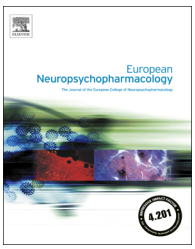
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Association of central serotonin transporter availability and body mass index in healthy Europeans



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Abstract
Serotonin-mediated mechanisms, in particular via the serotonin transporter (SERT), are thought to have an effect on food intake and play an important role in the pathophysiology of obesity. However, imaging studies that examined the correlation between body mass index (BMI) and SERT are sparse and provided contradictory results. The aim of this study was to further test the association between SERT and BMI in a large cohort of healthy subjects. *Methods*: 127 subjects of the ENC DAT database (58 females, age 52±18 years, range 20–83, BMI 25.2±3.8 kg/m^2, range 18.2–41.1) were analysed using region-of-interest (ROI) and voxel-based approaches to calculate [^{123}I]FP-CIT specific-to-nonspecific binding ratios (SBR) in the hypothalamus/thalamus and midbrain/brainstem as SERT-specific target regions. *Results*: In the voxel-based analysis, SERT availability and BMI were positively associated in the thalamus, but not in the midbrain. In the ROI-analysis, the interaction between gender and BMI showed a trend with higher correlation coefficient for men in the midbrain albeit not significant (0.033 SBR m^2/kg, p=0.1). *Conclusions*: The data are in agreement with previous PET findings of an altered central serotonergic tone depending on BMI, as a probable pathophysiologic mechanism in obesity, and should encourage further clinical studies in obesity targeting the serotonergic system.
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1. Introduction

Obesity rates have reached epidemic proportions worldwide, and might become the number one preventable public health threat for the 21st century (Sturm, 2002) with high socio-economic impact due to serious medical sequelae, e.g. an increase in type II diabetes mellitus. Despite rapid progress in identifying the social, environmental and genetic causes of overeating, the mechanisms by which these factors result in obesity are not resolved. Regarding the central mechanism thought to be relevant for obesity, the monoaminergic systems seem to play a pivotal role in reward processing (i.e. the dopaminergic pathways of the ventral tegmental area, nucleus accumbens, and frontal cortex) (Volkow et al., 2011), stress-mediation (in particular norepinephrine; Hainer et al., 2006), and the homoeostatic control of feeding. With respect to this, the modulation by serotonin of eating behaviour integrates homoeostatic and hedonic aspects as well as reward regulation at the intersection of the mesolimbic system, hypothalamus and brainstem (Hoebel, 1985). Changes in serotonergic functioning, as a main factor in the regulation of eating behaviour and energy balance, were shown in a variety of animal and clinical studies. Recently, a study in genetically engineered mice showed that knocking out the serotonin transporter (SERT) leads not only to hypophagia and hyperleptinemia but also to insulin resistance, hepatic steatosis, and obesity independent of food intake (Chen et al., 2012). Other studies on SERT knock-out mice also showed increased levels of abdominal fat and susceptibility to obesity (Homberg et al., 2010; Uçeyler et al., 2010). In addition, selectively bred polygenic obese rats had lower SERT binding when compared to polygenic diet-resistant rats (Ratner et al., 2012), whereas no change in SERT was seen in diet-induced obesity in outbred rats. This was not the case in a mouse model: diet resistant mice have lower SERT binding than diet-induced obese mice (Huang et al., 2004). Also, a recent imaging study showed that obesity is associated with high serotonin-4 receptor availability in the brain reward system (Haahr et al., 2012). Evidence for a serotonergic involvement in the pathophysiology of satiety and overeating also came from the efficacy of anorectic drugs. For example, sibutramine (Reductil) targeting the SERT as well as the norepinephrine transporter (NET) has an appetite-suppressing, anorexogenic effect (Hainer et al., 2006). Hence, both monoaminergic systems, and in particular the presynaptically located transporters, are likely to represent key biochemical substrates in the intrinsic control of eating, and their failure in function, or compensatory change in expression, are thought to underlie overeating.

