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" Obesity serum factors affect human platelets SERT: we need a cellular model to investigate "

Dr. Laura Fabbrini

Tutor:

Prof. Gino Giannaccini

Chief of PhD Course:

Prof. Antonio Lucacchini

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CHAPTER 1

1.1 The serotonin

The tryptophan derivative 5-hydroxytryptamine (5-HT) is an important signaling molecule in the brain and periphery (Sodhi MS et al. 2004; McLean PG et al. 2007). Serotonin present in enteric nerves is synthesized in the enteric nervous system (Yu PL et al. 1999). Serotonergic neurons constitute about 2% of all mesenteric neurons. Serotonin is released from the bowel when enteric nerves are stimulated (Gershon MD et al 2007). Serotonin is synthesized from essential amino acid tryptophan by tryptophan hydroxylase I (TpH-1), a rate limiting enzyme present in enterochromaffin cells (EC) (Fig.1), whereas enteric and central serotonergic neurons contain TpH-2 (Walther DJ et al. 2003; Cote F et al. 2003). EC cells produce and secrete far more serotonin than either central or peripheral serotonergic neurons to reach the GI lumen and blood (Tamir H et al. 1985). Overflowing serotonin from EC cells, which is taken up and concentrated in platelets, is virtually the sole source of blood serotonin; platelets lack TpH. Serotonin exerts its action by binding to its receptors (5-HT1 to 5- HT7) present in both intrinsic and extrinsic primary afferent neurons.

Serotonin in tissues metabolized mainly by enzyme monoamine oxidase. In the kidney and liver, monoamine oxidase and aldehyde dehydrogenase convert 5-HT to 5-hydroxyindole acetic acid (5-HIAA), which is excreted in the urine. Approximately 2–10 mg of 5-HIAA is excreted daily by the normal adult as a result of metabolism of endogenous 5-HT. Serotonin present in the food, metabolized before entering the blood stream.

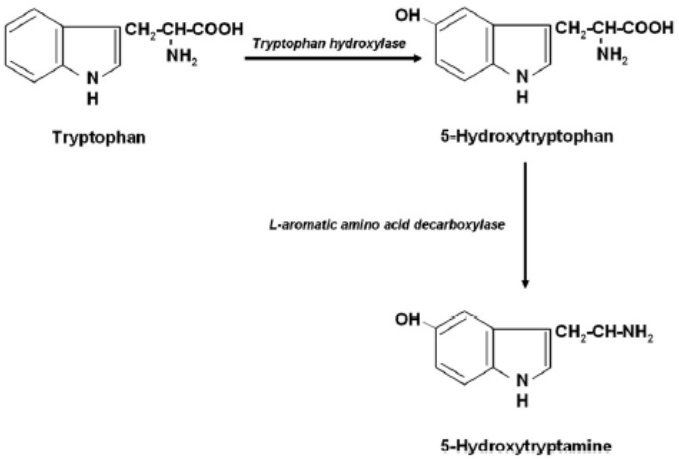

Fig.1 Serotonin synthesis

1.4 The serotonin transporter

Like many other neurotransmitters, 5-HT is predominantly inactivated by transporter-mediated clearance. Serotonin transporter (SERT) is encoded by a single gene in man as well as in commonly used rodent models (Ramamoorthy S et al. 1993). SERTs bear closest identity with norepinephrine transporters (NET, SLC6A2) with which they also share sensitivity to tricyclic antidepressants (Barker EL et al. 1995).

Topologically, SERT is a polytopic, integral membrane protein comprised of twelve transmembrane domains with cytoplasmic NH2 and COOH termini (Ramamoorthy S et al. 1993; Blakely RD et al. 1991; Hoffman BJ et al. 1991). The crystal structure of a bacterial SLC6A4 transporter, LeuTAa (Yamashita A et al. 2005; Chen JG er al. 1998; Torres GE et al. 2007), is consistent with the predicted topology of SERT (Henry LK et al. 2006; Henry LK et al. 2007) and has permitted the use of homology-based modeling approaches to provide a more realistic inspection of key helices that participate in 5-HT recognition as well as how 5-HT transport across membranes is achieved. SERT mediates secondary active, ion-coupled 5-HT transport, deriving energy for inward 5-HT transport largely from the Na^+ gradient.

SERT exhibits a coupling stechiometry of one 5-HT: one Na^+ and one Cl^-, with one K^+ molecule believed to be effluxed on a separate step of the transport cycle (Rudnick G et al. 1993).

