except for the 5-HT₃ receptor subtype, which is a cation channel, all other 5-HT receptors are heptahelical transmembrane proteins that exert their action through G-proteins on second messenger systems. The 5-HT₁ subclass, 5-HT₄, 5-HT₅, 5-HT₆ and

5-HT₇ all act through the adenylyl cyclase – cyclic adenosine monophosphate (cAMP) pathway. The receptors of the 5-HT₁ subclass (A, B, D, E and F) inhibit adenylyl cyclase, reducing the intracellular cAMP. 5-HT₄, 5-HT₆ and 5-HT₇ receptors cause activation of adenylyl cyclase and increase in the intracellular cAMP. The 5-HT₅ receptors have been shown to cause both inhibition and activation of adenylyl cyclase. The cAMP molecules cause activation of protein kinase A, which in turn activates other important signalling molecules. 5-HT₂ subclass (A, B and C) receptors have second messenger systems operating through phospholipase C, causing formation of inositol-*tris*-phosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to a receptor on the endoplasmic reticulum, triggering it to release Ca²⁺. The released Ca²⁺ and DAG then cause stimulation of the protein kinase C, which can lead to phosphorylation of the receptors (altering the properties of the G-proteins) or activation of transcription factors. The Ca²⁺ released from the endoplasmic reticulum can also stimulate the firing of neurons, lead to activation of K-channels antagonizing the effects of Ca²⁺, and lead to activation of tyrosine kinase - the mitogen activated protein kinase (MAPK) pathway activating many transcription factors (30).

5-HT can act as a classical neurotransmitter (strictly in the synaptic cleft, between the presynaptic axon and postsynaptic neurons). However, in many sites it acts through volume (also known as paracrine or diffuse) transmission, by diffusing to more remote receptor sites. 5-HT_{1A}, 5-HT_{1B} auto- and heteroreceptors as well as 5-HT_{2A} heteroreceptors receive their serotonergic input mainly by volume transmission (29).

Table 1. The 5-HT receptor subtypes.

Subtype	Type	Signal transduction	Localization	Function
5-HT _{1A}	GBR	Inhibition of AC	Raphe nuclei Hippocampus, cortex, septum	Autoreceptor Postsynaptic heteroreceptor Modulates anxiety and depression?
5-HT _{1B}	GBR	Inhibition of AC	Subiculum Substantia nigra, cerebral vasculature, trigeminal ganglion	Autoreceptor and postsynaptic heteroreceptor Modulation of locomotor activity and aggression? Vasoconstriction
5-HT _{1D}	GBR	Inhibition of AC	Cerebral vasculature, trigeminal ganglion	Vasoconstriction
5-HT _{1E}	GBR	Inhibition of AC	Entorhinal cortex, striatum	Vasoconstriction
5-HT _{1F}	GBR	Inhibition of AC	Dorsal raphe, hippocampus, cortex, striatum, cerebral vasculature, trigeminal ganglion Periphery	
5-HT _{2A}	GBR	Activation of PLC	Cerebral cortex Platelets, smooth muscle	Neuronal excitation Modulates cognitive process of working memory? Platelet aggregation Contraction
5-HT _{2B}	GBR	Activation of PLC	Stomach fundus	Regulation of neuronal excitability Anorexigenic effects Anxiolytic effects? Neuronal excitation, emesis
5-HT _{2C}	GBR	Activation of PLC	Hippocampus, prefrontal cortex, amygdala, striatum, hypothalamus, choroid plexus	
5-HT ₃	Ion channel		Area postrema, hippocampus, neocortex, amygdala, hypothalamus Peripheral nerves Pituitary gland, enteric nervous system	
5-HT ₄	GBR	Activation of AC	Hippocampus, striatum, substantia nigra, superior colliculus GI tract	Neuronal excitation Modulation of neurotransmitter release (5-HT, DA, ACh) Serotonergic regulation of cognition and anxiety?
5-HT _{5A}	GBR	Inhibition of AC	Hippocampus, neocortex, cerebellum, raphe nuclei	Unknown
5-HT _{5B}	GBR	Unknown	Hippocampus, neocortex, cerebellum, raphe nuclei	Unknown
5-HT ₆	GBR	Activation of AC	Neocortex, hippocampus, striatum, amygdala	Unknown
5-HT ₇	GBR	Activation of AC	Hypothalamus, thalamus Intestine	Modulation of circadian rhythms?

GBR= G-protein coupled; AC= adenylyl cyclase; PLC= phospholipase C (31,32)

2.1.5. The serotonin transporter

The serotonin transporter is reviewed in detail in section 2.2.

2.2. THE SEROTONIN TRANSPORTER (SERT)

2.2.1. Overview

The 5-HT signalling can be controlled by alterations in its synthesis, storage, release and inactivation. Inactivation of 5-HT is accomplished by the serotonin transporter (SERT, also known as 5-HTT and SLC6A4), which has an important role in controlling the amount of effective 5-HT in the extracellular space in the sites where 5-HT acts, e.g., the CNS, platelets, enterochromaffin cells, etc. In the CNS, SERTs terminate the action of 5-HT by removing it from the synaptic cleft or other extracellular sites, and returning it to the presynaptic neuron to be either recycled for use, or degraded by the MAO enzyme. By reuptake, SERT minimizes the duration of the neurotransmitter-receptor interaction, and makes receptor desensitization less likely to occur. In the CNS, most of the SERTs (90 % in the rat) are located in the 5-HT neurons, and smaller amount in non-5-HT neurons (33).

The natural endogenous substrate for SERT is 5-HT, and synthetic substrates include amphetamine and fenfluramine. Given the abundance of important processes that 5-HT modulates, it is logical that pharmacological antagonists for SERT have been developed. Tricyclic antidepressants (e.g., imipramine, clomipramine and amitriptyline) were the first SERT antagonists used; however, in addition to SERT, they bind also to norepinephrine transporters (NETs) and to a smaller degree also to dopamine transporters (DATs), and have problematic side effects. Also the abused drugs amphetamine, cocaine and ecstasy (MDMA) block all the monoamine neurotransmitter transporters. The selective serotonin reuptake inhibitors (SSRIs) block selectively SERTs and include drugs such as fluoxetine, sertraline and citalopram. While tricyclic antidepressants are sometimes still used in the treatment of depression, SSRIs have become more popular due to having less side effects and better safety profile. SSRIs are widely used in psychiatry; not only in the treatment of depression, but also of eating disorders, anxiety and obsessive-compulsive disease (10). Due to their effect on feeding behaviour, they also have some use as anti-obesity drugs (11).

The gene coding for SERT belongs to the SLC6 neurotransmitter transporter gene family. This gene family also contains transporters for dopamine (DA), norepinephrine (NE), GABA, glycine, and for some amino acids, as well as some transporters with unknown substrates (orphan transporters). These transporters share several structural similarities. All are comprised of a single subunit with amino- and carboxyl termini, have 12 plasma membrane spanning regions, and have a large extracellular loop between the 3rd and 4th transmembrane regions. The intracellular parts of these transporters possess several phosphorylation sites, suggesting that second messengers regulate transporter function and subcellular redistribution (34). These transporters are also functionally similar; they all couple the uptake of their respective substrates to the co-transport of Na⁺ and Cl⁻ ions and counter-transport of K⁺ to the extracellular space.

This transport process is dependent on maintenance of ion gradients across the cell membrane by $\text{Na}^+\text{K}^+\text{ATPases}$ (35).

The functional role of the SERTs has been studied using SERT knockout mice (mice devoid of SERTs). These mice show increased extracellular 5-HT levels (36), decreased whole brain tissue 5-HT levels (37), increased 5-HT synthesis (37), down-regulation of the function of 5-HT_{1A} and 5-HT_{1B} receptors (38) and no alterations in the other neurotransmitter systems (39). The SERT knockout mice also display several behavioral phenotypes, e.g., depression-like symptoms (40) and reduced aggression (41). However, these results cannot be directly applied on humans, as variations in SERT activity are more subtle and the causal relationships less well known.

2.2.2. Genetic variation of gene coding for SERT

The gene coding for the serotonin transporter (SLC6A4) is located in the chromosome 17q11.2. It consists of 14 exons and spans 37.8 kb, and encodes a 630 amino acid protein (42). Given that SERT is a key regulator of the bioavailability of 5-HT and the wide spectrum of functions and behaviors that 5-HT affects, any modulation in the expression or action of 5-HT would be expected to have consequences (43).

Polymorphic areas have been found both from the coding and the non-coding sequences of the SERT gene. In the coding sequences, some regions with single nucleotide polymorphism (SNP) are known; however, none of them has been found to associate with clinical disorders thus far (43). In contrast, the non-coding sequences are known to have regions with variable number tandem repeat (VNTR) polymorphism, and these polymorphisms have been associated with a predisposition to various psychiatric and neurological disorders. While polymorphism in the non-coding sequences has no effect on the structure of the coded protein, it can affect the amount of expression of the protein or the post-transcriptional properties of the gene, such as mRNA stability. It has been suggested that the VNTR regions act as both tissue-specific and stimulus-inducible regulators of SERT gene expression. They may fine-tune SERT function by altering the level of transporter mRNA, which in turn regulates the concentration of SERT in specific cells or in response to chemical, physiological or environmental challenges (43). It has been suggested that this kind of modulation of gene expression, altering neurotransmitter signalling in response to various challenges and stresses, may be correlated not only with a predisposition to various disorders but also to the variation between individuals (43). In an individual suffering of a specific disorder, e.g., major depression, SERT polymorphism may also affect the pharmacological response to SSRI treatment (44). The differential SERT expression caused by SERT polymorphism may also affect 5-HT levels during embryogenesis and early life (45). This may have long-standing implications, as temporary alterations in 5-HT homeostasis during development may modify the fine-wiring of brain connections and lead to permanent changes in adult behavior (2,46).

At present, several polymorphic regions are known in the SERT gene. Most investigated to date is the biallelic insertion/deletion found in the 5' promoter region of the gene 1.2 kb upstream of the start of the transcriptional site (47,48). This VNTR is called 5-HTT gene-linked polymorphic region (5-HTTLPR), and was initially identified as two variants containing either 14 (deletion/short, abbreviated as "S") or 16 (insertion/long, abbreviated as "L") copies of a 22 bp repeat. An individual can be homozygous (LL or SS) or heterozygous (LS) for these alleles. Many studies have found the level of SERT expression to be higher in the LL homozygotes as compared to carriers of one or two S alleles (48-50); dominant effect of the S allele has been suggested by some (48,49), while other studies have suggested an additive, not dominant effect of the 5-HTTLPR polymorphism (50). Further subgroups were later found to exist in this VNTR to give rise to altogether fourteen allelic variations of the region (51). One of these, a SNP with A to G substitution of the long variant (rs25531), has attracted more interest than others. Some studies have suggested that this L_G variant may be similar to S allele in causing reduced SERT mRNA levels as compared to L_AL_A homozygotes (52). However, only some of the most recent studies on 5-HTTLPR polymorphism have taken this SNP into account.

Association studies on 5-HTTLPR polymorphism have found increased risk of several psychiatric disorders in connection to the 5-HTTLPR variants. SS-homozygosity has been associated with increased risk to uni- and bipolar depression (53-55), anxiety (49,56,57), and predisposition to depression and suicide following stressful life-events (58,59). LL-homozygosity has been associated with increased risk of OCD (60) and increased intensity of hallucinations in schizophrenic persons (61). Structural and functional brain differences have also been reported between healthy carriers of the different 5-HTTLPR variants. As compared to LL-homozygotes, the carriers of S allele have been reported to have reduced volume and grey matter density in several frontal regions, and increased reactivity (as studied with fMRI) to both negative (in striatum and insula) and positive (in left frontal and posterior cingulate regions) stimuli (62). Increased amygdala reactivity to fearful stimuli is a very consistent finding in carriers of S allele (63-66), but has recently been suggested to result from reduced activation in response to neutral stimuli rather than from increased reactivity to fearful stimuli (62). Also the "functional connectivity" of amygdala with ventromedial prefrontal cortex (66) and perigenual anterior cingulate cortex (67) has been reported to differ between the 5-HTTLPR variants. It has been suggested that 5-HTTLPR genotype is a susceptibility factor for affective disorders by biasing the functional reactivity of the human amygdala in the context of stressful life experiences (64).

Studies done in rhesus monkeys, which have a polymorphism in the promoter region of 5-HTT gene functionally similar to human 5-HTTLPR polymorphism, have given light to the gene x environment interaction in association of this polymorphism. Several studies have suggested that carriers of the S allele are more vulnerable to stressful life events (being reared by peers instead of their mothers) in early life, leading to far reaching consequences such as aggressiveness (68) and higher use of alcohol and greater sensitivity to its effects (69,70). These monkeys also show lower CNS 5-HT

turnover (71). Human studies likewise have shown gene x environment effects of 5-HTTLPR polymorphism. Carriers of the S-allele who are exposed to childhood maltreatment or stressful life-events are more vulnerable to adult depression than LL homozygotes (58).

Several PET and SPET studies have been published on the effect of the 5-HTTLPR genotype on the brain SERT binding, but their results have been inconsistent. The first published study on this field used [¹²³I]-β-CIT as its ligand and reported elevated brainstem SERT binding in LL homozygotes as compared to S carriers (72), but later studies using the same ligand did not replicate this finding (73,74). A PET study using [¹¹C]-McN5652 as a ligand found also no difference in SERT binding between the genotypes (75). Three PET studies on 5-HTTLPR polymorphism grouped the L alleles further into L_A and L_G. A study using [¹¹C]-McN5652 found no differences between genotypes (76), whereas two studies using [¹¹C]-DASB found elevated SERT binding in L_AL_A carriers compared to carriers of S or L_G in the putamen (77) and the midbrain (78).

A second widely studied VNTR in the non-coding region of the SERT gene is called STIN2. It is located in intron 2 and can have 9 (STin2.9), 10 (STin2.10) or 12 (STin2.12) copies of a repeat sequence (79), giving rise to six possible genotypes. Also STIN2 VNTR can lead to differential levels of SERT expression, the STin2.12 having the greatest enhancing effect on the SERT expression (80). Despite somewhat discrepant results, association studies have linked also this polymorphism to a number of psychiatric and neurological disorders, e.g., STin2.9 allele to mood disorders (79,81), anxiety (82), and migraine with aura (83); STin2.12 to OCD (84) and migraine without aura (83); and STin2.10 homozygosity to predisposition to suicide (85). No brain imaging studies have been published to date on the effects of the STIN2 polymorphism.

2.2.3. Regulation of the SERTs

Like other Na⁺/Cl⁻ -dependent neurotransmitter transporters, also SERTs can be regulated both long-term (regulation at the gene level, on a timescale of days) and short-term (regulation at the protein level, on a timescale of seconds to minutes). Both long- and short term regulation can affect either transporter activity, transporter number on the cell membrane, or both (34). A lot has been discovered about the signalling mechanisms involved, especially in the acute regulation; less is still known about the factors that trigger these regulatory changes in the gene and protein level.

2.2.3.1. Short-term regulation

There are two different modes for short-term regulation of SERTs: regulation of the number of SERTs expressed on the plasma membrane (“trafficking-dependent regulation”) and regulation of the SERTs’ intrinsic activity (“trafficking-independent regulation”) (86). The term “trafficking“ is used when discussing SERT internalization

from the plasma membrane to the cytosol and externalization from the cytosol to the plasma membrane. For the regulation of the amount of effective 5-HT in the synaptic cleft and on other extracellular sites, only the amount of SERTs on the plasma membrane matters, not the total number of SERTs in the neuron (including SERTs in the cytosol as well as on the membrane). In the trafficking-independent regulation, the SERT activity is altered independent of changes in SERT density.

At present, less is known about the factors that trigger acute regulation at the protein level than about the following signalling mechanisms. Nevertheless, it has been shown that at least the following factors can trigger alterations in the status of the Na⁺/Cl⁻ -dependent neurotransmitter transporters:

1. Transient changes in the membrane potential; depolarization is associated with reduced 5-HT uptake and hyperpolarization with enhanced uptake (87,88).
2. Occupancy of SERT by its substrate or inhibitor; 5-HT and the pharmacological substrates amphetamine and fenfluramine prevent SERT internalization, whereas SERT inhibitors SSRIs and cocaine prevent the effect of substrates (89).
3. Ethanol; enhances SERTs' activity (90).
4. Presynaptic autoreceptors; 5-HT_{1B} activity has been suggested to increase SERT activity (91).
5. Presynaptic heteroreceptors; for example stimulation of Alpha-2 adrenergic heteroreceptors has been shown to lead to a rapid down-regulation of SERT activity (92).

