

RESEARCH ARTICLE

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The expression of platelet serotonin transporter (SERT) in human obesity

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Abstract

Background: Serotonin (5-HT) is a well-known modulator of eating behavior. However, the molecular mechanisms linking its action to body weight balance have been only partially elucidated. Since platelets are a suitable peripheral model to study 5-HT transport, metabolism and release, we herein evaluated the expression of the platelet 5-HT re-uptake system (SERT) by [³H]-paroxetine binding assay. A cohort of 114 unrelated individuals (34 males, 80 females; age, mean ± SD: 38.57 ± 12.47 years) without major psychiatric disorders, was recruited following a naturalistic design regarding age or gender and classified accordingly to their body mass index (BMI). Subjects were divided into 5 groups: normal-weight (NW), overweight (OW) and grade I-III obese (OB) individuals. For gender analyses, data were transformed into [³H]-paroxetine density (B_{max})/BMI ratios to overcome both the disparity of women vs. men number and anthropometric differences between sexes.

Results: [³H]-paroxetine B_{max} (SERT density, fmol/mg proteins) was reduced in platelet membranes of grade II ($p < 0.01$) and III ($p < 0.001$) obese subjects vs. controls and in overweight subjects ($p < 0.05$) vs. grade III obese individuals. Considering all patients together, a strong negative correlation between B_{max} and BMI ($r = -0.449$; $P < 0.0001$) was demonstrated. Conversely, [³H]-paroxetine K_D (dissociation constant, nM) did not differ among groups. No gender-related variation concerning B_{max} /BMI ratios was observed in this cohort of subjects.

Conclusions: The down-regulation of SERT in platelet membranes of severe human obesity ($BMI > 35 \text{ Kg/m}^2$) confirms the involvement of 5-HT system in body weight gain. Moreover, this findings may help to elucidate those monoamine-endocrine networks acting on fat storage, adipocyte signaling and energy balance. Targeting 5-HT/5-HT-related markers will possibly uncover the existence of human obesity subtypes.

Keywords: Human obesity, SERT expression, [³H]-paroxetine binding, Platelets

Background

Among neurotransmitters linked to appetite control, serotonin (5-HT) has a particular role: this endogenous amine is tightly involved in the regulation of feeding behavior at hypothalamic level, acting within the ventromedial and lateral nuclei [1-3]. In fact, the activity of 5-HTergic raphe and hypothalamic neurons is influenced by meal macronutrient composition and insulin secretion, as suggested by the findings that the tryptophan/large neutral amino acids concentration ratio (Trp:LNAAs) in plasma

(an index of Trp availability to brain uptake) and 5-HT synthesis are both increased after a carbohydrate-rich meal [4-8]. The same concept applies to protein-rich meals or meals containing proteins with high tryptophan content (e.g. α -lactalbumin) [9], demonstrating the impact of diet upon tryptophan uptake, 5-HT production and synaptic release. On the other side, glucocorticoid response influences monoamine/5-HT transmission and receptor function in the central nervous system (CNS), thus affecting feeding behavior and macronutrient choice [10-13]. These observations clearly suggest a link between stress-response, 5-HT function, weight gain and obesity. Several studies indicate that obesity has, in most cases, a polygenic background [14-16]. Among others, genes coding for

