FACULTY OF HEALTH AND MEDICAL SCIENCES
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PhD Thesis

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In Vivo PET Imaging of the Cerebral 5-HT_4 Receptors in Healthy Volunteers - In Relation to Appetite, Memory and Pharmacological Intervention

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Please note that the results of study 3 were revised after this thesis was handed in and therefore the results and discussion presented here should not be quoted. A revised manuscript will be presented at the defense January 11, 2013 and later published.

Thesis summary

This thesis focuses on the cerebral serotonin type 4 receptor (5-HT_4) in relation to appetite regulation, memory and the sensitivity to endogenous cerebral serotonin level. The 5-HT_4 receptor has diverse regulatory functions and has emerged as an interesting part of the cerebral serotonin system. Numerous animal studies have shown that the 5-HT_4 receptor plays a role in cognitive function, particularly in memory functions and the receptor is a pharmacological target for treatment of Alzheimer's Disease. Newer studies have also shown that stimulation of the receptor influences appetite regulation in areas of the brain reward system such as nucleus accumbens. Studies in rats have suggested that the receptor could function as a proxy of the endogenous brain serotonin level. For example the receptor level were down-regulated 16-47% after selective serotonin reuptake inhibitors (SSRI) treatment and serotonin depletion resulted in a receptor up-regulation.

Healthy volunteers were included in the studies of this thesis, and the aims were to

- examine the relationship between memory acquisition and consolidation and in the hippocampal 5-HT_4 receptor binding.
- determine the relationship between body mass index (BMI) and the cerebral 5-HT_4 receptor density in ventral striatum
- investigate how a reduction in body weight after 3-weeks of SSRI versus placebo intervention is associated with a change in ventral striatal 5-HT_4 receptor binding.
- examine how a 3-week SSRI or placebo intervention influences the cerebral 5-HT_4 receptor binding in general and relate a change in binding to the 5-HTTLPR polymorphism status.

For determination of cerebral 5-HT_4 receptor binding a 2-hour dynamic positron emission tomography (PET) scan with the radiotracer [^{11}C]SB207145 were performed and regional and voxel-level time-activity curves were extracted from grey matter. The non-displaceable 5-HT_4 receptor binding (BP_{ND}) was quantified with the simplified reference tissue model (SRTM).

The 5-HT_4 receptor binding was positively correlated with BMI in areas important for the brain reward system such as nucleus accumbens, ventral pallidum, orbitofrontal cortex and hippocampus. Furthermore, weight loss after 3 weeks of SSRI intervention was positively associated with the 5-HT_4 receptor binding in ventral striatum, including the nucleus accumbens. These findings suggest that the receptor may be involved in the hedonic part of food intake in humans.

The 5-HT_4 receptor binding in hippocampus was negatively associated with memory acquisition and consolidation, and confirms the role of the receptor in memory functions in humans.

We found that the 5-HTTLPR polymorphism of the serotonin transporter influences the effect of SSRI intervention on the levels of the 5-HT_4 receptor, however, we did not find evidence that the 5-HT_4 receptor could function as a biomarker for the endogenous brain serotonin level.

Resumé af Ph.D. afhandlingen

Fokus i denne ph.d. afhandling er den cerebrale serotonin type 4 receptor (5-HT_4) i forbindelse med appetit regulering, hukommelse og sensitivitet over for det endogene serotonin niveau. 5-HT_4 receptoren er en vigtig del af det cerebrale serotonin system og har forskellige regulerende funktioner. Talrige dyre forsøg har vist, at 5-HT_4 receptoren spiller en rolle for kognitionen, især for hukommelsesfunktionerne og receptoren er et farmakologisk mål for behandling af Alzheimer's sygdom. Desuden har nyere dyreforsøg har vist, at stimulering af receptoren påvirker appetitreguleringen i områder af hjernens belønningssystem så som nucleus accumbens. Det er også vist i rotter, at receptoren muligvis kan fungere som en markør for det cerebrale endogene serotonin niveau. For eksempel var receptoren nedreguleret med 16-47% efter behandling med selektive serotonin reuptake hæmmere (SSRI).

Vi inkluderede raske mennesker i denne afhandling, og formålet var at

- undersøge forholdet mellem hjernens hukommelsesfunktion og 5-HT_4 receptor bindingen i hippocampus.
- undersøge forholdet mellem body mass index (BMI) og antallet af cerebrale 5-HT_4 receptorer i den ventrale del at striatum.
- undersøge hvordan et vægttab efter 3-ugers SSRI behandling vs. placebo er associeret med ændringer i 5-HT_4 receptor bindingen i den ventrale del af striatum.
- undersøge hvordan 3 ugers SSRI eller placebo behandling påvirker 5-HT_4 receptor bindingen generelt og relatere en ændring i bindingen til 5-HTTLPR status.

For at bestemme bindingspotentialet for 5-HT_4 receptoren udførtes to timers dynamiske positron emission tomografi (PET) skanninger med radioliganden [^{11}C]SB207145. På både regionalt og voxel-niveau ekstraheredes tids-aktivitets-kurver fra hjernens grå substans og det non-displacerebare bidningspotentiale blev udregnet ved hjælp af simplified reference tissue model (SRTM).

5-HT_4 receptor bindingen var positivt korreleret med BMI i områder af hjernens belønningssystem så som ventral striatum, ventral pallidum, orbitofrontal cortex og hippocampus. Endvidere fandt vi at et signifikant vægttab efter 3 ugers behandling med SSRI

var positivt korreleret med ændringen i 5-HT_4 receptor niveauerne i ventral striatum. Disse resultater tyder på, at receptoren er involveret i den hedoniske del af føde indtagelse i mennesker.

5-HT_4 receptor bindingen i hippocampus var negativt associeret med hukommelses tilegnelse og konsolidering, og resultatet bekræfter, at receptoren spiller en rolle i hukommelsesfunktionen hos mennesker.

Vi fandt, at 5-HTTLPR polymorfismen i serotonin transporter genet påvirker effekten af SSRI behandling på 5-HT_4 receptor niveauerne, men vi fandt imidlertid ikke et klart bevis på, at 5-HT_4 receptoren kan fungere som en biomarkør for det cerebrale serotonin niveau.

List of papers

1. Haahr ME, Rasmussen PM, Madsen K, Marner L, Ratner C, Gillings N, Baaré WFC, Knudsen GM. Obesity is associated with high serotonin 4 receptor availability in the brain reward circuitry. NeuroImage 2012, doi:10.1016/j.neuroimage.2012.03.050

2. Haahr ME, Fisher P, Holst KK, Madsen K, Jensen CG, Marner L, Lehel S, Baaré W, Knudsen GM, Hasselbalch SG. The 5-HT_4 receptor levels in hippocampus correlate inversely with memory test performance in humans. Hum Brain Mapp. 2012 Jun 26. doi: 10.1002/hbm.22123.

3. Haahr ME, Fisher PM, Jensen CG, Frokjaer V, Nørremølle A, Madsen K, Baare W, Madsen J, Rabiner EA, Knudsen GM. The cerebral in vivo 5-HT_4 receptor binding before and after 3 weeks of SSRI intervention in healthy humans. Manuscript.

Additional papers

Ratner C, Ettrup A, Bueter M, Haahr ME, Compan V, le Roux CW, Levin B, Hansen HH, Knudsen GM. Cerebral Markers of the Serotonergic System in Rat Models of Obesity and After Roux-en-Y Gastric Bypass. Obesity 2012 Mar 26. doi: 10.1038/oby.2012.75.

Erritzoe D, Frokjaer VG, Haahr MT, Kalbitzer J, Svarer C, Holst KK, Hansen DL, Jernigan TL, Lehel S, Knudsen GM. Cerebral serotonin transporter binding is inversely related to body mass index. Neuroimage. 2010 Aug 1;52(1):284-9.

Madsen K, Neumann WJ, Holst K, Marner L, Haahr MT, Lehel S, Knudsen GM, Hasselbalch SG. Cerebral serotonin 4 receptors and amyloid-β in early Alzheimer's disease. J Alzheimers Dis. 2011;26(3):457-66.

Pinborg, L, Feng, L, Haahr, ME, Gillings, N, Dyssegaard, A, Madsen, J, Svarer, C, Yndgaard, S, Kjaer, T, Parsey, R, Hansen, H, Ettrup, A, Paulson, O, Knudsen, GM. No change in [^{11}C]CUMI-101 binding to 5-HT_{1A} receptors after intravenous citalopram in human. Synapse. 2012 Jun 23. doi: 10.1002/syn.21579

Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HT_4	serotonin type 4
AD	Alzheimer's Dementia
BP	binding potential
BP_{ND}	binding potential relative to the non-displaceable binding
B_{max}	receptor concentration
BMI	Body Mass Index
CART	Cocaine- and amphetamine-regulated transcript
CSF	cerebrospinal fluid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
FWHM	Full With Half Maximum
GABA	gamma-amino-butyric acid
HRRT	High Resolution Research Tomographs
ICD-10	International Classification of Diseases
KD	Dissociation Constant
MDI	Major Depression Index
MNI	Montreal Neurological Institute (definition of a standard brain)
MRI	Magnetic Resonance Imaging
MDMA	ecstasy
NAc	nucleus accumbens
NPAIRS	nonparametric, prediction, activation, influence, reproducibility, re-sampling
pCPA	parachlorophenylanine
PET	Positron Emission Tomography
PV	Partial Volume
RAVLT	Reys Auditory Verbal Learning Test
ROI	Regions of Interest
ROCFT	Rey-Osterrieth's Complex Figure Test
SCL-90-R	Symptom Checklist Revised
SERT	serotonin transporter
SPM	Statistical Parametric Mapping

SRTM	Simplified Reference Tissue Model
SSRI	Selective Serotonin Reuptake Inhibitors
SPECT	Single Photon Emission Computed Tomography
TACs	Time Activity Curves
VOI	Volumes of Interest
5,7-DHT	5,7-dihydroxytryptamine
5-HTP	5-hydroxytryptophan

Introduction

Cerebral serotonergic neurotransmission modulates normal brain functions, such as mood, sleep, emotions, sexual activity, appetite and memory and is involved in diseases such as schizophrenia, eating disorders, mood disorders and substance abuse. Fourteen receptors are associated with the cerebral serotonin system and this thesis focuses on the type 4 (5-HT_4) receptor. Recently it became possible to study the 5-HT_4 receptor in humans by development of the PET tracer [^{11}C]SB207145. With this tracer we can visualize and quantify the *in vivo* 5-HT_4 receptors in humans.

The background section of the thesis presents the serotonin system in general and the involvement of serotonergic neurotransmission and the 5-HT_4 receptor in particularly in appetite regulation and memory. Further, a proposed role of the 5-HT_4 receptor as a proxy for the extracellular serotonin level is described. Lastly the basic principles behind a positron emission tomography (PET) scan are presented with particular focus on the methods for visualizing the 5-HT_4 receptor.

The thesis includes three clinical *in vivo* PET studies that explore the regulation of the 5-HT_4 receptor in relation to appetite regulation with focus on the brain reward system and to memory functions in hippocampus and the last study investigate receptor changes after pharmacologically induced increases of the extracellular brain serotonin level.

Background

The cerebral 5-HT system and its receptors

The cerebral serotonin (5-hydroxytryptamine, 5-HT) system is implicated in several physiological functions such as mood and emotions, thermoregulation, sex, sleep, appetite, and memory (Sghendo and Mifsud, 2012). Disturbances in the serotonin system either in the global serotonergic neurotransmission and/or the distribution of pre- and postsynaptic receptors are believed to be implicated in a variety of human diseases such as schizophrenia, addiction, eating disorders, depression, obsessive compulsive disorder and Alzheimer's Dementia (Blier and de Montigny, 1999, Kaye et al., 2005, Rasmussen et al., 2010).

5-HT is synthesized from the amino acid tryptophan in neurons in the raphé nuclei in the brainstem from where they project to virtually every part of the brain (figure 1). The serotonin system have at least 14 pre- and postsynaptic receptors and based on variations in function and distribution the receptors are divided into seven major classes: 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 (Hannon and Hoyer, 2008). Some classes have several subtypes, for example the 5-HT_2 class is subdivided into 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. The 5-HT_3 receptor is an ionotropic receptor, whereas all the others are metabotropic G-protein-coupled receptors. Receptor diversity is further increased by post-genomic modifications, for example the 5-HT_4 receptor is transcribed from a complex gene and modified into more than 15 distinct splice variants, some of them displaying a preferential distribution within distinct peripheral and/or central tissues (Bockaert et al., 2004, De Maeyer et al., 2008). Taken together, the cerebral serotonin system is indeed one of the most complex signaling systems in the brain and often the precise role of serotonin or one of the receptor subtypes is frustratingly hard to define. However, this makes research in the area stimulating, challenging and full of surprises.

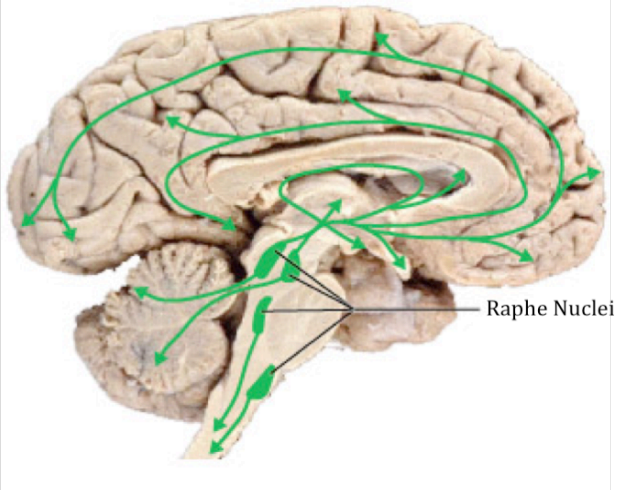

Figure 1. Projection from the serotonergic nerons in raphé nuclei (From http://learn.genetics.utah.edu/)

It is still impossible to measure acute or chronic fluctuations in endogenous serotonin levels *in vivo* in humans despite numerous attempts (Paterson et al., 2010). Therefore, any associations between the cerebral serotonin levels in humans and brain diseases or physiological functions are investigated with surrogate markers such as receptor quantities, genetic polymorphisms, post mortem receptor autoradiography or cerebrospinal fluid metabolites. It is possible biochemically to manipulate the extracellular level of cerebral 5-HT in humans. An increase can be obtained by blockade of the 5-HTT with selective serotonin reuptake inhibitors (SSRI) and a 5-HT depletion can be obtained by arresting the production of 5-HT by ingestion of mixture of amino acids that depletes the brain of its 5-HT precursor tryptophan.

The 5-HT$_4$ receptor

Interest in the 5-HT$_4$ receptor was prompted by increasing evidence of its involvement in a variety of physiological functions and diseases. The receptor was identified not more than two decades ago (Dumuis et al., 1988a, Dumuis et al., 1988b) and since then knowledge was gained from several studies on its anatomical distribution, as well as its pharmacological, biochemical, functional and genetic characteristics. The receptor has important peripheral functions within the gastro-intestinal tract (Kim, 2009), and in central processes such as memory (King et al., 2008), regulation of food intake (Jean et al., 2007), release of other neurotransmitters such as acetylcholine, GABA, dopamine and serotonin (Ge and Barnes,

1996, Matsumoto et al., 2001, Bianchi et al., 2002, Alex and Pehek, 2007). Therefore the receptor is a pharmacologic target especially as a treatment of Alzheimer's Dementia (AD) (Lezoualc'h, 2007) and 5-HT_4 receptor agonists could constitute a new category of antidepressants (Lucas et al., 2005, Lucas et al., 2007).

The cerebral 5-HT_4 receptors are coupled to G-proteins and positively linked to the adenylate cyclase (Hannon and Hoyer, 2008). The increase in cAMP levels leads to an activation of protein kinase A that mediates closure of potassium channels (Fagni et al., 1992, Ansanay et al., 1995) and inhibition of calcium release (Torres et al., 1996) and thus the receptor contributes mainly to neuronal excitability.

The 5-HT_4 receptors are expressed predominantly in basal ganglia and the limbic system including the hippocampus and amygdala, while the cortical expression is low and cerebellum is almost devoid of receptors (Waeber et al., 1996, Marner et al., 2010). With the introduction of the radioligand [^{11}C]SB207145 it is possible to visualize and quantify the human cerebral 5-HT_4 receptors *in vivo* (Marner et al., 2010). This will be described thoroughly in the end of this chapter.

Figure 2 shows a diagram of a serotonergic chemical synapse. Most of the 5-HT_4 receptors are located postsynaptically, and transmit the 5-HT signal to the postsynaptic neuron. However, a presynaptic localization is also likely on serotonergic but also cholinergic, GABAergic, dopaminergic terminals because of enhanced release of these neurotransmitters by 5-HT_4 receptor agonists (Bockaert et al., 2006). Also illustrated presynaptically is the serotonin transporter that removes serotonin from the synapse and is blocked by selective serotonin reuptake inhibitors (SSRIs).

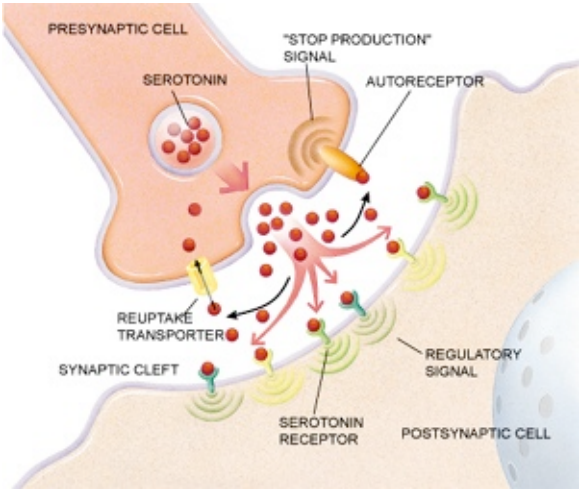

Figure 2. A serotonergic synapse (From www.kolonpharm.co.kr)

Appetite regulation and the serotonin system

Obesity is defined as a state of excessive accumulation of body fat that is a risk factor for development of several serious disorders, most notably type-2 diabetes, cardiovascular diseases and cancer. During the past 30 years the number of obese individuals has grown in epidemic speed in both high and low income countries (WHO, 1998), however, signs of a leveling off of the epidemic is seen in many countries (Rokholm et al., 2010) and in Denmark it might even be receding (Svendstrup et al., 2011). In spite of these good news obesity will continue to be a major health problem in many years to come. One difficulty is that even though weight loss can be rapidly achieved by caloric restriction and exercise, it is rarely sustained (Avenell et al., 2004, Bray, 2008). Currently, the only treatment of obesity, that provides a sizeable sustained weight-loss, is bariatric surgery (Edholm et al., 2012). However, this treatment also has severe side-effects such as cardiorespiratory failure, venous thromboembolism, wound infections, anastomotic leaks, and chronic gastrointestinal symptoms (Hofmann, 2010). For that reason, substantial effort is undertaken to develop new effective anti-obesity medications and the cerebral neurotransmitter systems that regulate appetite (such as serotonin, dopamine, GABA and nor-epinephrine) are some of the main targets.

The link between serotonin and feeding is likely to be phylogenetically ancient (Lent and Dickinson, 1988). Serotonin transmitter pathways exist in both brain and gastrointestinal peripheral sites crucial to feeding and intimate associations between nutritional relevant information and serotonergic function exist (Blundell, 1992). Serotonergic drugs can accelerate the onset of satiety (Blundell, 1986, Li et al., 1994), enhance basal metabolic rate, and inhibit carbohydrate craving (Moses and Wurtman, 1984, Laferrere and Wurtman, 1989).

More than two decades ago it was established that administration of agents that are either toxic to 5-HT neurons (e.g. 5,7-dihydroxytryptamine, 5,7-DHT) or prevent 5-HT synthesis (e.g. parachlorophenylanine, pCPA) increase food intake in rats with a subsequent increase in body weight (Saller and Stricker, 1976, Waldbillig et al., 1981). In contrast, increased central 5-HT levels following administration of the 5-HT precursor 5-hydroxytryptophan (5-HTP) or of the 5-HT releasing agent fenfluramine significantly decreased food intake (Clineschmidt, 1973, Barrett and McSharry, 1975, Blundell and Leshem, 1975, Duhault et al., 1975). In line with these early results, administration of the selective serotonin reuptake inhibitor fluoxetine was shown to induce significant weight loss within the first two weeks in both healthy young men (McGuirk and Silverstone, 1990) and obese individuals (Lawton et al., 1995, Ward et al.,

1999). Other 5-HT releasing agents such as fenfluramine, the serotonin and norepinephrine reuptake inhibitor sibutramine, the 5-HT1B/2C agonist mCPP, and the 5-HT1B/1D agonist sumatriptan also lead to significant weight loss (Halford et al., 2005).

Recently animal studies identified the 5-HT_4 receptor as an interesting modulator of food intake. First of all the receptor could influence the basal feeding pattern as a 5-HT_4 receptor antagonist increases food intake (Jean et al., 2007) even though results are not consistent (Francis et al., 2010). Furthermore, the receptor could be involved in the emotional eating component as the receptor mediate the appetite suppressant effect of the drug ecstasy (MDMA) in nucleus accumbens (NAc) through CART (cocaine- and amphetamine-regulated transcript) (Jean et al., 2007, Francis et al., 2010), an anorexigenic neuromodulator involved in reward and addiction (Vicentic and Jones, 2007). It is further shown that antagonism or knock down of the receptor in the NAc suppress the rewarding effect of MDMA and that over-expressing of 5-HT_4 receptor in NAc increases CART and satiety (Compan et al., 2010). Further, several studies link the receptor to the feeding response to stress (Compan et al., 2004b, Jean et al., 2010, Laurent et al., 2010).

Memory and the serotonin system

Growing evidence indicates that 5-HT modulate learning and memory e.g. in humans a low dose of SSRI stimulated short-term memory (Dumont et al., 2005) while 5-HT depletion impaired memory acquisition and retention (Sambeth et al., 2007) and moreover, a reduced 5-HT turnover is associated with impaired long-term memory (Schmitt et al., 2006). It was also shown that pharmacological intervention influencing the serotonin receptors could potentially improve memory and learning disabilities (Buhot et al., 2000, Cifariello et al., 2008, Perez-Garcia and Meneses, 2008).

The 5-HT_4 receptor has a relatively high density in the hippocampus (Waeber et al., 1996, Marner et al., 2010) and could play a key role as a modulator of cellular memory processes in hippocampus such as long-term depression and potentiation (Kemp and Manahan-Vaughan, 2004).

Episodic memory is the memory for events and experiences and involves three cognitive processes; encoding, consolidation and retrieval (Lezak et al., 2004). During encoding, the new information presented is acquired and learned. Consolidation is the processing of encoded information into long-term storage for later retrieval. Numerous animal studies over

the last two decades have shown with a rare consistency that stimulation of the 5-HT_4 receptor facilitates memory and learning (for an overview see table 1) at least when it comes to memory encoding. In all studies where 5-HT_4 receptor agonist was injected immediately before the acquisition phase, memory was enhanced. During the consolidation phase there was some inconsistency as the study of Meneses and Hong (Meneses and Hong, 1997) found impairment of memory ability when injecting the agonist in the consolidation phase. Nevertheless, the receptor is an interesting pharmacologic target in treatment of memory deficits, especially for Alzheimer's dementia (Madsen et al., 2010). Also due to evidence that stimulation of the 5-HT_4 receptor possibly leads to acetylcholine efflux and increase the non-amyloidogenic soluble form of amyloid precusor protein (Lezoualc'h, 2007, Mohler et al., 2007).

Table 1. Effects of 5-HT$_4$ receptor agonists in animal studies on various memory tasks.