The signalling mechanisms for acute regulation are complex and not fully understood. For the trafficking-dependent regulation, best studied are the different protein kinases and phosphatases that act by affecting SERT's phosphorylation status (34). The phosphorylation of SERT by activation of protein kinase C or inhibition of protein phosphatases 1 and 2A leads to internalization of SERTs and thereby decreases SERT density on the plasma membrane (93,94). One mechanism through which this is achieved is disruption of SERTs association with protein phosphatase 2A (PP2A) on the plasma membrane (95). Further trafficking-dependent mechanisms reducing SERT activity include SERTs association with neuronal nitric oxide synthase (nNOS) (96), and α 2-adrenergic receptor (α 2AR) activation (92), which both may contribute to SERT internalization. The p38 mitogen-activated protein kinase (p38 MAPK) is also involved in maintaining the basal phosphorylation of SERT, and its inhibition leads to reduced SERT insertion to the plasma membrane and thus reduced 5-HT uptake (97). SERT externalization leading to increased SERT densities may be achieved by activation of A3 adenosine receptor (A3AR), requiring protein kinase G (PKG) activation by a phospholipase C, Ca⁺⁺, and cGMP-dependent mechanisms (86,98,99).

Trafficking-independent elevation of SERT activity can be achieved by PKG activation (100), or by activation of p38 MAPK (101). p38 MAPK activation can be stimulated either by PKG or directly independent of PKG by inflammatory cytokines interleukin-1beta (IL-1beta) and tumor necrosis factor alpha (TNF-alpha) (102). Trafficking-independent reduction in SERT activity can be achieved by PKC activators (103).

2.2.3.2. Long-term regulation

Long-term regulation can be caused by physiological changes (for example in age and hormonal status) and gene-environment interactions. Also pharmacological agents, with chronic administration of substrates and antagonists of SERT as well as abused substances such as cigarettes, ethanol and abused drugs may cause long term changes in SERTs. The effects of pharmacological agents are easiest to study in experimental settings, and thus more is known about them than other factors. The long-term regulation of SERTs is thought to involve changes in gene transcription and mRNA translation/stability, but post-translational modifications, protein trafficking, cytoskeleton interactions and oligomerization may be involved, too (34). The SERT gene possesses binding sites for several transcription factors (104).

2.2.3.3. Factors that may cause long-term regulation of SERTs

Due to extensiveness of this field of study only areas relevant for SERT imaging will be reviewed.

2.2.3.3.1. Chronic drug administration

Studies with chronic SSRI treatment show down-regulation of SERTs (105,106).

2.2.3.3.2. Aging

Post mortem studies on the effect of healthy aging on SERT densities have had discrepant results (107-109). Radioligand binding studies by SPET and PET on healthy humans have most often reported 2-10% decline in SERT binding per decade (110-115), even though some studies show no effect of age (116-118).

2.2.3.3.3. Gender and sex steroids

A large body of data indicates sexual dimorphism in various aspects of the 5-HT system. Sexual dimorphism is also present in the prevalence of psychiatric disorders associated to disturbed 5-HT function, e.g. major depression (119).

Sex steroid effects have been studied in animal models, and these results vary slightly depending on the species studied. Receptors for 17β -estradiol (17β -E), progesterone (P) and androgens are found in the serotonergic dorsal raphe nuclei of mice, rats (120,121) and primates (122,123). Both ovarian hormones and testosterone seem to affect the serotonergic system. Male rats have higher basal tonic firing activity of serotonergic neurons in the dorsal raphe than female rats (124), and in female rats, this basal firing rate is higher during pregnancy and the postpartum period (124). Both testosterone and 17β -E increase the basal firing rate of the 5-HT neurons both in male and female rats (125), and metabolites of progesterone increase the basal 5-HT firing rate in female rats (126). Ovarian steroids have also been shown to modulate the

expression of different genes of the 5-HT system, increasing tryptophan hydroxylase mRNA (127) and reducing 5-HT_{1A} mRNA (128). Regarding the expression of SERTs, some discrepancy exists depending on the species studied; in ovariectomized monkeys, ovarian steroids reduced SERT mRNA (129), while in ovariectomized and castrated rats estradiol and testosterone increased SERT mRNA (130,131).

Human studies show that women as compared to men have higher blood 5-HT levels but lower 5-HIAA levels (132). Women also have lower rate of serotonin synthesis, as studied with α -[¹¹C]methyl-L-tryptophan and PET (133,134). The rate of 5-HT synthesis is also reduced more in women than in men as a consequence of acute tryptophan depletion (ATD) (133). Also the mood effects produced by ATD are stronger in women than in men (135).

Sex differences in the human brain SERT binding have been investigated by two SPET and PET studies, one reporting higher SERT binding in men (136) and the other in women (137). Women are reported to have higher availability of 5-HT_{1A} receptors in several brain areas (137-139). While some studies have found no effect of sex on the brain 5-HT_{2A} binding (140,141), other studies have reported an increase of 5-HT_{2A} binding in postmenopausal women following treatment with estradiol and progesterone (142,143).

Given that sex steroids seem to affect the 5-HT system, it is also possible that fluctuation of the levels of ovarian hormones during menstrual cycle causes alterations in the 5-HT system. Cyclic alterations of hypothalamic 5-HT levels (144,145) and 5-HT_{1A} binding (146) have been reported in rodents. In humans, such alterations have been reported in platelet 5-HT_{2A} binding (147), whereas results regarding the blood 5-HT levels (148,149) and platelet SERT binding (147,150) are discrepant. A recent PET study on 5 healthy women reported higher 5-HT_{1A} binding in the dorsal raphe in follicular as compared to luteal phase (151). The only study to date to investigate the within-subject variation in the brain SERT binding between luteal and follicular menstrual phases of healthy women did not detect significant variation in the region investigated (diencephalon-brainstem) (152).

2.2.3.3.4. Seasons and the amount of light

Variation of 5-HT function has been suggested as a consequence of day-to-day variation in the amount of sunlight (153) and variation of seasons (153,154). Hyposerotonergic state during the dark season has also been proposed as one etiologic factor in the Seasonal Depressive Disorder (SAD) (155,156). Studies investigating seasonal variation of 5-HT indices in healthy humans have had inconsistent results. The first study on this topic investigated *post mortem* human brains and reported seasonal variation in the hypothalamic 5-HT levels, with a maximum during fall and minimum in winter (154). Since then, other studies have investigated 5-HT indices in healthy living subjects. Studies on CSF and internal jugular vein 5-HIAA levels have suggested increased turnover of 5-HT in summer (153,157), whereas studies on platelet SERT binding have had inconsistent results, reporting either higher (158) or lower (159)

SERT densities in summer than in winter, or no circannual variation (160). Similar inconsistency applies to results of platelet 5-HT_{2A} binding studies (158,161,162) and neuroendocrine challenge tests (163). Only one prior imaging study has investigated seasonal variation in the central 5-HT system; this SPET study reported higher SERT binding in women investigated in summer as compared to different women investigated in winter (164).

2.2.3.3.5. Tobacco, alcohol and drugs of abuse

In animal studies, nicotine increases brain 5-HT release (165), which by activation of 5-HT_{1A} receptors leads to transient, short-term inhibition of the serotonergic neurons in the dorsal raphe nucleus (166). Nicotine also affects the brain [³H]paroxetine binding in a region-specific manner (167). Studies on the effect of cigarette smoking on the 5-HT system in humans are scant. One SPET study reported modestly higher (10%) SERT binding in the brainstem of smokers as compared to non-smokers (136). No other SPET or PET studies have been published on the effect of cigarette smoking on the brain 5-HT system.

Three studies have compared SERT binding of alcoholic subjects to that of healthy controls, reporting either decreased binding in the midbrain (168) and the brainstem (116), or no differences (169) between alcoholic and non-alcoholic subjects. No imaging studies have been published on the effect of social drinking on SERT binding.

Of the drugs of abuse, the effect of MDMA on the brain SERT binding has been investigated in several studies. At least two human studies have reported reduced SERT binding in the MDMA users (170,171), whereas a recent study examining baboons did not find differences between MDMA users and controls (172). The same study also reported increased SERT binding in cocaine-abusing baboons. In humans, PET or SPET studies investigating the effect of the drugs of abuse other than MDMA on the 5-HT system have not been published.

2.3. IMAGING OF THE BRAIN 5-HT SYSTEM

2.3.1 Emission tomography methods

2.3.1.1 Overview

The techniques for brain imaging applying radioactive ligands include single photon emission tomography (SPET) and positron emission tomography (PET). While both apply radioactive isotopes for non-invasive *in vivo* imaging, these methods differ with respect to the used radioactive isotopes, machinery, and image resolution.

Isotopes are different forms of an element, each having the same number of protons (the same atomic number) but different number of neutrons (different mass number), which makes the nucleus unstable. The unstable nucleus has excessive energy which is released in radioactive decay. In this process, the excess energy is emitted as newly-

created radiation particles (α , β^+ , and β^- -particles being the most common ones), or as electromagnetic waves (γ - or x-rays) (173).

Some radioactive isotopes occur naturally, and others are produced artificially. The radioisotopes used in SPET emit gamma rays, and are typically derived from the naturally occurring forms of an element rather easily. PET studies use positron emitters, which are always artificially produced. This is usually done in a cyclotron, although in future generators may be used increasingly for production of some positron emitters. The most common isotopes used in SPET are ^{99m}Tc and ^{123}I , while in PET most common are ^{11}C , ^{15}H , ^{18}F and ^{68}Ga . In both emission tomography methods, the radioactive isotope is combined to a pharmaceutical agent, thus forming a radioligand. It is usually given to the study subject as an intra venous injection. In the body, the radioligand is typically incorporated into a metabolic process or binds to a specific receptor, enabling imaging of specific molecules or metabolic processes (173).

Until recently, use of SPET has been considerably more common than PET, but recent rapid spread of PET machinery as well as the development of suitable ligands have shifted the emphasis to PET studies. SPET has the advantage of cheaper machinery and more easily available radioligands, while isotopes used in PET typically require a cyclotron in vicinity of the scanner for their production. However, positron emitters can be incorporated to virtually all organic compounds and thus allow wider range of targets. Also the imaging resolution achieved with PET (typically 5-7 mm) is superior to SPET (typically 10-14 mm).

2.3.1.2. Principles of SPET

As mentioned above, radioisotopes emitting gamma rays are used in SPET studies. The radioligand given to the study subject binds to its target sites in the body and emits gamma rays. These are detected by crystal planes (detectors) of the gamma camera, which absorb the gamma rays and scintillate in response to detected gamma radiation. The scintillation is detected by photomultiplier tubes, which transform the light photons to an electric signal, which is reconstructed into two-dimensional images by computer systems. This reconstructed image reflects the distribution and relative concentration of radioactivity in the organs and tissues imaged. In SPET imaging, the gamma camera acquires two-dimensional images from multiple angles, and computer systems reconstruct three-dimensional images from the two-dimensional ones (173).

2.3.1.3. Principles of PET

In PET studies, the radioactive nucleus of the radioligand emits positrons, which travel in tissue typically a distance of 1-2 mm before colliding to and combining with their counterparts electrons. This process is called annihilation, and in it the two particles vanish and are converted into energy in the form of two 512 keV gamma rays, which travel in opposite directions. These gamma rays are detected simultaneously by a ring consisting of several small gamma detectors and are then further processed into three dimensional images (173).

2.3.1.4. Quantification methods

2.3.1.4.1. Tracer kinetic modelling

Emission tomography scans measure total radioactivity in tissue. This consists not only of radioligand bound specifically to the target receptors, but also of free tissue activity (non-specific activity) as well as free radioactivity within vasculature. In order to compare the target receptors between subjects or groups, it is necessary to be able to make estimations of receptor-specific binding and for this tracer kinetic modelling is needed. These models describe the radioligands pharmacokinetics and rate constants for flux of the ligand between different compartments (**Figure 2**), and necessitate collection of kinetic data with input and output functions (174).

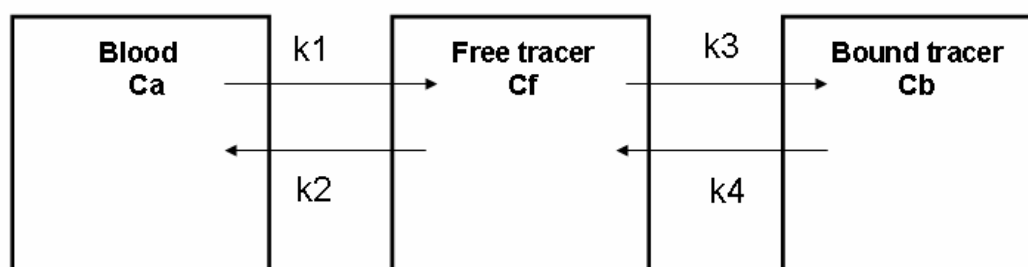


Figure 2. Standard compartmental model for receptor-binding ligands. Ca, Cf, and Cb represent time-dependent local activity of radioligand in blood (Ca), free in tissue (Cf) and bound in tissue (Cb). k1 to k4 represent the rate constants between compartments.

Full kinetic models involve serial measurements of concentration of the radioligand (corrected by its metabolites) from the plasma and dynamic/serial emission tomography scans for measurement of time-activity curves in a target regions. For derivation of receptor density (B_{max}) and equilibrium dissociation constant (K_d) at least two studies at different specific activities are needed. Instead of B_{max} and K_d , binding potential ($BP = B_{max}/K_d$) is usually the parameter of interest in clinical studies comparing receptor-specific binding between groups. In the absence of competing ligands, a modified binding potential BP' can be calculated from the measured parameters of a kinetic study as $BP' = f_2 B_{max}/K_d = k_3/k_4$, where f_2 is the fraction of free ligand in tissue (174,175). This model assumes that non-specific binding in tissue is a constant and can thus be disregarded.

Reference region approaches are simpler quantification methods that have been developed in order to avoid invasive full kinetic studies with arterial sampling. These methods compare radioactivity in a target region (containing the receptor of interest) to radioactivity in a reference region (devoid of specific receptor binding). Under equilibrium conditions the volumes of distribution can be obtained, usually by simple

measurement of tissue activity in target and reference regions. The two regions should differ with respect to bound tracer C_B but contain the same activity of free tracer C_F . In this setting BP' is related to the different distribution volumes in target (DV_{rec}) and reference (DV_{ref}) tissues, and to the ratio between activities of the target (C_t) and reference (C_f) tissues, and can be expressed as: $BP' = (DV_{rec}/DV_{ref}) - 1 = (C_t/C_f) - 1$ (174,175).

As actual equilibrium is often difficult to reach, an approximation using graphic presentation of kinetic data, the Logan plot (176) may be used. In this method, the integral of regional activity over current regional activity is plotted versus the integral of plasma activity over regional activity, and the slope of the curve approximates the regional tracer DV. In further simplification, arterial sampling can be substituted by deriving the input function from the time activity curve of the reference region. By comparing the slopes for the target (DV_{rec}) and the reference region (DV_{ref}), the BP can be calculated as $BP = (DV_{rec}/DV_{ref}) - 1 = \text{Distribution volume ratio (DVR)} - 1$ (174,177). The assumption of this model is that the flux from the free tissue compartment to arterial plasma compartment (k_2) remains constant in the target and reference regions.

Due to the longer physical half lives of SPET ligands as compared to PET ligands, kinetic studies with measurement times over several hours are usually needed. These are not well tolerated by study subjects, and thus more patient-friendly methods with single scans are usually favoured. The most popular is the use of the simple ratio method, where the ratio of specific to unspecific binding is expressed as Specific binding ratio (SBR) = (mean counts in the target region - mean counts in the reference region) / mean counts in the reference region. SBR is sometimes also called as Specific uptake ratio (SUR).

2.3.1.4.2. Definition of target regions

Brain studies applying emission tomography methods typically estimate radioligand binding in a predefined region or volume of interest (ROI or VOI), or alternatively, radioactivity can be assessed in each voxel.