1.5 Structure, expression and function of the SERT protein

SERT belongs to the superfamily of large-solute-carrier transporters and is designated as SLC6A4. Human SERT is a monomeric protein encompassing 630 amino acids and consists of 12 putative transmembrane domains (TMs), a large extracellular loop between TM3 and TM4 that has potential sites for N-glycosylation and large extracellular NH2- and C-terminal domains containing potential phosphorylation sites (Murphy D.L et al. 2004, Murphy D.L et al. 2008; Spiller R. 2007; Lesch K.P et al. 2005; Usala T. et al . 2008; Rudnick, G. 2006). Amino acids in TM1 and TM3 are major determinants for serotonin binding (Kilic F. et al. 2003) and phosphorylation and dephosphorylation processes regulate the membrane SERT density (Quick, M.W. 2002).

SERT expression has been demonstrated in the central nervous system (CNS) of various mammalian species (Gill R.K. et al. 2008). The distribution of serotonin transporter in human brain was evaluated by mean of binding assays using specific and selective radioligands. In particular, the highest concentrations of [3H]paroxetine-specific binding sites were found in the substantia nigra, hypothalamus, and hippocampus of human brain. Lower values were obtained in the basal ganglia and the thalamus. The specific binding was very low in cerebral and cerebellar cortices (Laruelle M. et al.198)

The neuroanatomical distribution of binding sites for [3H]imipramine and [3H]citalopram was assessed by in vitro autoradiography in select regions of the rat and human forebrain. To determine involvement of serotonin-containing terminals in the binding of [3H]imipramine and [3H]citalopram, binding of these compounds was measured in rats after destroying serotonin-containing neurons with 5,7-dihydroxytryptamine (5,7-DHT). Treatment with this neurotoxin decreased serotonin content by 90% and reduced [3H]citalopram binding to a similar extent. These results demonstrated that [3H]citalopram binding was a reliable marker for serotonin-containing terminals. Binding of [3H]imipramine was reduced by only 15-35% after 5,7-DHT treatment. These latter results suggested that only a small fraction of [3H]imipramine binding to brain sections was associated with serotonergic terminals under standard conditions used in autoradiographic studies with the ligand. Dose-response effects of fluoxetine and desipramine on displacement of [3H]imipramine binding in forebrain regions indicate that the ligand labels predominantly high capacity, low affinity binding sites. To determine the utility of the rat

brain as a model for [3H]imipramine and [3H]citalopram binding in the human brain, binding of the ligands was compared in human and rat hypothalamus, amygdala, and hippocampus. The pharmacological characteristics of [3H]imipramine and [3H]citalopram binding were similar in the rat and human brain. However, substantial species differences were observed in topographic patterns of [3H]imipramine binding within the hippocampus and hypothalamus. The distribution of [3H]citalopram binding sites within the amygdala and hypothalamus were also strikingly different in rats compared to humans. This work provides the first demonstration that marked species differences exist in the topography of serotonergic innervation and in the distribution of [3H]imipramine binding sites within the rat and human brain regions examined (Duncan GE. Et al. 1992).

At intestinal level, evidence of SERT expression was initially obtained in rats and guinea pigs (Chen J.X. et al. 1998). Recently, SERT mRNA has been detected in normal human stomach and intestine, with high density in small intestine (Meier Y. et al. 2007; Van Lelyveld N. et al. 2007).

SERT immunostaining has been observed mainly in human duodenal and ileal epithelial cells (Gill R.K. et al. 2008), and it has also been demonstrated in rectal biopsies from healthy humans (Coates M.D. et al 2004). However, information on SERT expression in the neuromuscular compartments of gastrointestinal tract in humans and animals is lacking.

SERT operates the reuptake of serotonin through a mechanism associated with co-transport of Na^+ and Cl^- and counter-transport of K^+. As a first step, serotonin, Na^+ and Cl^- bind a single site, accessible from the extracellular space. Then, a conformational change occurs that blocks the accessibility of the binding site from the extracellular side and opens access to it from the cytoplasm side. In this conformation, serotonin, Na^+ and Cl^- dissociate from SERT, and K+ binds to the same site to facilitate a conformational change back to the status that allows accessibility from extracellular space (Murphy D.L. et al. 2004).

In the digestive tract, serotonin is stored in enterochromaffin cells (ECs), which release the mediator in response to mechanical or chemical stimuli, and to a lesser extent in some intramural enteric neurons. After it is released by ECs, serotonin promotes peristalsis, secretion, vasodilation and sensory signalling in the gut via interaction with a complex array of serotonergic receptor subtypes. The primary targets of serotonin released from ECs are the mucosal projections of afferent neurons. These include extrinsic nerves, which transmit gut sensations to the CNS, and intrinsic primary afferent neurons, which initiate

peristaltic and secretory reflexes. Serotonin released from myenteric neurons is involved in the regulation of digestive motility (Gershon MD. Et al. 2007).