5-HT$_4$R agonists	Animal	Task	Outcome	Study
Encoding				
BIMU 1	Rats	Olfactory association learning	↑ short term memory	(Marchetti-Gauthier, 1997)
BIMU 1	Rats	Social recognition	↑ short term memory	(Letty et al., 1997)
BIMU 1/BIMU 8	Rats	Autoshaping task	↑ learning	(Meneses and Hong, 1997)
BIMU1/BIMU8	Mice	Passive avoidance	↑ learning (Antagonist, scopolamine, dicyclomine induced dysfunction)	(Galeotti et al., 1998)
RS 67333	Rats	Olfactory associative discrimination	↑ cognition (antagonist induced dysfunction)	(Marchetti et al., 2000)
RS 67333	Rats	Place and object recognition	↑ memory	(Lamirault and Simon, 2001)
RS 67333	Rats	Morris water maze	↓ swim distance	(Lelong et al., 2001)
RS 67333	Rats	Place recognition task	↑ acquisition	(Orsetti et al., 2003)
RS 67333/BIMU1	Mice	Y-maze	↑ working memory	(Lelong et al., 2003)
RS 67333	Rats	Rat spatial navigation	↑ cognition (Atropine induced dysfunction)	(Fontana et al., 1997)
RS 17017	Young/old Macaques	Delayed matching-to-sample	↑ mnemonic effects	(Terry et al., 1998)
SC 53116	Rats	Passive avoidance	↑ learning (Scopolamine induced dysfunction)	(Matsumoto et al., 2001)
SL65.0155	Rats	Tests of learning and memory	↑ memory	(Moser et al., 2002)
VRX-03011	Rats	Delayed spontaneous alternation	↑ memory	(Mohler et al., 2007)
Consolidation				
BIMU 1	Rats	Olfactory association Learning	↑ long term memory	(Marchetti-Gauthier, 1997)
BIMU 1/BIMU 8	Rats	Autoshaping task	↓ consolidation	(Meneses and Hong, 1997)
RS67333	Rats	Olfactory associative discrimination	↑ long term memory	(Marchetti et al., 2000)
RS67333	Aged rats	Place and object recognition	↑ long term memory	(Lamirault and Simon, 2001)
RS 67333	Rats	Place recognition task	↑ consolidation	(Orsetti et al., 2003)
SL65.0155	Aged rats	Olfactory associative discrimination	↑ long term memory	(Marchetti et al., 2011)
SL65.0155	Rats	Object recognition	↑ 24 h retention	(Moser et al., 2002)

The 5-HT$_4$ receptor as a marker of extracellular serotonin levels

There is evidence from both animal and human studies that the cerebral 5-HT$_4$ receptor is inversely regulated by extracellular 5-HT levels. Two weeks of the tricyclic antidepressant imipramine that leads to increases the extracellular serotonin levels attenuates the stimulatory effect of 5-HT$_4$ receptor agonism on hippocampal neuron excitability (Bijak, 1997, Bijak et al., 1997, Zahorodna et al., 2002), while chronic 5-HT depletion up-regulates 5-HT$_4$ receptor binding in the hippocampus and basal ganglia (Compan et al., 1996). We found in rats that chronic (2 and 3 weeks) but not acute administration of the SSRI paroxetine caused a 16-47% downregulation of 5-HT$_4$ receptor density in all investigated brain regions, including striatum and hippocampus, while subchronic 5-HT depletion increased the 5-HT$_4$ receptor in the dorsal hippocampus, hypothalamus, and lateral globus pallidus (Licht et al., 2009). This finding was replicated by another group: three weeks of fluoxetine treatment in rats decreased the 5-HT$_4$ receptor density in striatum and hippocampus (Vidal et al., 2009) and three weeks treatment with the non-selective 5-HT noradrenalin reuptake inhibitor, Venlafaxine, was also associated with a decrease of the 5-HT$_4$ receptor density in striatum and hippocampus (Vidal et al., 2010). Furthermore, genetically modified expression of the gene coding for the 5-HTT induced significant alterations in 5-HT$_4$ receptor density in mice: Mice over-expressing 5-HTT (i.e., resulting in reduced 5-HT signaling) have increased cerebral 5-HT$_4$ receptor density while knock-out of the gene coding for 5-HTT (i.e., resulting in increased 5-HT signaling) results in reduced 5-HT$_4$ receptor density (Jennings et al., 2011). Studies in humans go along the same line: whereas acute blockade of serotonin reuptake with citalopram, putatively resulting in increased 5-HT signaling, does not modify the cerebral 5-HT$_4$ receptor binding as assessed in vivo with positron emission tomography (PET) and the radioligand [^{11}C]SB207145 binding (Marner et al., 2010) then a recent study from our lab identified an association between 5-HTTLPR status and 5-HT$_4$ receptor binding. The 5-HTTLPR is a common genetic variant within the gene (SLC6A4) coding for the serotonin transporter (5-HTT) where the 'long' (L) allele exhibits increased serotonin transporter (5-HTT) transcription in vitro relative to the 'short' (S) allele, putatively affecting 5-HT signaling (Lesch et al., 1996). We found that S carriers showed reduced [^{11}C]SB207145 binding relative to L/L homozygotes, consistent with the S allele resulting in diminished 5-HTT production and subsequent increases in 5-HT levels leading to a decrease in 5-HT$_4$ receptor (Fisher et al., 2012). Taken together, these findings

indicate that the 5-HT$_4$ receptor could be used as a proxy for chronically, but not acutely altered cerebral 5-HT levels, and imaging of the receptor could potentially act as a biomarker for, e.g., responsiveness to antidepressive treatment.

Positron Emission Tomography (PET) in the brain

PET was first introduced in the 1950s and is a nuclear imaging method, that can produce three-dimensional images of functional processers in the body, be they physiological, biochemical or pharmacological. The basic principle is that a molecule of interest (a precursor) is labeled with a positron-emitting radioisotope. This radiotracer is injected into the body where it binds to a target structure of interest. The PET scanner is build of rings of detectors surrounding the subject studied. When the positron decays the PET scanner detects a true event, when the time delay of the incoming rays is less than 10 ns and further is on the response line between two detectors. The three dimensional image of the distribution of the radiotracer is reconstructed using e.g. iterative or filtered backprojection algorithms (Figure 3).

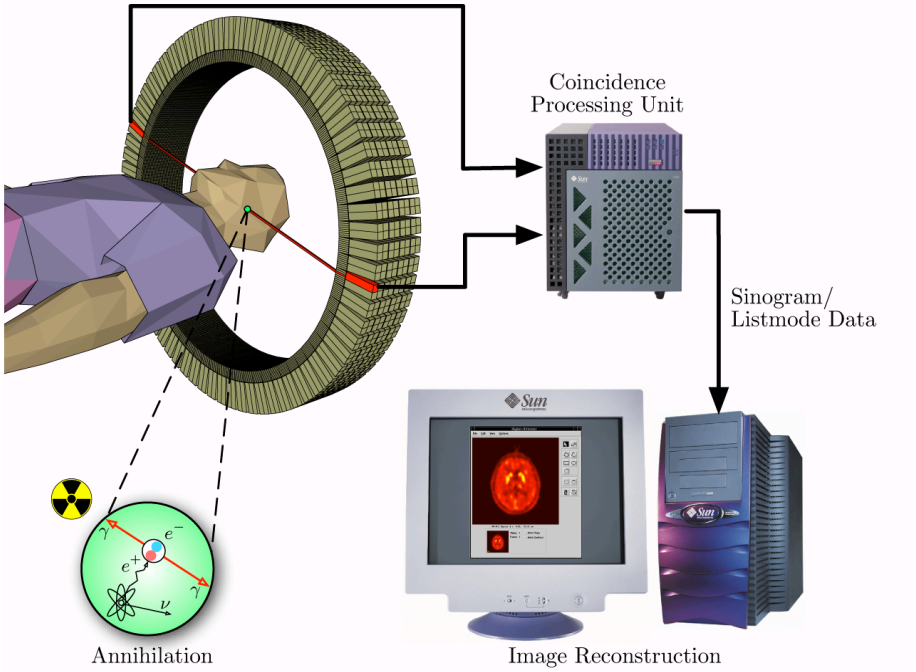

Figure 3. The principles of a PET scan: During the annihilation process two gamma photons are emitted in diametrically opposing directions. The PET scanner registers these photons as soon as they arrive at the detector ring and a processing unit decides if the photons can be registered as a coincidence event. All events are processed to construct the final 3D image data via image reconstruction procedures (from nucmed.wikispaces.com).

Radioligands

The most frequently used radioisotopes are [^{11}C], [^{15}O], and [^{18}F]. They have short physical half lives (^{11}C: 20.3 min., ^{15}O: 2.03 min., and ^{18}F: 109.8 min.) and therefore demands a cyclotron in the proximity of the PET imaging facility. Furthermore labeling of the precursor can be complex, and a high a fraction must be labeled to give sufficient specific activity, which is a measure of the radioactivity per unit mass of the precursor. If a large fraction of the unlabelled precursor is injected and compete with the radiolabeled tracer for the target sites, the measurements of e.g. neuroreceptors will be underestimated. If the upper limit of receptor occupancy is kept under 5-10%, then the impact on the receptor binding of the injected fraction of unlabelled precursor is minimized (Innis et al., 2007).

PET imaging can be used to trace the functional pathway of any compound in living humans, provided it can be radiolabeled with a PET isotope. Thus in theory, the specific processes that can be explored with PET are virtually limitless. However, a high-quality radiotracer for functional brain imaging must fulfill several requirements:

- Be able to cross the blood-brain barrier
- A favorable ratio between specific and non-specific binding (high signal-to-noise ratio)
- Binds to the target sites with high selectivity and sufficient affinity (with too high affinity the radiotracer is irreversible during the scan)
- Have relatively fast kinetics, so that equilibrium between association and disassociation to target sites is reached within the time window of a scan
- Tolerable radioactivity dosages
- Preferable a reference region with negligible number of target sites, thus representing the free and non-specific bound radiotracer only (to avoid arterial cannulation in a clinical setting)
- A sufficient specific radioactivity must be achievable so that less than 5-10% of target sites are occupied by unlabeled precursor
- No radiolabeled metabolites in the brain that disturb the measurements by binding to other irrelevant brain sites.

Additional requirements of a good tracer are low plasma protein binding and poor substrate for the brains efflux transporter p-glycoprotein (Syvanen et al., 2006).

Kinetic modeling

In a dynamic PET scan the number of events in specific regions or voxels is measured over time and time activity curves (TACs) are generated (figure 4) and to describe the physiological system a range of different kinetic models can be used. Determining the precise quantitative measure of neuroreceptors in the brain is a complex task, since the receptor density, B_{max}, cannot in a single bolus injection PET scan be separated from the dissociation coefficient (K_d). Therefore, the surrogate outcome of a neurorecetor PET study is the so-called binding potential, BP, which corresponds to the ratio of B_{max} to K_d (Innis et al., 2007).

The binding potential is a measure of the equilibrium concentration of specific binding as a ratio to a reference tissue. Different bindings potentials are defined depending on the reference tissue used (Innis et al., 2007):

- free plasma concentration - free non-protein bound - BP_F
- total plasma concentration - not corrected for protein binding - BP_P
- non-displaceable uptake – concentration in a reference region - BP_{ND}

If a true reference brain region exists devoid of specific binding and with similar non-specific binding as the rest of the brain, use of non-invasive reference tissue model becomes feasible (Hume et al., 1992). The often-used simplified reference tissue model (SRTM) (Lammertsma and Hume, 1996) assumes that

1) the non-displaceable distribution volume is the same in the region of interest and the reference tissue.
2) tracer kinetics in the target region (as well as the reference region) are difficult to distinguish between free and specific compartments and can be fitted satisfactorily with a one-tissue compartment model

Measuring the plasma input curve and using tracer plasma concentration as the reference tissue (obtaining the BP_P) is regarded as the gold standard for measuring the binding potential compared to reference tissue models. This is due to the vulnerability of the reference models to changes in non-displaceable binding that will cause bias in the BP_{ND}. However, invasive arterial cannulation and laboring measurements of radiolabeled metabolites hamper clinical use and therefore, tracers with a true reference region is often preferred in a clinical setting.

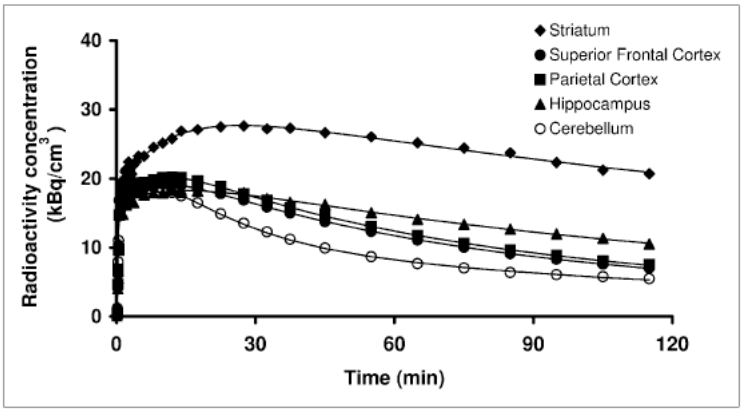

Figure 4. The time activity curves for grey matter of four regions of interest in a two-hour dynamic PET scan with the radiotracer [^{11}C]SB207145. Cerebellum is the reference region. (From Marner et al 2009)

PET imaging of the 5-HT$_4$ receptor

With the development of the PET tracer [^{11}C]SB207145 it is possible to measure the human cerebral 5-HT$_4$ receptors in vivo. This tracer is so far the only PET 5-HT$_4$ radioligand fully evaluated in humans but other tracers are in progress such as the high-affinity 5-HT$_4$ receptor antagonist SB207710 (Xu et al., 2010). This latter compound is also available in a radioiodinated form as a single photon emission computed tomography (SPECT) tracer and shows promising results in rats and monkeys (Varnas et al., 2003)

The [^{11}C]SB207145 was initially developed by GlaxoSmithKline Clinical Imaging Center (Gee et al., 2008), and was thoroughly validated in humans by our group (Marner et al., 2009). The [^{11}C]SB207145 has a high affinity to the 5-HT$_4$ receptor, but is still reversible within the time window of a two hour dynamic PET-scan. It has a relatively slow metabolic rate and high free fraction in plasma (mean around 27%). A blocking study with the 5-HT$_4$ receptor partial agonist, piboserod, revealed that the radioligand was selective for the 5-HT$_4$ receptor, that cerebellum was a suitable reference region devoid of specific binding, and that non-specific binding was constant across brain regions. The SRTM was validated against arterial input models (which is considered the golden standard) and showed good test–retest reproducibility (6-10% in moderate to high-binding regions and 12%-14% in low-binding regions) and reliability and therefore measurements of arterial input may be avoided. However, when compared to a two-compartment model the SRTM underestimated the BP_{ND} in the high-binding striatal regions with 20%-43%, so careful considerations should be implied for individual applications.

Analyses of PET images

PET images can in principal be analyzed in two different ways: With or without *a priori* defined regions of interest (ROI). With *a priori* defined regions one binding potential is obtained in the predefined regions such as parietal cortex or occipital cortex. This means that any variation in BP within the region is averaged and no within-regional details can be obtained. However, the advantage is that a limited number of analyses are made based on predefined theories of brain function. Without predefined regions a BP is generated in each voxel in a PET images and analyzed separately without any predefined theories on brain functionality. The method offers possibilities to illustrate variation in BP within regions. However, the disadvantage with a voxel-based analysis is the risk of false positive results as sometimes more than 100.000 analyses are made and therefore corrections for multiple comparisons are mandatory.

Regional analyses of PET images

A PET image has limited structural information and therefore ROI are often defined on magnetic resonance images (MRI). MRI can be segmented into grey matter, white matter and cerebrospinal fluid, and information can be extracted exclusively from the area that the target site is located in, for example serotonin receptors in grey matter. Different approaches exist to delineate ROIs e.g. they can be manually drawn, however user-independent methods using probability maps also exists (Svarer et al., 2005). With this method a template set of 10 MRIs is automatically co-registered to a new participants' MRI. The identified transformation parameters are used to define ROIs in the new participants' MRI space, and through coregistration, these ROIs are transferred onto the PET images (figure 5).

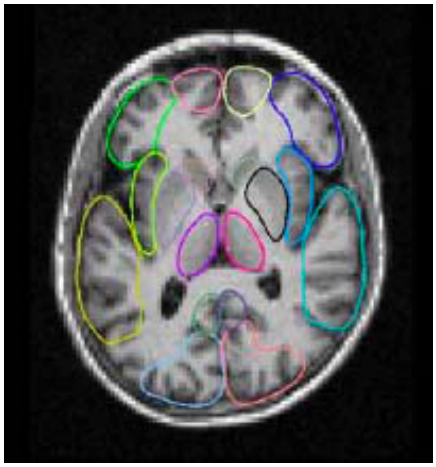

Figure 5. Automatic user-independent delineated regions of interest at the slice of an MRI (Svarer et al., 2005)

Voxel-based analyses

To enable a voxel based analysis parametric images for each included individual must be constructed. Parametric images contain a binding potential in each voxel and by warping these images into a common space a voxel level statistical analysis can be made. Parametric images from dynamic PET scans are obtained by applying kinetic modeling to the time activity curves in each voxel. One method developed by Gunn et al uses a basis function implementation of SRTM (Gunn et al., 1997), where *a priori* information obtained from a ROI analysis is used to robustly and fast generate BP_{ND}s in each single voxel in the PET image.

Aims and Hypothesis

Study 1 (paper 1):

This is the first study in humans to examine the relation between the cerebral 5-HT_4 receptor availability and obesity. We used a cohort of 28 healthy humans with varying body mass index (BMI). Based on the findings in previous animal studies our region of interest was primarily nucleus accumbens and secondly other regions of the reward system such as ventral pallidus, hippocampus and orbitofrontal cortex. We expected to find an association between BMI and the 5-HT_4 receptor levels in these regions of the brain.

Study 2 (paper 2):

To investigate the individual variation of the 5-HT_4 receptor binding in hippocampus in relation to memory encoding and consolidation in healthy young volunteers. Since 5-HT_4 receptor activation in animals generally have a facilitatory effect on memory functions, we hypothesized that the level of 5-HT_4 receptors would be positively correlated with the immediate and delayed recall in the memory tasks.

Study 3 (paper 3):

In a randomized double-blinded placebo-controlled design we measured the *in vivo* 5-HT_4 receptor binding in healthy men before and after pharmacologically increased extracellular 5-HT levels by means of the selective serotonin reuptake inhibitor fluoxetine. Based on an expected monotonic association between the 5-HT_4 receptor binding and the 5-HT levels, we hypothesized that the 5-HT_4 receptor binding would decrease in the intervention group. Further, since L/L carriers are associated with a higher level of 5-HTT we expected this association to be influenced by 5HTTLPR status: the L/L homocygotes would show a greater response to SSRI intervention than the S carriers.

Study 3A (not included in the papers):

In the set-up of study 3 we expected a weight loss in the fluoxetine-treated group and not the placebo-treated group. Based on our result in study 1 we further expected an association between a change in BMI and a change in 5-HT_4 receptor binding in ventral striatum including nucleus accumbens.

Methods and design

Subjects (all studies)

Healthy volunteers over 18 years were recruited by public advertisements or extracted from the civil registration system in Denmark. After the aims and designs of the specific studies had been explained, written informed consent was obtained from each participant according to the declaration of Helsinki II. The Ethical Committee of Copenhagen approved the studies. In total 61 healthy volunteers were included in this thesis. Some of the healthy subjects participated in more than one study (figure 6). 93 PET scans were done in total. All subjects were scanned in the period from 2006 to 2012.

Exclusion criteria were:

- Primary psychiatric disease (DSM IV or WHO ICD-10)
- Previous or current neurological disorder, other severe medical disorder or drug intake likely to influence the results
- Drug use (Any use within the last 3 months, lifetime use > 10 times for ecstasy, cocaine or heroine and > 50 times for cannabis)
- Alcohol intake > 14/week for women and 21/week for men
- Significant head injury
- MRI exclusion criteria (e.g., claustrophobia or metal implants)
- Pregnancy or breastfeeding at the time of PET scan

All healthy subjects had a normal neurological examination and unremarkable brain MRI. All subjects completed the Symptom Check List Revised (SCL-90-R) questionnaire (Derogatis, 1994) and Major Depression Inventory (MDI) (Forsell, 2005) to assess symptoms of depression, distress and psychopathology.

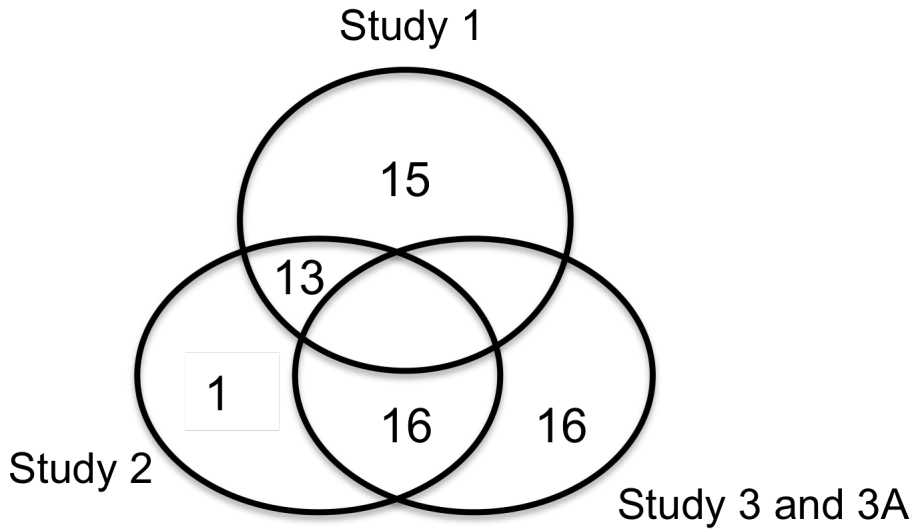

Figure 6. The distribution of the 61 healthy volunteers in this thesis

Demographics in study 1 (paper 1):

- 28 healthy participants (12 women)
- Mean age 41.1 ± 17.7 years (range 20.0-68.7 years)
- Mean BMI 26.5 ± 6.8 kg/m^2 (range 20.5 to 40.0 kg/m^2). Sixteen individuals had normal bodyweight (BMI<25 kg/m^2) and 12 were overweight or obese (BMI>25 kg/m^2).
- MDI median 3, range 0-11, SCL-90-R global score 0.08, range 0-0.43

Demographics in study 2 (paper 2):

- 30 healthy participants (6 women)
- Mean age 27.2 ± 6.3 years (range 20.0-44.7 years)
- Mean BMI 23.1 ± 2.0 kg/m^2 (range 19.1 to 28.6 kg/m^2)
- Total years of education 15.7 ± 2.1 years
- I.Q. score 104.4 ± 10.5 (range 90.5 to 123.5)
- MDI median 3, (range 0-21), SCL-90-R global score 0.11, (range 0-0.81)

Demographics in study 3 (paper 3) and 3A:

Table 2. Demographic data in study 3/3A

	Active		Placebo	
N	16		16	
Age (years)	25.7 ± 5.2		25.9 ± 3.9	
Education (years)	15.7 ± 1.9		15.8 ± 1.6	
Genotype - ss/sl/ll	3/8/5[1]		1/8/7[1]	
MDI	6 (0-21)		4.5 (0-12)	
SCL-90-R	0.13 (0-0.81)		0.15 (0-0.68)	
	Baseline	Rescan	Baseline	Rescan
BMI (kg/m²)	23.1 ± 1.9	22.6 ± 2.0[2]	23.4 ± 3.1	23.4 ± 3.0

[1] One person was L_AL_A. [2] $p<0001$ between baseline and rescan (paired t-test). MDI = Major Depression Index. SCL-90-R = Symptom Check List Revised. Data is given as mean ± standard deviation or median with range (MDI and SCL-90-R)

In study 2 and 3/3A one participant scored 21 in the MDI questionnaire but did not fulfill the ICD-10 criteria for a depression and consequently, he remained in the study.

Memory testing in study 2 (paper 2)

Episodic memory functions in humans can be measured with verbal or visual memory tasks. In study 2 we used Reys Auditory Verbal Learning Test (RAVLT) to assess episodic verbal memory and Rey-Osterrieth's Complex Figure Test (ROCFT) to evaluate visual non-verbal memory (Lezak et al., 2004). In RAVLT, a list of 15 emotionally neutral words was read aloud five times. After each presentation, the participant delivered a free immediate, verbal recall. After a 30 minutes delay the participant was asked to recall the list again. To reflect the cognitive process of memory encoding we used the total number of words the individuals acquired during the five first trials (immediate recall, maximum score 75) and to reflect memory consolidation we used the number of recalled words after 30 minutes (delayed recall, maximum score 15) (Vakil et al., 2010). In ROCFT, participants copied a complex geometric figure and then reproduced it from memory after 3 minutes and after a delay of 30 minutes. To measure memory encoding the scores of 3 minutes were used (immediate recall) and to measure memory consolidation the delayed scores at 30 minutes were chosen (delayed recall). In both memory tests it was not possible to eliminate retrieval from consolidation.