ROI (or VOI) based methods. If these methods are used, the regions of interests are decided on the basis of the prevailing knowledge about the distribution of the target receptor in the brain and on the functional relevance of these regions to the study questions. Careful selection of ROIs reduces the number of statistical analyses, thus increasing study power. Several methods are used in ROI definition. Some studies define their ROIs on the basis of anatomical boundaries given e.g., by an MR image (117,178) or the early phase radioligand image mimicking brain perfusion image (179). However, the limitation for using anatomical images for target region definition is the fact that many of the brain nuclei cannot be seen in the anatomical images. In this case a larger anatomical structure (containing also tissue other than the specific brain nuclei) may be selected for binding quantification. Alternatively, the ROIs can be placed directly on the SPET/PET images, where radioligand distribution shows the location of

the target sites. ROIs can then be either drawn manually based on the visual information provided by the image (180), or alternatively, fixed ROIs, e.g., of spheres with fixed area/volume can be placed over the predetermined brain regions (181).

Voxel-based methods. These methods typically use brain templates generated specifically for the radioligand in question (182-184). Individual brain images are registered and realigned to the template, thus generating stereotactic images from the individual brain scans. The stereotactic brain images can then be used for estimation of radioligand binding in each voxel, enabling voxel-by-voxel comparisons between subjects and study groups. Some nuclear medicine workstations offer programs for formation of brain templates. During recent years, Statistical Parametric Mapping (SPM) (Wellcome Department of Imaging Neuroscience, UCL, London, UK) (185) has become a popular tool for voxel-by-voxel analyses of brain images generated by PET, SPET and MRI studies.

2.3.2. Radioligands for imaging of the brain 5-HT system

2.3.2.1. 5-HT synthesis and neuronal activity

Two radioligands targeted to different steps in the 5-HT synthesis have been investigated as potential ligands for PET studies. ^{11}C -labeled α -methyl-L-tryptophan (^{11}C - α Mtrp) (186) is an analog of tryptophan, which is a substrate for tryptophan hydroxylase, the first enzyme in the two-step synthesis of 5-HT. It is in part further metabolized into ^{11}C - α -methyl-serotonin in 5-HT neurons. The second ligand, 5-Hydroxy-L- $[\beta$ - $^{11}\text{C}]$ tryptophan (^{11}C]HTP) is identical to the endogenous compound that will undergo conversion to 5-HT by aromatic L-amino acid decarboxylase, the second enzyme in the biosynthesis, and then further catabolism by MAO A enzyme to 5-HIAA. These ligands have been studied in healthy subjects (133,134,187), and there are also few studies in patients (188-190). Some groups have investigated 5-HT neuronal activity indirectly, by measuring fenfluramine-induced changes in cerebral glucose metabolism with ^{18}F -fluorodeoxyglucose (^{18}F FDG) and PET (191-193).

2.3.2.2. 5-HT receptors

Several radioligands exist for imaging of 5-HT_{2A} receptors, e.g. PET ligands [^{18}F]Setoperone (194), [^{18}F]altanserin (195) and [^{11}C]MDL 100,907 (196), as well as SPET ligand ^{123}I -R91150 (197). For 5-HT_{1A} receptor imaging, [*carbonyl*- ^{11}C]WAY-100635 (198) and [^{18}F]MPPF (199,200) have been used in PET studies, whereas no good SPET ligands for 5-HT_{1A} imaging are available at the moment. Both these receptors have been studied in patient samples, for example in major depression (201,202), schizophrenia (203) and eating disorders (204,205).

2.3.2.3. Serotonin transporters

In radionuclide imaging studies of the 5-HT system, serotonin transporters have been the most popular study object. This is perhaps due to existence of SERT ligands for

both SPET and PET imaging. Until the last couple of years, SERTs were mainly investigated using SPET. However, this is changing due to a rapid spread of PET machinery and development of SERT ligands for PET imaging.

For years, most SERT studies were done using the SPET ligand [¹²³I]methyl 3 beta-(4-iodophenyl) tropane-2 beta-carboxylate ([¹²³I]β-CIT). The problem with [¹²³I]β-CIT is that it is nonselective, i.e., has affinity for DATs, SERTs, and with a minor degree to NETs (206-209). Based on displacements studies, its binding in the striatum represents binding to DATs and binding in the thalamus and midbrain areas represents binding to SERTs (208,210). However, autoradiography studies show the existence of DATs (211) and NETs (212) in the midbrain and NETs in the thalamus (212). Furthermore, a PET study investigating non-human primates reported displacement of [¹¹C]β-CIT by NET ligands in the thalamus (209). The results gathered from [¹²³I]β-CIT studies may thus in theory be affected by binding to DATs and NETs also in the areas considered specific for SERT, i.e., the midbrain and thalamus. [¹²³I]2beta-carbomethoxy-3beta-(4-iodophenyl) [¹²³I]nor-β-CIT is an analogue of [¹²³I]β-CIT with high affinity to both SERTs and DATs, but with tenfold higher affinity to SERTs in comparison to [¹²³I]β-CIT (213). Similar to [¹²³I]β-CIT, its binding to midbrain and thalamus is considered to be specific for SERTs (214,215); nevertheless, due to its non-selectivity, binding to other monoamine transporters remains a possibility also for [¹²³I]nor-β-CIT.

Iodine 123-labeled 2-((2-((dimethylamino)methyl)phenyl)thio)-5-iodophenylamine ([¹²³I]ADAM) is a newer SPET ligand that binds selectively to SERTs, having 10,000-fold affinity for SERTs over DATs and NETs (216), and therefore its binding to other monoamine transporters is not a problem. Several groups have assessed its imaging characteristics in humans (217-223). However, also [¹²³I]ADAM possesses some problems; the optimal scanning time varies somewhat depending on the density of SERTs in the region (217,223), and thus kinetics may vary between individuals and perhaps even more between patients and healthy controls depending on their respective SERT densities. Its test-retest variability is not ideal, being 13% ± 11% in the midbrain, 16% ± 13% in thalamus, and around 20% in the regions with lower SERT binding (217); this variability increases the sample sizes that are needed to obtain significant results in clinical studies. Furthermore, according to recent report (223), the simple ratio method used for quantification of SERT binding by all previous studies applying [¹²³I]ADAM may overestimate the specific binding values in high binding regions. Use of more reliable methods (Logan model) would require longer acquisition times (120 min as compared to 30 min) (223) and may thus be impractical.

To date, three PET ligands for SERT imaging have been used in clinical human studies: [¹¹C]McN5652 (224), [¹¹C]DASB (225), and [¹¹C]MADAM (226). Of these, [¹¹C]McN5652 has weaker specific-to-non-specific binding ratios than [¹¹C]DASB (227), making it a less optimal tracer. [¹¹C]DASB and [¹¹C]MADAM both have mean test-retest difference less than 11 % in regions with high SERT densities (228-230), and the reported intra-class correlation coefficients (ICCs) for test-retest data are either acceptable (in raphe, due to large inter-subject variability) (228,230) or good (228-230). No test-retest data have been published on [¹¹C]McN5652. [¹¹C]McN5652 and

[¹¹C]DASB have been used in patient samples, [¹¹C]MADAM so far only in preclinical studies done in healthy subjects.

2.4. BULIMIA NERVOSA

2.4.1. Clinical characteristics and epidemiology

Bulimia Nervosa (BN) is an eating disorder characterized by body image distortions and alternating episodes of binge eating and inappropriate compensatory behaviors. Its diagnostic criteria (DSM-IV) (231) are given in **Table 2**. Based on the individual's compensatory behaviours, BN is further classified either as the purging subtype (including vomiting and misuse of laxatives, diuretics, or enemas) or non-purging subtype (including restricted eating and exercise).

Table 2. DSM-IV criteria for Bulimia Nervosa

DSM-IV Criteria for Bulimia Nervosa (307.51)
A. Recurrent episodes of binge eating. An episode of binge eating is characterized by both of the following: (1) Eating, in a discrete period of time (e.g., within any 2-hour period), an amount of food that is definitely larger than most people would eat during a similar period of time and under similar circumstances (2) A sense of lack of control over eating during the episode (e.g., a feeling that one cannot stop eating or control what or how much one is eating)
B. Recurrent inappropriate compensatory behavior in order to prevent weight gain, such as self-induced vomiting; misuse of laxatives, diuretics, enemas, or other medications; fasting or excessive exercise
C. The binge eating and inappropriate compensatory behaviors both occur, on average, at least twice a week for 3 months
D. Self-evaluation is unduly influenced by body shape and weight
E. The disturbance does not occur exclusively during episodes of anorexia nervosa
Specify type: Purging type: During the current episode of bulimia nervosa, the person has regularly engaged in self-induced vomiting or the misuse of laxatives, diuretics, or enemas Nonpurging type: During the current episode of bulimia nervosa, the person has used inappropriate compensatory behaviors, such as fasting or excessive exercise, but has not regularly engaged in self-induced vomiting or the misuse of laxatives, diuretics, or enemas

(231)
The lifetime prevalence of BN has been estimated to range from 1.1% to 4.6% (232-234). It has been estimated that only a small minority (6%) of the BN patients are treated in mental health care (235).

The onset of BN usually occurs in adolescence or early adulthood and is most frequently seen in women (13). Restrictive eating behavior often precedes the onset of BN, and about a quarter of BN patients have previously suffered from Anorexia Nervosa (236). Later on, dietary restriction is interrupted by episodes of binge eating (13). The disorder is often self-perpetuating, but 5–10 years later, between a quarter and

a half of individuals still have an eating disorder of clinical severity (234,237,238). Unlike Anorexia Nervosa, BN is not associated with increased mortality (16). Individuals with BN who engage in frequent vomiting may suffer from somatic problems such as electrolyte abnormalities, metabolic alkalosis, erosion of dental enamel, swelling of the parotid glands, and scars and calluses on the backs of their hands (239). Bulimia Nervosa patients also often suffer from other psychiatric morbidity at some point in their life. Common comorbid psychiatric conditions include e.g., major depression, substance and alcohol abuse, impulse control disorder, and personality disorders (240-242). Personality features of individuals with BN include some features shared with Anorexia Nervosa such as high harm avoidance, perfectionism, and low self-esteem. Features more specific to BN include higher novelty seeking, higher impulsivity, lower self-directedness, and lower cooperativeness (243,244).

2.4.2. Etiology

Both genetic and environmental factors are believed to be involved in the etiology of BN. Historically, eating disorders have been conceptualized as having sociocultural origins (idealization of slimness in the western world). Family history of eating disorders, depression, substance misuse (especially alcoholism) and obesity have been implicated as risk-factors, as well as premorbid experiences such as sexual abuse, family dieting, critical comments about eating, weight and body shape, and adverse parenting. Further implicated risk factors include premorbid personality characteristics such as low self-esteem, anxiety and anxiety disorders, and physical characteristics such as obesity and early menarche. Psychological theories include the need to feel in control of life as well as overvaluation of shape and weight (13).

Eating disorders often run in families, and there seems to be cross-transmission between different eating disorders, suggesting a shared familial liability (245). Twin studies have enabled more detailed analyses on the contribution of heritability (the cumulative effect of different genes), shared environmental factors (environmental influences to which both members of a twin pair are exposed) and unique environmental factors (environmental factors to which only one member of a twin pair is exposed) (246). The heritability estimates for BN range between 28-83% (13,247-250). The contribution of unique environmental factors is estimated to range between 17 % (248) to 38 % (249).

To date, only one linkage study for BN has been published, reporting significant linkage on chromosome arm 10p for a broad sample of families affected by BN (251). Association studies have explored the impact of several genes on BN, especially the genes affecting the 5-HT system. Most investigated is the association of BN and 5-HTTLPR polymorphism. Although one study found an association between the 5-HTTLPR genotype and BN (252), other studies have not replicated the finding (253,254). However, some studies have found an association between the 5-HTTLPR genotype and particular predisposing eating disorder-related behavioral and attitudinal

traits (254-256). Same applies for polymorphism of genes coding for 5-HT_{2A} receptor (257), 5-HT_{2C}-receptor (256), catecholamine-O-methyltransferase (COMT) (255), and tryptophan-hydroxylase-1 (258). Other investigated genes include e.g., genes coding for leptin (no association) (259), brain derived neurotrophic factor (BDNF) (discrepant findings) (260-262), and ghrelin (discrepant findings) (263,264).

2.4.3. 5-HT function in Bulimia Nervosa

Neurobiological alterations are found in subjects affected by BN, which has led to the question whether these alterations are a cause or consequence of the disorder. Most evidence exists on the role of 5-HT. The monoamine system has been the most obvious target for investigations as the medications acting on monoamine neurotransmitters are used in treatment of psychiatric disorders. However, it is possible that other systems have a role, too, and furthermore, that the alterations detected in the 5-HT system are not primary but instead reflect alterations in some other aspects of neurobiology.

Several study results have led to the 5-HT hypothesis in BN:

1. 5-HT has effect on food intake and body weight (11), which may contribute to appetite dysregulation in eating disorders.
2. BN is often accompanied by disturbances of mood and impulse control (240), in which disturbed 5-HT function has also been implicated (5,15).
3. Subjects who are ill with BN show several abnormalities in 5-HT metabolism and function as compared to healthy subjects. The neuroendocrine responses to intravenous 5-hydroxytryptophan and d,l-fenfluramine hydrochloride are blunted (265,266); the CSF levels of 5-HIAA are reduced in a subgroup of more frequently binge-eating in bulimic subjects (267,268); and the mood lowering effect of acute TRP depletion is increased (269) in BN. Three PET or SPET studies have previously investigated subjects ill with BN, reporting reduced SERT binding in the hypothalamus/thalamus (181), increased 5-HT_{1A} binding in the frontal, cingulate, temporal, and raphe regions (270), and normal 5-HT_{2A} receptor binding (271).
4. The SSRI medications, especially fluoxetine, are at the moment the most effective treatment for BN, despite the fact that not all affected subjects benefit from it (16).

It is not known whether the disturbances in 5-HT system in BN and in other eating disorders are a cause (i.e., genetic trait making a subject vulnerable for getting the disease) or a consequence of the dysregulated eating behavior (i.e., a state resulting from changes in nutritional status during the illness). The heritability of eating disorders (247-250) as well as the existence of particular premorbid temperament characteristics that persist after recovery suggest that there may be a trait for developing an eating disorder (6). The premorbid temperament characteristics include childhood perfectionism, obsessive-compulsive personality patterns, and anxiety (272-274). After recovery from BN, persistence of mild to moderate negative moods and obsessions with perfectionism and exactness has been reported (275). A neurobiological disturbance

may underlie the vulnerability for BN and remain after recovery. Studying individuals before the onset of illness is obviously difficult. Studying individuals after recovery is easier and has been done in BN subjects. These studies show some persistent alterations in the 5-HT system that may represent a trait and predispose a subject for BN. In recovered BN subjects, acute tryptophan depletion caused lowering of mood, increases in ratings of body image concern, and subjective loss of control of eating (276). PET studies have shown loss of normal age-related decline in the 5-HT_{2A} binding in subjects who have recovered from BN (277).

The problem of interpreting studies in recovered subjects lies in the fact that it is not possible to differentiate whether the detected finding is due to a trait or represents scarring caused by the state. Twin studies with discordant monozygotic twin pairs offer one possibility for overcoming this problem, as a trait effect should be found also in the genetically similar co-twin.

2.4.4. Treatment

According to a recent review, several studies have shown that fluoxetine (60 mg/day) reduces core bulimic symptoms (binge eating and purging) and associated psychological features in the short term. Single studies provide preliminary evidence on the beneficial effect of fluvoxamine, trazodone, desipramine, topiramate, ondansetron and brofaromine (16). Cognitive behavioral therapy (CBT) decreases core behavioral symptoms and psychological features in both the short and long term (16), and, according to another review, may be the most effective treatment for BN (278). How best to treat individuals who do not respond to CBT or fluoxetine remains unknown (16).

2.5. OBESITY

2.5.1. Overview

Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health. The most commonly used population measure of obesity is the body mass index (BMI), a person's weight (in kilograms) divided by his or her height squared (in metres). A person with a BMI of 30 or more is generally considered obese. A person with a BMI equal to or more than 25 is considered overweight (17).

Obesity is one of the main health issues of our time, and has spread so rapidly in recent decades that it has become popular to talk about it as "the obesity epidemic". In the year 2005, the prevalence of obesity in Finland was 17.8 % for women and 18.9 % for men, in the United Kingdom 24.2 % and 21.6 % respectively, and in the United States 41.8 % and 36.5 % (279). It affects individuals of all ages: the World Health Organization (WHO) estimated that globally in the year 2005 approximately 1.6 billion adults (age 15+) were overweight, at least 400 million were obese, and at least 20

million children under the age of 5 years were overweight. Furthermore, by the year 2015, approximately 2.3 billion adults are estimated to be overweight and more than 700 million obese (17). The rapid spread of obesity is often associated to the western sedentary lifestyle, with excessive food supplies and lack of exercise. However, it is not only a problem of the westernized world; despite problems of malnutrition and starvation, obesity is increasing rapidly also in the developing world (280). Overweight and obesity are major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer (17).