Only few studies have been performed that applied single-photon emission computed tomography (SPECT) or positron emission tomography (PET) with radiotracers for the SERT to unravel altered SERT availability in vivo in obesity or that looked into the association between BMI and SERT. Talbot et al. (2010) reported on a PET study with the highly SERT-selective radiotracer [^{11}C]DASB, which was initiated to investigate mechanisms underlying the clinical efficacy of sibutramine. They found SERT occupancy, by clinical doses of sibutramine, of modest magnitude

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supporting the assumption that SERT inhibition may be necessary for sibutramine's antiobesity effect in humans, and suggested that the hypophagic effect requires instead the co-inhibition of both SERT and NET. Given that the serotonergic system is a tonic, modulatory network of fibres stemming from the midbrain raphe nuclei, one might speculate that changes in either the brainstem SERT or the SERT at nerve terminals (e.g., in the diencephalon) are altered when external stimuli disturb the homoeostasis to maintain the serotonergic tone. Obesity and the body mass index (BMI) as a marker for overweight might therefore be associated with a change in regional SERT availability in human. First in vivo [^{11}C]DASB PET studies on SERT revealed contradictory findings, either a positive or an inverse correlation between SERT availability and BMI (Hesse et al., 2009b; Erritzoe et al., 2010), respectively. These studies in healthy volunteers were hampered by a lack of a larger sample of subjects with higher BMI (>35 kg/m^2) so that the curves may have been driven by some outliers in the upper range. A role for the SERT in obesity and BMI is thus plausible, but not yet conclusively demonstrated.

The objective of this study is to analyse extrastriatal SERT binding in an unique European database of [^{123}I]FP-CIT SPECT scans of healthy volunteers to test for an association between SERT availability and BMI with a large BMI range. Recent studies showed that [^{123}I]FP-CIT does not only bind to the dopamine transporter (DAT) in vivo, but also to extrastriatal SERTs (Booij et al., 2007). Based on the mentioned preliminary SERT PET studies in conjunction with BMI, we hypothesised that BMI and SERT binding ratios are correlated in healthy volunteers.

2. Experimental procedures

This project was part of the collaborative European Association of Nuclear Medicine (EANM) Research Ltd. (EARL) initiative "European Database of [^{123}I]FP-CIT SPECT scans of healthy controls (ENC-DAT)", which started in 2007 and was successfully completed in 2010 (13 European institutions in 10 European countries recruiting 151 subjects) (Dickson et al., 2012; Varrone et al., 2013).

2.1. Subjects

The subjects were healthy volunteers fulfilling the inclusion and exclusion criteria as previously published (Varrone et al., 2013; van de Giessen et al., 2013). In brief, attendees had an age between 20 and 90 years, no evidence or history of neurological and psychiatric disorders as excluded by a neurologist, motor complaints as assessed with the Unified Parkinson's Disease Rating Scale (UPDRS), a Symptom Checklist-90-R (SCL-90-R) score <63 to ensure only minimal psychological problems, a Beck Depression Inventory (BDI) score under 9 points, and no evidence for cognitive impairment, as assessed with the Mini-mental state examination (MMSE). Subjects on medication known to affect DAT or SERT binding were not included in the study. Urine based screening tests for drug abuse was obtained in all subjects to exclude the use of illegal substances. They all had an MRI scan without significant diffuse or confluent white matter hyperintensities in T2-weighted images for exclusion of significant structural pathologies and for anatomical co-registration with SPECT data (see below).

All subjects underwent a general physical examination, including weight and height measurement for BMI calculation. The protocol was in accordance with the declaration of Helsinki and approved by the Medical Ethical Committees of all participating centres and all subjects provided written informed consent.

2.2. SPECT acquisition and data processing

Each subject underwent one SPECT scan starting 3–4 h after intravenous application of $\sim$185 MBq [^{123}I]FP-CIT (DaTSCAN; GE Healthcare), prior to which they received thyroid blockade (see Varrone et al. (2013) for more details of the scanning protocols). SERT specificity of diencephalon-midbrain [^{123}I]FP-CIT binding was shown in vivo in human by displacement studies using the selective serotonin reuptake inhibitor paroxetine (Booij et al., 2007; Ziebell et al., 2010). Technical data and standardized data for the SPECT cameras that were used in the trial acquisition were described elsewhere (Dickson et al., 2012; Tossici-Bolt et al., 2011). All scans were reconstructed by the core lab on HERMES (HERMES Medical Solution, Stockholm, Sweden) and corrected for attenuation and scatter in the core centre.