Intervention regime and blinding in study 3 (paper 3) and 3A

Fluoxetine was the first selective inhibitor of the serotonin transporter (SSRI) introduced in the 1980'ies with little affinity for muscarinic, histaminic, serotonergic, or noradrenergic receptors (Stark et al., 1985). The half-life of fluoxetine is 1-4 days, while the main metabolite nor-fluoxetine with comparable pharmacologic activity, has a half-life of 4-16 days (Hiemke and Hartter, 2000). The most frequent adverse events associated with fluoxetine treatment are nausea, insomnia, nervousness, somnolence and sexual dysfunction (Zajecka et al., 1999). We preferred fluoxetine over citalopram first of all as fluoxetine treatment significantly effected 5-HT_4 receptor levels in rats (Vidal et al., 2009) and furthermore, some studies report that in order to increase extracellular 5-HT levels after chronic citalopram administration, an acute citalopram challenge is required on top of the chronic regime (Invernizzi et al., 1994, Ceglia et al., 2004, Muraki et al., 2008), and in some studies no raise in 5-HT levels is detected (Invernizzi et al., 1994, 1995, Invernizzi et al., 1997, Grignaschi et al., 1998, Pozzi et al., 1999, Ceglia et al., 2004, Muraki et al., 2008).

The participants were randomized based on a match by genotype, age and education by an un-blinded investigator to intervention with capsules containing fluoxetine (N=16) or placebo with no active drug (N=16). All capsules were identical regardless of content and delivered pre-packed. The participants received identical intervention regimes regardless of randomization group and the intervention began after the first PET scan and lasted for a total 3 weeks. To minimize side-effects the participants took the capsules in the evening and the initial dose of fluoxetine were 20mg/day for three days. Then the dose was increased to 40 mg/day until the last PET scan had been conducted 18-20 days later. To minimize discontinuation symptoms the participants continued on 20 mg/day for 5 days after the PET-scan (the placebo group also continued for 5 days). Three participants were excluded before the PET-scans were completed: two because of failure of radiotracer production and one (placebo group) did not show up for the PET rescan.

The participants and the remaining investigators were blinded to the intervention type until the outcome of the BP_{ND} and voxel-based analysis had been made. The subjects were contacted by a medical doctor 2 to 3 times during the 3 weeks of treatment to register side-effects and again after treatment termination to register any discontinuation symptoms. The side-effects were scored according to the UKU side-effect rating scale (Lingjaerde et al., 1987). S-fluoxetine was measured in the middle of the treatment period and again immediately before the PET rescan.

Genotyping in study 3 (paper 3)

Analysis was performed on blood samples that were drawn at the time of PET scanning and immediately frozen at -20 °C. DNA was extracted from blood using a Qiagen Mini Kit (Qiagen). Alternatively, analysis was performed on saliva samples, collected using the prepIT kit (DNA Genotek Inc). DNA was extracted according to the manufacturers instructions. The 5-HTTLPR short/long and rs25531 A/G polymorphisms in the serotonin transporter gene were genotyped by a method consisting of a polymerase chain reaction (PCR) amplification with the following primers: forward 5'-GGC GTT GCC GCT CTG AAT GC-3' and reverse 5'-CTG ACC CCT GAA AAC TGT GC-3', followed by MspI digestion and fragment analysis by electrophoresis on an ABI 7500 multiplex (Applied Biosystems) using a FAM-labeled forward primer. When using the fluorescence labeling and MspI digestion the following three alleles can be demonstrated: 5-HTTLPR l + rs25531/A (lA, 341 bp), 5-HTTLPR s + rs25531/A (sA, 298 bp), and rs25531/G (167 bp). In order to distinguish the 5-HTTLPR l + rs25531/G (lG) from the rare 5-HTTLPR s + rs25531/G (sG) allele, all samples showing a G allele were subsequently analyzed for 5-HTTLPR s or l allele by PCR amplification with the same primers followed by electrophoresis of the undigested product (l allele: 571 bp, s allele: 528 bp).

Imaging and receptor quantification

The following section briefly describes the methods for image acquisition, analysis and quantification of the 5-HT_4 receptor.

MRI (all studies)

Magnetic resonance imaging was conducted on a Siemens Magnetom Trio 3T MRI scanner (matrix 256x256; 1x1x1mm voxels). A high-resolution 3D T1-weighted, sagittal, magnetization prepared rapid gradient echo (MPRAGE) scan and a high-resolution 3D T2-weighted, variable flip angle, sagittal, Turbo Spin Echo scan were acquired matrix 256x256, voxel size 1x1x1 mm). All scans are corrected for spatial distortions due to non-linearity in the gradient system of the scanner (Jovicich et al., 2006). The T1 weighted MRIs were

segmented into grey matter, white matter and CSF using Statistical Parametric Mapping (SPM5; Wellcome Department of Cognitive Neurology, London, UK). Segmented images made it possible to extract PET data from grey matter voxels only.

PET imaging of 5-HT_4 receptors (all studies)

[^{11}C]SB207145 was synthesized using a fully automated radio-synthesis system as previously described (Gillings and Larsen, 2005, Gee et al., 2008). Immediately after a 20 seconds intravenous bolus injection of [^{11}C]SB207145 a 120 minutes dynamic 3D PET scan of 38 frames (6x5 s, 10x15 s, 4x30 s, 5x120 s, 5x300 s and 8x600 s) was initiated. Two different PET scanners were used in the studies in this thesis. All of the subjects in study 1 and 13 of the 30 subjects in study 2 were scanned on an 18 ring GE Advance scanner (General Electric, Milwaukee, WI, USA). The scanner had a spatial resolution of 6-8 mm full with half maximum (FWHM) and the images were reconstructed with filtered back projection and corrected for attenuation, dead time and scatter. The last 17 subjects in study 2 and all of the subjects in study 3/3A were scanned with a high resolution Siemens HRRT scanner with an approximate spatial resolution of 1-2 mm FWHM (Olesen et al., 2009). The HRRT images were reconstructed with the iterative 3D-OSEM-PSF point spread function reconstruction with attenuation map improvements (Sureau et al., 2008).

After reconstruction the frames were aligned to correct for movement during the scan using AIR 5.2.5 (Woods et al., 1992). The images from the HRRT scanner were only corrected for head movements if the median movement exceeded 3 mm. Flow-weighted PET images (the first 20 minutes of the scan) were automatically co-registered to the MRI using either the AIR algorithm for the scans from the GE Advance scanner (Woods et al., 1992) or using SPM for the scans from the HRRT scanner. The precision of each co-registration was evaluated by visual inspection in three planes.

5-HT_4 receptor quantification and image analyses (all studies)

The quantitative analysis to obtain the binding potential (BP) of the 5-HT_4 receptor was performed with the simplified reference tissue model (SRTM) (Lammertsma and Hume, 1996) using PMOD (PMOD Inc, Zürich, Switzerland). This method was found to be a reliable and reproducible method for quantification of [^{11}C]SB207145 receptor binding in humans

(Marner et al., 2009). Cerebellum was used as a reference region since blocking with a selective 5-HT_4 receptor compound prior to radiotracer administration did not alter the cerebellar binding (Marner et al., 2009).

For the regional analyses the regions of interest (ROI) were automatically delineated as described above (Svarer et al., 2005).

For the voxel-based analyses parametric images were constructed also with SRTM according to the method presented by Gunn et al (Gunn et al., 1997). The single-subject BP_{ND} parametric maps were processed and warped to MNI space within SPM8 (Wellcome Department of Imaging Neuroscience. London, UK) using the single-subject GM segmented MRI and MNI templates. Final voxel-size was 2x2x2 mm. The PET images from the Advance Scanner were spatially smoothed with a Gaussian kernel with 12 mm FWHM, since we found the best performance of this kernel in the nonparametric, prediction, activation, influence, reproducibility, re-sampling (NPAIRS) framework, for more information see (Strother et al., 2002). In study 1 a mask was constructed from the intersection between a mask based on PET images, and included only voxels with an average BP_{ND}>0.1, and a mask constructed from gray matter voxels with an average value>0.2. The final mask included 171661 voxels. In study 2 we were evaluating BP_{ND} variation within hippocampus and defined the region using the Wake Forest University Pickatlas (v3.0) (Maldjian et al., 2003, Maldjian et al., 2004). In study 3A ventral striatum was defined as a 10 mm sphere centered at MNI coordinates ±12,12,-10 . This definition was used in previous publications (Nikolova et al., 2012).

Methodological considerations

In this section, concerns related to the different methods employed in this thesis will be addressed.

BP_{ND} influenced by fluctuations in endogenous serotonin?

We believe that even large acute fluctuations in 5-HT will not affect the affinity of [^{11}C]SB207145 to the 5-HT_4 receptor since the affinity of 5-HT to the 5-HT_4 receptor is low with Ki values in the order of 0.1-1.2 μM (Paterson et al., 2010). In support of this, we showed in a previous publication that acute infusion of citalopram (leading to acute increases in interstitial 5-HT) does not alter cerebral [^{11}C]SB207145 binding (Marner et al., 2010).

Therefore, eventual acute differences in interstitial 5-HT will not affect the BP_{ND} (eq.1) through changing K_D.

Tracer availability

Cold mass

As previously described, injection of a too high mass of the unlabeled compound results in an underestimation of the binding potential because of competitive binding between labeled and unlabeled ligand. Therefore, the amount of given mass was assessed in all 3 studies. In a previous study with test-retest PET scans we determined the upper limit of mass dose of [^{11}C]SB207145 to yield occupancy of 5-10% of the receptor to be 4.5 ± 1.2 µg (Madsen et al., 2011c). In the three studies included in the thesis we kept the average injected mass below this limit (Study 1: 3.5 ± 1.7 (0.1-5.9), study 2: 1.9 ± 1.8 (0.1 to 5.9) and study 3/3A: see table 3. Nonetheless, we included cold mass as a covariate in the regression models in study 1 and 2 but it did not contribute to our finding and in study 1 we compared lean vs. overweight/obese but did not find any significant difference in the administered cold mass dose. In study 3/3A we observed a significant difference between baseline and rescans in the placebo group (p=0.04). Therefore, we corrected the BP_{ND}s with the population-based ID_{50} (the injected dose estimated to saturate 50% of the receptors) calculated in the paper by Madsen et al (Madsen et al., 2011c) using the equation

$$BP_{ND}^{C} = BP_{ND} \times (D + ID_{50}) / ID_{50}$$

where BP_{ND} is the measured binding potential and BP_{ND}^{C} is the corrected binding potential, D is the injected dose and ID_{50} is the injected dose estimated to saturate 50% of the receptors, in this case 85.8 µg (Madsen et al., 2011c)

Table 3. Injected cold dose of SB207145 and the non-specific binding in study 3/3A.

	Active		Placebo	
	Baseline	**Rescan**	**Baseline**	**Rescan**
Non-specific binding	106.4 ± 19.2	104.1 ± 16.3	110.0 ± 15.1	109.3 ± 18.5
Cold dose inj. (µg)	1.15 ± 0.90	1.28 ± 0.94	1.40 ± 0.65	2.14 ± 1.42[1]

[1] p=0.04 between baseline and rescan (paired t-test)

Non-specific binding

To rule out any difference in our cohorts in cerebellar uptake we calculated the non-specific binding. Since we used a reference region method to obtain the BP_{ND}, it was not possible to get a direct measure. We did however use the area under the reference region's time activity curve curves normalized to the injected dose.

In study 1 and 2 we included the non-specific binding in regression analyses and evaluated if the variable correlated with BMI or BP_{ND}. We did not find such correlations. In study 1 we additionally compared lean vs. overweight/obese but did not find any significant difference. In study 3/3A no significant differences were observed between baseline and rescans or between active and placebo group (see table 3).

Grey matter volumes

Gray matter volume could influence the measures of BP_{ND} and thereby the results of our studies. Particularly in study 1 since grey matter volumes also was found to correlate positively with measures of obesity (Horstmann et al., 2011). We assessed gray matter volume as defined from the MR SPM segmentation in regression models in study 1 and 2. Keeping in mind the limitations of this rough measure of gray matter volume we did not, however, find that this factor correlated contributed to our findings (study 1) or correlated with memory scores or BP_{ND} (study 2).

Body Mass Index (BMI)

Body composition was assessed with BMI (defined as weight divided by squared height). A BMI between 18.5- 25 kg/m^2 is defined as normal weight, between 20 to 25 kg/m^2 is defined as overweight, and more than 30 kg/m^2 is defined as obese. The measurement of BMI is inexpensive, convenient, and reliable, and errors due to inter- and intra observer variation are small. Further, it has low correlation to height and a high correlation to percent body fat, although significantly influenced by gender and age (Garrow and Webster, 1985, Deurenberg et al., 1991). To find out whether BMI in study 1 corresponded to another anthropometric estimate of the nutrition state in our cohort, the waist circumference was obtained 24 of the 28 individuals included. Using normal linear regression, a positive relation between BMI and this measure was detected ($p<0.0001$).

Since we believe that the BMI defined cut-off between normal-weight and obesity is rather arbitrary, we chose to study the association between BMI and the receptor rather than compared normal-weight vs. overweight/obese.

Obesity and comorbidity

Comorbidity between affective disorders and obesity exist: Individuals with BMI>30 kg/m^2 have significantly increased odds of developing mood, anxiety, and personality disprder as well as alcohol abuse (Petry et al., 2008). For this reason, and because of the suspected involvement of 5-HT$_4$ receptors in the pathophysiology of e.g. depression and cognitive functions, we speculated if our results was influenced by differences in psychiatric symptoms such as depression- or anxiety-like symptoms in lean as compared to overweight/obese. Therefore, the between group scores from SCL-90-R as well as MDI were compared in study 1. No significant differences were detected for any of these measures incl submeasures of SCL-90-R (part of these data are presented in paper 1). We further compared the alcohol drinking and smoking habits of lean vs. overweight/obese and found no significant difference.

Age and gender

Age and gender should be considered in analyses including the 5-HT$_4$ receptor binding level, since our lab recently found a decrease in receptor binding of 1% per decade in striatum and a 13% lower binding in women in the limbic system (Madsen et al., 2011b).

In study 1 we had a relatively large age span and included almost an equal number of men and women and therefore, we chose to keep the variables of age and gender in our analyses regardless of significance level. In study 2 we excluded subjects over 45 years and only a few women were included, therefore we excluded the variables from the model if they not significant, which was what we found (all p-values were larger than 0.23). Only men were included in study 3/3A and the two groups (active and placebo) were age-matched. Consequently, we did not take age and gender into account in our analyses.

Ceiling effects in Reys Auditory Verbal Learning Test

We found ceiling effects in RAVLT, which in young and well-educated cohorts is a prevalent finding with the applied testing procedures (Uttl, 2005, Van der Elst et al., 2005). The ceiling effect causes bias in regression parameters in a standard linear regression (under-estimation of the association between memory score and 5-HT_4 receptor BP_{ND}), and to limit this effect we conducted the analyses of RAVLT scores using Tobit regression (Tobin, 1958). The Tobit model was designed to estimate linear relationships between censored variables. In this study we have a censoring, where individuals with a RAVLT memory score at or above the threshold of 15 words, take on the value of that threshold, so that the true memory score might be equal to the threshold of 15 words, but it might also be higher. Therefore, we analyzed the data with a statistical model that take censoring of measurements into account to avoid inconsistent estimates present in an ordinary linear regression. Obviously, our study would have been stronger if we had used a memory test, which tested the participants within their full range of memory capabilities but the ceiling effects were only realized after the completion of the study. For this reason and because affective biases in memory may be related to serotonin (Mendelsohn et al., 2009), we are currently developing and testing the Cimbi Affective Memory Test (CAMT), a 24-word test administrating positive, negative and neutral words with procedures similar to RAVLT.

How to pool data from two different PET scanners

As mentioned, the subjects in study 2 were scanned with two different PET scanners: A GE Advance scanner with a spatial resolution of 6-8 mm FWHM and a HRRT scanner with a spatial resolution of 1-2 mm FWHM. The resolution in a PET image is influenced by movement artifacts, statistical noise and filtering and the images will appear blurred as a particular area in the brain will be contaminated by the activity in the adjacent areas, a phenomenon known as partial volume (PV) effect. Since the two scanners have different image resolution characteristics a bias between the two scanners in the BP_{ND} will appear: the images from the HRRT scanner will be less influenced by the PV effect, while the images from the Advance scanner will be under more substantial influence. To correct for PV effects and obtain comparable images from the two scanners three different approaches can be taken: 1) correction of the PV effect in the images from the Advance scanner by algorithms developed by e.g. Meltzer et al (Meltzer et al., 1990) or Muller-Gartner et al (Muller-Gartner et al., 1992),

2) Gaussian filtering of the HRRT scanner images to a resolution comparable to Advances scans, and 3) a post images analysis approach where the bias is corrected statistically by including scanner type as a variable in the statistical analysis.

My colleague Karine Madsen scanned four subjects (mean age 54, range 53-56) on both the GE Advance scanner and the HRRT scanner with [^{11}C]SB207145 and analyzed the data in five brain regions: striatum, hippocampus, amygdala, cingulate and neocortex (Madsen, 2010). The raw BP_{ND} was underestimated with a mean of 24% ± 14% in images from the GE Advance scanner compared to the HRRT scanner images. Approach number 1 was to apply a Muller-Gartner PV correction to the GE Advance scanner raw data and the mean difference in BP_{ND} between the two scanners was reduced to a mean of 0% ± 19%. However, this method showed considerable regional variation. This is not surprising since the PV effects is greater in thin areas with a large surface-area bordering low activity areas (e.g. neocortex) compared to more spherical areas surrounded by tissue with similar activity (e.g. hippocampus). This implies that PV correction works well in e.g. prefrontal cortex, while in hippocampus the bias between scanners in BP_{ND} was still 12.7%. Approach number 2 was to apply a Gaussian filter to the HRRT scanner data and thereby reduce the resolution to a comparable level to the Advance scanner data. Different filter sizes were applied and it was apparent that a filter of 10*10*10 mm gave BP_{ND} closest to the Advances scanner data. However, again considerable regional differences were observed. This method was relatively effective in hippocampus, but not in cortical and cingulate regions.

The last method was a statistical correction applied after images analysis was undertaken the usual way. With this approach the scanner type was included as a class variable in all analyses. The assumptions are that there is a linear bias between the two scanners giving a correction factor for each region dependent on the obtained BP_{ND}'s.

We chose this last approach in study 2 to correct for the two different scanner types because this method did take into account the regional bias and we therefore considered this method most reliable.

Test-retest data on the HRRT scanner

Some variation between two otherwise identical PET investigations is expected as the PET image is as mentioned subject to e.g. movement artifacts, statistical noise and partial volume

effects. To analyze the test-retest difference in the placebo group in study 3/3a, a relative test-retest difference was calculated per ROI as:

$$\Delta\% = 2 \times (\text{retest value} - \text{test value} / \text{retest value} + \text{test value}) \times 100$$

The mean of the Δ% is a measure of the systematic bias and the standard deviation of Δ% is a measure of the mean test-retest difference and characterizes the reproducibility (Marner et al., 2009). Reliability was determined with the Intraclass Correlation Coefficient (ICC) defined as:

$$ICC = MSS_{Between} - MSS_{Within} / MSS_{Between} + MSS_{Within}$$

$MSS_{Between}$ is the mean sum of squares between subjects, and MSS_{Within} is the mean sum of squares within subjects. An ICC score of -1 denotes no reliability, and +1 denotes maximum reliability.

In study 3/3A we studied the participants with [^{11}C]SB207145 PET 3-weeks apart (on a HRRT scanner) and matched to an acceptable degree the same-day investigations (on a GE Advance scanner) done by Marner et al (Marner et al., 2009) (see table 4), however, in hippocampus and neocortex we observed a smaller reliability.

Table 4. Test-retest data from same-day [^{11}C]SB207145 PET investigations on a GE Advance scanner (Marner et al., 2009) compared to 3-weeks apart investigations on a HRRT scanner.

	Striatum		**Hippocampus**		**Cortex***	
	Same-day Advance	3-week HRRT	Same-day Advance	3-week HRRT	Same-day Advance	3-week HRRT
Δ%	3.7	1.1	4.0	3.0	5.7	1.4
SD Δ%	6.1	10.4	9.9	13.4	13.6	12.2
ICC	0.76	0.78	0.88	0.31	0.77	0.48

Δ% = relative test-retest difference. SD Δ% = standard deviation of the relative test-retest difference. ICC = Intraclass correlation coefficient. * Neocortex in study 3/3A and parietal cortex in Marner et al

Statistics

The employed statistical models are briefly described in this section, while detailed information is included in the papers.

Study 1

Based on the animal literature our primary volume of interest was the ventral striatum including the NAc, which overlap two volumes as defined in the atlas according to Svarer et al (Svarer et al., 2005). Therefore, we chose a voxel-based analysis with familiy wise error (FWE) corrections for multiple comparisons as our primary analysis. To investigate if BMI predicted the 5-HT_4 receptor binding a whole brain voxel-wise regression analysis was conducted with BMI, age, and gender included in the model as covariates. The primary contrast of interest was the positive BMI t-contrast.

In parallel we did an analysis with a priori defined brain volumes of putamen, caudate, hippocampus and orbitofrontal cortex. Regional BP_{ND} was used as dependent variable in regression analyses including BMI, age and gender as covariates.

Study 2

The volumes of interest were left and right hippocampus, and we modeled the association between the 5-HT_4 receptor binding and memory functions using regression analyses. The four memory test scores used in the regression models were: 1) RAVLT immediate recall (sum of the five initial trials), 2) the RAVLT delayed recall, 3) the immediate recall in ROCFT, and 4) delayed recall score in ROCFT. These outcomes were one by one compared to the 5-HT_4 receptor BP_{ND} in left and right hippocampus with scanner type as covariate. In addition to the regional analyses we performed voxel-wise regression analyses to reveal effects of interest within the structure of hippocampus. RAVLT immediate and delayed recall was employed in the model as dependent variable and BP_{ND} and scanner type as covariates. We corrected for multiple comparisons using Monte Carlo simulation in 3dClustSim within AFNI (http://afni.nimh.nih/gov/afni) and the method yielded a cluster extent significance threshold of $k \geq 30$ voxels ($p < 0.05$).

Study 3

We primarily evaluated the average BP_{ND} of striatum since SSRI treatment in rats reduced 5-HT_4 receptor binding up to 37% (Licht et al., 2009, Vidal et al., 2009) and this region as a [^{11}C]SB207145 high-binding region also provides a good signal-to-noise ratio. Secondly, we examined 1) the bilateral hippocampus, since SSRI treatment in rats also decreased 5-HT_4

receptor binding in this region and 2) cortical effects using a large neocortical region providing a robust measurement despite low 5-HT_4 receptor binding.

We compared the baseline BP_{ND} with the rescan BP_{ND} in each region of interest to test the intervention effect on regional 5-HT_4 receptor binding. The percent change in BP_{ND} from baseline to rescans were calculated with the 95% confidence interval and used to compare the fluoxetine and the placebo group and the subgroups of 5-HTTLPR status.

Study 3A

Based on the findings in study 1 our volume of interest was the ventral striatum, including the NAc, as defined as a 10 mm (radius) sphere centered at (±12,12,-10; MNI space) using a parametric approach. This definition has been used in previous publications e.g. (Nikolova et al., 2012). The left and right spheres are ~515 voxels (at voxel-size = 2x2x2mm). We evaluated if the any baseline to rescan change in BMI correlated with any baseline to rescan change in 5-HT_4 receptor binding in ventral striatum and used Monte Carlo simulation in 3dClustSim within AFNI (http://afni.nimh.nih/gov/afni) for correction for multiple comparisons and the method yielded a cluster extent significance threshold of $k \geq 90$ voxels ($p < 0.05$).

Results and discussion

This section shows and discusses the main results of each aim in the studies. Further details are included in the papers except for the results of study 3A, which are only presented in the thesis.