Overweight and obesity develop ultimately from an imbalance between ingested and expended energy, but the mechanisms of the underlying causative processes are still not fully understood. Alongside peripheral factors secreted by the pancreas, the gut and the adipose tissue, neuropeptides and neurotransmitters participate in the control of energy homeostasis (18). At the moment, more than 40 orexigenic and anorexigenic signalling molecules are known, including hormones, neuropeptides, neurotransmitters, enzymes, other chemical messengers and their receptors (19). Most of these factors exert their influence in the hypothalamus. Alongside the many signalling molecules, feeding behavior is affected by the brain reward circuitry involving the drive to obtain a rewarding stimulus as well as the hedonic experience of getting the stimulus (281).

Although many factors underlie the causes of obesity, genetics is considered to play a significant role in regulation of energy balance and in the development of obesity (282). The influence of genetic factors is supported by several family and twin studies (283,284). The latest Human obesity gene map (year 2005) reported a total of 127 candidate genes in association with obesity-related phenotypes. At least 22 of these genes are each supported by at least five positive studies (285).

2.5.2. 5-HT and feeding behavior

Serotonin is believed to be one of the many signalling molecules affecting feeding behavior, having an anorexigenic effect (11). This is suggested by several lines of evidence: In laboratory animals, artificial reductions in the brain 5-HT levels lead to hyperphagia and weight gain (286), whereas increases in 5-HT levels lead to hypophagia (287-289). Medications that increase brain 5-HT levels reduce feeding behavior and body weight (290,291). Elevated levels of 5-HT have been implicated in cancer anorexia (292). Studies also show that hypo-caloric diet reduces brain 5-HT levels in animals (293). In healthy humans, dieting causes reduction in plasma tryptophan levels (and thus likely also brain 5-HT levels) (294,295) and increases the sensitivity of postsynaptic 5-HT_{2C} receptors (294). Reduced baseline 5-HT levels are reported in obese rats (296). In obese humans, plasma concentrations of tryptophan are reported to be low and do not normalize after weight reduction (297,298), suggesting persistent alterations in the serotonergic system. Taken together, the data suggest that reduced 5-HT transmission (caused by reduced extracellular 5-HT) is associated with increased appetite and obesity, whereas increased 5-HT transmission (caused by increased extracellular 5-HT) has a suppressive effect on feeding behavior and appetite.

Of the 5-HT receptors, the association of 5-HT_{2C} and 5-HT_{1B} receptors with feeding behaviour is the strongest. 5-HT_{2C} receptor-deficient mice exhibit abnormal control of feeding behavior, leading to development of overweight (299). Also 5-HT_{1B} receptor-deficient mice exhibit increased body weight (300) and attenuated responses to classic anorectic 5-HT compounds like d-fen (301). Both 5-HT_{2C} and 5-HT_{1B} receptors affect feeding behavior by modulating the hypothalamic melanocortin system, leading to downstream activation of the melanocortin 4 receptor. Stimulation of the 5-HT_{2C} receptors directly activates pro-opiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus, promoting the release of α -melanocyte stimulating hormone (α -MSH) (an anorexigenic factor) acting on melanocortin 4 receptor (302,303). Stimulation of 5-HT_{1B} receptors first inhibits the release of Agouti Related Protein (AgRP), which leads to decreased inhibitory synaptic input onto POMC neurons, and thus also promotes the release of α -MSH (304). Other 5-HT receptors, e.g., 5-HT_{1A}, 5-HT_{2B} and especially 5-HT₆ receptors have also been implicated as having association with feeding behavior (305).

Some studies have also found associations between obesity and genes related to 5-HT function. Three studies have found an association between the 5-HTTLPR genotype and obesity, reporting a higher frequency of obesity and higher mean BMI in carriers of the S allele: one study in Argentinean adolescents, showing association in both sexes (306); one study in Argentinean young adult men (307); and one longitudinal study in US citizens as adolescents and young adults, showing association in white and Hispanic men, but not in women or African American men (308). The last study also found higher BMI rise in carriers of the S allele when assessing change in BMI between two contacts (at about 16.5 and 22 years of age) (308). Genetic associations relating to the 5-HT system have also been reported with polymorphisms of the SLC6A14 gene (309,310), MAO-A gene (308,311), 5-HT_{2A} receptor gene (312,313) and 5-HT_{2C} receptor gene (314,315).

3. STUDY OBJECTIVES

1. To compare the repeatability of manual versus automated VOI placement in SERT binding quantification of [¹²³I]ADAM images.
2. To investigate the existence of seasonal variation in SERT binding
3. To investigate the SERT binding of subjects with and genetically predisposed to Bulimia Nervosa
4. To investigate the relationship between SERT binding and acquired obesity (as represented by body mass index)

4. METHODS

4.1. STUDY DESIGN

In study **I**, a brain template for the automated registration and realignment of [^{123}I]ADAM-images was created from [^{123}I]ADAM-scans of healthy women. Furthermore, a predefined volume of interest (VOI) map was created for the quantification of SERT binding. The repeatability of the automated procedure in quantifying SERT binding was tested by comparing SERT binding measurements between the results of initial fitting procedure to a second fitting performed after misalignment of the images. The repeatability of the automated procedure was tested in a group of women with Bulimia Nervosa.

SERT binding was also quantified using manual delineation of the VOIs. Manual VOI delineations of the midbrain and cerebellum (the reference region) were tested for intra- and inter-observer repeatability. Intra-observer repeatability was tested between repeated VOI definitions performed six months apart by a blinded observer (a nuclear medicine physician). Inter-observer repeatability was tested between the results of the first analysis by the first observer and results of VOI definitions done by a second blinded observer (a hospital physicist). For testing the intra-observer variability, we used [^{123}I]ADAM-scans of healthy subjects (co-twins of subjects with an eating disorder) and of the group of women with BN. Inter-observer repeatability of the manual VOI definitions was tested in the group of BN women.

In study **II**, the existence of seasonal variation in SERT binding was investigated by comparing the intra-subject variability of [^{123}I]ADAM binding in the midbrain and thalamus of healthy young adults between two repeated [^{123}I]ADAM-scans, performed in summer and in winter. Based on earlier results (164), we hypothesized higher SERT binding in summer as compared to winter.

In study **III**, we investigated SERT binding in women with lifetime BN and in healthy women genetically predisposed to BN (twin-sisters of bulimic women) by comparing them to healthy women with no predisposition for BN. For this purpose, we used a twin sample (including both mono- and dizygotic twin pairs). SERT binding in the midbrain and thalamus was investigated both in individuals (clustering caused by inclusion of twins was taken into account for) and in twin pairs (within-pair comparisons), using [^{123}I]ADAM as a SERT ligand. In *post hoc* analyses, a subgroup of purging BN women was compared with the healthy women. Based on earlier results (181), we hypothesized reduced SERT binding in the BN probands. Furthermore, we assumed that SERT transmission is a bulimia-related endophenotype, i.e., a heritable quantitative trait that is state-independent (manifest in the individual whether or not illness is active), and found more often in unaffected family members than in the general population. Therefore, we hypothesized that SERT availability would be clearly reduced in SERT rich brain regions in BN women compared to unaffected women, and

that the SERT availability of unaffected co-twins would be intermediate between that of probands and healthy women.

In study **IV**, we investigated the relationship between acquired obesity and SERT binding in a sample of monozygotic (MZ) twins. BMI was used as an index of obesity. SERT binding was measured in the midbrain and hypothalamus/thalamus areas using [¹²³I]nor-β-CIT as a ligand. The relationship between obesity and SERT binding was assessed both in individual data (clustering caused by inclusion of twins was taken into account for) and in twin pairs (within-pair comparisons, twin with higher BMI compared with co-twin with lower BMI). In *post hoc* analyses, the effects of sex and oral contraceptives on the SERT binding were investigated.

In studies **III** and **IV**, we investigated twins and applied twin study designs in their simplest form, by:

- 1) Comparing variables between probands, their unaffected co-twins and unrelated healthy subjects (study **III**)
- 2) Comparing some variables between genetically similar (MZ) twins (within-pair analyses) (study **IV**), when the effect of genetic factors is eliminated and the observed differences will be caused by unique environmental influences.

Twin studies investigating discordant twin pairs (one twin affected and the co-twin unaffected) and healthy control twin pairs offer an opportunity to explore the differences between genetic and environmental influences, given that the twins share either all or half their segregating genes with their affected co-twin, depending on whether the twin pair is monozygotic (MZ) or dizygotic (DZ). Moreover, twins are of the same age and have generally similar childhood and adolescent experiences. Observed differences between the affected twin and unaffected co-twin are likely due to state effects (caused by unique environmental effects), whereas differences observed between healthy twin sisters and unrelated controls would be trait-related (representing the effect of genes). Observed similarity of discordant twins would be explained by shared genetic liability as well as by shared environmental effects. Studies published in the last few years have shown that epigenetic transformations play an important role in the discordance of MZ twins (316).

4.2. STUDY SUBJECTS

The study subjects of studies **I-IV** were recruited in three separate procedures, two of which involved screening subjects from the Finnish twin study FinnTwin16, a longitudinal national twin cohort including virtually all Finnish twins born in 1975-1979 (317). The third subset of subjects were healthy young men recruited through and advertisement directed at male students.

4.2.1. Studies I-III

4.2.1.1. Recruitment of study subjects

In the studies **I-III**, we used [¹²³I]ADAM-scans done for subjects screened from the FinnTwin16 twin cohort. In addition, healthy male, non-twin subjects were recruited specifically for the study **II**.

Recruitment of twins for studies I-III: 2545 female twins were sent a self-report questionnaire screening for potential eating disorders at the age of 22-27 years. 292 screen-positive women, their 130 female co-twins, and 210 screen-negative women were then interviewed using a short version of the Structured Clinical Interview for DSM-IV (SCID) (318) (interview participation rate 85.2 %) (319). From the interviews, we obtained lifetime diagnoses of AN, BN, BED, MDD and OCD. After exclusion of twin pairs who were not eligible for the study (due to pregnancy, having psychiatric morbidity other than BN, or medications affecting the 5-HT system) we ended up with 15 twin pairs in which either one or both twins had DSM-IV criteria fulfilling lifetime BN, more broadly defined BN or other eating disorder. In 10 pairs one or both subjects had a DSM-IV criteria fulfilling lifetime BN; three pairs were concordant for BN and seven pairs were discordant for BN (one female-male pair). Five pairs (including two female-male pairs) were discordant for eating disorder symptoms not fulfilling the diagnostic criteria for BN; their brain scans were used only in study **I**. In addition, 10 healthy pairs from the screen-negative twins took part in the studies. All 25 twin pairs went through SPET imaging with [¹²³I]ADAM. Two healthy individuals from different healthy pairs were excluded from the analyses; one due to an earlier history of MDD and the other due to an unsuccessful radioligand injection. The scans performed to the remaining 48 subjects were used in studies **I-III** in different combinations. Furthermore, five women from 5 healthy twin pairs were scanned twice for the purposes of the study **II** on seasonal variation in SERT binding.

Recruitment of healthy male subjects for study II: For the study **II** on seasonal variation in SERT binding, we wanted to include healthy individuals from both sexes. As all the healthy twin pairs mentioned above were female-female pairs, we sought healthy young men to take part in the study through a separate recruitment process. This involved an advertisement of the study directed to healthy young adult male students. Nine men were initially investigated; one was excluded due to technical problems in the first scanning. Eight men were invited for a second scan a few months later; one of them declined the invitation, resulting in altogether seven healthy male subjects in study **II**.

4.2.1.2. Study subjects in each study I-III

In study **I**, all study subjects came from the twin sample. [¹²³I]ADAM scans of 15 healthy females were used for the formation of [¹²³I]ADAM brain template. For the repeatability analyses of the automated system, scans of 10 BN women were used. For intra-observer repeatability analyses of manual VOI definitions we used brain scans of (i) 11 healthy co-twins (including two males) of subjects affected by an eating disorder

and (ii) 10 women with BN. For inter-observer repeatability analyses of manual VOI definitions we used the brain scans of 10 women with BN

In study **II**, we performed [^{123}I]ADAM scans to 12 (five women and seven men) healthy subjects twice, in summer and in winter. Five of the subjects were healthy women from the twin sample (age in summer 26.8 ± 0.84 years and in winter 26.4 ± 1.14 years; body mass index (BMI) in summer $21.54 \pm 4.14 \text{ kg/m}^2$ and in winter $21.84 \pm 4.07 \text{ kg/m}^2$). Their first [^{123}I]ADAM scans had been performed in winter for the purposes of studies **I** and **III**, and they were then asked if they would be willing to be investigated for a second time, in summer. Only one healthy female per healthy twin pair was invited. Seven subjects were healthy males (age in summer 23.29 ± 1.60 years and in winter 23.57 ± 1.99 years; BMI in summer $23.99 \pm 2.30 \text{ kg/m}^2$ and in winter $24.16 \pm 2.58 \text{ kg/m}^2$) enrolled for the study in a separate process as described above. None of the study subjects were alcoholics and four smoked cigarettes regularly.

In study **III**, we investigated three groups of subjects:

- 1) *The BN women*; 13 women fulfilling the DSM-IV criteria of lifetime BN (seven women from BN-discordant twin pairs (one MZ pair and six DZ pairs) and six women from three BN-concordant twin pairs (2 MZ and 1 DZ pairs). Their ages were 24.8 ± 1.7 years and BMIs $22.9 \pm 3.2 \text{ kg/m}^2$.
- 2) *The unaffected female co-twins of the BN women*; six healthy women (one from MZ and five from DZ twin pairs). Their ages were 24.8 ± 2.1 years and BMIs $23.6 \pm 3.2 \text{ kg/m}^2$.
- 3) *The healthy women*; 18 women from 10 twin pairs (5 MZ and 5 DZ female-female pairs). Their ages were 25.3 ± 1.7 years and BMIs $22.2 \pm 3.5 \text{ kg/m}^2$.

Male co-twins were excluded from the study.

4.2.2. Study IV

The subjects for Study **IV** were also enrolled from the FinnTwin16 twin cohort. 658 MZ twin pairs were screened at age of 23–27 years based on their responses to questions on weight and height. 16 healthy MZ twin pairs (eight female and eight male pairs, ages 24–27 (25.42 ± 1.29) years) were selected on the basis of their intrapair difference in BMI so that twin pairs with both similar and divergent BMIs were included. One of the male twins had very high BMI due to muscularity (BMI 31.46 kg/m^2 and fat percentage 11 %) and as we wanted to use BMI as an index of obesity, we excluded him from the analyses of individual data and the twin pair from the analyses of pairwise data. For the remaining 31 individuals, the BMIs ranged from 19.1 to 31.9 kg/m^2 (mean $25.57 \pm 3.45 \text{ kg/m}^2$) and the intrapair BMI differences ranged from 0.31 to 8.1 kg/m^2 (mean $2.24 \pm 2.12 \text{ kg/m}^2$). The mean intrapair weight difference was $6.32 \pm 6.25 \text{ kg}$. All subjects were healthy and none of the women were pregnant. None

of the subjects used concomitant medications except oral contraceptives (n = 6, in 3 twin pairs). Two subjects were current smokers. None used alcohol daily, but seven subjects reported weekly use of alcohol.

4.3. ASSESSMENT OF CLINICAL, PSYCHIATRIC AND BEHAVIOURAL CHARACTERISTICS

In studies **I-III**, the subjects' somatic health was classified as normal based on interviews performed by MDs (AK, AK-R, ES). For subjects in study **IV**, a more detailed assessment of somatic health was made, including laboratory tests as well as clinical examination by an MD (KP). In each subject, body weight and height were measured, and the BMI was calculated as body weight (in kilograms) divided by height (in meters) squared. In study **IV**, body composition was measured by bioelectrical impedance analyser (STA/BIA with Bodygram software; Akern, Florence, Italy).