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proteins involved in 5-HT system such as the 5-HT transporter (SERT or 5-HT-T) [17-21], carriers for neutral amino acids (including tryptophan) [22] and 5-HT receptor subtypes [23-28] appear to be functionally relevant in either animal or human obesity. From diet studies conducted in rodents and humans, the interest at targeting specific 5-HT sites and, in particular, SERT [29,30] is strongly increased. Structurally, SERT is a glycoprotein belonging to the super-family of membrane-bound NaCl-dependent neurotransmitter transporters, characterized by 12 putative membrane spanning domains: it promotes 5-HT clearance (re-uptake) from the extracellular milieu and modifies the sensitization state of 5-HT receptors within the nervous system or non-neural districts (gut, platelets, lymphomonocytes) [31,32]. It is a pharmacologically active site, the target of re-uptake inhibitors as tricyclic antidepressants (TCA) and Selective Serotonin Reuptake Inhibitors (SSRIs) or 5-HT releasers like fenfluramine and 3,4-methylenedioxy-N-methamphetamine (MDMA) [33]. Both SERT expression and 5-HT uptake function are finely tuned by protein-kinases activities and gene transcription which control, following cell necessities, conformational changes of the membrane-bound SERT protein and/or the degree of SERT partition between cytoskeleton and plasma membrane [34-37].

Data on SERT expression/affinity in peripheral districts of overweight/obese subjects are currently not available. Platelets are a valuable peripheral model that mimics 5-HT transport, metabolism and release in the CNS, since they have been characterized for many years as a surrogate of impaired 5-HT activity in subjects with psychiatric disorders, eating behavior and ageing [38-44]. Therefore, the present study aimed to evaluate human platelets SERT number or affinity according to different categories of body mass index (BMI) or genders.

Results

Subjects' groups

As shown in Table 1, 114 individuals were recruited in the study and divided into 5 main BMI groups: 28 normal weight subjects (NWs), 18 overweight (OWs), 17 class I obese (OB-Is), 19 class II obese (OB-IIs) and 32 class III obese (OB-IIIs) individuals. ANOVA analysis showed

a significant difference among BMIs of the groups ($p < 0.0001$), without noticeable age variations ($p > 0.05$).

[³H]-paroxetine binding experiments

Equilibrium saturation and Scatchard analysis of [³H]-paroxetine specific binding showed a single population of high-affinity recognition sites in platelet membranes from all the subjects under investigation, clearly indicating the labeling of a single protein. The specific binding was about 90% of total binding at the K_D concentration. The [³H]-paroxetine B_{max} (fmol/mg protein) values, corresponding to SERT expression in platelet membranes, were: 1311 ± 51.29 (min.-max: 767-1795) in NWs; 1215 ± 59.44 (min.-max: 665-1685) in OWs; 1137 ± 70.36 (min.-max: 700-1700) in OB-Is; 986.4 ± 89.73 (min.-max: 344-1675) in OB-IIs; 906.8 ± 58.51 (min.-max: 336-1737) in OB-IIIs. The [³H]-paroxetine K_D values (nM), corresponding to the SERT protein affinity state for the specific ligand, were: 0.092 ± 0.009 (min.-max: 0.028-0.20) in NWs; 0.073 ± 0.0085 (min.-max: 0.025-0.16) in OWs; 0.076 ± 0.009 (min.-max: 0.03-0.15) in OB-Is; 0.085 ± 0.009 (min.-max: 0.038-0.19) in OB-IIs; 0.077 ± 0.007 (min.-max: 0.025-0.22) in OB-IIIs. Individual results for [³H]-paroxetine B_{max} and K_D , obtained from the 5 BMI groups of subjects, are reported in Figure 1(a,b). ANOVA analysis showed a significant difference between the [³H]-paroxetine B_{max} means of the 5 BMI groups ($p < 0.0001$); after the *post-hoc* Bonferroni correction test, B_{max} mean values were significantly reduced in OB subjects class II-III (BMI $> 35 \text{ kg/m}^2$) vs. NWs ($p < 0.01$ and $p < 0.001$, respectively) (Figure 1a); B_{max} values were also decreased in OB-IIIs respect to OWs ($p < 0.05$) (Figure 1a).