Study 1 and study 3A

We found a significant positive association between BMI and the 5-HT_4 receptor density in brain areas involved in reward: bilateral NAc/ventral pallidum and left orbitofrontal cortex (700 voxels), the left hippocampal region (167 voxels) (figure 7).

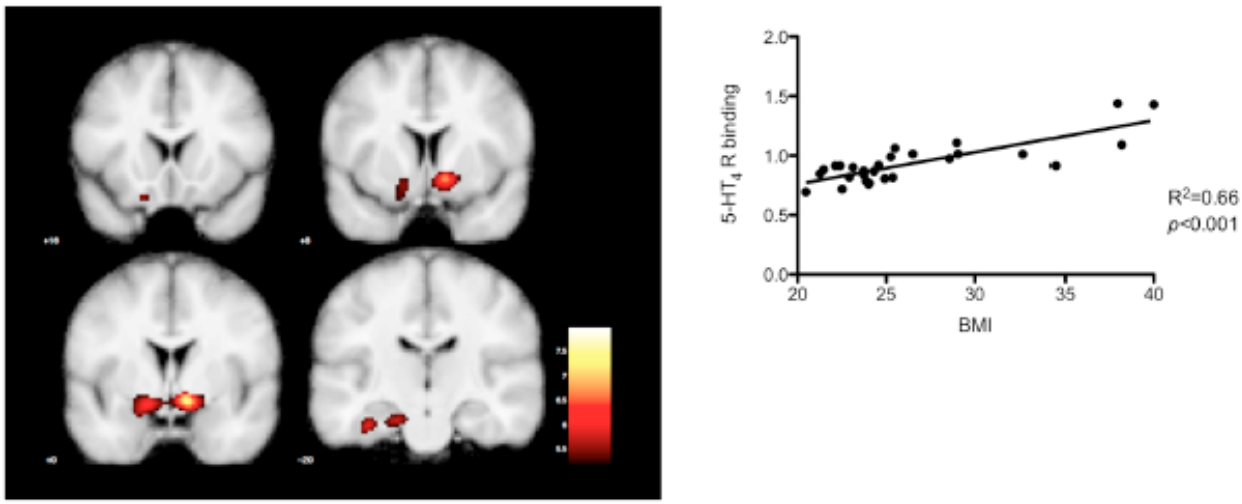

Figure 7. On the left a statistical parameter map shows voxels with a significant positive association between $5HT_4R$ BP_{ND} and BMI in the 28 included individuals. The color bar depicts t-values of suprathreshold (p<0.05 with Family Wise Error correction) voxels. On the right a plot of the positive association between BMI and the mean 5-HT_4 receptor binding in the 700 voxels cluster including bilateral nucleus accumbens and ventral pallidum.

In study 3A we found a significant decrease in BMI in the fluoxetine group (baseline BMI 23.1 ± 1.9 vs. rescan BMI 22.6 ± 2.0, p<0.001), whereas no change was observed in the placebo group (baseline BMI 23.4 ± 3.1 vs. rescan BMI 23.4 ± 3.0, p=0.46). In the parametric analysis we found a statistically significant cluster of 195 voxels in the right ventral striatum (peak in MNI coordinates 18,10,-10), where the change in BMI in the fluoxetine group correlated positively with the change in 5-HT_4 receptor binding the ventral striatum (see figure 8). We also saw a cluster of 54 voxels in the left ventral striatum (peak in MNI coordinates -12,6,-18), however insignificant after correction for multiple comparisons (see figure 8). The finding implies that the larger the weight loss, the larger decrease in the 5-HT_4 receptor binding.

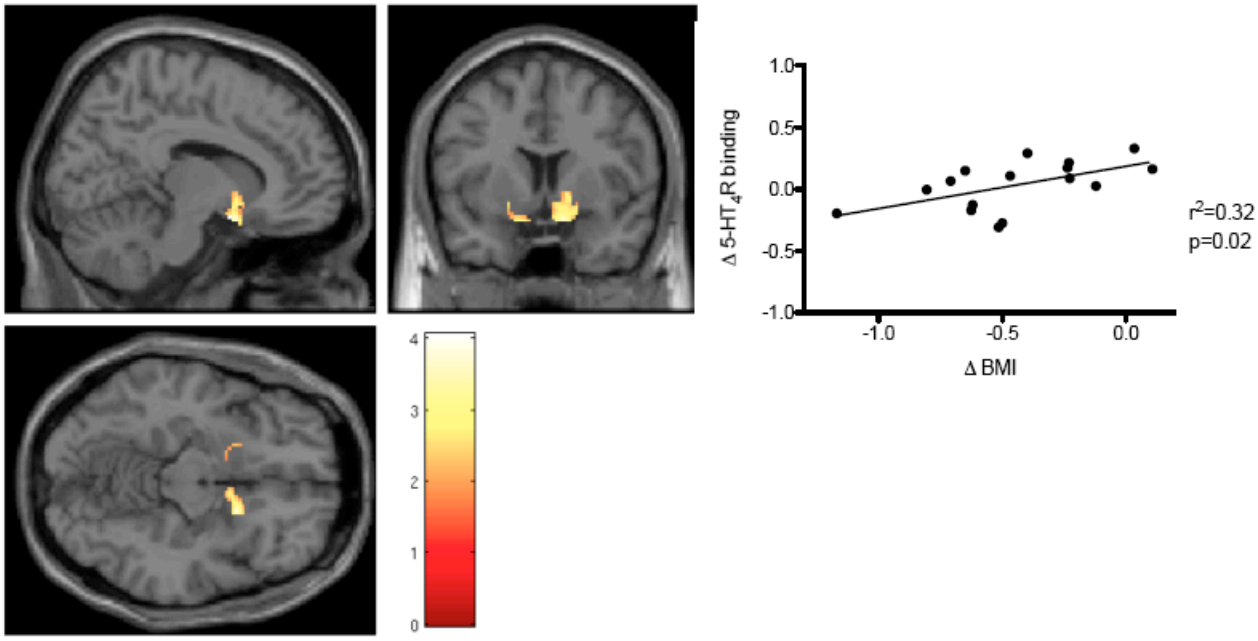

Figure 8. On the left a statistical parameter map shows voxels with a significant positive association between change in BMI and change in $5HT_4R$ BP_{ND} in the 16 fluoxetine-treated lean individuals in study 3A. The color bar depicts t-values of suprathreshold (p<0.05, uncorrected). On the right a plot of the positive association between the change in BMI and the mean change in 5-HT_4 receptor binding in the 197 voxels in the right ventral striatum.

The NAc is regarded as a "hedonic hotspot" that enhance "liking" reactions, defined as the pleasure component of a food reward (Berridge and Kringelbach, 2008, Berridge et al., 2010). The serotonin system may be involved in the hedonic processes of feeding, e.g. acute tryptophan depletion in obese women lead to significantly increased sweet calorie intake (Pagoto et al., 2009) and serotonergic projections from the dorsal raphe nuclei are found to influence the NAc (De Deurwaerdere and Spampinato, 1999). Our findings in the NAc of higher 5-HT_4 receptor availability in obese humans in study 1 and a positive association between a decrease on BMI and a decrease in 5-HT_4 receptor binding in study 3A suggest a specific role of the 5-HT_4 receptor in this hotspot in feeding. This is in line with observations in animal studies. First of all our studies in humans corroborate our previous finding in rats: We modeled the dietary-genetic aspect of the human obesity syndrome using rats selectively bred for polygenic high and low weight gainers (Levin et al., 1997) and found a higher level of 5-HT_4 receptors in NAc in the obese rats compared to the lean rats (Ratner et al., 2012). Moreover, stimulation of the 5-HT_4 receptor in the NAc either with BIMU8 or with the 5-HT releasing drug MDMA induces satiety and anorexia by inducing the reward and addiction

related protein CART (cocaine- and amphetamine-regulated transcript) (Jean et al., 2007). Further, 5-HT_4 receptors in NAc are specific mediators of MDMA-induced appetite suppression (Francis et al., 2010). Inhibition of the 5-HT_4 receptor in NAc elevates food intake in fed but not food-deprived mice (Jean et al., 2007), possibly because of a lower 5-HT level in NAc in the food-deprived state (Verhagen et al., 2009). Taken together, these animal studies suggest that stimulation of the 5-HT_4 receptor in NAc has a limiting effect on food intake, at least in conditions with high 5-HT level such as in the fed state (Verhagen et al., 2009) or in MDMA stimulated conditions (Baumann et al., 2008). Therefore, we speculate that the significant weight loss in the lean healthy SSRI-treated volunteers could be mediated through the 5-HT_4 receptor in NAc and the positive association between a decrease in BMI and a decrease in 5-HT_4 receptor binding may be compensatory as to limit further weight loss.

Obesity in humans is associated with lower cerebral 5-HT levels (Bjorntorp, 1995, Strombom et al., 1996), implying that the receptor in the obese individuals may be insufficiently stimulated to reduce the drive to eat. Furthermore, 5-HT depletion was found to significantly increase 5-HT_4 receptor binding in NAc in pigs (Ettrup et al., 2011) and we therefore speculate that the higher 5-HT_4 receptor level in the obese individuals in NAc may be a compensatory up-regulation due to the lower 5-HT levels and further that the receptor requires a higher endogenous stimulation to elicit satiety. Insufficient 5-HT_4 receptor signaling could also in turn result in lower 5-HT levels in NAc, given that CART stimulates 5-HT release in NAc, either via direct excitatory effects of CART on serotonergic terminals in NAc or by serotonergic projections from the dorsal raphe nuclei (Ma et al., 2007). In this context we also speculate, that since 5-HT_4 receptor signaling was found to increase striatal dopamine release, (Porras et al., 2002) a mechanism involved in reward (Norgren et al., 2006), the higher availability of the 5-HT_4 receptor in obesity may be involved in the impairments of the dopamine circuits thought to be involved in both addiction and obesity (Volkow et al., 2011).

In study 1 we also found a highly significant association between the 5-HT_4 receptor and obesity in the left hippocampal region; a brain region viewed as an associative brain structure in reward learning (Berridge and Kringelbach, 2008) and has also been suggested central to energy dysregulation (Davidson et al., 2005). In an imaging study the region has also been linked to memories of desired foods (Pelchat et al., 2004). The 5-HT_4 receptor has robustly been shown to be involved in learning and memory in animal studies (King et al., 2008) and its stimulation also increases 5-HT extracellular levels in the rat hippocampus (Ge and Barnes,

1996). Our finding that obesity is associated with high 5-HT_4 receptor levels in the left hippocampal area suggests a role for the 5-HT_4 receptor, not only in the hedonic component of food intake but also in the learning part of food representations.

Positive associations between the 5-HT_4 receptor level and obesity were furthermore found in study 1 in the orbitofrontal cortex (OFC). The OFC is a central region in food reward processing (Berridge and Kringelbach, 2008) and is thought to be involved in the integration of food-related sensory, cognitive and reinforcing information in the human brain (Morris and Dolan, 2001, Rolls, 2005). Moreover, a decrease in left OFC activation correlated with subjective pleasantness rating when the food was eaten to satiety (Kringelbach et al., 2003), suggesting that the saliency value of the food is represented in the OFC. Orbitofrontal 5-HT is found to prevent a salient stimuli in biasing the response to a specific task (Walker et al., 2009). This is in line with the observation that a pharmacologic increase of the brain 5-HT level reduces activation in the orbitofrontal cortex when looking at chocolate stimulus (McCabe et al., 2010). We speculate that the high level of 5-HT_4 receptor receptors in this brain area indicate an attenuated serotonergic function in obesity.

Study 2

In thirty young and healthy individuals we found a significant negative correlation of the 5-HT_4 receptor expression in left and right hippocampus and immediate recall in RAVLT (p=0.009 and p=0.010 respectively) and further a significant negative correlation between the right hippocampal 5-HT_4 receptor expression and delayed recall in RAVLT (p=0.014) whereas this relationship was not found for the left hippocampus (p=0.25) (table 5 and figure 9). Linear regression analyses including ROCFT scores were not significant (p>0.29) (see paper 2).

Memory score	Region	Estimate ± SE	95% CI	P-value
RAVLT immediate recall	Left hippocampus	-37.9 ± 14.5 /BP_{ND}	-66.4 to -9.4	0.009
	Right hippocampus	-37.3 ± 14.5 /BP_{ND}	-65.8 to -8.8	0.010
RAVLT delayed recall	Left hippocampus	-4.6 ± 4.0 /BP_{ND}	-12.5 to 3.3	0.25
	Right hippocampus	-9.6 ± 3.9 /BP_{ND}	-17.2 to -1.9	0.014

Table 5 Results of Tobit regression (RAVLT) analyses of left and right hippocampus with the memory scores as outcome variable and 5-HT_4 receptor binding and scanner type as explanatory variables.

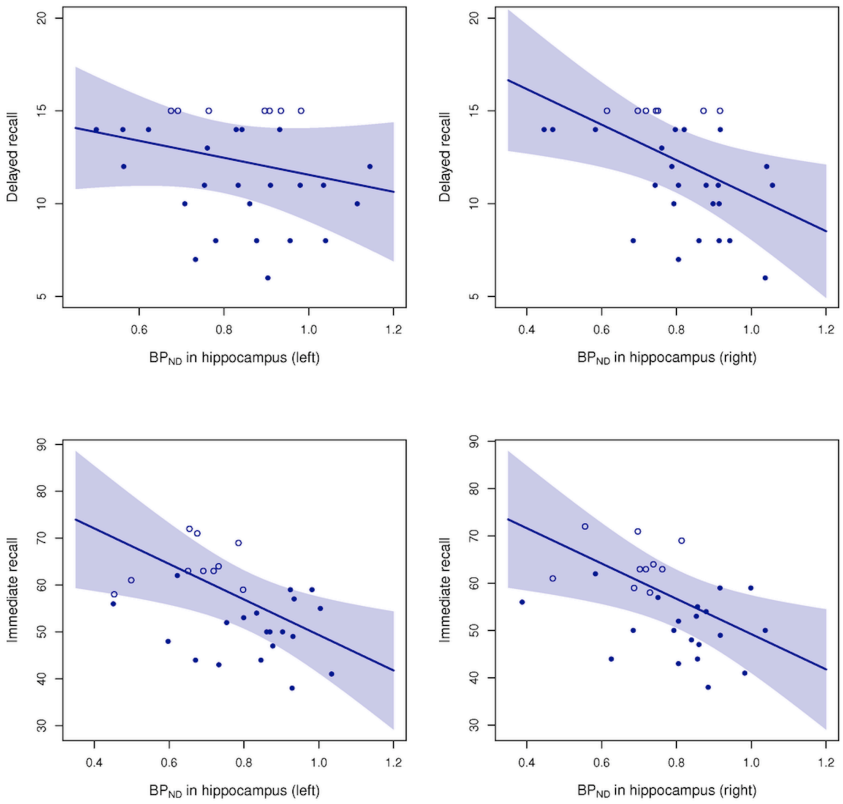

Figure 9 Plots showing estimated linear associations between RAVLT immediate recall and delayed recall and the left and right regional BP_{ND} in hippocampus. BP_{ND} values are adjusted for the estimated scanner effect. The shaded region is the pointwise 95% confidence limits. The censored observations—the unfilled circles—all scored 15 in the delayed recall or in one or more of the five immediate recall sessions.

Our results suggest that the 5-HT_4 receptor in humans is involved in memory encoding and consolidation and that fewer hippocampal 5-HT_4 receptors are representative of a better episodic memory function. As receptor stimulation according to the animal literature generally has a facilitatory effect on memory acquisition and as receptor expression is augmented when rats undergo memory training (Manuel-Apolinar et al., 2005), the result does not call for a straightforward interpretation. However, most of the animal studies examined functional effects of agonism or antagonism of the receptor, which could potentially explain the difference between the present clinical study and the experimental studies.

As shown in the methods section the binding potential of the 5-HT_4 receptor is a composite measure of receptor expression and affinity (Lammertsma and Hume, 1996). Since [^{11}C]SB207145 has not been found sensitive to acute endogenous serotonin release (Marner et al., 2010) and since the affinity is assumed to be equivalent in hippocampus and the reference region, the BP_{ND} in this study is proportional to the receptor density in hippocampus. The 5-HT_4 receptor is expressed in hippocampus in central locations, where both intrinsic hippocampal and extrinsic cortical and subcortical circuits are modified such as the CA1, the dentate gyrus and the subiculum (King et al., 2008). The precise hippocampal neuronal localization has not yet been specified, but lesion studies indicate that the receptor is present on glutamatergic neurons (Vilaro et al., 2005). Furthermore, stimulation of the 5-HT_4 receptor leads to modulation of the acetylcholine and GABA release in hippocampus, so the receptor is likely to be localized on these neurons, even though indirect effects cannot be excluded (Matsumoto et al., 2001, Bianchi et al., 2002, Bockaert et al., 2006). As the *in vivo* binding of [^{11}C]SB207145 cannot differentiate the type of neurons or the specific location of the 5-HT_4 receptor within hippocampus, a low receptor density in hippocampus cannot simply be linked to low neuronal activity and functions. Further, stimulation of the receptor is also not in a simple way linked to better memory. For example, in healthy volunteers a low dose of selective serotonin reuptake inhibitors (SSRI) improved short-term memory, while a high dose impaired it (Dumont et al., 2005) and, as mentioned in the introduction, 5-HT_4 receptor agonists impaired memory function in young rats while it improved memory functions in old rats (Lamirault and Simon, 2001). Therefore, various explanations of the results in this study can be proposed. Acute tryptophan depletion impairs episodic memory (Sambeth et al., 2007, Mendelsohn et al., 2009), and therefore efficient serotonergic innervation and adequate 5-HT interstitial levels in hippocampus seem important for memory functions. Since 5-HT_4 receptor binding was found to be down-regulated after chronic SSRI

treatment (Licht et al., 2009, Vidal et al., 2009), high serotonin levels in hippocampus may reduce the levels of the 5-HT_4 receptor. This might explain the paradoxical finding that the subjects in this study with a high memory performance have lower levels of the receptor. On the other hand, since subchronic serotonin depletion was found to cause an up-regulation of the 5-HT_4 receptor (Licht et al., 2009) subjects with an inefficient memory function may improve the functioning of the serotonin system by up-regulating the 5-HT_4 receptor, that both may improve memory directly and indirectly by stimulating 5-HT release in hippocampus (Ge and Barnes, 1996, Licht et al., 2010). However, hypothetically this up-regulation is insufficient to boost memory function to the level of the better performing subjects. We also speculate that the relation between the serotonin system and memory may be inversely u-shaped as it is assumed for dopaminergic modulation of cognitive functions (Cools and D'Esposito, 2011): both low and high 5-HT_4 receptor binding may reflect levels of serotonin outside the optimal range for memory function. However, to find evidence for this hypothesis one would need to include individuals that were performing less well on the memory tasks presented.

We previously investigated the relation between the RAVLT memory scores and the serotonin transporter (SERT) and found no correlation (Madsen et al., 2011a). The reason for these differing results may be that SERT is considered to be a marker of general serotonergic innervation with complex postsynaptic actions, while the 5-HT_4 receptor is likely to be directly involved in memory function as previously show in the introduction.

It was clear from the voxel-based analysis within hippocampus that the association between memory functions and receptor expression was found both in ventral (significant cluster with MNI coordinates -30, -16, -16) and dorsal (significant cluster with MNI coordinates -30, -32, -8) hippocampus (figure 10). Ventral and dorsal hippocampus have been assigned to dissimilar functional roles: Dorsal hippocampus is mainly participating in cognitive functions while ventral hippocampus is mainly involved in emotions, stress and affect (Fanselow and Dong, 2010). This is in line with the roles of the 5-HT_4 receptor in the brain. As shown both from the previous animal literature and the present study the receptor is likely to play a role in memory and learning processes, however, the receptor is also likely to be involved in emotional processes as studies has linked the receptor to regulation of the hedonic part of food intake (Jean et al., 2007, Compan et al., 2010, Francis et al., 2010) and emotional processes such as stress (Compan et al., 2004a).

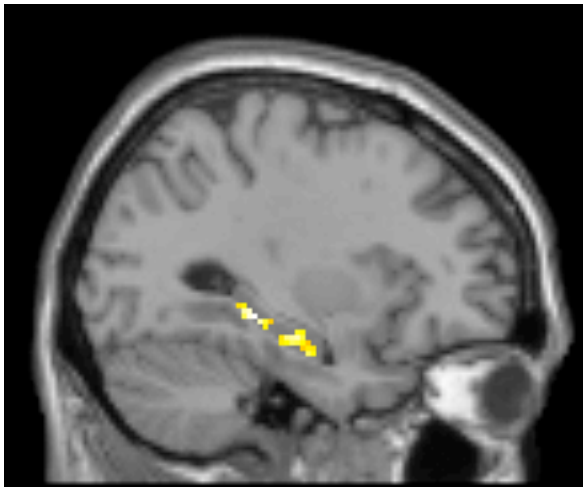

Figure 10 Statistical parametric map overlaid on a T1-weighted MR image showing the two significant clusters of negative associations between [^{11}C]SB207145 BP_{ND} and RAVLT immediate recall in left hippocampus.

In contrast to the verbal memory performance we did not find significant correlations between the non-verbal memory task and hippocampal 5-HT_4 receptors even though the memory tests correlated strongly ($p<0.001$). These results could be explained by the specific functions of the 5-HT_4 receptor. It has been shown that neuronal networks of encoding and retrieval can be distinguished by their reaction to specific stimuli (e.g. verbal *vs.* visual) even though interactions between the systems are evident (Nyberg et al., 2000). Our results suggest that the 5-HT_4 receptor primarily affects the verbal memory network and not the visual. It is not possible to confirm this result in the existing literature, as 5-HT_4 receptor agonism seems to improve all the various animal memory tests, at least in the acquisition phase. However, in animals it is difficult to precisely imitate the visual and verbal memory tasks used in humans, as species differences may be considerable. Thus, rather than drawing any specific conclusions, we recommend that future human studies seek to clarify these associations between serotonergic tone, 5-HT_4 receptors and verbal and visual memory.

Study 3

Based on previous animal studies that showed that 5-HT_4 receptor levels show a monotonic response to 5-HT levels (Licht et al., 2009, Vidal et al., 2009, Jennings et al., 2011), we expected fluoxetine intervention to decrease 5-HT_4 receptor binding. We only found a trend to such an effect when the considering all fluoxetine-treated participants (see table 6). No significant changes were observed within the placebo group or between the fluoxetine and

the placebo group (all p-values were >0.45).

Table 6. 5-HT$_4$ receptor BP$_{ND}$ in striatum, hippocampus and neocortex in the fluoxetine group (N=16).

	Striatum	Hippocampus	Neocortex
Baseline[1]	3.76 ± 0.39	1.15 ± 0.14	0.75 ± 0.20
Rescan[1]	3.61 ± 0.46	1.13 ± 0.14	0.77 ± 0.19
Mean change in % (95% CI)[2]	-3.60 (-9.95 to 2.76)	-1.03 (-7.28 to 5.2)	3.94 (-2.87 to 10.75)
Paired t-test (1-tailed)	0.09	0.28	0.16
Exact p[3]	0.098	0.77	0.45

[1] Mean BP$_{ND}$ ± standard deviation. [2]Calculated as ((BPND rescan/BPND baseline)-1)*100. [3] Wilcoxon Matched-Pairs Signed Rank Test. Striatum = Volume-weighted mean BP$_{ND}$ of putamen and caudatus. CI = Confindence Interval

However, when subdividing the fluoxetine treated participants based on the 5-HTTLPR status we found a significant decrease of striatal 5-HT$_4$ receptor binding in the L/L homocygotes (see figure 11).