For the twin subjects in studies **I** and **III**, a detailed psychiatric and behavioural assessment was done. Initial screening was done by sending 2545 female twins a questionnaire including questions on current height and weight, ideal weight, minimum and maximum weight at current height, purging, and weight loss behaviors, as well as assessment of body image by use of three subscales (body dissatisfaction, drive for thinness, and bulimia) of the Eating Disorder Inventory (320). Secondly, the screen-positive women were telephone-interviewed with short version of SCID-I interview (318) for obtaining the lifetime diagnoses of AN, BN, BED, MDD and OCD. Thirdly, face-to-face interviews with EATATE (274) and Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) (321,322) were done for the BN women and for their healthy co-twins and the healthy control twin pairs. The EATATE is a semi-structured clinical interview based upon the Eating Disorders Examination (323) but adapted to assess lifetime symptoms of ED following the Longitudinal Interval Follow-up Evaluation (324). SSAGA is a fully structured, poly-diagnostic psychiatric interview that is used to make substance use disorder and psychiatric disorder diagnoses according to DSM-III-R criteria (325)

For those healthy females participating also in study **II**, psychiatric interviews were performed once, before the first [¹²³I]ADAM scan. For the male subjects in study **II**, no structured psychiatric interviews were performed. Their classification as mentally healthy was based on self-report.

For subjects in study **IV**, structured psychiatric interviews (SSAGA) (321) were performed to exclude lifetime psychiatric diagnoses.

4.4. ASSESSMENT OF ZYGOSITY

The zygosity of all twin pairs was confirmed by genetic blood marker tests using the highly polymorphic, multiple genetic marker set used in the Paternity testing laboratory, National Public Health Institute, Helsinki, Finland.

4.5. SERT IMAGING

4.5.1. Radioligands

4.5.1.1. [¹²³I]ADAM

Iodine 123-labeled 2-((2-((dimethylamino)methyl)phenyl)thio)-5-iodophenylamine ([¹²³I]ADAM) (MAP Medical Technologies Oy, Tikkakoski, Finland) was used to assess SERT binding in studies **I-III**. Thirty minutes before its intravenous injection, the subjects were given 400 mg of potassium perchlorate orally to reduce [¹²³I]-uptake in the thyroid and the salivary glands. Injected radioactivity of [¹²³I]ADAM varied between 139-231 MBq. All radioligand injections were given at 8 o'clock a.m. following an overnight fast.

[¹²³I]ADAM binds selectively to SERTs, with an affinity to SERTs more than 1000-fold over its affinity to dopamine transporters (DATs) and norepinephrine transporters (NETs) (216). Its binding is greatest and least variable in a test-retest setting in the midbrain and thalamus and reaches pseudoequilibrium at 4-6 h post injection (217). Its effective dose is similar to the other commonly used radioligands (220,221).

4.5.1.2. [¹²³I]nor-β-CIT

Iodine 123-labeled 2β-carbomethoxy-3β-(4-iodophenyl) ([¹²³I]nor-β-CIT), supplied by MAP Medical Oy, Tikkakoski, Finland, was used for investigating SERT binding in study **IV**. The mean injected dose of 196.3 MBq (range 187-206 MBq) was given to subjects as an intra venous injection, following an overnight fast. Potassium perchlorate was given to subjects before the radioligand injection for reduction of [¹²³I]-uptake in the thyroid and the salivary glands.

[¹²³I]nor-β-CIT is an analogue of [¹²³I]β-CIT with a high affinity to both SERTs and DATs. It has a tenfold higher affinity to SERTs in comparison to [¹²³I]β-CIT (213). In the midbrain and hypothalamus/thalamus its binding is considered specific, but not selective, to SERTs. In an autoradiography displacement study in post-mortem human brains, the SERT blocker citalopram fully inhibited [¹²³I]nor-β-CIT:s binding to the 5-HT rich thalamus (214), suggesting that [¹²³I]nor-β-CIT:s binding in the hypothalamus/thalamus is specific to SERTs. In a study in living humans, administration of the SERT blocker citalopram to one of the study subjects reduced [¹²³I]nor-β-CIT:s binding in the midbrain by 52% (215), supporting the view that [¹²³I]nor-β-CIT binds to SERTs also in the midbrain. The reported effective dose equivalent of [¹²³I]nor-β-CIT is 0.035 mSv/MBq (215).

4.5.2. SPET procedures

4.5.2.1. SPET studies using [¹²³I]ADAM

Data acquisition and reconstruction into images:

The SPET scans in studies **I-III** took place in the Nuclear Medicine Laboratory of the Helsinki University Central Hospital. The acquisitions were carried out 10 minutes and 5 hours after the radioligand injection. We used a Philips Picker Prism3000XP three-headed gamma camera with ultra-high-resolution fan-beam collimators (Philips Medical Systems, Cleveland, OH, USA). The fan-beam focus of the collimator was 535 mm and the radius of rotation, measured from the surface of the collimator, varied within 130-160 mm, depending on the patient. The acquisitions were performed using a 120° orbit in a stepwise mode. The subject's head was positioned to the centre of rotation in the head locker using a crossed laser beam system for repositioning. For repeated scans, the same positioning information (position and height of the bed) were used. A symmetrical energy window for ¹²³I was used (159 keV; 20 % wide, 143 keV – 175 keV). We used a 128 x 128 matrix size with 120 projection angles (40 projections/detector). The data were collected for 45 s per projection angle, resulting in an average of 50 kilocounts (kcts) per projection in the acquisitions at 10 min and about 20 kcts at 5h.

All reconstructions and image analyses were done on HERMES software system (Hermes Medical Solutions, Stockholm, Sweden), using iterative reconstruction program HOSEM (OS-EM V5.201 by R. Larkin). The decay-corrected reconstructed transverse slices were oriented to the orbitomeatal line. The number of subsets was 8 with 6 iterations. Attenuation correction was performed during the reconstruction using Chang's first-order approximation with the linear attenuation correction ($\mu = 0.110 \text{ cm}^{-1}$), which was based on an ellipse contour of the brain. The images were post-filtered using a Butterworth filter with cut-off frequency of 1.2 cm^{-1} and order 15.

Creation of [¹²³I]ADAM template and the predefined VOI map:

A brain template for [¹²³I]ADAM-images was created in study **I** and later used for automated registration and formation of stereotactic [¹²³I]ADAM images from individual [¹²³I]ADAM scans in studies **I-III**. For the formation of the template, we used [¹²³I]ADAM-scans performed for 15 healthy women. The subjects had earlier been investigated by magnetic resonance imaging (MRI) (Siemens Vision 1.5T with MP_RAGE sequence; Siemens AG, Erlangen, Germany). An MRI scan of one arbitrarily chosen study subject was chosen as a starting point to which her [¹²³I]ADAM scan was then co-registered. Registration was performed with the application MultiModality on HERMES using an automatic algorithm with 6 degrees of freedom (size changes were restricted) and "Mutual Information" method as measure of similarity. The [¹²³I]ADAM-scans of the remaining 14 subjects were then spatially co-registered with the first one using BRASS (Brain Registration and Analysis of SPECT Studies) software (Hermes Medical Solutions, Stockholm, Sweden) on a HERMES, using 9 degrees of freedom and Mutual Information. The 15 co-registered SPET scans

were normalized to total counts and averaged to build a template containing mean values and distributions in every pixel. Anatomically standardized normal reference template was created using the Modelgen software (Hermes Medical Solutions, Stockholm, Sweden).

The template was then used in conjunction with the – intrinsically co-registered – sample MRI to define a set of volumes of interests (VOIs) for all further analyses. Initially a VOI map with several regions was generated, but due to [¹²³I]ADAM's reproducibility issues (217), we only used the midbrain and thalamus VOIs as our target regions and the cerebellum VOI as a reference region (326) in studies **II** and **III**. The automated VOI quantification was performed using the anatomically standardized (stereotactic) images. In studies **II** and **III**, we chose to adjust the location of the midbrain VOI slightly manually, if its automated positioning did not seem exact in visual inspection. The possible moving of the midbrain VOI was then done without altering its size or the transverse slice, to which it was placed by the automated procedure.

Repeatability analyses:

In study **I**, the misalignment of the [¹²³I]ADAM scans following the initial automated registration of the scans to the template was performed using randomly selected values generated in Excel2000 (Microsoft Corporation, USA). Misalignment parameters varied between 10 pixels in translation in X, Y and Z directions, 10 % in scaling and 10° in rotation. Because YZ rotation might vary more depending on patient positioning, this parameter was defined to vary up to 20°.

In study **I**, we also tested the intra- and inter-subject variability in manual VOI drawing. For this purpose, [¹²³I]ADAM images were first reconstructed, attenuation corrected and reoriented to the orbitomeatal line. Every two consecutive transaxial slices were then summed together, and the VOIs were drawn onto these summed slices. The midbrain VOIs were drawn onto images obtained from the acquisitions at 5 hours. The cerebellum VOIs were initially drawn onto the brain perfusion phase images obtained at 10 minutes, after which the VOI was transferred to the 5 hour images for quantification of SERT binding. The lower threshold of 30% was used in all VOI definitions.

Estimation of SERT binding:

In studies **I-III** using [¹²³I]ADAM, SERT binding was estimated by using the simple ratio method applying the formula Specific Binding Ratio (SBR) = (mean counts in the target area - mean counts in cerebellum) / mean counts in cerebellum.

4.5.2.2. SPET studies using [¹²³I]nor-β-CIT

Data acquisition and reconstruction into images:

The SPET scans in the study **IV**, applying [¹²³I]nor-β-CIT as a SERT ligand, were performed at the Department of Clinical Physiology and Nuclear Medicine of the Kuopio University Hospital. The acquisitions were performed at 5 minutes, 6 hours and

24 hours after the injection of the ligand by a dedicated Siemens MultiSPECT 3 gamma camera with fan-beam collimators (Siemens Medical Systems; Hoffman Estates II1., USA). Head positioning was monitored by using two position lasers. The SPET scans were decay-corrected and reconstructed with Butterworth-filtered back-projection in a 128×128 matrix with a pixel size of 3×3 mm, and attenuation-corrected with Chang's algorithm. The attenuation correction was uniform with the attenuation coefficient of 0.11 cm⁻¹. The SPET slices were consecutively summarized to the slice thickness of 6 mm and re-aligned using a Siemens semi-automatic brain quantification program and the Talairach coordinates (327). The slices were rotated and re-aligned so that transaxial (x-direction), sagittal (y-direction) and coronal (z-direction) slices were at right angles to each other.

VOI placement was done using a semi-automatic brain quantification program of Siemens. The lower threshold of 60 % of the maximum count was used to reduce the volume averaging and partial volume errors. Target VOIs (included in the present work) were the midbrain and hypothalamus/thalamus, and the cerebellum VOI was used as a reference region (326).

Estimation of SERT binding

In study **IV** using [¹²³I]nor-β-CIT, we applied a graphical reference tissue method (328,329) to estimate the specific binding in terms of distribution volume ratios (DVRs). For calculating DVR, the integrated tissue radioactivity from time zero to T, normalized to tissue activity at time T, was plotted versus the integrated cerebellar time-activity data, which were also normalized to tissue activity at time T. This plot becomes linear when pseudoequilibrium is reached and the slopes for the midbrain and hypothalamus/thalamus data equal DVR. DVR is related to binding potential through formula BP = DVR - 1 (329). The model is based on the assumption that flux from the free tissue compartment to arterial plasma compartment remains constant in the target and reference regions.

4.5. STATISTICAL ANALYSES

In studies **II-IV**, the data were analyzed using the Stata software (release 8.2; Stata Corp., College Station, TX, USA). All data were normally distributed.

In study **I**, the inter- and intra-observer repeatability of the SERT binding quantification based on manual VOI definition and the repeatability of the SERT binding quantification based on automated method were investigated (i) graphically by using a Bland-Altman plot, where the difference between measurements is plotted against their mean value; and (ii) by calculation of coefficient of repeatability values (s), determined as twice the standard deviation of differences between analyses (330,331). The difference between the automated registrations for each patient was also evaluated comparing the initial registration to the second registration (following misalignment of the images) on a voxel-by-voxel basis, and calculating the mean

variation of the two registered studies, which describes the percentage difference or change in voxels between the two registrations.

In study **II**, the paired nonparametric Wilcoxon tests were used for within-subject comparisons of the summer and winter data, and the multiple linear regression analyses were used to test the effects of multiple explanatory variables on a response variable.

In studies applying twin design (studies **III** and **IV**), the analyses were done both for individual and for pair-wise data. In individual data, the bias caused by inclusion of genetically similar subjects was taken into account for by adjusting for clustering of twins within pairs using estimation procedures for complex survey methods. In Stata, the procedures SVYMEAN, SVYTEST (Wald test) and SVYREG (multiple linear regression analysis) permit derivation of the proper standard errors, variances, confidence intervals and P values, correcting for dependency within a twin pair. For comparison of more than two groups, we used one-way analysis of variance, and then appropriate two-group tests.

For within-pair comparisons of the variables, we used the paired nonparametric Wilcoxon tests. In study **III**, Wilcoxon tests were used to compare the BN women with their healthy co-twins and in study **IV**, they were used to compare the twin with higher BMI to his/her leaner co-twin. In studies **III-IV**, we also calculated the mean intrapair differences between the twin and the co-twin for various variables and then computed Spearman correlations between the difference variables; for this analysis the unit of observation is the twin pair and all observations are statistically independent of each other.

Data are shown as mean \pm standard deviation, except for multiple regression analyses, where β -coefficients \pm standard errors are shown. P values less than 0.05 were considered statistically significant.

5. RESULTS

5.1. COMPARISON OF REPRODUCIBILITY OF MANUAL AND AUTOMATED QUANTIFICATION TECHNIQUES FOR SERT BINDING IN STUDIES WITH [¹²³I]ADAM

For the automated method, the Bland-Altman plot of the midbrain SBRs yielded from two automated registrations and VOI placements can be seen in **Figure 3**. The coefficient of repeatability for the specific binding ratio (SBR) in the midbrain VOI was $s=0.13$. The mean variation between the first and second automated fitting procedures (describing the percentage difference in all voxels between the two registrations) was $0.38 \pm 0.15\%$.

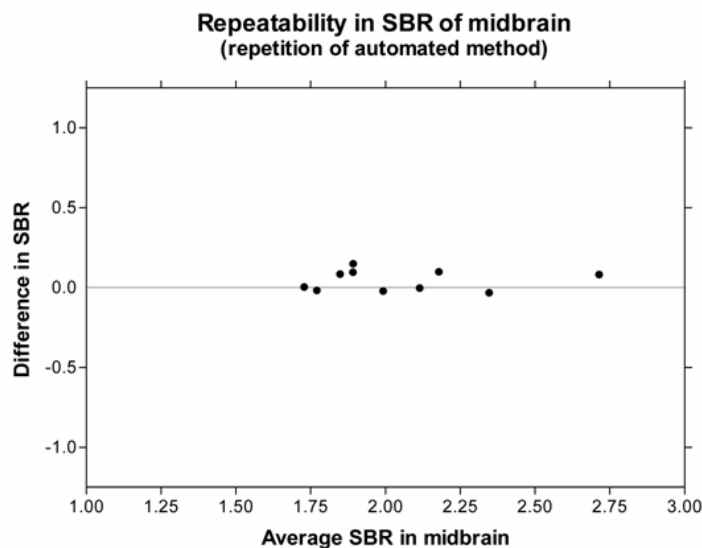


Figure 3. Repeatability of the midbrain SBR by the automated method.

For the SBRs yielded by manual VOI definition, the Bland-Altman plots for intra- and inter-observer repeatability are shown in **Figure 4**. The intra-observer coefficient of repeatability for analyses of the 10 BN subjects was $s=0.70$. For the same study subjects, the inter-observer coefficient of repeatability was $s=1.00$.