Thus, changes in SERT activity, resulting from genetic or environmental factors as well as pharmacological modulations, are expected to alter functions regulated by serotonergic pathways.

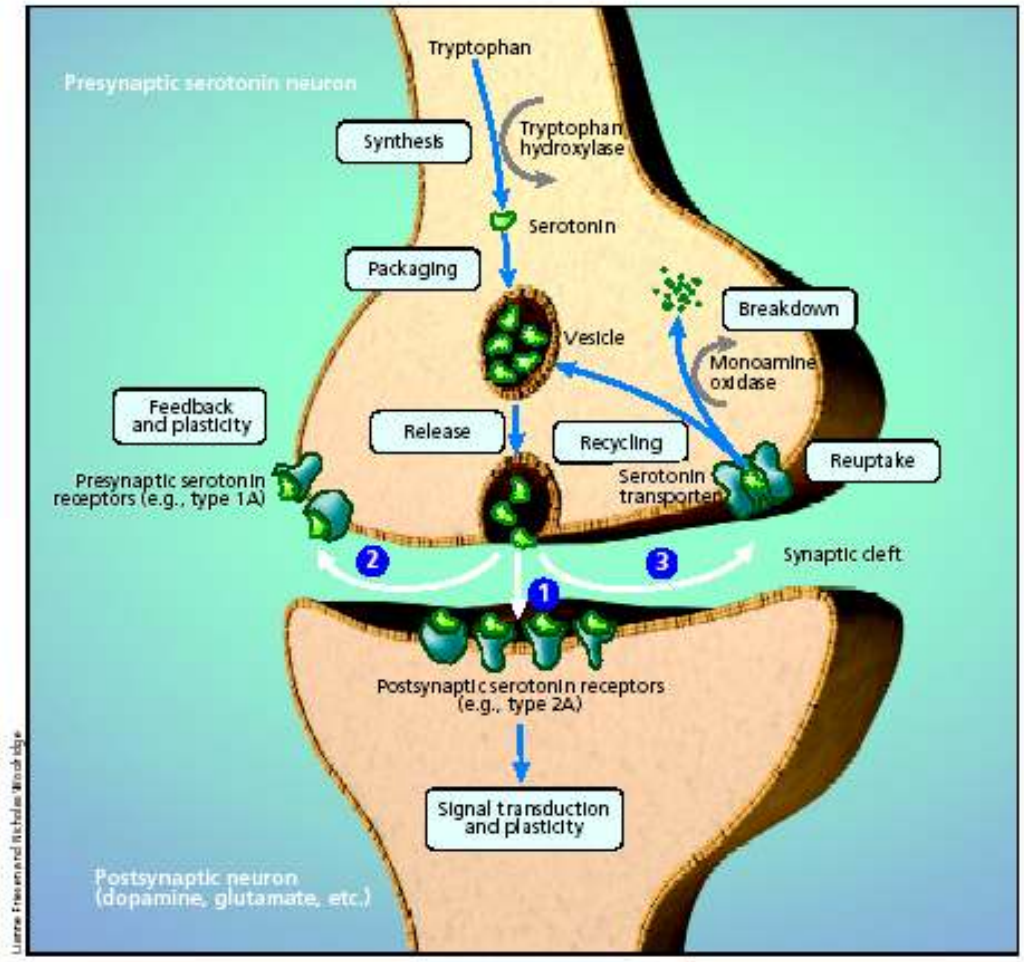

Fig.2: Serotonergic synapse

1.4 Molecular structure and transcription of the SERT gene

A single SERT gene has been identified in the human genome. It is located on chromosome 17q11.2, covers 37.8 kb, comprises 14 exons, encompasses a 630 amino acid open reading frame and has three putative polyadenylation sites. The start codon is located in exon-2, the protein is coded by the nucleotide sequence running from exon-2 to exon-14 and exon-1 consists of a noncoding sequence (Ramamoorthy, S. et al 1993; Battersby, S. et al. 1992).
After initial reports indicating a 14-exon structure, the human SERT gene was shown to encode two variants of exon-1 (1A and 1B) (Bradley, C.C. et al 1997). Alternative splicing yields two mRNA species comprising exons 1A+2 and 1A+1B+2, and transcription of both mRNAs is controlled by a promoter containing highly polymorphic sequences (Figure 3), as discussed below. A third mRNA species comprising exons 1B+2 was not identified (Bradley, C.C. et al 1997). However, a recent study of normal human intestine has revealed three SERT mRNA species, which could arise from alternative splicing (Gill, R.K. et al. 2008), supporting the hypothesis that the human SERT gene has the potential to generate more than two mRNA variants. Evidence for multiple exon-1 sequences was also found in rodents (Ozsarac, N. et al. 2002; Sakai, K. et al. 2003). Rat SERT RNA transcript contains three noncoding exons (1A, 1B and 1C), and alternative splicing generates mRNAs with the exonic combinations 1A+2, 1A+1B+2 or 1C+2. mRNAs comprising 1A+2 or 1A+1B+2 