2.3. Region-of-interest (ROI) analysis

For ROI analysis, the SPECT data were transferred to a HERMES workstation and manually co-registered with the MRI scans by using the HERMES MultiModality software following a previously described procedure (Hesse et al., 2003, 2009a). Briefly, the individual MRI scan was reoriented towards the anterior-posterior commissure lines based on a normal standardized MRI. Then, the individual SPECT data were co-registered onto the realigned individual MRI in all three (x, y, and z) planes and the ROIs, which are atlas-based predefined in the normal standardized MRI, were adjusted to the individual anatomy. The uptake in each ROI with the highest mean count density in adjacent slices comprising the entire brain structure, i.e. the SERT-rich regions thalamus/hypothalamus (diencephalon) and midbrain/brainstem was determined (one continuous ROI from the thalamus level to the upper brainstem providing two peaks of mean count density, one at the thalamus/hypothalamus and one at the upper brainstem level, Figure 1). The occipital cortex was used as the reference region representing non-specific binding to calculate the specific-to-nonspecific binding ratio (SBR), which is the (activity in target-ROI divided by the activity in occipital cortex) minus 1.

Statistical analysis on the interaction between ROI (SBR) and BMI data considering age, gender, and scan start (after injection) was performed using either PASW/SPSS 20 (IBM, Armonck, NY) or the software R (http://www.r-project.org/, R Development Core Team, 2011), version 2.14.0, and is based on a linear model including camera-type as a factor, scan start, gender (Ruhé et al., 2009), age (Hesse et al., 2003), BMI (as continuous variables) and the higher terms gender $\times$ age and gender Note please retain the same space before multiplication symbol as given in the previous line $\times$ BMI. Terms are then dropped in a stepwise fashion based on Akaike's information criterion and the final model is assessed using a marginal t-statistic. p-Values of <0.05 were considered to be statistically significant.

2.4. Voxel-based analysis

Voxel-based analysis was additionally applied using SPM8 running on MATLAB 7.5 for Windows (MathWorks, Natick, MA) (Figure 2). The procedure has been described in detail elsewhere (van de Giessen et al., 2013). To obtain individual parametric maps of SERT SBR, the uptake in the occipital cortex was extracted for each scan and SBR calculated for the full brain by (activity per voxel divided by the activity in occipital cortex) minus 1. The ROI for the occipital cortex was drawn manually on the mean scan in ITK-SNAP (version 2.1, PICSL, University of Pennsylvania). A regression analysis was performed in SPM8 with the SBR maps as dependent variable, BMI as independent variable and age, gender, scan time (3 or 4 h post-injection), and camera-type as covariates. Accordingly, comparison of images between groups (BMI ≤ 25 kg/m^2 versus BMI >25 kg/m^2 and BMI ≤ 25 kg/m^2 versus BMI ≥ 30 kg/m^2) was done with ANCOVA with SBR maps as dependent variable, BMI group as independent variable and age, gender, scan time,