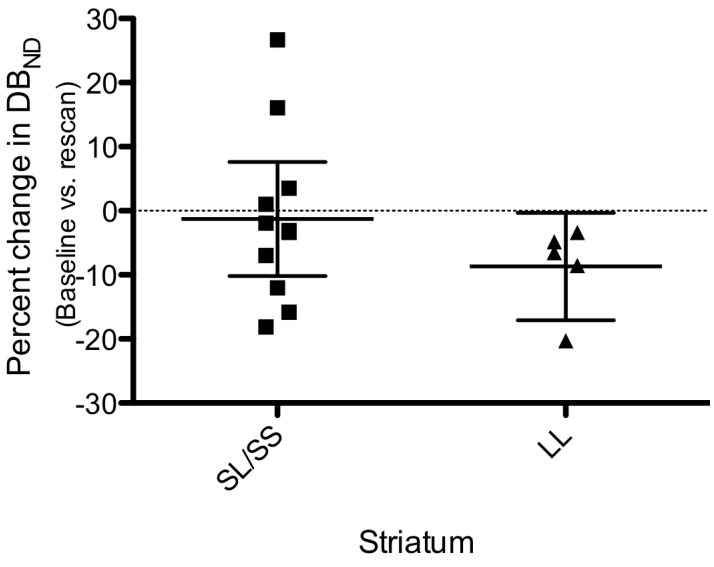

Figure 11. Estimate of the percent difference in the striatal [^{11}C]SB207145 binding when subdividing the fluoxetine group into S-carriers (N=11), LL homocygotes (N=5). The LL homocygotes show a significant decrease in 5-HT$_4$ receptor binding (-8.7% [95% CI -17.1 to -0.3] p=0.045) compare to the S carriers (-1.3% [95% CI -10.2 to 7.6, p=0.76]

The finding is consistent with our hypothesis, and that the L/L homocygote carriers have an elevated 5-HTT production and thereby a larger effect of fluoxetine intervention on the 5-HT levels leading to a greater decrease in 5-HT$_4$ receptor levels relative to S carriers and our

finding provide evidence for an effect of 5-HTTLPR status on serotonergic neurotransmission. L/L carriers have been linked in humans to a higher 5-HTT levels in striatum (Praschak-Rieder et al., 2007, Kalbitzer et al., 2010) and in the midbrian (Reimold et al., 2007). In mice an elevated level of 5-HTT was found to effect the levels of the 5-HT_4 receptor possibly due to the more efficient 5-HT uptake: Over-expression of the gene coding for the 5-HTT resulted in increased 5-HT_4 receptor binding while homozygous knock-out of the gene coding for the 5-HTT resulted in a down-regulation of 5-HT_4 receptor binding measured with [^{3}H]SB207145 (Jennings et al., 2011). L/L carriers was also found to have an enhanced effect of SSRI treatment and a study from Smits et al concluded that pretreatment genotyping of depressed patients may lead to a greater number of patients experiencing early remission (Smits et al., 2007). Recently we investigated the association between the 5-HTTLPR status and the levels of 5-HT_4 receptor and the L/L carriers was found to have higher level of 5-HT_4 receptor relatively to S carriers consistent with a higher level of the 5-HTT leads to a lower level of 5-HT leading to more 5-HT_4 receptor (Fisher et al., 2012). Taken together these studies show that the significant effect of SSRI treatment in the L/L carriers in striatum may arise from L/L carriers having a larger available pool of 5-HT_4 receptor at baseline to facilitate a larger decrease in 5-HT_4 receptor binding when influenced by a raised 5-HT level due to SSRI treatment that in itself may induce a larger effect on L/L carriers due to their larger amount of 5-HTT.

Our finding that L/L carriers showed a decrease in 5-HT_4 receptor binding is consistent with a model wherein decreased 5-HT_4 receptor levels reflects increased 5-HT levels. It is, however, important to note this reflects an indirect evaluation 5-HT levels and 5-HT release in humans and of how 5-HTTLPR status may contribute to individual differences in 5-HT levels. Currently, there is no validated method available for quantifying *in vivo* 5-HT levels and 5-HT release in humans (Paterson et al., 2010) and the emergence of such a method (similar to displacement of dopamine D2 receptors with radiotracers such as [^{11}C]raclopride) could provide more direct evidence supporting our current findings.

We observed the significant association between L/L carriers and change in [^{11}C]SB207145 binding in striatum after 3 weeks of fluoxetine intervention, whereas we only found a trend to such an effect of fluoxetine intervention on the 5-HT_4 receptor levels in the S carries in striatum and no effect in all fluoxetine-treated individuals in hippocampus or neocortex despite previous animal study reporting such an effect. Several reasons might explain this translational inconsistency. Both the 5-HT_4 receptor regulation and effects of the 5-HTTLPR

polymorphism may differ on a cellular and/or regional level. Several reports show a specific effect of 5-HT_4 receptor agonism on a cellular level in hippocampus modulating tissue specific functions in memory formation such as long-term potentiation and long-term depression (Kulla and Manahan-Vaughan, 2002, Kemp and Manahan-Vaughan, 2004, Marchetti et al., 2004). Moreover, in previous studies we used [^{11}C]SB207145 PET and linked regulation of the 5-HT_4 receptor to memory function in hippocampus (Haahr et al., 2012a), appetite regulation in reward related areas such as nucleus accumbens (Haahr et al., 2012b), and the female gender to 5-HT_4 receptor availability in the limbic system (Madsen et al., 2011b). It is, however, important to note that these associations may or may not arise from individual differences in the extracellular 5-HT level. Also the 5-HTTLPR genotype status has a region specific effect on the 5-HTT levels. For example Praschak-Rieder et al. and Kalbitzer et al used [^{11}C]DASB PET and both found an increased level of the 5-HTT in L_AL_A carriers only in striatum lending an explanation of why our findings were restricted to striatum. As the *in vivo* binding of [^{11}C]SB207145 measured with PET cannot differentiate the type of neurons or the specific cellular location of the 5-HT_4 receptor future studies aimed at understanding cell specific 5-HT_4 receptor expression may provide insight into whether this is a possible source of our observed region-specific effects or if the finding in striatum was driven by the better signal-to-noise ratio implying larger statistical power associated with a more robust measure.

Another reason could be species differences between rodents and humans in the effect of SSRI on extracellular serotonin levels. Disparities between species that influence pharmacological sensitivity are observed in the serotonin system anatomy, receptor distribution and innervations (Lewis et al., 1986, Bruinvels et al., 1994). In mice chronic fluoxetine administration significantly increased the level of serotonin measured with microdialysis over the range of 7 to 28 days (Popa et al., 2010), however, in primates this effect was not observed in a similar microdialysis study measuring 5-HT content from 3 to 21 days: After an initial increase in extracellular serotonin the levels decreased towards baseline after 7 days (Smith et al., 2000).

The doses in all the mentioned animal studies were 10-30 fold greater than our human study. However, 40 mg/day used in this study is an effective clinical dose and the serum concentrations of fluoxetine and the active metabolite nor-fluoxetine were in our cohort within the same range as reported in depressive patients (Jannuzzi et al., 2002).

We observed no differences in side-effects in the fluoxetine and the placebo group. Eight out of 16 individuals from the fluoxetine intervention group versus nine out of 16 in the placebo

group reported no unusual symptoms at any contact in the intervention period. Seven participants in the active group versus five in the placebo group had a few (UKU scores < 5) symptoms such as nausea, insomnia, nervousness, and somnolence. One person in each group had an UKU score of > than 5 and beside the nonspecific symptoms also had sexual dysfunction (fluoxetine group UKU=10) and concentration problems (placebo group UKU score 8). None of the participants had any discontinuation symptoms.

Conclusions

This thesis presents some of the first studies in humans of the *in vivo* levels of the cerebral 5-HT_4 receptor using [^{11}C]SB207145 PET. The findings of a higher availability in NAc of the 5-HT_4 receptor in obese individuals and that this availability seems to be influence by bodyweight changes corroborate previous animal studies and provide further insight into how the brain serotonin system and specifically the 5-HT_4 receptor is involved in the regulation of food intake. Studies find that the serotonin system may be involved in the hedonic processes of feeding, and our findings in the hotspot of pleasure, nucleus accumbens, suggest that the 5-HT_4 receptor is particularly involved in the hedonic processes of feeding. In this context we speculate if stimulating the cerebral 5-HT_4 receptor may reduce reward-related overeating in humans. In study 3A we longitudinally studied the effect of weight loss induced by SSRI treatment and found an association between weight loss and decrease in 5-HT_4 receptor binding in NAc. Since receptor stimulation in animals studies limit food intake, we speculated that the weight loss may be mediated through the 5-HT_4 receptor. However, future studies are needed to provide evidence for this hypothesis: A study in which a 5-HT_4 receptor antagonist is given in conjunction with the SSRI would decipher whether the change in 5-.HT_4 receptor binding is a cause or an effect of the weight loss. Other important and interesting studies to undertake could be to test for associations between individual preferences for particular food components and markers of serotonergic neurotransmission e.g. to measure the 5-HT_4 receptor binding in relation to carbohydrate-cravers vs. non-carbohydrate-cravers or to measure other food preferences and possibly compare the results to fMRI with palatable food cues or decision making tasks. It would also be interesting to examine the 5-HT_4 receptor levels in anorectic persons and test if the correlation between the 5-HT_4 receptor levels and body weight also is significant in the opposite extreme of the weight

scale. Particularly, since animal studies link anorexia, the 5-HT_4 receptor and the reward mechanisms in the NAc (Jean et al., 2007, Compan et al., 2010).

Study 2 in this thesis was the first study in humans to examine the association between the 5-HT_4 receptor in hippocampus and memory functions: an important study given the numerous animal studies linking the receptor to memory functions. The study provides evidence that the 5-HT_4 receptor is associated with memory functions in hippocampus. Since 5-HT_4 receptor stimulation generally enhances memory performance across various domains in animals, it was an unexpected finding that the 5HT_4 receptor level correlated negatively with measures of memory function. This finding may be explained by the complex interactions between the intrinsic serotonergic tonus and receptor functions in the hippocampus. We speculate that stimulation of the human 5-HT_4 receptor could improve memory functions, however further studies in humans are needed to elucidate the functional significance of this study. It would also be interesting in a future study to examine the gender difference in memory and learning – women have a better episodic memory performance than men (Van der Elst et al., 2005) – and the levels of the 5-HT_4 receptor in hippocampus or how the receptor level develops with age in relation to cognitive abilities.

In study 3 we found evidence for an effect of 5-HTTLPR status on serotonergic neurotransmission in that LL homocygotes show reduced 5-HT_4 receptor levels if 5-HT levels are increased as opposed to S carriers. Our findings corroborate previous animal studies showing a monotonic regulation of the 5-HT_4 receptor relative to fluctuations in 5-HT levels and provide evidence for differential regulation of the serotonin system dependent on the 5-HTTLPR status. However, we did not find evidence for that the 5-HT_4 receptor is a sensitive proxy for the extracellular serotonin level at least not with a 3-week increase in cerebral 5-HT level. Undoubtedly, a breakthrough in serotonin research will appear the day a radiotracer is found that is sensitive to changes in the 5-HT level. We recently investigated the promising 5-HT_{1A} receptor agonist [^{11}C]CUMI-101, but failed to show these properties (Pinborg et al., 2012).

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Appendix (paper 1, 2 and 3)

Paper 1

Haahr ME, Rasmussen PM, Madsen K, Marner L,
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Obesity is associated with high serotonin 4 receptor availability in the brain reward circuitry ☆

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ABSTRACT

The neurobiology underlying obesity is not fully understood. The neurotransmitter serotonin (5-HT) is established as a satiety-generating signal, but its rewarding role in feeding is less well elucidated. From animal experiments there is now evidence that the 5-HT_4 receptor (5-HT_4R) is involved in food intake, and that pharmacological or genetic manipulation of the receptor in reward-related brain areas alters food intake.
Here, we used positron emission tomography in humans to examine the association between cerebral 5-HT_4Rs and common obesity.
We found in humans a strong positive association between body mass index and the 5-HT_4R density bilaterally in the two reward 'hot spots' nucleus accumbens and ventral pallidum, and additionally in the left hippocampal region and orbitofrontal cortex.
These findings suggest that the 5-HT_4R is critically involved in reward circuits that regulate people's food intake. They also suggest that pharmacological stimulation of the cerebral 5-HT_4R may reduce reward-related overeating in humans.

Introduction

When, why, and how much we eat is a complex process that depends on the brains' ability to integrate inhibitory and excitatory signals from the organism. Excitatory signals reflect the need for energy and nutrients. Inhibitory signals arise from satiety generating signals after food intake. In addition, there is an important emotional component influencing why and how much we eat (Berridge et al., 2010). For example, food may serve to alleviate stress (Dallman et al., 2003).

Several neurotransmitters (e.g. 5-HT, dopamine, noradrenaline, GABA and opiates) are involved in feeding (Berthoud, 2002). 5-HT is well-established as a satiety generating signal in the brain (Blundell, 1992; Lam and Heisler, 2007) and serotonergic disturbances can perpetuate excess eating and lead to obesity. However, the specific role of the cerebral 5-HT system is unclear and exactly how the 5-HT transporter or the 14 receptors of the 5-HT system are involved in feeding needs to be further elucidated. So far only a few human in vivo neuroimaging studies of the cerebral 5-HT system in relation to obesity have been undertaken (Erritzoe et al., 2009; Erritzoe et al., 2010).

Recently our lab validated the radiotracer [^{11}C]SB207145 for use with positron emission tomography (PET) (Marner et al., 2009) and it became possible to study the cerebral 5-HT type 4 receptor (5-HT_4R) in humans in vivo.

The cerebral 5-HT_4Rs is located with high density in structures such as striatum and globus pallidum including NAc and ventral pallidum, with intermediate density in hippocampus and low density in neocortex (Marner et al., 2010; Varnas et al., 2003). The receptor has been found to influence cognitive functions and affective symptoms and interestingly, studies have shown that the 5-HT_4R seems to exert control over dopamine transmission (Parga et al., 2007; Porras et al., 2002). Animal studies have identified the 5-HT_{2C}, 5-HT_{1B}, and 5-HT_6 receptors as influencing food intake and these receptors are considered as pharmacological anti-obesity targets (Garfield and Heisler, 2009). However, newer animal studies also identify the 5-HT_4R as a regulator of food intake. In a recent study we saw that

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inbred obesity-susceptible rats had higher 5-HT_4R level in nucleus accumbens (NAc) than inbred obesity-resistant lean rats irrespective of diet (Ratner et al., 2012) and it was also found that the receptor is involved in appetite when it comes to food-related reward and addiction by mediating the appetite suppressant effect of ecstasy (MDMA) in NAc (Francis et al., 2010; Jean et al., 2007). In line with this observation, 5-HT_4R knock-out mice were found to be desensitized to stress-induced anorexia (Compan et al., 2004).

In the present study we examined for the first time the relationship between the cerebral 5-HT_4R availability and common obesity in a cohort of humans with varying body mass index (BMI). Based on the findings in previous animal studies our a priori region of interest was primarily nucleus accumbens and secondly other regions of the reward system such as striatum, hippocampus and orbitofrontal cortex. We expected to find an effect of BMI on the receptor levels in these regions of the brain.

Methods

Participants

Twenty-eight healthy adults (12 females) with a median age of 38.4 years (range 20.0–68.7 years) were recruited by public advertisements or from the Civil Registration System in Denmark. After the study aim and design had been explained, written informed consent was obtained from each participant according to the declaration of Helsinki II. The study was approved by the Ethical Committee of Copenhagen under protocols (KF) 01 274821, (KF)-2007-0028, and (KF) 01 2006-20. In a subset of the normal-weighted participants, we first independently assessed the soundness of the quantification methods (Marner et al., 2009) and age and sex effects on 5-HT_4R binding (Madsen et al., 2011).

The participants were examined by a physician and had a normal physical and neurological examination. They further had a normal blood screen and an unremarkable MRI. The exclusion criteria were as follows: Drug use within the last 3 months, i.e., use of cocaine, heroin, amphetamine or ecstasy more than 10 times, use of cannabis more than 50 times, use of prescribed anti-obesity medication such as Rimobant or Orlistat for the last 6 months, neurological disorder or major psychiatric disease, previous or current use of antipsychotics or antidepressants or other medication that significantly affect brain function (e.g. limited use of NSAID were permitted), uncontrolled cardiovascular or endocrinological disease. All subjects completed the Symptom Check List Revised (SCL-90-R) questionnaire (Derogatis, 1994) and Major Depression Inventory (MDI) (Forsell, 2005) to assess symptoms of depression, distress and psychopathology and all of the 28 individuals scored substantially below the cut-offs for caseness in scales (Forsell, 2005) (MDI: median 3, range 0–11 and SCL-90-R global score: median 0.08, range 0–0.43). We found no statistically significant deference between obese/overweight and normal-weight in these scores $p = 0.42$ (MDI) and $p = 0.74$ (SCL-90-R).

In 27 individuals (including one treated for Type II diabetes) hemoglobin 1Ac were found below 6.5%. We did not measure tryptophan levels or control for food intake preceding the PET scan, but this is unlikely to have effected the results of this study since [^{11}C]SB207145 binding is not affected by acute changes in cerebral 5-HT levels (Marner et al., 2010).

Imaging and quantification of the 5-HT_4Rs

GlaxoSmithKline kindly provided the precursor for preparation of [^{11}C]SB207145. The tracer was synthesized using a fully automated radio-synthesis system as previously described (Gee et al., 2008; Gillings and Larsen, 2005; Marner et al., 2009). Immediately after an intravenous bolus injection of [^{11}C]SB207145 a 120 min dynamic PET scan (6×5 s, 10×15 s, 4×30 s, 5×120 s, 5×300 s and 8×600 s) was initiated using an eighteen ring GE-Advance scanner (Milwaukee, WI) with an approximate in-plane resolution of 6 mm. The mean activity of [^{11}C]SB207145 injected was 481 ± 148.5 (s.d.) MBq and the mean cold mass was 3.5 ± 1.7 (s.d.) μg. The images were reconstructed with filtered back projection and corrected for attenuation, dead time and scatter.

In all subjects magnetic resonance (MR) imaging of the brain was obtained with a Siemens 3.0 T Trio scanner. A high-resolution 3D T1-weighted, sagittal, magnetization prepared rapid gradient echo (MPRAGE) scan and a high-resolution 3D T2-weighted, variable flip angle, sagittal, Turbo Spin Echo scan were acquired. The acquisition parameters were for the T1-weighted image: TE/TR/TI = 3.04/1550/800 ms; bandwidth = 170 Hz/Px; echo spacing = 7.7 ms; flip angle = 9°; field of view = 256 mm; matrix 256×256; 192 slices and the T2-weighted image: TE/TR = 354/3000 ms; 1 slab; bandwidth = 752 Hz/Px; echo spacing = 3.58 ms; turbo factor = 197; and field of view = 282 mm. Voxel size was 1×1×1 mm. All scans are corrected for spatial distortions due to non-linearity in the gradient system of the scanner (Jovicich et al., 2006).

The frames of the dynamic PET scan were aligned to correct for head-motion artifacts using the AIR routines (version 5.2.5). A flow-weighted mean emission image of the first 20 min was automatically aligned to the same individuals' T1-weighted brain MRI. The T2 weighted images served for brain masking purposes.

The quantitative analysis to obtain the binding potential (BP) of the 5-HT_4R was performed with the simplified reference tissue model (SRTM) (Lammertsma and Hume, 1996) using PMOD (PMOD Inc, Zürich, Switzerland). This model calculates the BP_{ND} based on the ratio between the radioligand uptake in a reference tissue devoid of specific binding and the radioligand uptake in the voxel/region of interest. The BP_{ND} obtained from the SRTM is not affected by the metabolism or the free fraction of the tracer (Lammertsma and Hume, 1996). A blocking study confirmed that [^{11}C]SB207145 binds specifically to the 5-HT_4R and that cerebellum is a suitable reference region devoid of specific 5-HT_4R binding (Marner et al., 2009). The SRTM was validated in humans against arterial input and was found reliable and reproducible for quantification of [^{11}C]SB207145 (Marner et al., 2009). The model estimates the non-displaceable BP (BP_{ND}), which is defined as:

$$BP_{ND} = f_{ND} * B_{max}/K_d \quad (1)$$

where f_{ND} is the free fraction of tracer in non-displaceable tissue compartment, B_{max} is the receptor concentration and K_d is the equilibrium dissociation constant for the tracer.

Image processing and statistical analysis

Parametric images of the BP_{ND} were calculated with SRTM and the parametric images were further processed and analyzed with SPM8 (Wellcome Department of Imaging Neuroscience. London, UK) in the following order: 1) the T1-weighted images were spatial normalized to the Montreal Neurological Institute (MNI) T1 template using the unified segmentation and registration procedure (Ashburner and Friston, 2005) with standard parameters. This procedure also provided tissue segmentations in MNI space. 2) The PET images were re-sliced into MNI space using the estimated warp fields with 2 mm isotropic voxels. 3) The PET images were spatially smoothed with a Gaussian kernel with 12 mm full-width half-maximum. 4) A mask was constructed from the intersection between a mask based on PET images, and included only voxels with an average $BP_{ND} > 0.1$, and a mask constructed from gray matter voxels with an average value > 0.2 (after a spatially smoothing with a 8 mm full-width half-maximum Gaussian kernel). The final mask included 171661 voxels. 5) A voxel-wise multiple regression was conducted with BMI, age, and gender included in the model as covariates. Age and gender

were included in the model since our lab recently found a decrease in receptor binding of 1% per decade in striatum and a 13% lower binding in women in the limbic system (Madsen et al., 2011). The primary contrast of interest was the positive BMI t-contrast. Significance threshold was set to $p<0.05$, and all voxel level p-values were Family Wise Error corrected for multiple comparisons.

In parallel, we also obtained volumes of interest (VOI) of putamen, caudate, hippocampus and orbitofrontal cortex by automatic delineation on each subjects MRI in a user-independent fashion with the Pvelab software package (Svarer et al., 2005). Regional BP_{ND} was calculated with the SRTM and used as dependent variable in regression analyses including BMI, age and gender as covariates. Analyses were done in AnalystSoft, StatPlus:mac, version 2008.

Results

The BMI of the 28 included individuals ranged from 20.5 to 40.0 kg/m^2 with an average of 26.5 ± 6.8 (s.d.) kg/m^2. Sixteen individuals had normal bodyweight (BMI<25 kg/m^2) and 12 were overweight or obese (BMI>25 kg/m^2). Demographic data is provided in Table 1.

In the whole brain voxel-wise multiple regression analysis with correction for multiple comparisons, we found a significant positive association between BMI and the 5-HT_4R density in a large cluster of 700 voxels including bilateral NAc (peak p-value<0.001), ventral pallidum (peak p-value<0.01) and in the left central part of orbitofrontal cortex (peak p-value<0.05) (Table 2, Figs. 1 and 2). One additional significant cluster of 161 voxels was found in the left hippocampal region (peak p-value<0.05) (Table 2 and Fig. 1).

The regional analyses corroborated with the voxel-wise analyses that we found significant associations in putamen, orbitofrontal cortex and hippocampus, but not the caudate (Table 3).

Discussion

In this first in vivo study of obesity and human cerebral 5-HT_4R we find an increased 5-HT_4R availability in relation to BMI in brain areas involved in reward: the NAc, ventral pallidum, the left hippocampal region and left orbitofrontal cortex.

The NAc and ventral pallidum are intimately connected and are regarded as "hedonic hotspots" that work together to enhance "liking" reactions, defined as the actual pleasure component of a food reward (Berridge and Kringelbach, 2008; Berridge et al., 2010). In these two areas of the brain the signals of opioid, endocannabinoid, benzodiazepine-GABA, or orexin are already known to amplify the hedonic impact of palatable foods (Berridge et al., 2010). Serotonergic projections from the dorsal raphe nuclei are also found to influence the NAc (De Deurwaerdere and Spampinato, 1999). Our present finding of a higher 5-HT_4R availability in NAc and ventral pallidum in humans suggests a specific role of the 5-HT_4R in these hotspots in feeding. This is in line with observations in animal studies. First of all this study in humans is in line with our finding in rats: We modeled the dietary-genetic aspect of the human obesity syndrome using rats selectively bred for polygenic high and low weight gainers (Levin et al., 1997). In this model we also found a higher level of 5-HT_4Rs in NAc in the obese rats compared to the lean rats (Ratner et al., 2012). Moreover, stimulation of the 5-HT_4R in the NAc either with BIMU8, a receptor agonist, or with the 5-HT releasing drug MDMA induces satiety and anorexia by inducing the reward and addiction related protein CART (cocaine- and amphetamine-regulated transcript) (Jean et al., 2007). Further, 5-HT_4Rs in NAc are specific mediators of MDMA-induced appetite suppression (Francis et al., 2010). Inhibition of the 5-HT_4R in NAc elevates food intake in fed but not food-deprived mice (Jean et al., 2007), possibly because of a lower 5-HT level in NAc in the food-deprived state (Verhagen et al.,

Table 1
Demographic data of the normal weight and overweight/obese individuals. Data are mean ± s.d. and range in brackets. Groups are compared with two-sample t-tests.