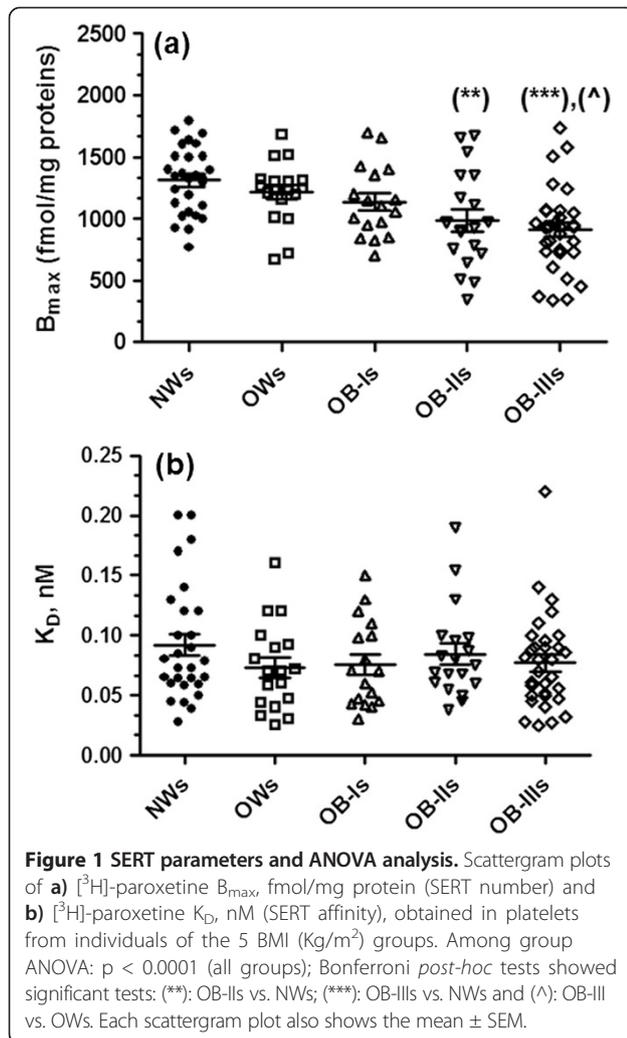
Correlation analyses and gender impact

Among-groups differences in SERT expression were additionally sustained by the significant negative correlation between [³H]-paroxetine B_{max} and BMI both in the whole cohort ($r = -0.449$, $p < 0.0001$; Figure 2a) and by gender sub-analysis in women ($r = -0.4178$; $p = 0.0001$; Figure 3a) and men ($r = -0.52$; $p = 0.0017$; Figure 3b). No significant gender related differences in subjects' variables (Table 2), as well as in B_{max} /BMI ratio (fmol m^2 /mg Kg) were found ($p = ns$), (Figure 4). The B_{max} /BMI ratio was: 39.12 ± 3.11

Table 1 The 5 groups of BMIs

	Controls (NWs, n = 28)	Overweight (OWs, n = 18)	Obese I (OB-Is, n = 17)	Obese II (OB-IIs, n = 19)	Obese III (OB-IIIs, n = 32)
Age (y)	35.11 ± 2.24 (20.0-61.0)	39.42 ± 3.10 (21.0-59.0)	39.76 ± 3.22 (16.0-63.0)	36.16 ± 2.92 (20.0-61.0)	41.94 ± 2.06 (22.0-59.0)
BMI (Kg/m ²)	21.39 ± 0.39 (18.3-25)	27.07 ± 0.26 (***) (25.4-28.8)	32.67 ± 0.30 (***) (30.1-34.7)	37.52 ± 0.34 (***) (35.5-39.8)	46.34 ± 0.67 (***) (40.0-54.8)