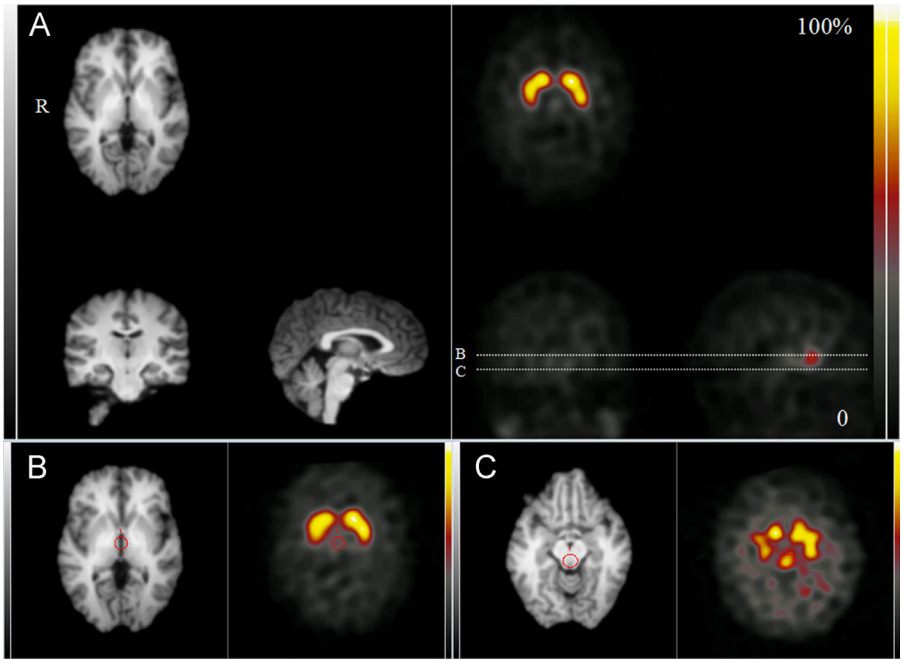


Figure 1 Co-registration of the SPECT to the individual MRI data for region-of-interest (ROI) analysis with HERMES MultiModality using mutual information algorithm and manually adapted realignment in three-dimensions (A). The dotted lines display the levels of axial slices. Bottom row illustrates co-registered data side-by-side with the target ROI (1) at the level of the hypothalamus (B) and at the midbrain level (C) indicating two peaks of mean count density within the one continuous placed region.

and camera-type as covariates. All analyses were confined to either diencephalon or brainstem using explicit masking with the target ROI drawn on the mean scan using intensity thresholding in ITK-SNAP (Figure 2). p-Values <0.05, family-wise error corrected (FWE corr), were considered significant.

3. Results

The final sample eligible for this ENC DAT sub-study (good image quality, full scatter windows, no truncation of data, i.e. scanning of the entire midbrain or occipital cortex) consisted of 127 subjects (58 female) with a mean age of 52.3 ± 18.3 years (range 20–83) and mean BMI of $25.2 \pm 3.8\ kg/m^2$ (range 18.2–41.1). We did not include one male subject for the entire analysis, because midbrain ROI partly fell outside the scanned part of the brain (as it was registered on the mean brain), which would have affected the voxel-based analysis. In the stratified groups, age differed between individuals with BMI $\leq 25\ kg/m^2$ ($n=66$; 47.5 ± 19.7 years) and BMI $>25\ kg/m^2$ ($n=61$; 57.4 ± 15.3 years; $p=0.001$) or BMI $\geq 30\ kg/m^2$ ($n=12$; 56.7 ± 15.7 years; $p=0.046$). SBR in the diencephalon and midbrain did not differ between the groups that were assessed 3–4 h post-injection ($p=0.63$ for the thalamus, and $p=0.98$ for the midbrain, data not shown in detail).

3.1. ROI analysis

The analysis of the diencephalon SBR data shows that the effect of different cameras is large, which resulted in a correction for Infinia camera (GE Healthcare, Fairfield, CT) of 0.5 ($p=0.0006$, 95% CI [0.2, 0.8]) and for the Varicam camera (GE Healthcare, Fairfield, CT) of -0.4 ($p=0.009$, 95% CI [-0.8, -0.1]) as part of the linear model.

Age was found to be negatively associated with a coefficient of -0.006 SBR/year ($p=0.02$, 95% CI [-0.010, -0.001]). The age dependence was almost entirely driven by two data points, however, and the p-value changed to 0.08 and 0.1 after removing these two points (see Figure 3). BMI and SBR of the diencephalon showed no significant association ($p=0.1$ for the full model), especially after removing the two aforementioned outliers ($p=0.8$).

The SBR for the midbrain also showed strong effects for the same two cameras with the correction of 0.37 for Infinia ($p=0.009$, 95% CI [0.1, 0.6]) and -0.33 for Varicam ($p=0.03$, 95% CI [-0.6, -0.03]). There was a weak and non-significant association with age with a coefficient of -0.004 SBR/year ($p=0.1$), whereas BMI showed little evidence for an association ($p=0.6$) although the interaction in the full model between gender and BMI showed a slight trend, whereby men's SBR values increase more quickly with BMI for men than for women (coefficient for men is: $0.033\ SBR\ m^2/kg$, $p=0.1$ and zero for women by construction), see Figure 4 for an illustration using linear regression.