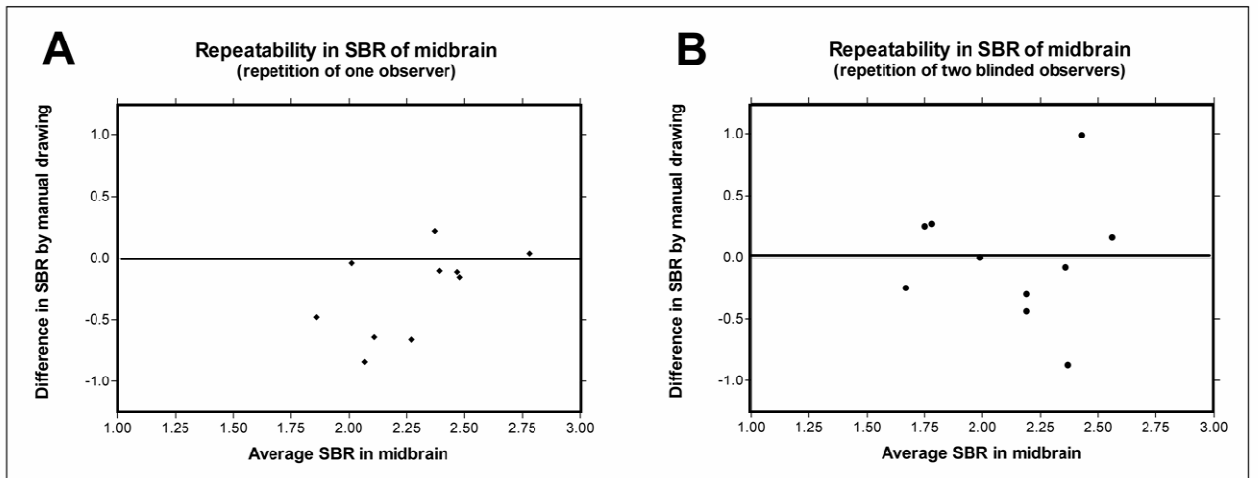


Figure 4. Intra- and inter-observer repeatability of the manual VOI drawing.

5.2. Seasonal variation in SERT binding of [¹²³I]ADAM

Table 3. The specific binding ratios of [¹²³I]ADAM at five hours after injection

	Midbrain _s	Midbrain _w	p-value*	Thalamus _s	Thalamus _w	p-value*
All subjects (n=12)	2.04 ± 0.30	2.04 ± 0.25	0.81	1.56 ± 0.33	1.52 ± 0.31	1.00
Women (n=5)	2.04 ± 0.18	2.03 ± 0.14	0.89	1.68 ± 0.45	1.35 ± 0.17	0.22
Men (n=7)	2.04 ± 0.38	2.04 ± 0.33	0.87	1.48 ± 0.22	1.64 ± 0.34	0.31

s=summer, w= winter, * Wilcoxon signed-rank test

Table 3 summarizes the main findings of SERT binding of [¹²³I]ADAM at summer and winter. No systematic variation in SERT binding of [¹²³I]ADAM was evident in either the midbrain or thalamus. This was true for both sexes. **Figure 5** further illustrates the lack of systematic differences between summer and winter scans; in both investigated areas, half of the subjects had higher SERT binding in summer and the other half in winter. Non-specific binding of [¹²³I]ADAM (as measured by the cerebellar activity) was also similar in summer (93.03±15.51 in counts/pixel/kBq) and winter (93.64±14.21 counts/pixel/kBq) scans (p=0.92).

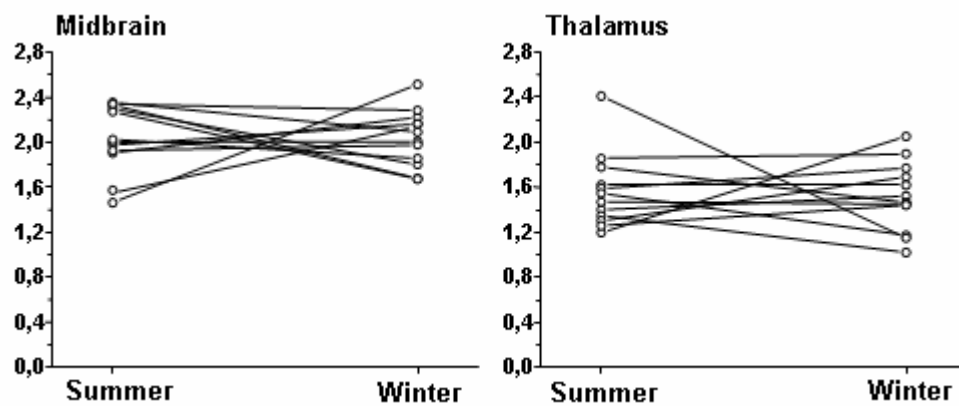


Figure 5: The specific binding ratios (SBRs) of SERT binding of [¹²³I]ADAM in summer and winter in the midbrain and thalamus.

The subjects' BMIs were similar at both scans (summer: 22.97 ± 3.27 kg/m², winter: 23.19 ± 3.33, p=0.08). [¹²³I]ADAM dose, BMI, and smoking had no significant effect on the SERT binding in either season.

5.3. SERT AVAILABILITY IN SUBJECTS AFFECTED BY OR GENETICALLY PREDISPOSED TO BULIMIA NERVOSA

5.3.1. Demographic variables and behavioural assessments

The BN women, their unaffected sisters and the healthy twin women had similar ages (24.8 ± 1.7 y, 24.8 ± 2.1 y, and 25.3 ± 1.7 y, $p= 0.81$) and BMIs (22.9 ± 3.2 kg/m², 23.6 ± 3.2 kg/m², and 22.2 ± 3.5 kg/m², $p= 0.68$). In the group of 13 women with lifetime BN, 11 were currently symptomatic, while two had been asymptomatic for three and five years. The mean age of onset of BN was 18.3 years (SD: 2.9 y, range: 13 - 21 y) and its mean duration was 6.5y (SD: 4.4 y, range: 6 months - 14 y). Nine of the 13 BN women suffered from the purging subtype of BN, and four were diagnosed with the non-purging subtype. Compensatory behaviors other than vomiting included diuretics and Ipecac abuse, excessive exercise, and fasting. Five individuals had a past history of anorexic symptoms, but had been asymptomatic for more than 6 months prior to our study. One of the BN women had current MDD, and seven BN women had a history of MDD. None of the healthy sisters or healthy control women had past or present psychiatric morbidities.

5.3.2. SERT binding in women with BN, their unaffected sisters and non-related healthy twin women

As shown in **Table 4**, the BN women, their unaffected sisters and the unrelated healthy control women had similar SBRs of [¹²³I]ADAM to SERTs both in the midbrain and thalamus. The unaffected sisters and the other healthy controls did not differ in their SBRs in the midbrain ($p= 0.68$) or thalamus ($p= 0.65$), and therefore we combined these two groups of healthy women. When BN women were compared with all the healthy women combined, no significant differences were found either in the midbrain (BN: 2.23 ± 0.22 , all healthy: 2.06 ± 0.30 , $p= 0.08$) or thalamus (BN: 1.45 ± 0.23 , all healthy: 1.42 ± 0.28 , $p= 0.45$). Removal of the two currently unsymptomatic women from the group of probands did not affect these results (data not shown). SERT binding in the cerebellum (i.e., non-specific uptake) did not differ between the groups (98.99 ± 17.53 counts/pixel/kBq for the subjects with lifetime BN, 93.38 ± 21.42 counts/pixel/kBq for the healthy co-twins and 96.52 ± 15.26 counts/pixel/kBq for the healthy controls, $p=0.73$).

Table 4. SBRs for women with lifetime BN, their healthy co-twins and healthy control women

	BN women (n=13)	Twin-sisters (n=6)	Controls (n=18)	p-value*
Midbrain	2.23 ± 0.22	2.02 ± 0.29	2.07 ± 0.31	0.21
Thalamus	1.45 ± 0.23	1.38 ± 0.25	1.43 ± 0.29	0.86

*Significant differences between the groups were calculated using ANOVA: p-values were corrected for clustered sampling caused by inclusion of twins.

In *post hoc* comparison of the subgroup of purging BN women and the healthy women, the BN probands had significantly increased SERT binding in the midbrain. No differences were evident in the thalamus (**Table 5**).

Table 5. SBRs: Comparison of the subgroup of purging BN women and all the healthy women

	Women with purging BN (n=9)	All healthy women (n=24)	p-value*
Midbrain	2.26 ± 0.19	2.06 ± 0.30	0.03
Thalamus	1.50 ± 0.25	1.42 ± 0.28	0.45

* t-test; p-values were corrected for clustered sampling caused by inclusion of twins.

5.3.3. The effect of past psychiatric comorbidities on individual data

The effects of MDD and past history of AN on the results were also explored. When subjects were grouped on the basis of MDD history, the subjects with (n=7) and without (n=30) MDD history had similar SBRs both in the midbrain ($SBR_{MDD} 2.20 \pm 0.22$, $SBR_{no-MDD} 2.10 \pm 0.30$, $p=0.38$) and thalamus ($SBR_{MDD} 1.48 \pm .28$, $SBR_{no-MDD} 1.42 \pm .26$, $p= 0.63$). Regarding past AN, the mean SBRs for SERT binding were very similar for the BN women with (n=5) and without (n=8) past history of AN both in the midbrain ($SBR_{AN} 2.25 \pm 0.26$, $SBR_{no-AN} 2.21 \pm 0.22$) and thalamus ($SBR_{AN} 1.50 \pm 0.28$, $SBR_{no-AN} 1.42 \pm 0.21$). In multiple linear regression analysis, history of AN or MDD had no effect on the results, whereas the effect of purging remained significant (**Table 6**).

Table 6. Multiple regression analysis examining the relationship of SERT binding with purging BN and past histories of Major Depression and Anorexia Nervosa

MIDBRAIN: $R^2 = 0.10$, $F(3,17) = 3.92$, $p = 0.03$	β	S.E. of β	p-value*
Purging BN	-0.22	0.09	0.02
History of major depression	0.08	0.10	0.44
History of AN	-0.08	0.09	0.38

THALAMUS: $R^2 = 0.03$, $F(3,17) = 1.02$, $p = 0.41$	β	S.E. of β	p-value*
Purging BN	-0.10	0.06	0.14
History of major depression	0.02	0.09	0.80
History of AN	-0.04	0.12	0.72

*p-values were corrected for clustered sampling caused by inclusion of twins.

5.3.4. Within-pair comparisons of SERT binding

In within-pair comparisons, there were no differences in SBRs between a twin and her co-twin in neither of the brain regions in either the group of BN-discordant pairs (n=6 pairs) or the group of healthy twin pairs (n=8 pairs). The mean intrapair differences for

SBRs (SBR of twin 1-SBR of twin 2 in a given VOI) were also similar for the BN-discordant twin pairs and the healthy control twin pairs. Due to small number of pairs (n= 2), within-pair comparisons were not done for the pairs discordant for purging lifetime BN.

5.4. RELATIONSHIP BETWEEN BODY MASS INDEX AND THE BRAIN SERT BINDING

5.4.1. BMI and SERT binding in individuals

In individual data (the bias caused by inclusion of MZ twins taken into account for), BMI, weight or fat percent did not correlate with SERT binding in either the hypothalamus/thalamus or midbrain.

The effect of sex and oral contraceptives:

In *post hoc* analyses, the effects of sex and oral contraceptives on SERT binding were analyzed. In individual data, men as compared to women had significantly higher SERT binding in the midbrain ($DVR_{men}: 1.37 \pm 0.14$, $DVR_{women}: 1.26 \pm 0.12$, $p= 0.04$). No significant differences between the sexes were evident in the hypothalamus/thalamus ($DVR_{men}: 1.24 \pm 0.16$, $DVR_{women}: 1.10 \pm 0.21$, $p=0.09$).

The 6 women using oral contraceptives (OCs) as compared to the 10 women without OCs had lower SERT binding in the hypothalamus/thalamus ($DVR_{OC}: 0.94 \pm 0.08$, $DVR_{without-OC}: 1.20 \pm 0.20$, $p= 0.01$). No significant differences were detected in the midbrain ($DVR_{OC}: 1.30 \pm 0.14$, $DVR_{without-OC}: 1.24 \pm 0.10$, $p= 0.45$). Exclusion of the women with oral contraceptives did not change the outcome of comparison of SERT binding between the sexes. Men still had higher SERT binding in the midbrain than the remaining ten women ($DVR_{men}: 1.37 \pm 0.14$, $DVR_{women}: 1.24 \pm 0.10$, $p= 0.01$), while similar SERT binding persisted in the hypothalamus/thalamus ($DVR_{men}: 1.24 \pm 0.16$, $DVR_{women}: 1.20 \pm 0.19$, $p=0.63$).

5.4.2. BMI and SERT binding in twin pairs

The results of within-pair comparisons are shown in **Table 7**. When twins were analyzed as pairs, comparing the twin with higher BMI to the co-twin with lower BMI, the twins with higher BMI had significantly higher SERT binding in the hypothalamus/thalamus than their co-twins. When men and women were analysed separately, the heavier women differed significantly from their leaner co-twins ($p= 0.01$), but no differences were found in men ($p= 0.61$). No significant within-pair differences in SERT binding were found in the midbrain.

The calculated intrapair differences in SERT binding did not correlate significantly with intrapair differences in BMI, weight, or body fat percentage in either the midbrain or hypothalamus/thalamus.

Table 7. BMIs, weights, fat percentages and SERT binding in pair-wise data.

	Twin with higher BMI	Co-twin with lower BMI	*p-value	Intrapair difference
All twin pairs (n=15 pairs)				
BMI (kg/m ²)	26.76 ± 3.61	24.52 ± 3.08	0.0007	2.24 ± 2.12
Weight (kg)	77.96 ± 12.74	71.64 ± 11.96	0.0012	6.32 ± 6.25
Percent whole body fat	28.91 ± 8.41	26.34 ± 8.29	0.0125	2.57 ± 3.27
SERT binding (DVR**):				
Hypothalamus/thalamus	1.21 ± 0.23	1.12 ± 0.16	0.04	0.09 ± 0.18
Midbrain	1.34 ± 0.15	1.30 ± 0.14	0.29	0.04 ± 0.16
Men (n= 7 pairs)				
BMI	26.46 ± 4.18	24.16 ± 3.50	0.02	2.30 ± 1.70
Weight	84.19 ± 15.20	76.79 ± 12.98	0.02	7.41 ± 5.68
Fat percent	22.77 ± 5.16	19.76 ± 4.13	0.09	3.01 ± 3.90
SERT binding (DVR**):				
Hypothalamus/thalamus	1.26 ± 0.22	1.22 ± 0.08	0.61	0.04 ± 0.23
Midbrain	1.41 ± 0.14	1.36 ± 0.14	0.24	0.05 ± 0.20
Women (n= 8 pairs)				
BMI	27.02 ± 03.31	24.84 ± 2.86	0.01	2.18 ± 2.56
Weight	72.51 ± 07.33	67.14 ± 9.59	0.03	5.38 ± 6.95
Fat percent	34.26 ± 06.93	32.10 ± 6.45	0.05	2.18 ± 2.81
SERT binding (DVR**):				
Hypothalamus/thalamus	1.17 ± 0.24	1.04 ± 0.16	0.01	0.13 ± 0.13
Midbrain	1.28 ± 0.12	1.24 ± 0.12	1.00	0.03 ± 0.13

*Wilcoxon signed-rank test; Data are shown as mean ± SD;

**DVR= distribution volume ratio

6. DISCUSSION

6.1. METHODOLOGICAL CONSIDERATIONS

6.1.1. Definition of volumes of interest

VOI definition is one potential influencer of outcomes in imaging studies of the brain neurotransmitter systems, especially in small and macroscopically invisible nuclei such as the serotonergic raphe nuclei. Perhaps due to this lack of visualization in anatomical imaging methods such as MRI and computed tomography (CT), in many studies the VOIs of SERT rich areas have been drawn manually onto images on the basis of visual information of the SPET images. However, this method of VOI definition is liable to both intra- and inter-observer variability. Differences between observers caused by their “handwriting” are likely even in the setting of agreed count thresholds for VOI definitions, as shown in study I. Furthermore, even in case of one observer, handwriting can change especially if time passes between the occasions. In case of a highly experienced observer, the intra-observer variability is likely to be smaller, but may nevertheless have some impact on study results. In order to reduce the variability caused by VOI drawing, some studies have used VOIs of fixed size, e.g., spherical VOIs of a certain size (e.g., (181)). In this way, the volume in which activity is measured is standardized, reducing one element of variability. If these VOIs are placed manually on the basis of visual information of the SPET data, some observer dependent variability is likely to remain.

One way of standardizing VOI definition is to base it on boundaries of anatomical structures. This can be achieved by co-registration of individual MR images and SPET/PET images and by drawing the VOIs based on anatomical boundaries visible in MRIs. Unless some automated procedures for VOI definition are used, even this method has an element of subjectivity caused by manual definition of regions. Furthermore, small errors occurring in co-registration of MR and SPET images are possible. The MRI-based demarcation of some structures such as raphe nuclei is not possible due to their invisibility in MRIs.

The disadvantage of VOI analyses is the need for prior determination of the volumes of interest, which may leave some significant effects undetected. Another disadvantage is the difficulty of inter-study comparisons (332).