	Normal weight	Overweight/obese	P-value
N	16 (7 female)	12 (6 female)	
Weight	70.5 ± 11.7 (54.4–92)	94.2 ± 15.1 (69.2–119)	<0.001
Height	174 ± 11.1 (155–194.5)	174.5 ± 7.6 (162.5–182.5)	0.89
BMI (kg/m^2)	23.1 ± 1.3 (20.5–24.9)	31.1 ± 5.4 (25.3–40.0)	<0.001
Age (years)	41.3 ± 15.4 (20.6–68.0)	41.0 ± 19.9 (20.0–68.7)	0.97
Smoking (cigarettes/day)	1.6 ± 4.4 (0–15)	4.3 ± 5.4 (0–15)	0.15
Alcohol (units/week)	5 ± 5 (0–16)	6 ± 4 (2–15)	0.99

Table 2
Clusters in the voxel-wise analysis showing a significant positive association in humans between 5-HT_4R binding and BMI adjusted for age and gender and Family Wise Error corrected for multiple comparisons ($p<0.05$) Reported maxima within each cluster are >8 mm apart.

	Number of voxels	Regions	Peak coordinates			T
			x	y	z	
Cluster 1	700	Nucleus accumbens	10	4	−10	7.96***
		Ventral pallidum	−14	0	−12	6.11**
		Orbitofrontal cortex	−16	16	−20	5.50*
Cluster 2	161	Hippocampal region	−36	−22	−26	5.95*
		Hippocampal region	−18	−18	−22	5.91*

*** $p<0.001$.
** $p<0.01$.
* $p<0.05$.

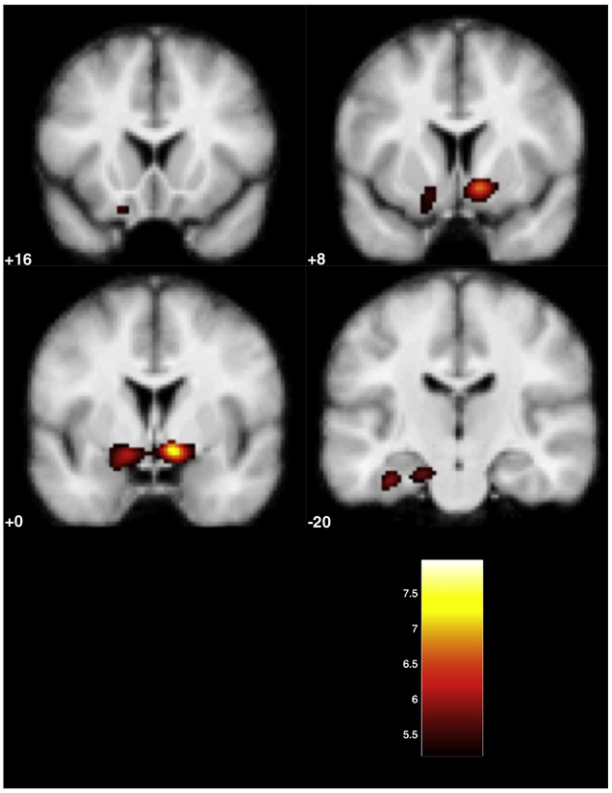

Fig. 1. Positive association in the voxel-wise analysis between 5-HT_4R BP_{ND} and BMI in humans (age and gender corrected). The statistical parameter map is projected onto an averaged anatomical image of the 28 subjects. The yellow color shows the areas of at least $p<0.05$ with Family Wise Error correction for multiple comparisons. The numbers denote the coronal slice in MNI space.

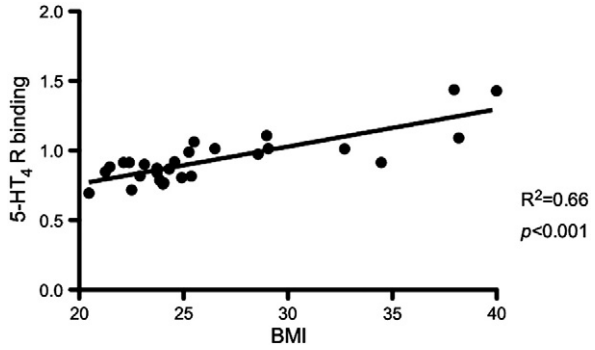

Fig. 2. Plot showing the association between BMI and the mean 5-HT_4R binding in the 700 voxels that include bilateral nucleus accumbens and ventral pallidum (age and gender corrected).

2009). Taken together, these animal studies suggest that stimulation of the 5-HT_4R in NAc has a limiting effect on food intake, at least in conditions with high 5-HT level such as in the fed state (Verhagen et al., 2009) or in MDMA stimulated conditions (Baumann et al., 2008). Obesity in humans is associated with lower cerebral 5-HT levels (Bjorntorp, 1995; Strombom et al., 1996), implying that the receptor may be insufficiently stimulated to reduce the drive to eat. Furthermore, 5-HT depletion was found to significantly increase 5-HT_4R binding in NAc in pigs (Ettrup et al., 2011) and we therefore speculate that the higher 5-HT_4R level in NAc may be a compensatory up-regulation due to the lower 5-HT levels and further that the receptor requires a higher endogenous stimulation to elicit satiety. Insufficient 5-HT_4R signaling could also in turn result in lower 5-HT levels in NAc, given that CART stimulates 5-HT release in NAc, either via direct excitatory effects of CART on serotonergic terminals in NAc or by serotonergic projections from the dorsal raphe nuclei (Ma et al., 2007). In this context we also speculate that since 5-HT_4R signaling was found to increase striatal dopamine release, (Porras et al., 2002) a mechanism involved in reward (Norgren et al., 2006), the higher availability of the 5-HT_4R in obesity may be involved in the impairments of the dopamine circuits thought to be involved in both addiction and obesity (Volkow et al., 2011).

We also found a highly significant association between the 5-HT_4R and obesity in the left hippocampal region; a brain region viewed as an associative brain structure in reward learning (Berridge and Kringelbach, 2008) and has also been suggested central to energy dysregulation (Davidson et al., 2005). In an imaging study the region has also been linked to memories of desired foods (Pelchat et al., 2004). The 5-HT_4R has robustly been shown to be involved in learning and memory in animal studies (King et al., 2008) and its stimulation also increases 5-HT extracellular levels in the rat hippocampus (Ge and Barnes, 1996). Our finding that obesity is associated with high 5-HT_4R levels in the left hippocampal area suggests a role for the 5-HT_4R, not only in the hedonic component of food intake but also in the learning part of food representations.

Positive associations between the 5-HT_4R level and obesity were furthermore found in the orbitofrontal cortex (OFC). The OFC is a central region in food reward processing (Berridge and Kringelbach, 2008) and is thought to be involved in the integration of food-related sensory, cognitive and reinforcing information in the human brain (Morris and Dolan, 2001; Rolls, 2005). Moreover, a decrease in left OFC activation correlated with subjective pleasantness rating when the food was eaten to satiety (Kringelbach et al., 2003), suggesting that the saliency value of the food is represented in the OFC. Orbitofrontal 5-HT is found to prevent a salient stimuli in biasing the response to a specific task (Walker et al., 2009). This is in line with the observation that a pharmacologic increase of the brain 5-HT level reduces activation in the orbitofrontal cortex when looking at chocolate stimulus (McCabe et al., 2010). We speculate that the high level of 5-HT_4R receptors in this brain area indicates an attenuated serotonergic function in obesity.

We believe that even large fluctuations in 5-HT will not affect the affinity of [^{11}C]SB207145 to the 5-HT_4R since the affinity of 5-HT to the 5-HT_4R is low with Ki values in the order of 0.1–1.2 μM (Paterson et al., 2010). In support of this, we showed in a previous publication that acute infusions of citalopram (leading to acute increases in interstitial 5-HT) does not alter cerebral [^{11}C]SB207145 binding (Marner et al., 2010). Therefore, eventual differences in interstitial 5-HT will not affect the BP_{ND} (Eq. (1)) through changing K_D. To ensure that the results were not explained by body weight related differences in tracer availability, the non-specific binding of the radiotracer and cold mass injected were included one by one in the regression model, but they were not found to contribute to our finding. To explore if differences in gray matter volume (Horstmann et al., 2011) contributed to our finding we post hoc included gray matter volume as defined from the MR SPM segmentation in the regression model. Keeping in mind the limitations of this rough measure of gray matter we did not, however, find that this factor contributed to our findings.

Table 3
Positive associations in the regional analyses between 5-HT_4R BP_{ND} and BMI (age and gender corrected).

Region	Estimate ± SE (BP_{nd} unit per kg/m^2)	R^2	P-value
Orbitofrontal ctx	0.007 ± 0.002	0.45	**0.001**
Hippocampus	0.007 ± 0.004	0.42	**0.03**
Putamen	0.02 ± 0.008	0.41	**0.03**
Caudate	0.008 ± 0.01	0.33	0.63

Conclusions

The findings in this study provide insight into how the brain 5-HT system and specifically the 5-HT_4R is involved in the regulation of food intake in humans and our data suggest that the receptor is especially involved in the hedonic processes of feeding. We speculate that stimulation of cerebral the 5-HT_4R could be considered in the treatment of human hedonic overeating.

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Paper 2

Haahr ME, Fisher P, Holst KK, Madsen K, Jensen CG, Marner L, Lehel S, Baaré W, Knudsen GM, Hasselbalch SG.

The 5-HT_4 receptor levels in hippocampus correlate inversely with memory test performance in humans.

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The 5-HT$_4$ Receptor Levels in Hippocampus Correlates Inversely With Memory Test Performance in Humans

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Abstract: The cerebral serotonin (5-HT) system is involved in cognitive functions such as memory and learning and animal studies have repeatedly shown that stimulation of the 5-HT type 4 receptor (5-HT$_4$R) facilitates memory and learning and further that the 5-HT$_4$R modulates cellular memory processes in hippocampus. However, any associations between memory functions and the expression of the 5-HT$_4$R in the human hippocampus have not been investigated. Using positron emission tomography with the tracer [^{11}C]SB207145 and Reys Auditory Verbal Learning Test we aimed to examine the individual variation of the 5-HT4R binding in hippocampus in relation to memory acquisition and consolidation in healthy young volunteers. We found significant, negative associations between the immediate recall scores and left and right hippocampal BP$_{ND}$, ($p = 0.009$ and $p = 0.010$ respectively) and between the right hippocampal BP$_{ND}$ and delayed recall ($p = 0.014$). These findings provide evidence that the 5-HT$_4$R is associated with memory functions in the human hippocampus and potentially pharmacological stimulation of the receptor may improve episodic memory. *Hum Brain Mapp 00:000–000, 2012.* © 2012 Wiley-Periodicals, Inc.

Key words: cerebral 5-HT$_4$ receptor; memory; hippocampus; Positron Emission Tomography; Reys Auditory Verbal Learning Test; human experimentation

INTRODUCTION

Learning and memory is essential for normal human behavior and disabilities in these cognitive functions are characteristic for a variety of psychiatric and neurologic diseases such as addiction, anxiety, depression, schizophrenia, and neurodegenerative diseases. Current treatments of memory and learning deficits are far from optimal and substantial effort is undertaken to find therapeutic approaches for example for preventing Alzheimer's Dementia [Frautschy and

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Cole, 2010]. Because of its implications in memory and learning [Schmitt et al. 2006] one of the potential therapeutic targets is the serotonin (5-HT) system. Pharmacological intervention influencing the serotonin receptors can potentially improve memory and learning disabilities [Buhot et al., 2000; Cifariello et al., 2008; Perez-Garcia and Meneses, 2008].

Numerous animal studies have repeatedly shown that stimulation of the 5-HT type 4 receptor (5-HT_4R) facilitates memory and learning, and accordingly the receptor is an interesting pharmacologic target for treatment of memory deficits [Bockaert et al., 2004]. Pre-task systemic injections in animals of the relatively nonselective 5-HT_4R agonists BIMU-1 and BIMU-8 or the more selective partial agonists such as RS17017 and RS6733 improved performance in a variety of memory tasks such as olfactory associative learning [Marchetti et al., 2000, 2004; Marchetti-Gauthier et al., 1997], social memory [Letty et al., 1997], autoshaping [Meneses and Hong, 1997], place and object recognition [Lamirault and Simon, 2001], the Morris water maze [Lelong et al., 2001], and matching-to-sample [Terry et al., 1998]. Furthermore, the new highly selective and potent partial 5-HT_4R agonist, VRX-03011, also enhanced memory in the delayed spontaneous alternation task [Mohler et al., 2007]. In all these studies the agonists were injected immediately before the memory task and therefore 5-HT_4R agonism seems to improve memory acquisition. The evidence for the 5-HT_4R's involvement in memory consolidation is less clear: For example, post-task injection of a 5-HT_4R agonist impaired performance in an autoshaping task [Meneses and Hong, 1997], but improved performance in aged rats in an object recognition task [Lamirault and Simon, 2001]. In addition, administration of the 5-HT_4R antagonists SDZ205557 and GR125487 immediately after termination of the training session weakened passive avoidance memory [Galeotti et al., 1998]. With the radioligand [^{11}C]SB207145 and positron emission tomography (PET) it has recently become possible to visualize and quantify the human cerebral 5-HT_4R in vivo [Marner et al., 2009].

Episodic memory is the memory for events and experiences and involves three cognitive processes; encoding, consolidation, and retrieval [Lezak et al., 2004]. During encoding, the new information presented is acquired and learned. Consolidation is the processing of encoded information into long-term storage for later retrieval. The hippocampus is a crucial structure for both memory encoding and consolidation [Cipolotti and Bird, 2006; Desgranges et al., 1998]. In functional neuroimaging studies of humans, hippocampal activations are consistently observed during both memory encoding and retrieval [Nyberg et al., 2000]. The 5-HT_4R has a relatively high hippocampal density [Marner et al., 2010; Waeber et al., 1996] and plays a key role as a modulator of cellular memory processes in the hippocampus: 5-HT_4R agonists modulate long-term potentiation and long-term depression [Kemp and Manahan-Vaughan, 2002, 2004; Marchetti et al., 2004] and further facilitates the neuronal excitability of pyramidal cells through cAMP mediated closure of potassium channels and regulation of calcium release [Andrade and Chaput, 1991; Fagni et al., 1992; Mlinar et al., 2006; Torres et al., 1996].

Episodic memory functions in humans can be measured with verbal or visual memory tasks. In this study we used Reys Auditory Verbal Learning Test and Rey-Osterrieth's Complex Figure Test, to examine episodic memory function in relation to the individual variation of the in vivo 5-HT_4R binding in hippocampus in healthy young volunteers. On the basis of the experimental studies mentioned above, we hypothesized that the level of 5-HT_4Rs would be positively correlated with the immediate and delayed recall in the memory tasks.

MATERIALS AND METHODS

Participants

Thirty healthy adults (6 females, 24 males) were recruited through newspaper advertisements. Since the memory test scores are reported to decrease with age we only included participants below 45 years of age (mean age 27.2 $\pm$ 6.3 (s.d.) years; range, 20.0–44.7 years), to avoid confounding effects of aging. Written informed consent was obtained according to the Declaration of Helsinki II, and the Copenhagen Region Ethics Committee approved the study. Thirteen of the participants had previously been included in other studies of the 5-HT_4R in humans [Madsen et al., 2010a; Marner et al., 2009, 2010].

Exclusion criteria included significant medical history (including obesity), drug or alcohol abuse, psychiatric disorders or head trauma. All participants had a normal neurological examination and an unremarkable magnetic resonance imaging (MRI) scan of the brain. All participants had completed some education after high-school with a mean of 15.7 $\pm$ 2.1 (s.d.) years of education in total. IQ in the cohort was investigated with two subscales (the number series and verbal analogies) of the Intelligenz-Struktur-Test 2000 R [Amthauer 2001; Neubauer et al., 2005] and found to be a little over average for the general population (mean 104.4 $\pm$ 10.5 (s.d.); range, 90.5–123.5).

Memory Testing

Two experienced neuropsychologists conducted memory testing on a day separate from the day of the PET scan (mean time-interval: 45.1 $\pm$ 37.6 (s.d.) days).

Two memory tests were used: Rey Auditory Verbal Learning Test (RAVLT) to assess episodic verbal memory and Rey-Osterrieth's Complex Figure Test (ROCFT) to evaluate visual nonverbal memory [Lezak et al., 2004]. In RAVLT, a list of 15 emotionally neutral words was read aloud by the neuropsychologist with a 2-s interval in the same sequence five times. After each presentation of the list, the participant delivered a free immediate, verbal recall with a time limit of 60-s. These five presentations were followed by presentation and recall of an interference list and after a 30-min delay the participant was asked to recall the first list again. To reflect the cognitive process of

memory encoding we used the total number of words the individuals acquired during the five first trials (immediate recall, maximum score 75) and to reflect memory consolidation we used the number of recalled words after 30-min (delayed recall, maximum score 15) [Vakil et al., 2010]. In our set-up it was not possible to separate the cognitive process of retrieval from encoding and consolidation, since we did not have a recognition trial at the end of the test.

In ROCFT, participants copied a complex geometric figure and then reproduced it from memory after 3-min and after a delay of 30-min. A 36-point scoring system was used to evaluate the reproductions. To measure memory encoding the scores of 3-min were used (immediate recall) and to measure memory consolidation the delayed scores at 30-min were chosen (delayed recall). Again, in this test it was not possible to eliminate memory retrieval from memory encoding and consolidation.

PET and MR Imaging

[^{11}C]SB207145 was synthesized using a fully automated radio-synthesis system as previously described [Gee et al., 2008; Gillings and Larsen, 2005]. Immediately after an intravenous bolus injection of [^{11}C]SB207145, (mean 490.3 ± 143.0 (s.d.) MBq; range, 206–617 MBq) a 120 min dynamic 3D PET scan (6 × 5 s, 10 × 15 s, 4 × 30 s, 5 × 120 s, 5 × 300 s, and 8 × 600 s) was initiated using either an eighteen ring GE-Advance scanner (GE, Milwaukee, WI) with an approximate in-plane resolution of 6 mm ($n = 13$) or a High Resolution Research Tomograph (HRRT) with an approximate in plane resolution of 1.5 mm ($n = 17$) [Olesen et al., 2009]. The images from the Advance scanner were reconstructed with filtered back projection and corrected for attenuation, dead time and scatter. The HRRT scans were reconstructed using the iterative PSF reconstruction with attenuation map improvements [Comtat 2008; Sureau et al., 2008].

MRI was conducted on a 3T Siemens Magnetom Trio scanner (Erlangen, Germany). High-resolution 3D T1-weighted (matrix 256 × 256; 1 × 1 × 1 mm^3 voxels) and 2D T2-weighted sequences were acquired and corrected for spatial distortions and nonuniformity [Jovicich et al., 2006; Sled et al., 1998]. The T1-weighted brain MRIs were segmented into gray matter, white matter, and cerebrospinal fluid using SPM5 (Wellcome Department of Cognitive Neurology, London, UK) and each voxel was assigned to the tissue class with the highest probability and this segmentation was subsequently used for delineation of the region of interest. The T2 weighted images served for brain masking purposes.

Quantification of Hippocampal 5HT$_4$Rs

The frames of the dynamic PET scan were aligned to correct for head-motion artifacts of more than 3 mm using the AIR routines (version 5.2.5). A flow-weighted mean emission image was automatically aligned to the same individuals MRI using either AIR routines (GE Advance scans) or SPM5 (HRRT scans).

The quantitative analysis to obtain the binding potential (BP) of the 5-HT$_4$R was performed with the simplified reference tissue model (SRTM) [Lammertsma and Hume, 1996] using PMOD (PMOD Inc, Zürich, Switzerland) since it was found to be a reliable and reproducible method for quantification of [^{11}C]SB207145 receptor binding in humans [Marner et al., 2009]. The cerebellum was used as a reference region since blocking with a selective 5-HT$_4$R compound prior to radiotracer administration did not alter the cerebellar binding [Marner et al., 2009]. The model estimates the nondisplaceable BP (BP_{ND}), which is defined as:

$$BP_{ND} = f_{ND} * B_{max}/K_d$$

where f_{ND} is the free fraction of tracer in nondisplaceable tissue compartment, B_{max} is the receptor concentration, and K_d is the equilibrium dissociation constant for the tracer.

Regional Analysis

The regions of interest in the present study were left and right hippocampus. The gray matter tissue concentration of radioactivity in the regions of interest was obtained by automatic delineation on each participants MRI in a user-independent fashion with the Pvelab software package [Svarer et al., 2005].

Statistics

We modeled the association between the 5-HT$_4$R binding and memory functions using regression analyses. The four memory test scores used in the models were: (1) RAVLT immediate recall (sum of the five initial trials), (2) the RAVLT delayed recall, (3) the immediate recall in ROCFT, and (4) delayed recall score in ROCFT. These outcomes were one by one compared to the 5-HT$_4$R BP_{ND} in left and right hippocampus. Ceiling effects were found for both RAVLT scores (immediate and delayed recall), which are often observed in young and well-educated cohorts [Uttl, 2005]. The ceiling effect causes bias in regression parameters in a standard linear regression (under-estimation of the association between memory score and 5-HT$_4$R BP_{ND}), and to limit this effect we conducted the analyses of RAVLT scores using Tobit regression [Tobin, 1958]. The Tobit model was designed to estimate linear relationships between censored variables. In this study we have a censoring, where individuals with a RAVLT memory score at or above the threshold of 15 words, take on the value of that threshold, so that the true memory score might be equal to the threshold of 15 words, but it might also be higher. In every regression analyses we adjusted for the influence of the type of PET scanner used by including the

TABLE I. Results of Tobit regressions (RAVLT) and linear regressions (ROCFT) analyses of left and right hippocampus with the memory scores as outcome variable and 5-HT$_4$R binding and scanner type as explanatory variables

Memory score	Region	Estimate (β) ± SE	R^2	95% CI	*P*-value
RAVLT immediate recall	Left hippocampus	−37.9 ± 14.5 /BP_{ND}	0.23[a]	−66.4 to −9.4	0.009
	Right hippocampus	−37.3 ± 14.5 /BP_{ND}	0.21[a]	−65.8 to −8.8	0.010
RAVLT delayed recall	Left hippocampus	−4.6 ± 4.0 /BP_{ND}	0.05[a]	−12.5 to 3.3	0.25
	Right hippocampus	−9.6 ± 3.9 /BP_{ND}	0.17[a]	−17.2 to −1.9	0.014
ROCFT immediate recall	Left hippocampus	−1.9 ± 5.4 /BP_{ND}	0.02	−13.1 to 9.2	0.71
	Right hippocampus	−3.7 ± 5.7 /BP_{ND}	0.03	−15.4 to 8.0	0.52
ROCFT delayed recall	Left hippocamp−us	−5.1 ± 5.1 /BP_{ND}	0.05	−16.0 to 4.9	0.29
	Right hippocampus	−4.7 ± 5.4 /BP_{ND}	0.04	−15.8 to 6.3	0.39

[a]McKelvey-Zavoina Pseudo-R^2 defined as ratio of variance of prediction and the latent response [Veall and Zimmermann, 1994]. RAVLT, Reys Auditory Verbal Learning Test; ROCFT, Rey-Osterrieth's Complex Figure Test; SE, standard error; CI, confidence interval.

scanner type as a class variable. Age, gender and IQ were furthermore included in the regression models and eliminated if nonsignificant. Additionally, we used linear regressions to evaluate whether BP_{ND} correlated to the nonspecific binding in the brain (computed as the area-under-the curve in the reference region) or injected amount of cold dose/kg. Further, we evaluated with linear regressions if BP_{ND} or memory scores correlated with gender corrected grey matter volumes. A significance level of $P = 0.05$ was adopted throughout the analyses, and all tests were two-tailed. All analyses were carried out in SAS (v. 9.1 SAS Institute Inc.) or R (v. 2.1.1, The R Foundation for Statistical Computing).