are thought to be regulated by the same promoter but have different expression patterns (1A+2 mRNA occurs mainly in the CNS and adrenal medulla; 1A+2 and 1A+1B+2 mRNAs occur in the stomach and heart). The 1C+2 mRNA species has been found only in the intestine and is likely to be controlled by a distinct promoter (Ozsarac, N. et al. 2002). Of note, rat exon-1C shares 68% identity with a region of human SERT gene upstream of exon-1B, suggesting the existence ofexon-1C in humans. The identification of a SERT mRNA variant containing exon-1C in rodents (Ozsarac, N. et al. 2002) has opened interesting perspectives for interpreting the role played by SERT in human gut physiology and IBS pathophysiology.
Indeed, if confirmed in humans, the predominance of 1C+2 mRNA transcripts in the intestine would imply that: (i) the polymorphic promoter, which regulates the expression of 1A+2 mRNA in CNS, might not control SERT expression in the gut; and (ii) as a consequence, polymorphisms in the promoter linked to exon-1A might affect SERT

expression in the CNS but not in the gut. Thus, studies designed to determine the transcriptional regulation of enteric SERT, as well as the exact expression pattern of alternatively spliced SERT mRNA transcripts in human intestinal tissues, would contribute to clarifying the role of SERT in human gut physiology and IBS pathophysiology.

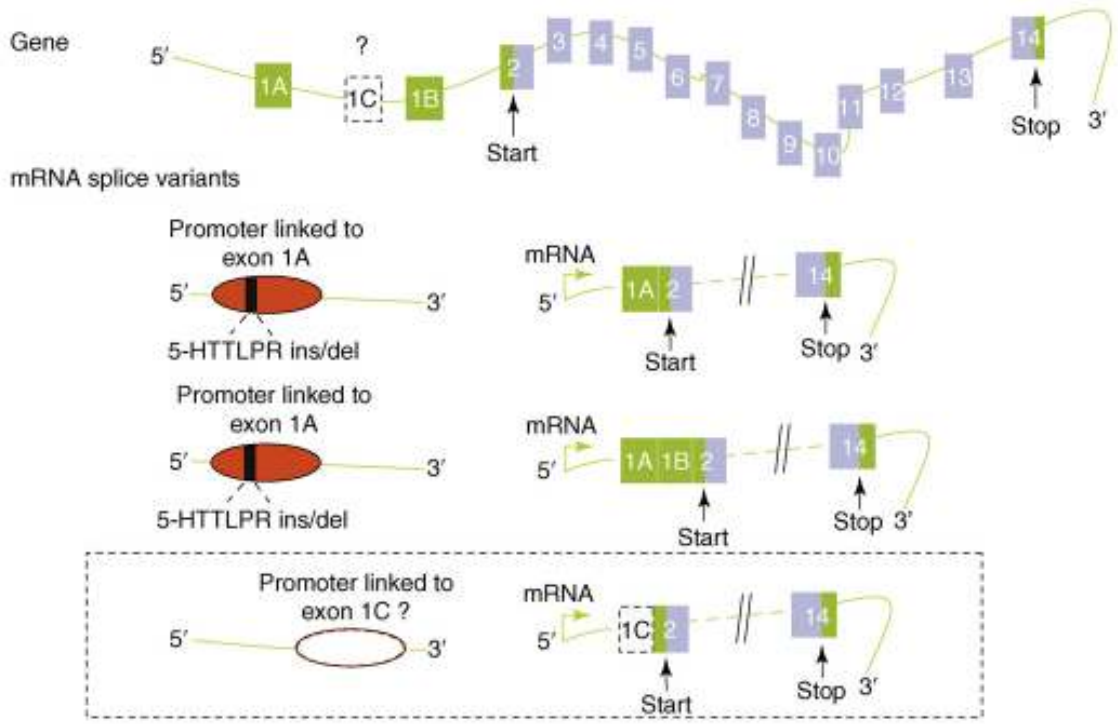

Fig.3 Schematic representation of the molecular structure of the serotonin transporter (SERT) gene in humans

1.5 Polymorphisms of the SERT gene

In the human SERT gene, nonsynonymous mutations, which result in changes of amino acid sequence, occur as single nucleotide polymorphisms (SNPs), and their frequency in the general population is lower than 1% (Leabman M.K. et al. 2007).