Radioligand specific brain templates are used increasingly for the automated registration and realignment of individual images (182-184). They can be used for formation of stereotactic images, which can thus be compared in a more standardized way, either with predefined VOIs or on a voxel-by-voxel basis. However, human brains differ in size and some individual variation may exist in the sizes of anatomical brain structures, which introduces a bias for binding estimation. In the fitting process the brain images are adjusted to fit the template, which may cause small alteration in the size of the structures, thus diluting or concentrating count densities. Voxel-based

methods, applying software such as the Statistical Parametric Mapping (SPM) (Wellcome Department of Imaging Neuroscience, UCL, London, UK) (185), offer the advantage of not limiting binding estimation to predefined regions. The downside of SPM is that the original images need to be smoothed in order to meet the requirements of the statistical assumptions and to compensate for the anatomical differences between subjects (332).

When investigating small structures, partial volume effect (PVE) can affect the measured binding estimates. To a degree, it can affect all the above mentioned quantification techniques, but especially manual VOI drawing of small structures. The PVE is caused by two distinct phenomena (333). First is the image blurring caused by limited spatial resolution of the imaging system, causing spillover (and sometimes spill in) of activity between regions. This usually makes a small structure appear larger but dimmer. The significance of the spillover is greatest in small structures with high count densities, and leads to an underestimation of the true activity. The second phenomenon causing PVE is image sampling. This is caused by the fact that SPET and PET images consist of voxels, which do not match the actual contours of the tracer distribution. Most voxels therefore include different types of tissues, and the signal intensity in each voxel is the mean of the signal intensities of the underlying tissues included in that voxel. Although PVE influences especially binding estimates based on manual VOI delineation, it has some effect even in count densities of single voxels, and to a degree has effect on all methods. Some correction methods for PVE exist (333), but are not used routinely in SPET. No PVE corrections were performed in studies **I-IV**, which can have some effect on our results.

In study **I**, we validated the automatic registration and realignment of [¹²³I]ADAM-images to a template and found it as a reproducible method. Use of the template-based registration and predefined VOI map produced reproducible SERT binding estimates, unlike manual VOI delineation. However, visual inspection revealed that VOI placement is sometimes not quite accurate, especially in the midbrain. In studies **II** and **III**, we thus decided to adjust the placement of the midbrain VOI manually, if considered necessary in inspection. In study **IV**, VOIs were drawn manually by highly experienced analyst. As discussed above, both these methods have their drawbacks. However, none of the methods described above are perfect and without the possibility of biasing factors.

6.1.2. The radioligands

Two SERT ligands were used in this thesis; [¹²³I]ADAM and [¹²³I]nor-β-CIT. Both have benefits over [¹²³I]β-CIT, which until recently was the most popular SERT ligand in SPET studies. [¹²³I]β-CIT has affinities for DATs, SERTs and to a minor degree to NETs (206-209), but its binding in the midbrain and hypothalamus/thalamus has been considered as specific for SERTs (208,210). However, considering that especially in the midbrain there are nuclei containing both DATs and NETs (211,212), the possibility remains that binding to DATs and NETs affects the results considered to

represent SERTs. The ligand used in study IV, [¹²³I]nor-β-CIT, has also affinities for DATs and SERTs (213), but its affinity to SERTs is relatively higher than that of [¹²³I]β-CITs, and it may thus be better suited for SERT imaging. [¹²³I]ADAM on the other hand has over 1000-fold affinity for SERTs over other monoamine transporters (216), and its binding is thus considered as selective for SERTs. With its affinity profile, it competes with the SERT ligands used in PET studies, i.e. [¹¹C]DASB, [¹¹C]McN5652 and [¹¹C]MADAM (334). However, the better resolution of PET as compared to SPET favours the use of PET ligands in SERT imaging. [¹²³I]ADAM has also some other properties that affect its suitability for SERT imaging. Its binding kinetics varies somewhat depending on the SERT density of the region (217,223), and theoretically, inter-individual differences in SERT densities can cause differences in its kinetics, too. In patients, these differences may be even greater and affect the SERT binding estimates between study populations.

The test-retest variability of [¹²³I]ADAM is not ideal, being 13% ± 11% in the midbrain, 16% ± 13% in the thalamus, and around 20% in the lower binding regions (217), and for this reason, we limited our SERT measurements to the midbrain and thalamus. Variability values expressed as percentages have not been published for SERT binding of [¹²³I]nor-β-CIT, but its test-retest difference (mean absolute difference between test and retest) for the midbrain distribution volume ratios is reported to very low ($VD_{\text{test}}: 1.27 \pm 0.11$, $VD_{\text{re-test}}: 1.27 \pm 0.14$, mean difference: 0.00 ± 0.08) (335). Of the other ligands for SERT imaging, [¹¹C]DASB and [¹¹C]MADAM have slightly less variability in their binding, both having mean test-retest difference less than 11 % in regions with high SERT densities (228-230). No test-retest variability results have been published for SERT binding of [¹²³I]β-CIT and [¹¹C]McN5652. The intra-class correlation coefficients for [¹¹C]DASB in the midbrain and thalamus areas are either of the same magnitude (228) as for [¹²³I]ADAM (217) or better (229), while for [¹¹C]MADAM, the reported ICCs in these two areas are worse than for [¹²³I]ADAM (230). For [¹²³I]nor-β-CIT, the ICC (in the midbrain) is slightly better than for [¹²³I]ADAM (335).

In some study subjects, lipophilic metabolites of [¹²³I]ADAM have been reported, but as SERT blocker citalopram had no effect on their blood concentrations, they are unlikely to bind to SERTs (223). Also for [¹²³I]nor-β-CIT at least one clearly lipophilic metabolite has been reported (215). However, no studies have been published on the effect of its metabolites on the specific binding estimates.

Most studies (including ours) using [¹²³I]ADAM as ligand have used the simple ratio method for the estimation of SERT binding. Initially, SERT binding quantification with a simple ratio method and by simplified reference tissue model (SRTM) (336) were compared to a full kinetic modelling with arterial blood sampling in baboons (337). Strong correlations between the full kinetic modelling and both non-invasive methods were detected. SRTM slightly underestimated and ratio method slightly overestimated SERT binding (337). Preliminary results (published as an abstract) in humans also showed a correlation between the SRTM and full kinetic modelling (338), supporting the use of non-invasive SERT quantification. Catafau et al (217) found good

correlations between the SRTM and ratio methods. However, the first paper comparing full kinetic modelling with several non-invasive quantification methods in humans was published only recently. That study reported only a moderate correlation between the ratio method and full kinetic modelling ($r = 0.94$) and considerable overestimation of specific uptake ratios (on average by $10\% \pm 28\%$). However, ratios were calculated from scans made at 200-240 min after injection, which may be a bit early considering the reported pseudoequilibrium of [^{123}I]ADAM binding at 240-360 min after injection (217), and thus affect the reported finding. Better agreement was reached using the Logan model and acquisition time 0-120 min (223). Nevertheless, some uncertainty remains on the validity of the ratio method in quantification of SERT binding in studies using [^{123}I]ADAM.

For [^{123}I]nor- β -CIT, initially the simple ratio method was used for SERT binding estimation (215). This has later been replaced by calculation of DVRs, using graphical reference tissue method without arterial sampling (112,328,329). No studies comparing these non-invasive methods with full kinetic modelling with arterial sampling have been published; some uncertainty thus remains on their agreement with full kinetic modelling when using [^{123}I]nor- β -CIT.

6.1.3. Relationship between 5-HT levels and SERT binding

Despite the fact that numerous studies have been published on imaging of the brain SERTs, there is no firm consensus on the relationship between SERT binding and the synaptic 5-HT levels. The earliest SPET studies on SERT binding using [^{123}I] β -CIT as a ligand found reduced SERT binding in conditions with presumed hyposerotonergic state and responsive to SSRI medication, e.g., MDD (339), seasonal affective disorder (340), alcoholism (116), and BN (181). Our initial hypotheses were also based on this assumption. However, even though some later studies using the same ligand and more SERT-selective ligands have had similar results (115,335,341,342), others have found no differences between respective study populations (117,169,343,344) or have had opposite results (345,346).

Theoretically, several different relationships are possible between extracellular 5-HT levels and the SERT levels. For example, reduced 5-HT levels could be associated with either reduced or elevated binding of the radioligand to the transporter by the following mechanisms. Binding could be reduced:

1. If reduction of extracellular 5-HT is caused by loss of serotonergic nerve terminals, leading to reduction in both SERT density and 5-HT (as in case of DAT binding in Parkinson's disease in which loss of presynaptic dopaminergic neurons causes reduced DAT binding (347)).
2. If reduced 5-HT leads to reduction in SERT expression or increased internalization of the SERTs from the plasma membrane. Acute reductions in 5-HT have been associated with reduced 5-HTT mRNA in animal studies (348,349), and this might reduce SERT expression and binding. Furthermore, in vitro studies have shown that SERT occupancy by 5-HT prevents the

internalization of SERTs from the plasma membrane (89), in which case reduced extracellular 5-HT could lead to internalization of SERTs into the cytosol and possibly to reduction in SERT binding.

Alternatively, SERT binding might be increased:

1. If increase in SERT density is the factor causative to or adding to the reduction of extracellular 5-HT. This view is supported by studies with SERT knockout mice, in which mice lacking SERTs show increased extracellular 5-HT (36).
2. If reduced 5-HT increases SERT's affinity for its ligands.
3. If there is reduced endogenous competition by 5-HT for the binding sites. Such competition has been found to exist for [¹²³I]-β-CIT (350), whereas results regarding selective SERT-ligand, [¹¹C]DASB, are inconsistent (351-355). SERT ligands may differ from one another in this respect, making conclusions regarding the relationship even more difficult. To date, no studies have been published on endogenous competition by 5-HT with [¹²³I]nor-β-CIT or [¹²³I]ADAM.

Further complexity to this picture may come from the lipophilicity of the SERT ligands. Lipophilicity is a necessary quality for a radioligand targeted to the brain, as it needs to cross the blood-brain barrier. However, due to lipophilicity, the radioligands used in brain imaging might also permeate the plasma membrane and enter the cell, and it has been suggested that radioligand binding techniques cannot discriminate cytosolic from surface transporter pools (356). This may be of importance in clinical studies, as the trafficking of SERTs between the plasma membrane and cytosol is one of the main events in the short term regulation of SERTs (34) and thus disease specific alterations particularly affecting this SERT distribution are possible. It is possible that radioligand studies are insensitive for this kind of alterations in the SERT densities, and differences may exist again between different SERT ligands.

The complexity of relationship between 5-HT levels and SERT binding highlights the necessity for more studies addressing this relationship. Furthermore, this should be done for each radioligand separately, a fact which has been much neglected until recently.

6.1.4. Other methodological considerations

Most studies investigating the brain SERT binding have used cerebellum as the reference region. Based on earlier *post mortem* study results, cerebellum is nearly devoid of SERTs, showing considerably lower [³H]Paroxetine binding than other brain regions (326). It has therefore been considered as suitable reference region for estimating SERT binding in SPET and PET studies. However, displaceable SERT binding in the cerebellum has been shown for some SERT ligands, such as [¹¹C]DASB and [¹¹C]McN5652 (334), both in rat and monkey cerebellum. For [¹¹C]DASB, also a human *post mortem* study reported displaceable SERT binding in the cerebellum,

showing highest specific SERT binding in the cerebellar vermis, followed by cerebellar grey matter and cerebellar white matter (357). It is presently thought that cerebellar vermis should not be included in the reference region, at least when using [¹¹C]DASB. In our studies, cerebellar vermis was included in the reference region. In rat cerebellum, displaceable [¹²³I]ADAM binding was detected, while no displaceable [¹²³I]ADAM binding was evident in the monkey cerebellum (334). However, a recent study reported no displacement of SERT binding of [¹²³I]ADAM from the cerebellum following infusion of citalopram, indicating that cerebellar binding of [¹²³I]ADAM represents non-specific binding and supporting its use as a reference region (223). For [¹²³I]nor-β-CIT, pre-treatment with citalopram did not displace the radioligand from the cerebellum in monkeys (214); in humans, displacement data has been published only for regions with specific binding, the midbrain and striatum (215). For some reason, even selective SERT ligands seem to differ from one another in their binding to cerebellum (334). One suggested explanation is that different SERT ligands bind to different classes of SERTs, differing either in their affinity states or in their subcellular organization (e.g., plasma membrane vs. cytosol) (334).

In all our studies comparing SERT binding between groups (studies **II-IV**), sample sizes were relatively small, but similar to sample sizes in many other SPET and PET studies. In reality, sample sizes are limited by the costs of an investigation as well as difficulties in finding study subjects that fit the inclusion and exclusion criteria. In studies **II-IV**, the sample sizes were smaller than we initially aimed for. In study **II**, three (out of 15) subjects dropped out, two of them because of technical problems in the SPET acquisitions. In study **III**, the number of BN cases was limited by the exclusion criteria (e.g., medications, tattoos (the subjects also went through MRIs), and by BN symptoms that in structured psychiatric interviews did not fulfil the diagnostic criteria. Also the number of unaffected co-twins was smaller than expected; the number of twin pairs concordant for BN was a surprise, and exclusion of male co-twins decreased the sample size of this group further. In study **IV**, only a small number of weight-discordant twins were found despite screening thousands of twins. Therefore, these studies have the possibility of type II error due to small sample sizes. However, in study **II** we don't consider this very likely, as there was not even a trend towards of difference in SERT binding between summer and winter scans. In study **III**, limited study power may have contributed to our inability to detect differences between BN women and the healthy women, and in study **IV**, to our inability to detect correlation between BMI and SERT binding in the individual data.

We could not time all the SPET scans of the women to a particular phase of their menstrual cycle, as is often done in PET and SPET studies investigating the serotonergic system. Animal studies suggest that ovarian hormones may affect the 5-HT system cyclically (145). In our studies timing the SPET scans to a particular phase of menstrual cycle was not possible, as the women in these studies were twins and the initial study designs were set to investigate twin-co-twin differences. We therefore prioritized scanning both twins at the same day instead of investigating them at a particular phase of their menstrual cycles. In study **II**, in which five unrelated women were studied twice, our aim was to time the second scan to the same phase of the

menstrual cycle as the first one, but for logistic reasons, this was possible only for three women. Therefore, although one SPET study found no differences in SERT binding in women scanned in their luteal phases as compared to the same women scanned at follicular phases (152), we cannot exclude the influence of the phase of menstrual cycle on the SERT binding estimates in women.

6.2. Seasonal variation in the brain SERT binding

In study **II**, we did not detect significant systematic variation between summer and winter data in SERT binding of healthy subjects in the SERT rich regions midbrain and thalamus. Our study was the first study to investigate within-subject seasonal variation in the brain SERTs. Some studies investigating platelet SERT (158-160) or 5-HT_{2A} (158,161,162) binding have investigated seasonal within-subject variation, but their results have been inconsistent. Furthermore, two studies investigating the relationship between platelet SERT binding and the brain SERT binding found no obvious correlation between the central and peripheral measures (339,358), indicating that peripheral measures cannot be directly interpreted to represent respective central measures.

Our results differ from the only previous study investigating the brain SERT binding (164), which reported lower SERT binding in the thalamus/hypothalamus in winter as compared to summer. However, the studies differ in the following aspects:

1. We used a SERT-selective ligand, whereas the other study applied [¹²³I]β-CIT. Even though [¹²³I]β-CIT's binding in the SERT rich regions thalamus, hypothalamus and midbrain has been considered as specific for SERTs (208,210), it nevertheless possesses affinities for DATs, and to some extent also to NETs (206-209). Our results thus reflect more reliably binding to SERTs, while theirs may have been affected by binding to other monoamine transporters.
2. We investigated both sexes, and the other study investigated only women. However, this is unlikely to explain the discrepancy between the study results, as our study outcome was similar for both sexes.
3. We investigated within-subject variation, and the other study investigated different subjects at different time points. Therefore, our study has less confounding variables.

Small sample size was a weakness for both of these studies; in their case five women were investigated in summer and six women in winter, while in our case five women and seven men were investigated in both seasons. Even if our sample size is bigger, it may not be big enough considering the reported test-retest variability of [¹²³I]ADAM (13% ± 11% in the midbrain and 16% ± 13% in thalamus (217)). Similar consideration for [¹²³I]β-CIT is prevented by the fact that no test-retest data for its SERT binding has been published. However, we do not believe limited study power explains our lack of significant seasonal variation, as we did not detect even any trend suggesting systematic seasonal variation.