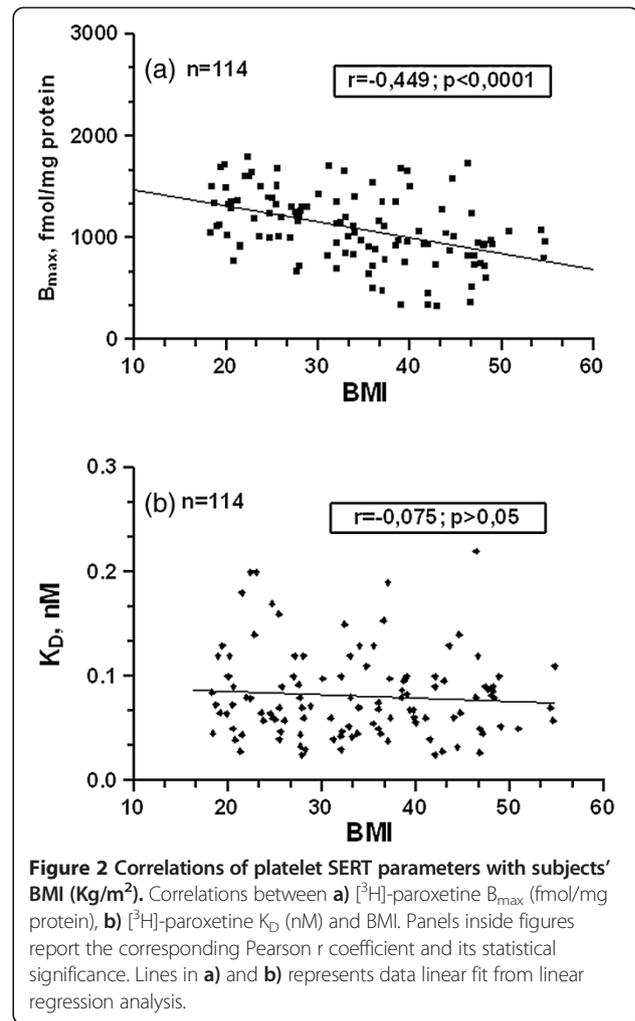
Data are presented as mean \pm S.E.M.; in parenthesis sample ranges, minimum and maximum values are shown. ANOVA BMI and *post-hoc* Bonferroni test: (***) $p < 0.001$, NWs vs. OWs, OB-I/II/IIIs.



(min.-max: 9.33-74.80) in men and 36.53 ± 2.21 (min.-max:7.92-87.06) in women. No significant variation was reported in SERT affinity (K_D) among the BMI based groups (Figures 1b and 2b).

Discussion

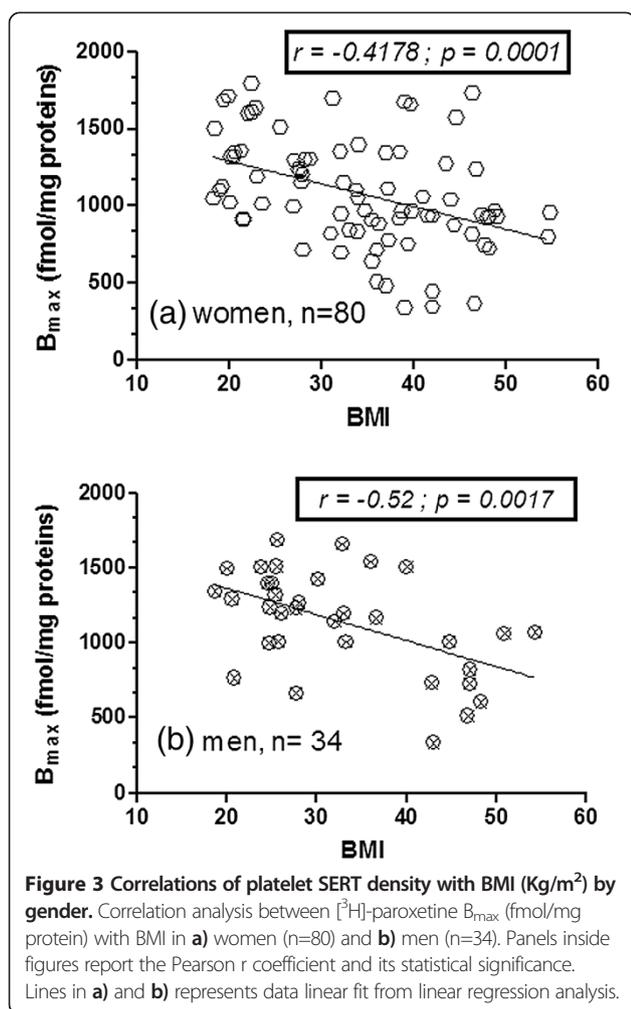
Serotonin (5-HT), primarily produced in CNS raphe nuclei and gut, plays a wide-ranging modulatory role at the level of several homeostatic responses. In particular, CNS 5-HT regulates many amongst the main individual adaptive-relational abilities to react to environmental changes, such as feeding behavior, thermoregulation, motor activity, libido, cognition, impulsivity, aggressiveness, nociception and mood. Besides, 5-HT also acts on peripheral tissues and organs, modulating the immune and flogistic responses, as well as blood stem cells differentiation, hemodynamic function and intestinal peristalsis [45]. Despite 5-HT has been extensively studied in recent years, the link between the expression of 5-HT transporter