3.2. Voxel-based analysis

There was no significant correlation with BMI and midbrain SERT, or with age and gender. But in the thalamus, a positive correlation between BMI and thalamus SERT in a cluster of 6 voxels (FWE corr, maximum t-value in cluster (max t) $=3.79$, $p=0.020$) (Figure 5), and a negative correlation between age and thalamus SERT in a cluster of 42 voxels (FWE corr, max $t=5.38$, $p=0.002$) were found. Comparing

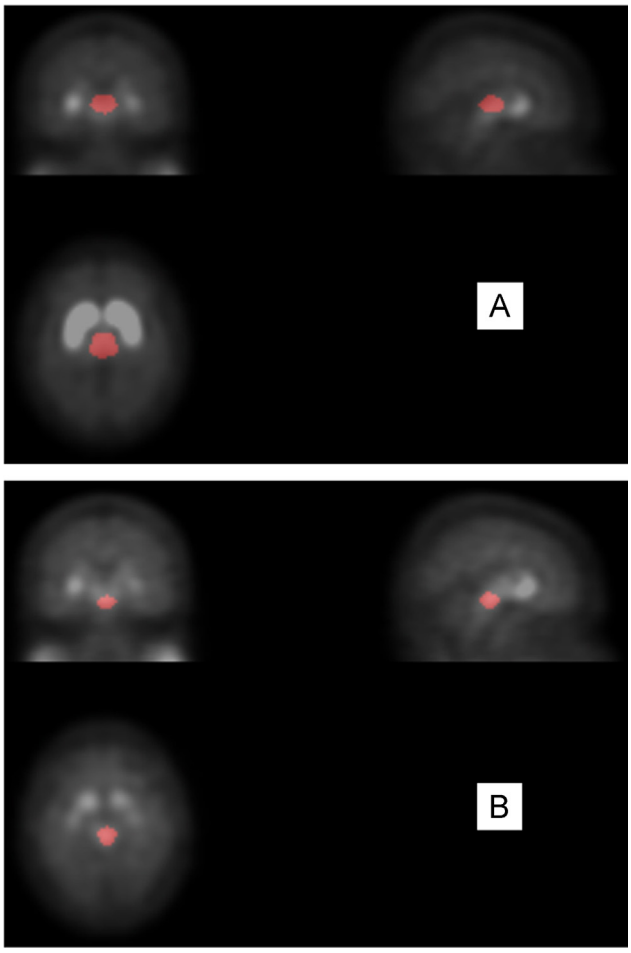


Figure 2 Mean scan with thalamus mask (A) and the midbrain mask (B) for voxel-based SPM analysis.

subjects with BMI ≤ 25 kg/m^2 and subjects with BMI >25 kg/m^2 there was no group difference in midbrain binding. Comparable results were found for the group comparison between subjects with BMI ≤ 25 kg/m^2 and subjects with BMI ≥ 30 kg/m^2, again there was no group difference. In the thalamus, binding is higher in the BMI >25 kg/m^2 group than in BMI ≤ 25 group (cluster size: 11 voxels, FWE-corr, max $t=4.07$, $p=0.009$), and age and thalamus binding did show a negative correlation (38 voxels, FWE-corr, max $t=5.23$, $p=0.001$). For BMI ≥ 30 kg/m^2 versus BMI ≤ 25 kg/m^2, the SERT binding is higher in the ≥ 30 kg/m^2 group in the thalamus (3 voxels, FWE-corr, max $t=3.76$, $p=0.025$) and again a negative correlation between age and binding (29 voxels, FWE-corr, max $t=4.69$, $p=0.002$) was revealed.