Voxel-Based Analysis

In addition to the regional analyses we performed voxel-based analyses to reveal effects of interest within the structure of hippocampus. The parametric time-activity curves from the HRRT scanner and the GE Advance scanner were smoothed with a 6 mm and a 4 mm full-width-half-maximum (FWHM) Gaussian kernel respectively to obtain the same level of noise in the images. To generate parametric images of the BP_{ND} in each voxel, we used the basis function implementation of SRTM [Gunn et al., 1997]. Then the single-subject BP_{ND} parametric maps were warped to MNI space within SPM8 (http://www.fil.ion.ucl.ac.uk/spm) using the single-subject GM segmented MRI and the SPM8 apriori grey.nii image as the template. Final voxel-size was $2 \times 2 \times 2$ mm^2. The parametric images from the GE Advance scanner were additionally smoothed with a 12 mm FWHM Gaussian kernel after normalization. We defined hippocampus using the Wake Forest University Pickatlas (v3.0) [Maldjian et al., 2003, 2004]. A voxel-wise Tobit regression analysis was conducted with each of RAVLT immediate and delayed recall as dependent variable and 5-HT$_4$R BP_{ND} and scanner type as covariates. We corrected for multiple comparisons using Monte Carlo simulation in 3dClustSim within AFNI (http://afni.nimh.nih/gov/afni). In the hippocampal volume the method yielded a cluster extent significance threshold of $k \geq 30$ voxels ($P < 0.05$).

RESULTS

Our regional Tobit regression analyses of left and right hippocampus revealed significant, negative associations between the RAVLT immediate recall scores and left and right hippocampal $BP_{ND,}$ ($P = 0.009$ and $P = 0.010$, respectively) (Table I and Fig. 1). Correspondingly, the right hippocampal BP_{ND} and RAVLT delayed recall showed a significant, negative association ($P = 0.014$), whereas this relationship was not found for the left hippocampus ($P = 0.25$) (Table I and Fig. 1). The model assumptions of linearity and distribution were found reasonable when assessed by inclusion of polynomial terms of the predictor and by graphical comparison of the Kaplan-Meier estimator and the model-specific distribution of the residuals. Linear regression analyses between ROCFT immediate and delayed recall scores and binding potentials in left and right hippocampus were not significant ($P > 0.29$). Age, gender, and IQ were included in the regression models, but eliminated from the analyses, as they did not contribute significantly to the model (All *P*-values > 0.23). The voxel-level Tobit analyses of associations between left hippocampal BP_{ND} and RAVLT immediate recall revealed two inversely associated clusters, which were significant after corrections for multiple comparisons (MNI coordinates −30, −16, −16, $z = 3.23$, 51 voxels, $P < 0.05$; MNI coordinates −30, −32, −8, $z = 3.72$, 31 voxels, $P < 0.05$) (Fig. 2). We also identified two clusters within the right hippocampus where RAVLT immediate recall was inversely associated with 5-HT$_4$R binding, however these did not survive correction for multiple comparisons (MNI coordinates 32, −16, −18, $z = 3.58$, 24 voxels, $P > 0.05$; MNI coordinates 30, −26, −12, $z = 3.36$, 14 voxels, $P > 0.05$). No clusters in left or right hippocampus were associated with RAVLT delayed recall after corrections for multiple comparisons. We found zero positively associated

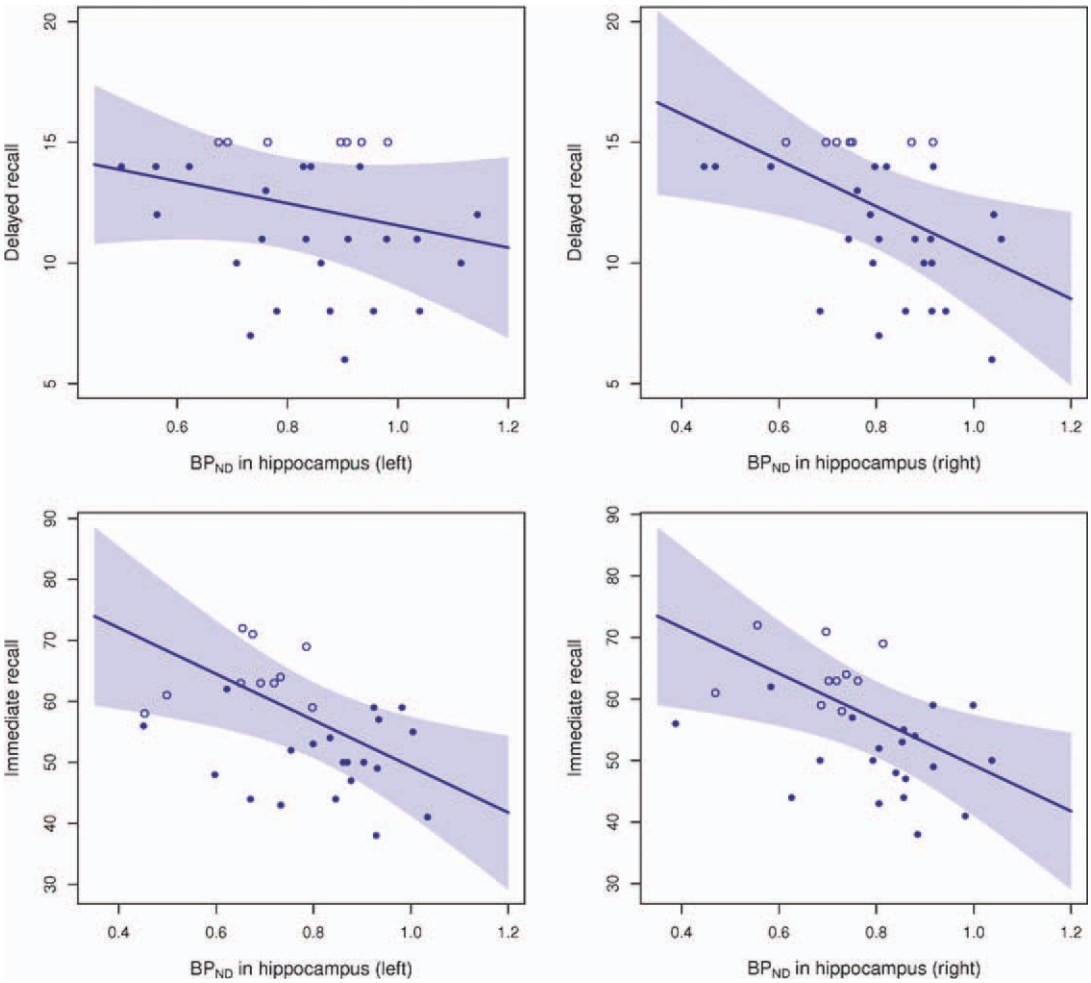

Figure 1.

Four plots showing estimated linear associations between RAVLT immediate recall and delayed recall and the left and right regional BP_{ND} in hippocampus. BP_{ND} values are adjusted for the estimated scanner effect. The shaded region is the pointwise 95% confidence limits. The censored observations—the unfilled circles—all scored 15 in the delayed recall or in one or more of the five immediate recall sessions.

voxels between RAVLT immediate or delayed recall and 5-HT_4R binding.

Since the BP_{ND} value of the hippocampus was associated with verbal but not nonverbal memory performance, we examined the interrelationships between these two kinds of memory test scores. In a univariate linear regression immediate and delayed recall scores were strongly correlated in both RAVLT ($P < 0.001$) and ROCFT ($P < 0.001$). Further, we observed strong correlations between verbal and nonverbal immediate recall scores ($P < 0.001$) as well as verbal and nonverbal delayed recall scores ($P < 0.001$).

We did not see any significant correlation between (1) gray matter volumes and BP_{ND} or memory scores or (2) BP_{ND} and nonspecific binding or cold dose/kg injected (All P-values > 0.26).

DISCUSSION

This is the first human in vivo study to examine relationships between 5-HT_4R-binding and memory functions. In 30 young and healthy individuals we found a significant negative correlation of the 5-HT_4R expression in left and right hippocampus and immediate recall and further a significant negative correlation between the right hippocampal 5-HT_4R expression and delayed recall. Our results suggest that the 5-HT_4R in humans is involved in memory encoding and consolidation and that fewer hippocampal 5-HT_4Rs are representative of a better episodic memory function. As receptor stimulation according to the animal literature generally has a facilitatory effect on memory acquisition and as receptor expression is augmented when rats undergo memory training [Manuel-Apolinar et al.,

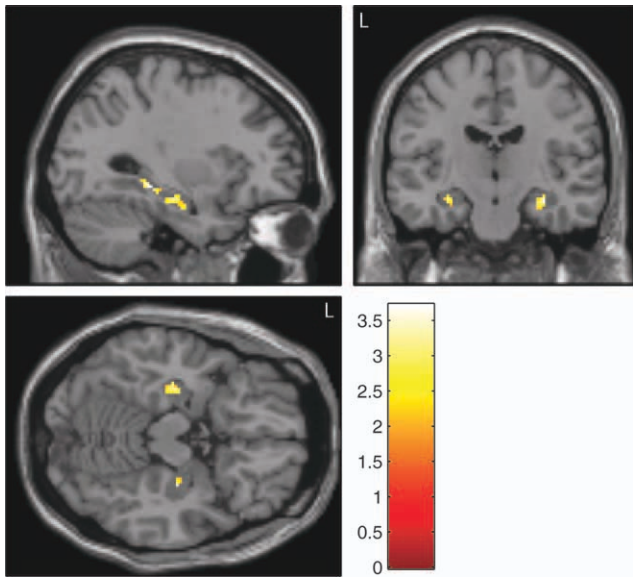

Figure 2.

Statistical parametric map overlaid on a T1-weighted MR image showing the negative associations between 5HT$_4$R BP_{ND} and RAVLT immediate recall in hippocampus ($P < 0.05$). Two clusters in the left hippocampus were significant after corrections for multiple comparisons while two clusters within the right hippocampus were below the cluster extent threshold. The color bar depicts the T values. MNI coordinates for the sagittal image is $X = -30$, the coronal image is $Y = -16$ and the axial image is $Z = -16$.

2005], the result does not call for a straightforward interpretation. However, most of the animal studies examined functional effects of agonism or antagonism of the receptor, which could potentially explain the difference between the present clinical study and the experimental studies.

As shown in the methods section the binding potential of the 5-HT$_4$R is a composite measure of receptor expression and affinity [Lammertsma and Hume, 1996]. Since [^{11}C]SB207145 has not been found sensitive to acute endogenous serotonin release [Marner et al., 2010] and since the affinity is assumed to be equivalent in hippocampus and the reference region, the BP_{ND} in this study is proportional to the receptor density in hippocampus. The 5-HT$_4$R is expressed in hippocampus in central locations, where both intrinsic hippocampal and extrinsic cortical and subcortical circuits are modified such as the CA1, the dentate gyrus and the subiculum [King et al., 2008]. The precise hippocampal neuronal localization has not yet been specified, but lesion studies indicate that the receptor is present on glutamatergic neurons [Vilaro et al., 2005]. Furthermore, stimulation of the 5-HT$_4$R leads to modulation of the acetylcholine and GABA release in hippocampus, so the receptor is likely to be localized on these neurons, even though indirect effects cannot be excluded [Bianchi et al., 2002; Bockaert et al., 2006; Matsumoto et al., 2001]. As the in vivo binding of [^{11}C]SB207145 cannot differentiate the type of neurons or the specific location of the 5-HT$_4$R within hippocampus, a low receptor density in hippocampus cannot simply be linked to low neuronal activity and functions. Further, stimulation of the receptor is also not in a simple way linked to better memory. For example, in healthy volunteers a low dose of selective serotonin reuptake inhibitors (SSRI) improved short-term memory, while a high dose impaired it [Dumont et al., 2005] and, as mentioned in the introduction, 5-HT$_4$R agonists impaired memory function in young rats while it improved memory functions in old rats [Lamirault and Simon, 2001]. Therefore, various explanations of the results in this study can be proposed. Acute tryptophan depletion impairs episodic memory [Mendelsohn et al., 2009; Sambeth et al., 2007], and therefore efficient serotonergic innervation and adequate 5-HT interstitial levels in hippocampus seem important for memory functions. Since 5-HT$_4$R binding was found to be down-regulated after chronic SSRI treatment [Licht et al., 2009; Vidal et al., 2009], high serotonin levels in hippocampus may reduce the levels of the 5-HT$_4$R. This might explain the paradoxical finding that the participants in this study with a high memory performance have lower levels of the receptor. On the other hand, since subchronic serotonin depletion was found to cause an up-regulation of the 5-HT$_4$R [Licht et al., 2009] subjects with an inefficient memory function may improve the functioning of the serotonin system by up-regulating the 5-HT$_4$R, that both may improve memory

directly and indirectly by stimulating 5-HT release in hippocampus [Ge and Barnes 1996; Licht et al., 2010]. However, hypothetically this up-regulation is insufficient to boost memory function to the level of the better performing subjects. This is in line with a recent study of Alzheimer's disease from our group, where accumulation of β-amyloid and thereby possible decline of interstitial serotonin levels was associated with an up-regulation of the 5-HT_4Rs [Madsen et al., 2010b]. In this context we also speculate that the relation between the serotonin system and memory may be inversely u-shaped as it is assumed for dopaminergic modulation of cognitive functions [Cools and D'Esposito, 2011]: both low and high 5-HT_4R binding may reflect levels of serotonin outside the optimal range for memory function. However, this study lacks variation in memory scores, with few low scoring subjects, to find evidence for this hypothesis.

We previously investigated the relation between the RAVLT memory scores and the serotonin transporter (SERT) and found no correlation [Madsen et al., 2011]. The reason for these differing results may be that SERT is considered to be a marker of general serotonergic innervation with complex postsynaptic actions, while the 5-HT_4R is likely to be directly involved in memory function as previously show in the introduction.

It was clear from the voxel-based analysis within hippocampus that the association between memory functions and receptor expression was found both in ventral and dorsal hippocampus, which have been assigned to dissimilar functional roles: Dorsal hippocampus is mainly participating in cognitive functions while ventral hippocampus is mainly involved in emotions, stress and affect [Fanselow and Dong, 2010]. This is in line with the roles of the 5-HT_4R in the brain. As shown both from the previous animal literature and this study the receptor is likely to play a role in memory and learning processes, however, the receptor is also likely to be involved in emotional processes as studies has linked the receptor to regulation of the hedonic part of food intake [Compan et al., 2010; Francis et al., 2010; Jean et al., 2007] and emotional processes such as stress [Compan et al., 2004].

It was evident from the overlapping confidence intervals (Table I) that the difference between left and right binding potentials would not statistically reach a 5% significance level. However, the RAVLT delayed recall scores were only significant in the right hippocampus. This finding could be due to differences in cognitive processes in the left and right hippocampus as some studies suggest that encoding processes may be left-lateralized and recall processes right-lateralized [Desgranges et al., 1998]. Another explanation may be that the 5-HT_4Rs differ in functionality with regard to memory encoding and consolidation as the previously mentioned animal literature suggest. This may result in differential regulation of the receptor dependent on the cognitive process.

In contrast to the verbal memory performance we did not find significant correlations between the nonverbal memory task and hippocampal 5-HT_4Rs even though the memory tests correlated strongly. These results could be explained by the specific functions of the 5-HT_4R. It has been shown that neuronal networks of encoding and retrieval can be distinguished by their reaction to specific stimuli (e.g., verbal vs. visual) even though interactions between the systems are evident [Nyberg et al., 2000]. Our results suggest that the 5-HT_4R primarily affects the verbal memory network and not the visual. It is not possible to confirm this result in the existing literature, as 5-HT_4R agonism seems to improve all the various animal memory tests, at least in the acquisition phase. However, in animals it is difficult to precisely imitate the visual and verbal memory tasks used in humans, as species differences may be considerable. Thus, rather than drawing any specific conclusions, we recommend that future human studies seek to clarify these associations between serotonergic tone, 5-HT_4Rs and verbal and visual memory.

One should consider the limitations of this study. We found ceiling effects in RAVLT, which in young and well-educated cohorts is a prevalent finding with the applied testing procedures [Uttl, 2005; Van der Elst et al., 2005]. We corrected for this bias by using a censored regression model to avoid inconsistent estimates present in an ordinary linear regression in these kinds of data. However, if the data was analyzed using an ordinary linear regression model, we still found significant correlations between the immediate recall scores and the 5-HT_4R levels in left hippocampus ($P = 0.04$) and right hippocampus ($P = 0.02$) and between RAVLT delayed scored and the receptor level in right hippocampus ($P = 0.03$). Another issue was that the subjects were scanned on two different PET scanners. We compensated for this by including scanner type in the regressions models, but it is not possible to exclude that some scanner effects were still present. However, we would point out that scanner type was insignificant in the regression models in all analyses and we did not observe any tendencies toward main effects of scanner type on immediate or delayed memory scores.

In conclusion, this is the first study in humans to examine the association between the 5-HT_4R in hippocampus and memory functions. The study provides evidence that the 5-HT_4R is associated with memory functions in hippocampus and supports an association between 5-HT_4R and the previously reported asymmetry of hippocampal function in memory. Since 5-HT_4R stimulation generally enhances memory performance across various domains in animals, it was an unexpected finding that the 5HT_4R level correlated negatively with measures of memory function. This finding may be explained by the complex interactions between the intrinsic serotonergic tonus and receptor functions in the hippocampus. Further studies in humans are needed to elucidate the functional significance of this study; however, we speculate that stimulation of the human 5-HT_4R could improve memory functions.

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Paper 3

Haahr ME, Fisher PM, Jensen CG, Frokjaer V, Nørremølle A, Madsen K, Baare W, Madsen J, Rabiner EA, Knudsen GM.

The cerebral in vivo 5-HT_4 receptor binding before and after 3 weeks of SSRI intervention in healthy humans.

Manuscript.

The cerebral in vivo 5-HT$_4$ receptor binding before and after 3 weeks of SSRI intervention in healthy humans.

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Introduction

The cerebral 5-HT type 4 receptor (5-HT_4R) is a post-synaptic G protein-coupled receptor positively linked to the adenylate cyclase that mediates closure of potassium channels (Fagni et al., 1992) and inhibition of calcium release (Torres et al., 1996), and stimulation of the receptor generally has excitatory neuronal effects (Bockaert et al., 2004). There is a high density of the 5-HT_4 receptor in striatum, globus pallidus, nucleus accumbens, and substantia nigra, an intermediate density in the hippocampal formation and the superficial layers of the neocortex, whereas cerebellum is practically devoid of receptors (Reynolds et al., 1995, Marner et al., 2010). Experimental studies have suggested several potential beneficial effects from central 5-HT_4 receptor agonism: enhanced memory acquisition and consolidation (King et al., 2008), an increase in the non-amyloidogenic soluble form of amyloid precusor protein (Lezoualc'h, 2007), reduced appetite (Jean et al., 2007), and a faster treatment response in depression (Lucas et al, 2007).

There is now evidence from both animal and human studies that the cerebral 5-HT_4R is inversely regulated by extracellular 5-HT levels. Two weeks of the tricyclic antidepressant imipramine that leads to increases the extracellular serotonin levels attenuates the stimulatory effect of 5-HT_4R agonism on hippocampal neuron excitability (Bijak, 1997, Bijak et al., 1997, Zahorodna et al., 2002), while chronic 5-HT depletion up-regulates 5-HT_4R binding in the hippocampus and basal ganglia (Compan et al., 1996). We found in rats that chronic (2 and 3 weeks) but not acute administration of the SSRI paroxetine caused a 16-47% downregulation of 5-HT_4R density in all investigated brain regions, including striatum and hippocampus, while subchronic 5-HT depletion increased the 5-HT_4R in the dorsal hippocampus, hypothalamus, and lateral globus pallidus (Licht et al., 2009). This finding was replicated by another group: three weeks treatment with another SSRI, fluoxetine, in rats decreased the 5-HT_4R density in striatum and

hippocampus (Vidal et al., 2009) and three weeks treatment with the non-selective 5-HT nor-adrenalin reuptake inhibitor, venlafaxine, was also associated with a decrease of the 5-HT_4R density in striatum and hippocampus (Vidal et al., 2010). Furthermore, genetically modified expression of the gene coding for the serotonin transporter (5-HTT) induced significant alterations in 5-HT_4R density in mice: Mice over-expressing 5-HTT (i.e., resulting in reduced 5-HT signaling) have increased cerebral 5-HT_4R density while knock-out of the gene coding for 5-HTT (i.e., resulting in increased 5-HT signaling) results in reduced 5-HT_4R density (Jennings et al., 2011). Studies in humans go along the same line: Whereas acute blockade of serotonin reuptake with citalopram, putatively resulting in increased 5-HT signaling, does not modify the cerebral 5-HT_4R binding as assessed in vivo with positron emission tomography (PET) and the radioligand [^{11}C]SB207145 binding (Marner et al., 2010) then a recent study from our lab identified an association between 5-HTTLPR status and 5-HT_4R binding. The 5-HTTLPR is a common genetic variant within the gene (SLC6A4) coding for the serotonin transporter (5-HTT) where the 'long' (L) allele exhibits increased 5-HTT transcription in vitro relative to the 'short' (S) allele, putatively affecting 5-HT signaling (Lesch et al., 1996). Fisher et al found that S carriers showed reduced [^{11}C]SB207145 binding relative to L/L homozygotes, consistent with the S allele resulting in diminished 5-HTT production and subsequent increases in 5-HT levels leading to a decrease in 5-HT_4R (Fisher et al., 2012). Taken together, these findings indicate that the 5-HT_4R could be used as a proxy for – chronically, but not acutely – altered cerebral 5-HT levels, and imaging of the receptor could potentially act as a biomarker for, e.g., responsiveness to antidepressive treatment.

In a randomized blinded placebo-controlled design we measured the *in vivo* 5-HT_4R binding in healthy men before and after pharmacologically increased extracellular 5-HT levels by means of fluoxetine. This drug increases the extracellular 5-HT levels by blocking the 5-HTT.

It is well-tolerated and has little affinity for serotonergic, muscarinic, histaminic, or noradrenergic receptors (Stark et al., 1985).

Based on the expected monotonic association between the 5-HT_4R binding and the 5-HT levels, we hypothesized that the 5-HT_4R binding would decrease in the intervention group. Further, since L/L carriers are associated with a higher level of 5-HTT we expected the monotonic 5-HT_4R response would be influenced by 5HTTLPR status: the L/L homocygotes would show a greater response to SSRI intervention than the S carriers.

Methods

Participants and interviews

Thirty-two eligible healthy males were recruited through newspaper and internet advertisements. All were Caucasian except one who were half Caucasian and half Negroid. Due to our hypothesis regarding the effect of genotype on 5-HT_4R levels we oversampled for 5-HTTLPR L_AL_A homocygotes by genotyping participants before inclusion. Written informed consent was obtained according to the declaration of Helsinki II and the study was approved by the Ethical Committee of Copenhagen. Seventeen of the datasets in this study was also included in previously published studies (Fisher et al., 2012, Haahr et al., 2012a).

The participants were examined by a physician and had a normal physical and neurological examination. They further had a normal blood screen and an unremarkable brain magnetic resonance imaging (MRI). The exclusion criteria were as follows: Drug use within the last 1 months, i.e., use of cocaine, heroin, amphetamine or ecstasy more than 10 times, use of cannabis more than 50 times, neurological disorder or major psychiatric disease according to ICD-10, previous or current use of antipsychotics or antidepressants or other medication that

significantly affect brain function (e.g. limited use of NSAID were permitted).

On the day of the PET scan we assessed symptoms of perceived stress and depression (Derogatis, 1994) by means of Cohen's Perceived Stress (Active: median 7.5 range 1-20; placebo: median 8, range 0-17, Mann Whitney U p=0.51), SCL-90-R (Active: 0.13, 0-0.81; placebo median 0.15, range 0-0.68, Mann Whitney U p=0.87) and Major Depression Index (MDI) (Active: median 6, range 0-21; placebo: 4.5, range 0-12, Mann Whitney U p=0.08). We saw a tendency to a higher MDI score in the active group and specifically one participant scored 21 in the questionnaire. However, he did not fulfill the ICD-10 criteria for a depression, since he was negative for any of the three core symptoms.