(SERT), the pivotal protein regulating its extra- and intracellular concentrations, and human obesity has been supported by few studies. By single-photon emission tomography (SPECT) analysis in midbrain areas of obese women affected by binge eating disorder (BED), a reduction in SERT density has been reported [46], and this reduction was rescued by SSRI therapy [47].

A more recent *in vivo* PET study, using a iodinate tracer ([¹²³I]-nor-β-CIT) in midbrain areas of monozygotic twins, has shown a higher SERT density in co-twins with higher BMI [48]. The latter study was conducted in the Finnish population, (presenting a reduced genetic variance than other human ethnic groups) and selected twins were prevalently women.

Conversely, other PET investigations on unrelated healthy volunteers using a different SERT ligand ([¹¹C]-DASB), have shown a negative correlation between cerebral SERT expression and BMI [49,50]. Our study clearly demonstrates a reduced SERT number in platelet membranes of severely obese subjects (> 35 kg/m²) and a negative correlation between platelet SERT B_{max} and BMI

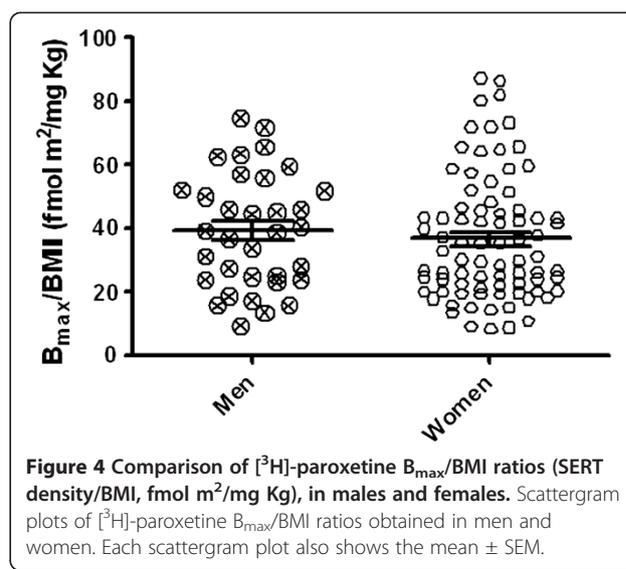


in human obesity. Instead, the lack of significant changes in the SERT affinity parameter K_D suggests a comparable SERT protein conformation in lean and obese individuals. All these studies substantiate the link between 5-HT activity, SERT expression and weight gain, but discrepancies are present. An explanation of this discrepancy can be found putting all these data in the context of SERT regulatory pathways.