4. Discussion

In vivo human data of SERT availability in obesity or its correlation with BMI are still sparse and rather contradictory. Since SERT represent a major target of anorectic pharmacotherapy as mentioned above, studies were encouraged to clarify whether there is an association between SERT availability and BMI as a marker of obesity. The main finding of this study was that a firm result regarding the association between SERT availability and BMI was not

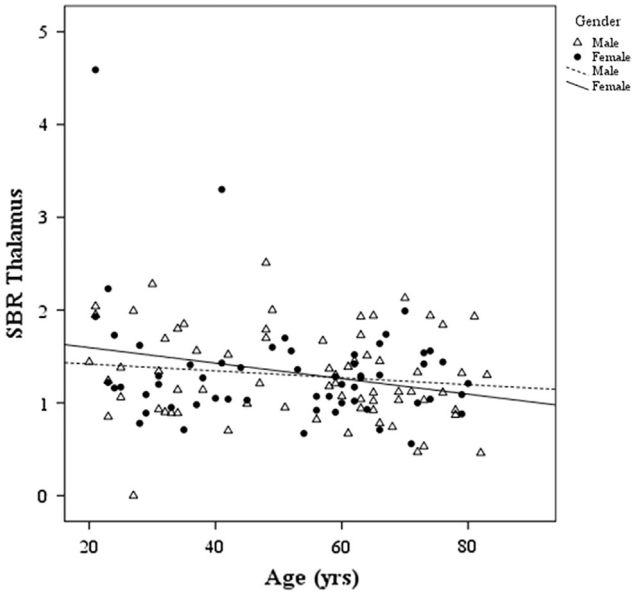


Figure 3 Negative correlation between SERT availability and age in the thalamus/hypothalamus. The line represents the fit for all values in female and the dotted line the fit for all male values. Note the spread of values reducing R^2 (0.06 in female, 0.02 in male subjects) and the two outliers as mentioned in the text.

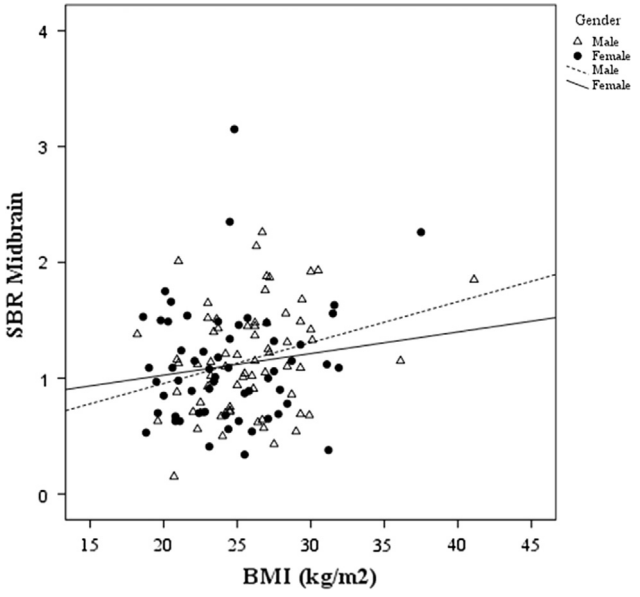


Figure 4 Correlation between SERT availability and BMI in the midbrain. The line represents the fit for all values in female and the dotted line the fit for all male values. Note the spread of values reducing R^2 (0.02 in female, 0.08 in male subjects).

obtained by the study data. However, the results of both voxel-based and ROI-analysis gave some clues for future research on the relationship between BMI and the brain serotonin system. In particular, voxel-based analysis indicates a positive correlation between SERT and BMI in the thalamus. In the ROI analysis the interaction between gender and BMI showed a trend with a higher coefficient

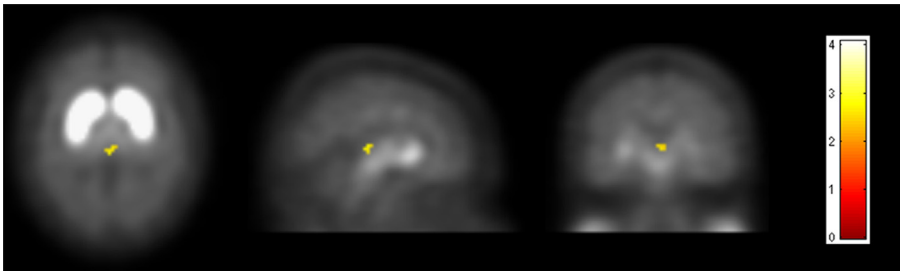


Figure 5 SPM analysis demonstrating the correlation between thalamic uptake indicating SERT availability and body-mass-index (BMI).

for men but this was of weaker significance compared with the voxel-based results.