Intervention regime

The participants were randomized based on a match by genotype, age and education by an un-blinded researcher (GMK) to capsules containing fluoxetine (N=16) or placebo with no active drug (N=16). All capsules were identical regardless of contents and delivered pre-packed by GMK. The participants received identical intervention regimes regardless of randomization group and the intervention began after the first PET scan. To minimized side-effects the participants took the capsules in the evening and the initial dose of fluoxetine were 20mg/day for three days. Then the dose was increased to 40 mg/day until the last PET scan had been conducted 18-20 days later. To minimize discontinuation symptoms the participants continued on 20 mg/day for 5 days after the PET-scan (the placebo group also continued for 5 days).

The participants and the remaining investigators (MEH, PMF, VF and personnel at the scanner) were blinded to the intervention type until the outcome of the BP_{ND} and voxel-based

analysis had been made. Three participants were excluded before the PET-scans were completed: two because of failure of radiotracer production and one (in the placebo group) did not show up for the PET rescan.

All participants were contacted by a medical doctor (VF or MEH) 2 to 3 times during the 3 weeks of intervention to ensure adherence to the protocol and to register side-effects. After intervention termination the participants were also contacted to register discontinuation symptoms. The side-effects were scored according to the UKU side-effect rating scale (Lingjaerde et al., 1987). S-fluoxetine was measured in the middle of the treatment period and again immediately before the PET rescan.

[^{11}C]SB207145 positron emission tomography

[^{11}C]SB207145 was synthesized using a fully automated radio-synthesis system as previously described (Marner et al., 2009). An intravenous bolus injection of [^{11}C]SB207145 was given over 20 seconds. Immediately after the injection a 120-min dynamic 3D PET scan (6x5 s, 10x15 s, 4x30 s, 5x120 s, 5x300 s and 8x600 s) was acquired using a High Resolution Research Tomograph (HRRT) with an approximate in plane resolution of 1.5 mm (Olesen et al., 2009). The scans were reconstructed using the iterative PSF reconstruction with attenuation map improvements (Sureau et al., 2008).

Structural MRIs were acquired using a Siemens Magnetom Trio 3T MR scanner. A high-resolution 3D T1-weighted MP-RAGE (matrix = 256 x 256, resolution = 1x1x1 mm voxels) and a T2-weighted Turbo Spin Echo structural image (matrix = 256x256, resolution = 1.1x1.1x1.x mm voxels) was acquired. The T1-weighted image was segmented into gray matter, white

matter, and cerebrospinal fluid using SPM5 (http://www.fil.ion.ucl.ac.uk/spm/). The T2-weighted image served for brain-masking purposes.

Quantification of the 5-HT$_4$R binding

If the median head-motion exceeded 3-mm the frames of the dynamic PET scan were aligned to correct for head-motion artifacts using the AIR routines (version 5.2.5). Each PET frame was aligned to a single PET frame with sufficient structural information using the scaled least squares cost-function in AIR (frame 26: 15 to 20 minutes post-injection). A flow-weighted (early) mean emission image was automatically aligned to the same individuals MRI using SPM5. Accurate co-registration was confirmed by visual inspection across all planes.

The tissue concentration of radioactivity in the volumes of interest (VOI) was obtained by automatic delineation of regions on each subjects MRI in a user-independent fashion with the Pvelab software package (http://www.nru.dk/downloads) (Svarer et al., 2005).

Regional time activity curves (TAC) were constructed and kinetic modeling was performed with the simplified reference tissue model (SRTM) with cerebellum as reference region. This method was found reliable and reproducible for quantification of [^{11}C]SB207145 in humans (Marner et al., 2009). The model estimates the regional *in vivo* measure of binding potential defined as $BP_{ND} = f_{ND} * B_{avail} * (1/K_D)$, where f_{ND} is the fraction of free versus non-displaceable radioligand concentration in the brain tissue, B_{avail} is the concentration receptors available for binding and $1/K_D$ is the inverse of the dissociation constant. Kinetic modeling was performed using the PMOD software version 2.95, build 2 (PMOD Inc., Zurich, Switzerland).

Genotyping

Analysis was performed on blood samples that were drawn at the time of PET scanning and immediately frozen at -20 °C. DNA was extracted from blood using a Qiagen Mini Kit (Qiagen). Alternatively, analysis was performed on saliva samples, collected using the prepIT kit (DNA Genotek Inc). DNA was extracted according to the manufacturers instructions. The 5-HTTLPR short/long and rs25531 A/G polymorphisms in the serotonin transporter gene were genotyped by a method consisting of a polymerase chain reaction (PCR) amplification with the following primers: forward 5'-GGC GTT GCC GCT CTG AAT GC-3' and reverse 5'-CTG ACC CCT GAA AAC TGT GC-3', followed by MspI digestion and fragment analysis by electrophoresis on an ABI 7500 multiplex (Applied Biosystems) using a FAM-labeled forward primer. When using the fluorescence labeling and MspI digestion the following three alleles can be demonstrated: 5-HTTLPR l + rs25531/A (lA, 341 bp), 5-HTTLPR s + rs25531/A (sA, 298 bp), and rs25531/G (167 bp). In order to distinguish the 5-HTTLPR l + rs25531/G (lG) from the rare 5-HTTLPR s + rs25531/G (sG) allele, all samples showing a G allele were subsequently analyzed for 5-HTTLPR s or l allele by PCR amplification with the same primers followed by electrophoresis of the undigested product (l allele: 571 bp, s allele: 528 bp).

Data analysis

The fluoxetine and the placebo group were compared with and Mann Whitney U Test for independent samples and baseline versus rescan data in both groups were compared with related samples Wilcoxon Signed Rank Sum Test (the distribution in striatum in the fluoxetine group did not pass the D'Agostino & Pearson test of normality).

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We primarily evaluated the average BP_{ND} of striatum since SSRI treatment in rats reduced 5-HT_4R binding up to 37% (Licht et al., 2009, Vidal et al., 2009) and this region as a [^{11}C]SB207145 high-binding region also provides a good signal-to-noise ratio. Secondly, we examined 1) the bilateral hippocampus, since SSRI treatment in rats also decreased 5-HT_4R binding in this region and 2) cortical effects using a large neocortical region providing a robust measurement despite low 5-HT_4R binding.

We compared the baseline BP_{ND} with the rescan BP_{ND} in each region of interest to test the intervention effect on regional 5-HT_4R binding. The percent change in BP_{ND} from baseline to rescans were calculated with the 95% confidence intervals and used to compare the fluoxetine and the placebo group and the subgroups of 5-HTTLPR status.

To analyze the test-retest difference in the placebo group, a relative test-retest difference was calculated per region of interest (ROI) as:

$$\Delta\% = 2 \times (\text{retest value} - \text{test value} / \text{retest value} + \text{test value}) \times 100$$

The mean of the Δ% is a measure of the systematic bias and the standard deviation of Δ% is a measure of the mean test-retest difference and characterizes the reproducibility (Marner et al., 2009). Reliability was determined with the Intraclass Correlation Coefficient (ICC) defined as:

$$ICC = MSS_{Between} - MSS_{Within} / MSS_{Between} + MSS_{Within}$$

$MSS_{Between}$ is the mean sum of squares between subjects, and MSS_{Within} is the mean sum of squares within subjects. An ICC score of -1 denotes no reliability, and +1 denotes maximum reliability.

A p-level of 0.05 was adopted in all regional analyses and done in SPSS 20 (IBM) and all voxel-based analyses were done in SPM8 (http://www.fil.ion.ucl.ac.uk/spm).

Results

Demographical, genotype, and tracer data are shown in table 1. Five participants in the active group were 5-HTTLPR homocygotes for the long allel (LL) whereof one was L_AL_G when genotyping the rs25531 A/G polymorphisms. Eight subjects were SL carriers and three were SS carriers (table 1). We observed a significant decrease in BMI within the intervention group (from 23.1 22.6 kg/m^2 to 22.6 kg/m^2, p=0.0001). We further saw a significant increase in cold dose given in the placebo group between baseline and rescan (from 1.40 µg to 2.14 µg, p=0.04). Even though this is within previously established [^{11}C]SB207145 dose limits (Madsen et al., 2011b), the paired design in this study is particularly sensitive to such a difference, and we therefore corrected all BP_{ND}s using the population-based ID_{50} (the injected dose estimated to saturate 50% of the receptors) calculated by Madsen et al (Madsen et al., 2011c).

We found a trend to a statistically significant association between a 3-week fluoxetine intervention and decreased in 5-HT_4 binding in striatum (p=0.098) but not in hippocampus (p=0.77) or neocortex (p=0.45) (table 2 and figure 1), and no significant changes were observed within the placebo group (all p-values were >0.30). Further, no significant differences was observed when the fluoxetine group was compared to the placebo group (all

p-values >0.27). However, when we subdivided the fluoxetine group based on the 5-HTTPLR status, we found a significant decrease in the 5-HT_4R binding in striatum in the L/L homocygotes (p=0.045) compared to the S carriers (p=0.48) (figure 2).

The relative test retest difference in the 16 placebo scans was -1.1% in striatum, -3.0% in hippocampus, and -1.4% in neocortex. The average test retest difference (SD of Δ%) was in 10.4% in striatum, 13.4% in hippocampus and 12.2% in neocortex. The ICC for the three regions was 0.78 for striatum, 0.31 for hippocampus, and 0.48 for neocortex.

Eight in the fluoxetine group versus nine in the placebo group reported no unusual symptoms at any contact in the intervention period. Seven participants in the active group versus five in the placebo group had a few (UKU scores < 5) symptoms such as nausea, insomnia, nervousness, and somnolence. One person in each group had an UKU score of > than 5 and beside the nonspecific symptoms also had sexual dysfunction (fluoxetine group UKU=10) and concentration problems (placebo group UKU score 8). None of the participants had any discontinuation symptoms.

In the 32 included participants S-fluoxetine and S-nor-fluoxetine were measured between the two scans (normalized to day 10) to 338.7 ± 137.0 nmol/l and S-norfluoxetine to 273.5 ± 72.0 nmol/l and immediately before the rescan S-fluoxetine to 495.2 ± 200.3 nmol/l and S-norfluoxetine to 451.5 ± 139.4 nmol/l. Non of the placebo treated participants had fluoxetine or nor-fluoxetine in serum.

Discussion

We used [^{11}C]SB207145 *in vivo* PET to study the effect of 3 weeks fluoxetine intervention on 5-HT_4R binding in healthy young men. Based on previous animal studies that showed that 5-

HT_4R levels show a monotonic response to 5-HT levels (Licht et al., 2009, Vidal et al., 2009, Jennings et al., 2011), we expected fluoxetine intervention to decrease 5-HT_4R binding. We only found a trend to such an effect when the considering all fluoxetine treated participants. However, when subdividing the fluoxetine treated participants based on the 5-HTTLPR status we found a significant decrease of striatal 5-HT_4R binding in the L/L homocygotes. The finding is consistent with our hypothesis, and that the L/L homocygote carriers have an elevated 5-HTT production and thereby a larger effect of fluoxetine intervention on the 5-HT levels leading to a greater decrease in 5-HT_4R levels relative to S carriers and our finding provide evidence for an effect of 5-HTTLPR status on serotonergic neurotransmission.

L/L carriers have been linked in humans to a higher 5-HTT levels in striatum (Praschak-Rieder et al., 2007, Kalbitzer et al., 2010) and in the midbrian (Reimold et al., 2007). In mice an elevated level of 5-HTT was found to effect the levels of the 5-HT_4R possibly due to the more efficient 5-HT uptake: Over-expression of the gene coding for the 5-HTT resulted in increased 5-HT_4R binding while homozygous knock-out of the gene coding for the 5-HTT resulted in a down-regulation of 5-HT_4R binding measured with [^{3}H]SB207145 (Jennings et al., 2011). L/L carriers was also found to have an enhanced effect of SSRI treatment and a study from Smits et al concluded that pretreatment genotyping of depressed patients may lead to a greater number of patients experiencing early remission (Smits et al., 2007). Recently we investigated the association between the 5-HTTLPR status and the levels of 5-HT_4R and the L/L carriers was found to have higher level of 5-HT_4R relatively to S carriers consistent with a higher level of the 5-HTT leads to a lower level of 5-HT leading to more 5-HT_4R (Fisher et al., 2012). Taken together these studies show that the significant effect of SSRI treatment in the L/L carriers in striatum may arise from 1) L/L carriers having a larger available pool of 5-HT_4R at baseline to facilitate a larger decrease in 5-HT_4R binding when

influenced by a raised 5-HT level due to SSRI treatment, and 2) SSRI treatment may induce a larger effect in L/L carriers due to their larger amount of 5-HTT than in S carriers.

Our finding that L/L carriers showed a decrease in 5-HT_4R binding is consistent with a model wherein decreased 5-HT_4R levels reflects increased 5-HT levels. It is, however, important to note this reflects an indirect evaluation 5-HT levels and 5-HT release in humans and of how 5-HTTLPR status may contribute to individual differences in 5-HT levels. Currently, there is no validated method available for quantifying *in vivo* 5-HT levels and 5-HT release in humans (Paterson et al., 2010) and the emergence of such a method (similar to displacement of dopamine D2 receptors with radiotracers such as [^{11}C]raclopride) could provide more direct evidence supporting our current findings.

We observed the significant association between L/L carriers and change in [^{11}C]SB207145 binding in striatum after 3 weeks of fluoxetine intervention, whereas we only found a trend to such an effect of fluoxetine intervention on the 5-HT_4R levels when including also the S carriers in striatum and no effect in all fluoxetine-treated individuals in hippocampus or neocortex despite previous animal study reporting such an effect. Several reasons might explain this translational inconsistency. Both the 5-HT_4R regulation and effects of the 5-HTTLPR polymorphism may differ on a cellular and/or regional level. Several reports show a specific effect of 5-HT_4R agonism on a cellular level in hippocampus modulating tissue specific functions in memory formation such as long-term potentiation and long-term depression (Kulla and Manahan-Vaughan, 2002, Kemp and Manahan-Vaughan, 2004, Marchetti et al., 2004). Moreover, in previous studies we used [^{11}C]SB207145 PET and linked regulation of the 5-HT_4R to memory function in hippocampus (Haahr et al., 2012a), appetite regulation in reward related areas such as nucleus accumbens (Haahr et al., 2012b), and the female gender to 5-HT_4R availability in the limbic system (Madsen et al., 2011a). It is however important to

note, that these associations may or may not arise from individual differences in the extracellular 5-HT level. Also the 5-HTTLPR polymorphism have a region specific effect on the 5-HTT levels. For example Praschak-Rieder et al. and Kalbitzer et al used [^{11}C]DASB PET and both found an increased level of the 5-HTT in L_AL_A carriers only in striatum lending an explanation of why our findings were restricted to striatum. As the *in vivo* binding of [^{11}C]SB207145 measured with PET cannot differentiate the type of neurons or the specific cellular location of the 5-HT_4R future studies aimed at understanding cell specific 5-HT_4R expression may provide insight into whether this is a possible source of our observed region-specific effects or if the finding in striatum was driven by the better signal-to-noise ratio implying larger statistical power associated with a more robust measure.

Another reason could be species differences between rodents and humans in the effect of SSRI on extracellular serotonin levels. Disparities between species that influence pharmacological sensitivity are observed in the serotonin system anatomy, receptor distribution and innervations (Lewis et al., 1986, Bruinvels et al., 1994). In mice chronic fluoxetine administration significantly increased the level of serotonin measured with microdialysis over the range of 7 to 28 days (Popa et al., 2010), however, in primates this effect was not observed in a similar microdialysis study measuring 5-HT content from 3 to 21 days: After an initial increase in extracellular serotonin the levels decreased towards baseline after 7 days (Smith et al., 2000).

The doses in all the mentioned animal studies were 10-30 fold greater than our human study. However, 40 mg/day used in this study is an effective clinical dose and the serum concentrations of fluoxetine and the active metabolite nor-fluoxetine were in our cohort within the same range as reported in depressive patients (Jannuzzi et al., 2002).

The binding potential estimated in this study is defined as $BP_{ND} = f_{ND} * B_{avail} * (1/K_D)$ and change

in BP_{ND} could possibly occur through changing affinity (K_D) with fluctuating 5-HT levels. This is not likely since the affinity of 5-HT to the 5-HT_4R is low with K_i values in the order of 0.1-1.2 μM (Paterson et al., 2010). In support of this, we showed in a previous publication that acute infusions of citalopram (leading to acute increases in interstitial 5-HT does not alter cerebral [^{11}C]SB207145 binding (Marner et al., 2010). Therefore, eventual differences in interstitial 5-HT will not affect the BP_{ND} through changing K_D.

Our method for quantification required the use of the cerebellum as a reference region for estimation of non-specific binding and free ligand concentration. There is a possibility that fluoxetine could induce differences in tracer kinetics for the cerebellum. Nevertheless, the proxe measure of the non-specific binding, the area under the cerebellar time–activity curves did not differ significantly in the two groups of volunteers before and after fluoxetine and placebo.

Even though all doses of SB207145 given in both groups were below the established tracer dose limits (Madsen et al., 2011c) we found a significant difference between baseline and rescans in the placebo group. To limit the effect of this difference on the BP_{ND} we chose the correct all BP_{ND}s using the ID_{50} obtained in the study by Madsen et al (Madsen et al., 2011b). However, analyzing the data without this correction did not change the results of the analysis (data not shown).

Conclusion

In conclusion, we find evidence for an effect of 5-HTTLPR status on serotonergic neurotransmission in that LL homocygotes show reduced 5-HT_4R levels if 5-HT levels are increased as opposed to S carriers. Our findings corroborate previous animal studies showing

a monotonic regulation of the 5-HT_4R relative to fluctuations in 5-HT levels and provide evidence for differential regulation of the serotonin system dependent on the 5-HTTLPR status.

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Table 1. Demographic, genotype, and tracer data. Active vs. placebo baseline data and within group differences (baseline vs. rescan) were compared with two-sample and paired t-tests respectively. There was no significant difference in baseline data. Between baseline and rescans there were significant differences in BMI in the fluoxetine group and in cold dose injected given in the placebo group.

	Intervention			
	Active		Placebo	
N	16		16	
Age (years)	25.7 ± 5.2		25.9 ± 3.9	
Education (years)	15.7 ± 1.9		15.8 ± 1.6	
Genotype - ss/sl/ll	3/8/5[1]		1/8/7[1]	
	Baseline	Rescan	Baseline	Rescan
BMI (kg/m2)	23.1 ± 1.9	22.6 ± 2.0[2]	23.4 ± 3.1	23.4 ± 3.0
Non-specific binding	106.4 ± 19.2	104.1 ± 16.3	110.0 ± 15.1	109.3 ± 18.5
Cold dose inj. (µg)	1.15 ± 0.90	1.28 ± 0.94	1.40 ± 0.65	2.14 ± 1.42[3]

Data is given as mean ± standard deviation. Cold dose inj. is the mass of SB207145 injected. Non-specific binding is the area under the time activity curve in cerebellum.
[1] One person was L_AL_G, [2] $p<0.001$, [3] $p=0.04$

Table 2. 5-HT_4R BP_{ND} in striatum, hippocampus and neocortex in the fluoxetine group (N=16).

	Striatum	Hippocampus	Neocortex
Baseline[1]	3.76 ± 0.39	1.15 ± 0.14	0.75 ± 0.20
Rescan[1]	3.61 ± 0.46	1.13 ± 0.14	0.77 ± 0.19
Mean change in % (95% CI)[2]	-3.60 (-9.95 to 2.76)	-1.03 (-7.28 to 5.2)	3.94 (-2.87 to 10.75)
Exact p[3]	0.098	0.77	0.45

[1] Mean BP_{ND} ± standard deviation, [2] BP_{ND} rescan/BP_{ND} baseline-1*100, [3] Wilcoxon Matched-Pairs Signed Rank Test. Striatum = Bilateral volume-weighted mean BP_{ND} of putamen and caudatus. CI = Confidence Interval

Figure 1. Upper panel shows an estimate of percent change in [^{11}C]SB207145 binding and the lower panel displays the baseline BP_{ND} versus the rescan BP_{ND} in striatum, hippocampus and neocortex in the fluoxetine and the placebo group. There was a trend to a significant decrease in striatum (p=0.098) and no significant effect in hippocampus (p=0.77) and neocortex (p=0.45).

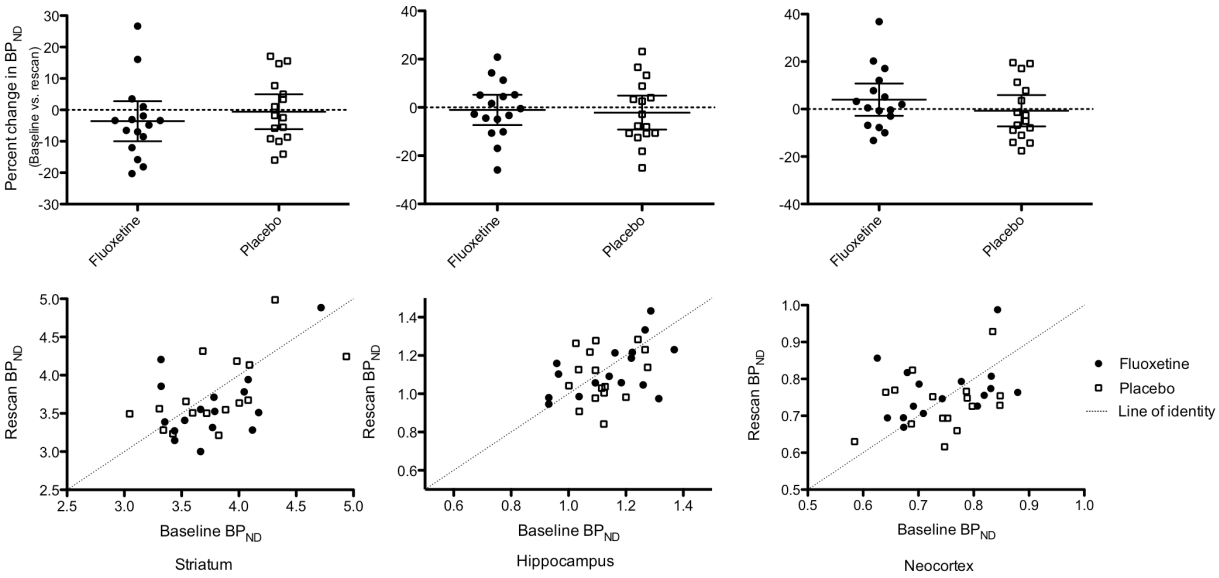
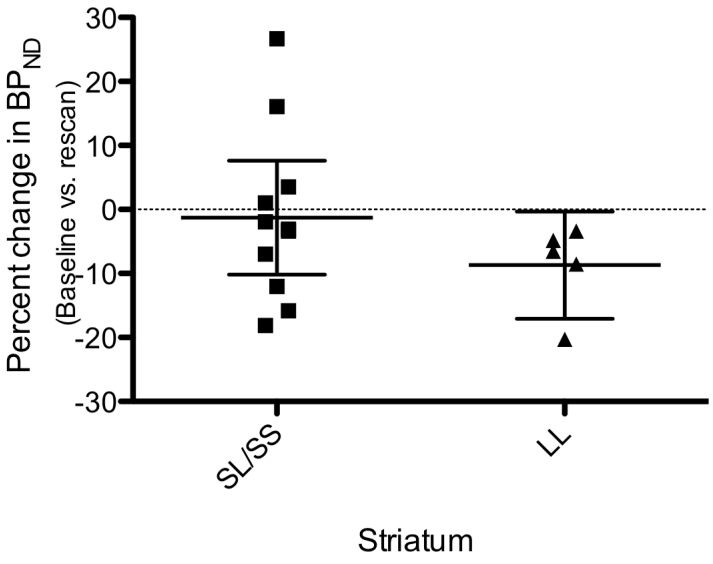


Figure 2. Estimate of the percent difference in the striatal [^{11}C]SB207145 binding when subdividing the fluoxetine group into S-carriers (N=11), LL homocygotes (N=5). The LL homocygotes show a significant decrease in 5-HT$_4$R binding, p=0.043, compare to the S carriers, p=0.48 (Wilcoxon Signed Rank Test).



One-sample t-test (LL group):
Mean change -8.7%
95% CI -17.1 to -0.3
p=0.045