Although we did not find systematic within-subject differences in SERT binding between summer and winter, the results of the two scans showed considerable variability for some of the participants. This may be explained by the test-retest variability of [¹²³I]ADAM, or by some other intra-subject changes in some unknown/unmeasured variables affecting SERTs. The subjects' BMIs or ages did not explain this variability.

Our study had some limitations that may have caused the fact that no seasonal variation was detected. Most importantly, there was no uniform and repeated psychiatric screening for the study subjects. Structured psychiatric interviews were performed only for women, who were interviewed by a psychiatrist before the winter scan but not before the summer scan; therefore, we cannot exclude some changes in their psychiatric status having taken place between the scans. For males, no structured psychiatric interviews were performed. Their classification as mentally healthy was based by their self-report and an interview by an MD, and some mild psychiatric problems may thus have not been detected. Therefore, mild psychiatric problems may have existed in some of the men in either or both scans, and in the women in the summer scans. Nevertheless, the within-subject study design reduces the likelihood of many other confounding variables. Secondly, four of our 12 study subjects smoked cigarettes. One study has reported an influence of smoking on SERT binding (136); however, to our knowledge, no studies using selective SERT ligands have attempted to replicate this finding. Again, the fact that within-subject changes were investigated is likely to reduce the influence of this possible confound. Thirdly, we cannot exclude with certainty the use of drugs affecting the 5-HT system in our study subjects, as we did not do blood screening to exclude their use, but instead trusted the subjects' self-report of non-use. Fourthly, we could not time the scans of the two female subjects to the same phase of their menstrual cycle in both study occasions. We thus cannot exclude changes in their SERT binding caused by alteration of the levels of the ovarian hormones, even though this is unlikely based on the results of an earlier study investigating the variation in SERT binding between follicular and luteal phases (152).

6.3. SERT BINDING IN SUBJECTS AFFECTED BY OR GENETICALLY PREDISPOSED TO BULIMIA NERVOSA

Our *a priori* hypothesis of clearly reduced SERT binding in the women with BN as compared to their unaffected co-twins or unrelated healthy women was not borne out. There are several possible explanations for this result:

1. *SERTs are not affected in BN, but disturbances exist in other parts of the serotonergic system.* The evidence favouring involvement of the serotonergic system in BN includes blunted neuroendocrine responses to drugs with 5-HT activity (265), worsening of symptoms after acute tryptophan depletion (269), the beneficial effect of fluoxetine in BN (16), and differences found in the brain PET- and SPET-studies in

SERTs (181) and 5-HT_{1A}-receptors (270). The evidence favouring particularly the involvement of SERTs is scant; the beneficial effect of fluoxetine (16); alterations in the SERT binding of platelets in BN (359); and a SPET study reporting reduced SERT binding in the hypothalamus/thalamus in BN women (181). However, even though the SSRI medications are effective in BN and many other psychiatric diseases, their effect is not believed to be a direct effect on SERTs; one suggested mechanism is desensitization of somatodendritic 5-HT_{1A} autoreceptors in the midbrain raphe nucleus, increasing 5-HT in critical brain regions (360), and other mechanisms are possible, too (see below). Also, the results obtained from the previous SPET study may have been affected by the ligand's ([¹²³I]β-CIT) binding to DATs and NETs (213). The platelet SERT binding results do not necessarily correlate with SERT binding in the brain (358). Of the genetic association studies involving SERTs, one study has found an association between 5-HTTLPR genotype and BN (252), but other studies have not replicated the finding (253,254).

2. *SERTs are affected in BN, but in other regions than were investigated in our study.* The ligand used in our study, [¹²³I]ADAM, has acceptable test-retest repeatability in the midbrain and thalamus, whereas in other regions its binding is more variable (217). We thus investigated SERT binding in these two regions only. The midbrain region containing the raphe nuclei has the highest SERT density (361) and [¹²³I]ADAM binding (217), and is therefore of special interest. The hypothalamus/thalamus is the region with the second greatest [¹²³I]ADAM binding (217) and is of interest also for its proximity to hypothalamus (these areas cannot be distinguished reliably by SPET), which is the brain area involved in the control of feeding behavior (362). Nevertheless, alterations in SERT density in BN are possible in other brain regions.

3. *Serotonergic system is not primarily affected, but the previously reported alterations are secondary to some other neurobiological alterations.* So far, the investigations on the neurobiology of BN have mainly concentrated on the serotonergic system, but other neurotransmitters, neuropeptides, intracellular signalling cascades etc. may also be involved. Regarding the effect of fluoxetine (16), effects other than direct effects on the serotonergic system are possible. In addition to desensitization of somatodendritic 5-HT_{1A} autoreceptors in the midbrain raphe nucleus (360), suggested mechanisms of action of the SSRI medications include modulation of the homeostasis between different neurotransmitters (363) and activation of the various intracellular signalling pathways and their downstream targets that promote expression of genes promoting neurogenesis (364).

4. *SERTs are affected in the investigated regions, but we failed to detect differences due to methodological reasons.* Firstly, the considerable test-retest variability of [¹²³I]ADAM binding (217) may necessitate bigger sample sizes than were investigated in our study. When BN probands were compared with all healthy subjects (including unaffected co-twins of the probands) there was a nonsignificant trend towards higher SERT binding in the midbrain (p=0.08). This difference might have been significant with a bigger sample. Secondly, a recent study questioned the validity of the simple

ratio method in the estimation of SERT binding in [¹²³I]ADAM studies (223); it is thus possible that differences would have been evident had we used other quantification methods, e.g. the Logan reference tissue model (223). Thirdly, due to the nature of the sample (population-based rather than clinic-based), the BN probands in our study may have been affected by milder disease than in the previous SPET study (181), thus affecting the discrepancy between the results. Fourthly, the fact that we could not control for the phase of the menstrual cycle might have affected our study results, causing more variation in SERT binding.

Even though our *a priori* hypothesis was not borne out, in *post hoc* analyses we found a significantly higher SERT binding in the midbrain region of the purging BN women (n=9) as compared to all healthy women (n=24). This suggests that purging and non-purging BN patients may differ neurobiologically, with respect to their SERT function or some other phenomenon reflecting to SERT binding. Purging bulimics have been found to differ from non-purging ones in certain personality variables (e.g. lower self-directedness, organization, personal standards, and higher novelty seeking) (365); neurobiological differences may thus be possible, too. It is also possible that serotonergic or SERT alterations are not specific for BN as such, but instead found in subjects with particular characteristics associated with BN. For example, some studies not finding an association between BN and 5-HTTLPR polymorphism have found an association between the 5-HTTLPR genotype and particular predisposing eating disorder-related behavioral and attitudinal traits (254-256). We did not investigate the association of SERT binding and particular personality characteristics. A further possibility is that increased SERT binding in the purging probands is secondary to purging bulimic behaviour, e.g., through alterations in nutritional status affecting 5-HT levels or SERT expression.

Our *a priori* hypothesis was reduced SERT binding in BN. Instead, in the *post hoc* analyses we found higher SERT binding in the purging BN probands, a finding opposite to the initial hypothesis. The possible alternatives for the relationships between 5-HT and SERT levels are discussed in section 6.1.3. It is possible that this relationship is radioligand specific, perhaps affected by differences in endogenous competition by 5-HT, or by differences regarding SERT subclasses the ligand binds to (334). Radioligand specific differences might thus explain the opposite results of the present and the previous study (181). At the moment, the reasons behind our finding can only be speculated. Considering the prevailing theory of reduced 5-HT firing in BN, our finding might represent secondarily increased affinity of the SERTs or reduced competition for the binding sites. Alternatively, the purging BN women might have primarily increased SERT expression, causing reduced extra-cellular 5-HT levels, reduced serotonergic firing, and the symptoms associated with purging BN, or the increase in SERT expression might be secondary to purging bulimic behavior.

We also hypothesized a BN associated trait in SERT binding. If such trait existed, the unaffected co-twins of BN probands would have had SBR values intermediate between the BN probands and unrelated controls, given that our sample included both MZ and DZ twin pairs. Against our initial hypothesis, the unaffected co-twins had

similar SERT binding as unrelated healthy controls. However, the small number of unaffected co-twins (made smaller by the unexpectedly many concordant pairs as well as by exclusion of male co-twins), reduces the reliability of this conclusion. The small number also prevented further subgroup analyses based on zygosity or the BN subtype. Therefore, we cannot suggest whether the increased SERT binding in purging BN is caused by genetic or environmental factors. The initial decision to include two currently asymptomatic women in the group of BN women was based on the assumption of a genetic trait. In *post hoc* analyses, removal of these two subjects did not affect our results.

Our sample of BN women fits well to the prevailing view of BN subjects, with the onset of their symptoms (around 18 years of age) (14), with their past history of anorexic symptoms (five subjects) (233,236) and history of major depression (seven with past MDD and one with current MDD) (14,232). Histories of AN and MDD could be considered as confounds when studying SERT binding in BN. However, finding a big enough sample of subjects without past co-morbidities is extremely difficult. In a large population-based sample, 94.5% of subjects with lifetime BN met criteria for at least 1 of the core DSM-IV disorders, and 70.7 % for a mood disorder (including MDD, dysthymia and bipolar disorders) (14). Our sample is thus well representative of BN patients. In statistical analyses of our data, past AN or MDD had no effect on our results. The ratio of purging vs. non-purging BN cases in our sample was 2.25:1; this ratio is bigger than in another population sample (reporting the ratio of life-time prevalence as 1:1) (233), but smaller than is reported in clinical sample (233).

6.4. Association between SERT binding and BMI

In study **IV**, we investigated the association between BMI and SERT binding in a sample of monozygotic twins, who were analyzed as individuals and as pairs. Individual data was used to study associations between variables caused by both genetic and non-genetic factors. Within-pair comparisons eliminated the effect of genes and shared environmental experiences on investigated relationships.

Our main finding was that twins with higher BMI had higher SERT binding than their co-twins in the hypothalamus/thalamus. The fact that the association was evident in within-pair comparisons of genetically similar co-twins but not in individual data suggests that the association is not caused by genetic factors, but is a product of environmental influences.

As discussed earlier in section 6.1.3., there is no clear consensus on the association of brain 5-HT levels and SERT binding in the brain imaging studies. The relationship may vary between different ligands, depending on whether the ligand competes with the endogenous substrate or not. Furthermore, even for one ligand the finding may represent different things in different settings. The measured SERT binding could theoretically be affected by: the actual number of 5-HT neurons; the number of SERTs

(affected e.g., by genetic polymorphism, epigenetic effects on SERT expression, and by various regulatory mechanisms acting in the short or long term); or by alterations in the 5-HT levels, causing alteration in SERT binding as a result of feedback mechanisms, altered competition with the endogenous ligand, or altered affinities. Not enough is known about this relationship to allow a reliable interpretation of study results, and it should be emphasized that all efforts to do so remain speculative.

Based on the existing data on the association of 5-HT with feeding behavior, 5-HT has long been considered as an anorexigenic signalling factor causing hypophagia and promoting the feeling of satiety (11). The precise mechanisms of its actions are still not fully understood but seem to involve multiple 5-HT receptors and downstream targets of other signalling systems involved in the regulation of feeding behavior (302,304). Several studies have also found differences relating to the 5-HT system between obese and lean animals and humans. Obese rats have lower brain 5-HT levels compared to lean rats (296). In humans, obese subjects have been found to have lower plasma levels of tryptophan than lean subjects (297,298), which is thought to lead to lower levels of brain 5-HT, which could lead to impaired satiety and enhanced feeding behavior. So differences may exist in the supply of 5-HT between the obese and lean subjects. Alternatively, alterations affecting primarily the brain are possible, e.g. alterations in different aspects of the 5-HT system, as has been suggested, e.g., by genetic association studies that have found associations between BMI and overweight with polymorphism of various genes affecting the 5-HT system (306-309,313,314).

Against this background, two interpretations for the increased SERT binding in the subjects with higher BMI seem plausible: Increased SERT binding is secondary to reduced levels of brain 5-HT (secondary to reduced supply of tryptophan) causing increased affinity and/or reduced endogenous competition for SERT binding; this interpretation remains speculative as we did not measure plasma tryptophan levels. Alternatively, SERT densities could be increased and cause reduced synaptic 5-HT levels and neuronal firing, leading to impaired satiety and weight gain. This would be unlikely to represent direct genetic influences, as we found different SERT binding between members of genetically similar twins. At the moment, there is more evidence to favour the first interpretation than the second one (297,298).

Recent studies have shed light on the reasons behind discordance of monozygotic twins. Despite being genetically identical, the twins may differ significantly in their phenotype. The twins may develop under influence of different environmental factors already during their fetal life, and later on the environmental effects add on. Epigenetic influences (affecting gene expression without affecting the DNA sequence) may be behind the discordance of MZ twins. Epigenetic differences between MZ twins have been detected for example in histone acetylation and DNA methylation. These differences are significantly more common in older than younger twins, and in twins who have been exposed to more environmental differences (316). In our study, epigenetic differences might be behind differences in levels of tryptophan and/or 5-HT, or cause differences in SERT expression. However, it remains unknown, whether differences in adiposity and weight gain are the cause or consequence of different

SERT binding. Instead of reduced 5-HT levels causing impaired satiety and increased weight gain, differences in external factors such as diet might cause epigenetic differences (366) affecting the 5-HT system. Furthermore, differences in personality characteristics relating to 5-HT system and associating with weight gain are possible.

When the within-pair data were analyzed separately for both sexes, the intra-pair difference in SERT binding in the hypothalamus/thalamus was significant only in women. Sexual dimorphism of the 5-HT system has been observed in several studies. In humans, the rate of the brain 5-HT synthesis is reported to be lower in women, and also the effects of tryptophan depletion on the rate of 5-HT synthesis (134), as well as its mood effects (135), are stronger in women than in men. Sex differences are also reported in the brain 5-HT_{1A} (137,138) and SERT binding (136,137), and different SERT binding between men and women was seen in our data, too (higher SERT binding in men in the midbrain area). Sex-related differences also exist in the prevalence and presentation of MDD (119), in which disturbed 5-HT function is implicated (5), and a previous study reported sex-specific differences in SERT binding between depressed men and women (341). Our results suggest that the association of BMI and SERT binding is also different between men and women. One possible explanation could be that if women have a relatively lower 5-HT function (as suggested by e.g., lower rates of 5-HT synthesis (134) and greater vulnerability to effects of tryptophan depletion (135)), also the effects of lower levels of plasma tryptophan (297,298) are more pronounced and evident in SERT binding in women. Sexual dimorphism has also been found in appetite, women having a relatively stronger preference for carbohydrates over proteins and fats than men (367). This difference in appetite has been suggested to relate to 5-HT, which affects the intake of carbohydrates more than it affects the intake of proteins or fats (362).

In individual data, no correlation between BMI and SERT binding was evident. This may be explained by the multitude of factors (genetic and environmental) that may cause differences in SERT binding between individuals. In MZ pairs, confounding due to genetic effects is eliminated in twin-co-twin comparisons and also many environmental factors are similar for monozygotic twins reared together.

Despite of being a result *post hoc* analysis, it is of interest that women using oral contraceptives had significantly reduced SERT binding in the hypothalamus/thalamus as compared to women without oral contraceptives. Exclusion of women with oral contraceptives did not affect our study results. To our knowledge, no previous PET or SPET studies on SERTs have addressed the effect of oral contraceptives or other exogenous synthetic estrogens. Considering how common these medications are, their influence may be a potential confound in studies of the brain 5-HT system and should therefore be addressed in future studies.

7. SUMMARY AND CONCLUSIONS

The main conclusions of the work presented in this thesis can be summarized as follows:

1. Definition of volumes of interest (VOIs) for purposes SERT binding quantification of [¹²³I]ADAM images is more reproducible using automated placement of predefined VOIs on stereotactic images than using manual VOI-drawing on non-stereotactic images (**I**).
2. SERT binding in the midbrain and thalamus regions is similar in summer and winter, suggesting that seasonal variation is not a significant confound in SERT imaging studies of these regions (**II**).
3. In a population-based sample, SERT binding in the midbrain and thalamus of women affected by Bulimia Nervosa is not different from their healthy twin sisters or other healthy women. In purging BN women SERT binding in the midbrain region may be increased as compared to healthy women (**III**).
4. SERT binding in hypothalamus/thalamus is increased in acquired obesity in women. This association is not genetic but a consequence of environmental influences (**IV**).

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