These results are in agreement with previous findings of a positive correlation between BMI and SERT availability in a [^{123}I]nor-β-CIT SPECT study in monozygotic twin pairs with acquired obesity (Koskela et al., 2008). It is interesting that this study also reported a significant effect in the thalamus but not in the midbrain. Like [^{123}I]FP-CIT, [^{123}I]nor-β-CIT binds in the striatum predominantly to the DATs, but in extrastriatal areas to the SERT, so, these radiotracers have comparable properties. Our present observations with this non-selective SPECT radiotracer are also in line with our own preliminary data of a positive correlation between SERT availability and BMI, measured with the SERT-selective [^{11}C] DASB (Hesse et al., 2009b). Contrarily, the study by Erritzoe et al. (2010) indicated a negative association between BMI, also in subcortical brain areas. The main reason for this discrepancy may be the low numbers of obese subjects in all studies, which makes single data points highly influential. In obesity, however, alterations of the presynaptic serotonergic function, i.e. the SERT, and changes of serotonergic tone were observed in recent animal studies, not only in the brain (Ratner et al., 2012) but also in the gut (Bertrand et al., 2012). For example, the study of Huang et al. (2004) showed increased SERT in diet-induced obesity, but the results of small laboratory animal studies are not consistent, even in the direction of the changes in SERT expression (Homberg et al., 2010; Uçeyler et al., 2010).

From a pathophysiological point of view, higher SERT availability at higher BMI theoretically indicates a higher SERT recruitment in healthy persons, most likely due to changes in extracellular serotonin. This serotonin imbalance either due to food overload or overactive reward and homoeostatic circuits (stress-induced) may lead to higher serotonin recruitment as well, and high SERT can also be a compensatory upregulation in the case of high serotonin levels (Ramamoorthy et al., 2011). Nevertheless, at this moment we do not know whether higher SERT availability can be a compensatory mechanism to chronic lower or higher extracellular serotonin concentrations. Indeed, (sub) acute lowering did not induce changes in SERT binding in humans (Praschak-Rieder et al., 2005), but this does not exclude that chronic changes in serotonin concentration may influence SERT binding. For studying the role of serotonin in obesity, it may be of interest to develop tools to assess extracellular serotonin concentrations, but this approach has not been successful yet (Pinborg et al., 2012). Low serotonin levels are associated with hyperphagia and weight gain (Lam et al., 2010). So, it can also be hypothesised that high SERT availability is a susceptibility for high BMI, as high SERT concentration leads to low synaptic serotonin levels and thus to hyperphagia. Since serotonin is a tonic, modulatory network and the major mode of transmission for serotonin in the brain is volume transmission with widespread serotonergic innervation from the raphe nuclei, SERT availability is not necessarily simply correlated with either downregulation or upregulation of the presynatic serotonin concentration. One can rather assume that input from internal and external sources differentially activate serotonergic tone in obese versus lean controls.

It is speculative to which part of the thalamus the presently observed significant small area in the thalamus belongs. This particular area, however, seems to involve the more midline part of the thalamus (the paraventricular thalamus, the pulvinar) rather than the hypothalamus. Interestingly, this part of the thalamus is responsible for the control response to chronic stress mediated by the serotonergic system and consequently the expression of SERT may play a role (reviewed by Price and Drevets (2010)). As the study by Koskela et al. (2008) did apply ROI analysis and not voxel-based analysis, it is unknown whether the reported effect in the thalamus was located in the same thalamic region in that study. Because we find this effect only in the voxel-based analysis in clusters that are not very large, future studies are necessary to replicate this finding.