Table 2 Gender effect on subject's variables

	Men, $n = 34$	Women, $n = 80$
Age (years)	37.71 ± 2.28 (20.00-63.00)	38.94 ± 1.36 (15.00-61.00)
BMI (kg/m^2)	32.74 ± 1.74 (18.71-54.30)	34.05 ± 1.11 (18.30-54.80)
[^3H]-paroxetine B_{max} (fmol/mg protein)	1142 ± 58.31 (336-1685)	1085 ± 39.02 (344-1795)
[^3H]-paroxetine K_D (nM)	0.071 ± 0.006 (0.028-0.170)	0.085 ± 0.005 (0.025-0.220)

Data are presented as mean \pm S.E.M. and ranges (minimum and maximum values).



As introduced before, protein SERT expression is a model of “fine-tuned” regulation of membrane-bound proteins. Beside undergoing a short-term up and down-regulation, SERT presence in cell membranes can be long-term modulated through positive and negative signals, allowing long-lasting cell adaptation to the extracellular content of 5-HT or other related stimuli. The balance between the converging short and long-term regulatory pathways of SERT defines its expression and affinity states during developmental stages, under physiological and pathological conditions.

We have previously shown that SERT protein expression in platelets (in plasma membrane and intracellular pools) is regulated by megakaryoblast cell differentiation processes [51]. We have also reported an up-regulated translocator protein TSPO expression in discrete brain regions of *ob/ob* mice, without appreciable changes in SERT number either in the brain or in platelets [52]. Since leptin has been found to down-regulate SERT expression [53], we hypothesized that *ob/ob* animals, during their development, can modulate SERT expression through the activation of alternative regulatory pathways, without excluding modified SERT reserve and 5-HT responsiveness. In the present study, a reduced platelet SERT in severe obese subjects (grade II and III) has been shown. This finding mirrors at the peripheral levels what previously reported in the brain [50]. In contrast to mutant leptin-lacking *ob/ob* mice, a link between human obesity, often associated with high serum leptin [54,55] and SERT regulatory cascades leading to its reduction or internalization can be hypothesized. The implications of regulatory mechanisms on reduced SERT expression in obesity is indirectly supported by studies conducted on double knockout SERT(-/-)/brain derived neurotrophic factor (BDNF) (+/-) mice [56,57] revealing the regulatory role

of either other monoamine protein markers or trophic factors on 5-HT physiology and activity on body weight balance. Nevertheless, currently, a clear explanation for the lower SERT expression found in platelets of severe obese individuals is lacking. Platelet 5-HT can be part of a network involving adipokines, cytokines and inflammatory responses [58]. This is supported by the report of adipocytes expressing 5-HT receptor subtypes [59] and, more recently, even SERT [60], suggesting that adipose tissue and 5-HT system interact with each other. It is possible that the reduced SERT expression is due to impaired 5-HT synthesis and activity in obese subjects [3,61], as reported for neurotic behaviors and personality traits, and that altered SERT/5-HT receptors and/or SERT regulation underscore obesity. In this study, none of the recruited subjects had a present or past history of a major psychiatric disorder, but some of them could present personality traits that could be possibly linked to susceptibility to obesity [57,62]. On the other side, imbalanced appetite hormones, adipokines or gut hormones could counter-regulate SERT expression.

The controversy between reduced SERT expression in obese subjects and increased midbrain SERT in acquired obesity, as reported in monozygotic co-twins with a higher BMI [48], can be explained by different SERT regulatory processes during gene-environment interactions. Specifically, the selection criteria applied in the Finnish study could have included higher BMI co-twins under particular lifestyles and/or changes of dietary habits leading to SERT up-regulation, as observed in rodent models of acquired obesity [63]. At the same time, considering the experimental design of the Finnish study, selected twins could also bear a genotype linked to vulnerability to stress as SERT-reducing obese subjects [64]. Moreover, of note, our investigation and that by Erritzoe et al. (2011) [50] much differ from the Finnish study [48] in terms of: a) evaluated BMI ranges; b) employed technical procedures (e.g., PET vs. *in vitro* binding experiments carried in membranes; different SERT binding tracers); c) sample size of recruited subjects.

Despite the well-known gender-related differences in obesity and fat distribution, we did not find appreciable differences in B_{\max}/BMI ratios in males vs. females, suggesting a gender-independent effect of BMI on SERT expression in platelets of severe obese individuals.

Conclusions

Analyzing the biggest cohort of the literature so far, our study demonstrates, for the first time, that SERT density is reduced in plasma membranes of circulating platelets of severe (class II/III, $\text{BMI} > 35 \text{ kg/m}^2$) obese subjects, without gender-related differences. Nevertheless, the complexity of SERT regulation needs to be investigated further. A multivariate statistical elaboration in normal,

overweight and obese subjects is currently in progress in order to better define the contribution of energy metabolism/adipocyte function on the modulation of platelet SERT (number and function) in obese individuals. Moreover, we suggest to better evaluate the role of 5-HT in body weight balance through the measure of other parameters such as 5-HT re-uptake function, intra-platelet/bloodstream 5-HT levels, intra-platelet SERT content, plasma large-neutral amino acids, BDNF, TSPO as well as the binding and sensitization state of 5-HT receptor subtypes in obese subjects. Microarray gene, peptide/protein analyses and metabolomics would be helpful to identify involved signals, effectors and regulatory cascades, also in other SERT expressing districts such as the gut or adipose tissue. The targeting of 5-HT-related gene/proteins and other monoamine or endocrine biomarkers would help to detect different subtypes of human obesity, possibly triggered by distinct biological causes, allowing the development of novel therapeutic strategies.

Methods

Chemicals

[³H]-paroxetine (specific activity: 15.5 Ci/mmol) was purchased from Perkin-Elmer, Life Science, Milan, Italy. All other reagents were of the best analytical grade.

Subjects

One hundred and fourteen (Italian) subjects (34 M; 80 W; age: 38.57 ± 12.47 years) with a BMI ranging between 18.30 and 54.80 Kg/m^2 ($33.54 \pm 9.923 \text{ Kg/m}^2$) were enrolled for the present study. Normal weight subjects were recruited from the medical and laboratory staff of the Endocrinology Center. Overweight and obese ($\text{BMI} > 25 \text{ Kg/m}^2$) subjects were recruited among the patients of the Obesity Center, Endocrinology Unit 1, University of Pisa. Exclusion criteria were: active cancer, heart, liver or kidney diseases; presence of hematological or neurological illnesses, a positive history for substance abuse and psychiatric (Axis I) disorders assessed by Structured Clinical Interview for DSM-IV Axis-I diseases (SCID/I diagnostic criteria).

Subjects assuming substances acting on SERT, other psychotropic agents or estro-progestinic drugs and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) were admitted to the study after a 3 months and 10 long days withdrawal, respectively. Assuming hypotensive or interfering with carbohydrate-lipid metabolism (insulin, oral hypoglycemic compounds, statines) drugs was an exclusion criteria as well.

Height was measured, while subjects were standing, using standardized techniques and equipment. Body weight was measured by a precision instrument and electronic scale ($\pm 0.1 \text{ Kg}$). A regular informed consent approved by the Ethics Committee of the Pisa University was signed by all subjects after reading a full explanation of the project.

Platelet sampling

To avoid catecholamine release as well as circadian rhythm interference, peripheral venous blood (30 ml) was drawn from fasting subjects in clinostat position between 8.30 and 10 a.m. Blood was collected into plastic tubes containing 5 ml of anticoagulant (2.2% sodium citrate, 1.2% citric acid) and centrifuged at low-speed (150 g) for 15 min at 23°C to separate the platelet rich plasma (PRP). Platelets were then precipitated from PRP by an ensuing centrifugation at 1,500 g for 15 min at 23°C and counted automatically with a flux cytometer (Cell-dyn 3500 system; Abbott, Milano, Italy). Platelets were then washed by centrifugation for 10 min at 10,000 g, 4°C and resulting pellets stored at -80°C until assay, performed within 1 week.