For the interpretation of the present results, however, some drawbacks and limitations have to be mentioned. To overcome the fact that the delineation of the SERT target areas in the diencephalon and midbrain (raphe) is difficult because they are small, and also the specific-to-nonspecific binding ratios in these areas are not that high, we used different (independent) approaches to analyse the data which are either voxel-based (without MRI co-registration) or based on anatomically re-aligned SPECT scans. With both methods the tendency to higher values at higher BMI was shown in SERT-rich brain areas although the regions differed depending on the method used. One reason for the discrepancy might be that the ROIs were including the whole target structure, while the voxel-based analysis provided significant clusters only in a small volume of the thalamus. So, FWE correction allows considering significant clusters of 3–6 voxels, which is at the border of the spatial resolution of gamma cameras. Such explanation is more likely than that different equilibrium conditions in the thalamus and midbrain might have influenced the study results. In a recent

study in healthy controls, we showed that specific-to-nonspecific [^{123}I]FP-CIT binding ratios in the midbrain and diencephalon were significantly higher 2 h compared to 1 h after injection and remained stable between 2 and 3 h after injection (Koopman et al., 2012). Consequently, 3 h after injection is a reasonable time-point to assess extrastriatal SERT binding with [^{123}I]FP-CIT SPECT in vivo, although it has not been formally tested if this ratio is also stable up to 4 h p.i. As a fact, the binding behaviour of [^{123}I]FP-CIT in the diencephalon–brainstem is slightly different from that in the striatum (see van de Giessen et al. (2013)) representing mainly DAT Although radiotracers derived chemically from cocaine like [^{123}I]FP-CIT and [^{99m}Tc]TRODAT are frequently used to assess striatal DAT binding e.g., in studies on obesity (van de Giessen et al., 2013; Chen et al., 2008), SERT findings obtained with non-selective radiotracers should be confirmed by studies using selective SERT tracers to further disentangle the obese phenotype and underlying monoaminergic mechanism.

Another limitation of this study is the age difference between the BMI groups. We therefore added age as a covariate to all analyses. Furthermore, BMI was positively correlated with SERT availability in the thalamus. Because age was negatively correlated with SERT availability and BMI and age are negatively associated with each other, it is unlikely that the presently reported positive association between BMI and thalamus SERT availability was actually due to age differences between groups.

Gender differences are also believed to play a major role in regulating body-weight, as suggested by recent imaging data on brain structure as well as performance (Horstmann et al., 2011) and they may affect SERT/brainstem monoamine transporter availability (Staley et al., 2001), but the mechanisms (e.g. neuroendocrine effects) are not fully understood yet. Nevertheless, gender was added as a covariate to all analyses as well. In the ROI analysis, however, the effect of gender was small.

Furthermore, the trend for a gender $\times$ BMI interaction effect on SERT binding in the thalamus in the ROI analysis should be interpreted with caution, as the effect size was small.

Genetic contribution to SERT binding in obesity is of interest, since the SERT promoter polymorphism might be related to obesity (Fuemmeler et al., 2008; Lan et al., 2009; Sookoian et al., 2008). Further, hypermethylation of the SERT promoter region is associated with obesity (Zhao et al., 2013). For this study, no genetic data were available, but it would be very interesting to incorporate this in future studies. These studies shall also include psychological, stress-related and social variables, which all might correlate with SERT outcome measures and hence should be controlled for in the analyses. Last but not least as is the case in all the available in vivo SERT PET and [^{123}I]FP-CIT imaging studies, the ENC-DAT study was also hampered by the limited number of heavily obese subjects, which as mentioned in every case seem to be the crucial data in determining the fitting curve.

In conclusion, the data are in agreement with previous PET findings of an altered central serotonergic tone depending on BMI, as a probable pathophysiologic mechanism in obesity, and should encourage further clinical studies in obesity targeting the serotonergic system.

Role of funding source

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Contributors

The study was a sub-project of the multicentric ENC-DAT trial. Each site contributed to this analysis, and the principal investigators of each site are mentioned. Swen Hesse and Elsmarieke van de Giessen performed the ROI analysis and the voxel-wise analysis, respectively. They wrote the first draft of the manuscript. David Petroff, Karsten Winter, and Franziska Zientek helped with the statistical analyses and graphical representations.

Conflict of interest

Jan Booij is a consultant of GE Healthcare. Swen Hesse and Osama Sabri received honoraria and travel grants from GE Healthcare. The other authors declared no conflict of interest.

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