Platelet membrane preparation

At the time of the assay, platelets were re-suspended in 10 volumes (*w:v*) ice-cold 5 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA and protease inhibitors (benzamidine 160 µg/ml, bacitracine 200 µg/ml; trypsin soy inhibitor 20 µg/ml). After homogenization by Ultraturrax, samples were centrifuged at 48,000 g for 15 minutes at 4°C. The ensuing pellets were washed twice in 10 volumes (*w:v*) ice-cold 50 mM Tris-HCl buffer (pH 7.4) by a centrifugation step, as above indicated. The final membrane pellets were suspended in the assay buffer consisting in a 50 mM Tris-HCl buffer (pH 7.4), containing 120 mM NaCl and 5 mM KCl. Protein content was determined by the Bradford's method (Bio-rad), using γ -globulins as the standard.

[³H]-Paroxetine binding assay

SERT binding parameters (maximal binding capacity, B_{max} , fmol/mg protein; dissociation constant, K_D , nM) were evaluated in platelet membranes by measuring the specific binding of [³H]-paroxetine. The [³H]-paroxetine B_{max} represents the specific density (number) or the degree of SERT protein expression on platelet membranes of each enrolled subject, while K_D being the main index of ligand-to-protein affinity. Saturation experiments were conducted as follows: 100 µl of membranes (corresponding to 50–100 µg proteins/tube) were incubated in assay buffer (50 mM Tris-HCl, 5 mM KCl, 120 mM NaCl, pH 7.4) with five increasing concentrations of [³H]-paroxetine (0.08–1.5 nM) in a final assay volume of 2 ml. Non-specific binding was performed, for each [³H]-paroxetine concentration point, in the presence of 10 µM fluoxetine, as cold displacer. Incubation was performed at 22–24°C for 60 min and halted by rapid filtration using Whatman GF/C glass fiber filters in a Brandell filtration apparatus. Filters were then washed three times with 5 ml ice-cold buffer assay, put into pony vials and measured for radioactivity (dpm) through a liquid phase

scintillation β -counter Packard 1600 TR. Specific binding was obtained by subtracting residual binding in the presence of 10 µM fluoxetine from total binding.

Data analysis

For statistical analyses, the subjects were divided into groups according to BMI classes: normal-weight (NW, controls), overweight (OW) and grade I-III obese (OB) individuals [65]. Equilibrium-saturation binding data, maximum binding capacity (B_{max} , fmol/mg of protein) and dissociation constant (K_D , nM), were calculated by the iterative curve-fitting computer programs EBDA-LIGAND (Kell for Windows, v. 6.0) [66] and Graph-Pad Prism (version 3 and 5, San Diego, CA, USA). Results of descriptive statistical analyses were reported as the mean \pm the Standard Error of the Mean (S.E.M.), when not differently indicated in the text. For inferential analyses relating SERT expression and affinity with obesity ANOVA followed by the Bonferroni *post-hoc* test as well as *t*-test Student for unpaired data (gender influence) were used; Pearson correlations analyses and linear regression tests between platelet [³H]-paroxetine binding parameters and BMI values were also performed in men and women separately between platelet [³H]-paroxetine binding parameters and BMI values were also performed in men and women separately. For gender-specific *t*-test analysis, binding densities were normalized for BMI in women and men separately, obtaining B_{max}/BMI values (fmol \times m² height squared/mg proteins \times Kg body weight). For all statistical analyses, Graph-Pad Prism software was used and the significance threshold was set at $p = 0.05$.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GG and AL conceived the study, participated to its design and coordinated all the study steps; FS, MM and PV participated to the study development while FS, AM, MMr and PF recruited and clinically evaluated the subjects; LB, LF, LS and ML conducted binding assays; CP and SB were responsible of blood sampling and processing; LP, LF, LG and MC elaborated experimental results; LP, LB and AM wrote the manuscript. All authors read and approved the final manuscript.

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