The possible relationships between nonsynonymous polymorphisms, changes in SERT function and implications in pathological conditions, including IBS and other digestive disorders, remain undetermined. A variable number of tandem repeats are present in intron-2 of the SERT gene. The most frequent variants consist of 16- or 17-bp sequences repeated nine (STin2*9), ten (STin2*10) or 12 (STin2*12) times, and there is evidence that the STin2*12 allele is associated with higher efficiency of SERT transcription than STin2*10 (Fiskerstrand C.E. et al. 1999).

The human SERT promoter linked to exon-1A contains a polymorphism, named 'serotonin transporter length polymorphic region' (5-HTTLPR), which consists of a 43-bp deletion or insertion resulting in a short (S) or long (L) allele (Wendland J.R. et al. 2006). In North American/European Caucasian populations, the distribution of 5-HTTLPR genotypes is 32% LL, 49% LS and 19% SS, whereas the SS genotype is represented in 57% of Asians (Smits K.M. et al 2004; Serretti A. et al. 2007). The SERT gene also contains an insertion or deletion polymorphism (381 bp between 5-HTTLPR and transcription start site) that might confer instability to the promoter and might be responsible for variations in SERT expression caused by the presence of transcription-factor-binding sites, including activator protein 1 (AP1), Elk1 and nuclear factor- kB (Flattem N.L et al. 2000) which can be activated in the presence of pathological conditions, such as inflammation. 5-HTTLPR has been proposed to influence SERT gene transcription because, in cells transfected with SERT promoter, SS homozygosity and LS heterozygosity resulted in a lower transcriptional activity (and thereby lower SERT expression and lower uptake efficiency) than LL homozygosity (Heils A. et al. 1996). In line with these findings, lymphoblasts from subjects with the LL genotype were found to contain higher concentrations of SERT mRNA transcripts than cells obtained from SS or SL subjects (Lesch K.P. et al. 1996). Subsequently, 5-HTTLPR alleles were shown to differently influence transcription rate, expression and function of SERT in brain (Hranilovic D. et al. 2004). Based on these observations, clinical investigations have examined possible associations of 5-HTTLPR

genotypes with pathophysiology, clinical features or therapeutic response to SSRIs in various disorders.

Advances in SERT genetics suggest that initial assumptions on molecular structure and transcriptional activity of 5-HTTLPR are too simplistic and deserve reconsideration. According to current evidence, repetitive sequences within 5-HTTLPR can generate four subvariants of allele S, six subvariants of allele L and several additional allelic variants (Delbruck S.J.W. et al. 1997; Michaelovsky E. et al. 1999; Rasmussen H.B. et al.2007).

Hu et al. (Hu, X. et al. 2005) have described an A!G SNP within the 43 bp insertion of 5-HTTLPR that confers the ability to enhance SERT transcription to the A variant of the L allele (LA) while rendering the transcriptional activity of the G variant (LG) similar to that of the S allele. Kraft et al. (Kraft J.B. et al. 2005) have also identified an A!G SNP (designated as rs25531), which has now been recognized as the same 298 SNP discovered by Hu et al. (Hu X. et al. 2005). A subsequent analysis of 5-HTTLPR showed that the boundaries of the 43 bp insertion or deletion segment can be set in such a way that the A!G SNP can lie within both the L and S allele, thereby allowing the S allele to exist as an SA or SG variant. Genotyping analysis has indicated a low to moderate frequency for LG and SG alleles (6.5% and 0.25%, respectively). However, Kraft et al. found that rs25531, but not 5-HTTLPR, was associated with variations in therapeutic effects of SSRIs, and therefore it has been argued that 5-HTTLPR and rs25531 should be regarded as two independent polymorphisms. To complicate the picture further, reporter gene experiments in transfected cells have not confirmed the differential influence of 5-HTTLPR alleles on SERT transcription (Sakai K. et al. 2002). Moreover, studies in humans have also failed to demonstrate significant correlations between 5-HTTLPR genotypes and SERT expression in human brain (Shioe K. et al. 2003; Lim J.E. et al. 2006). These observations raise relevant questions about the validity of previous association studies, including those on IBS, and suggest the possibility that SERT gene transcription could be influenced by polymorphisms in partial linkage disequilibrium with 5-HTTLPR.

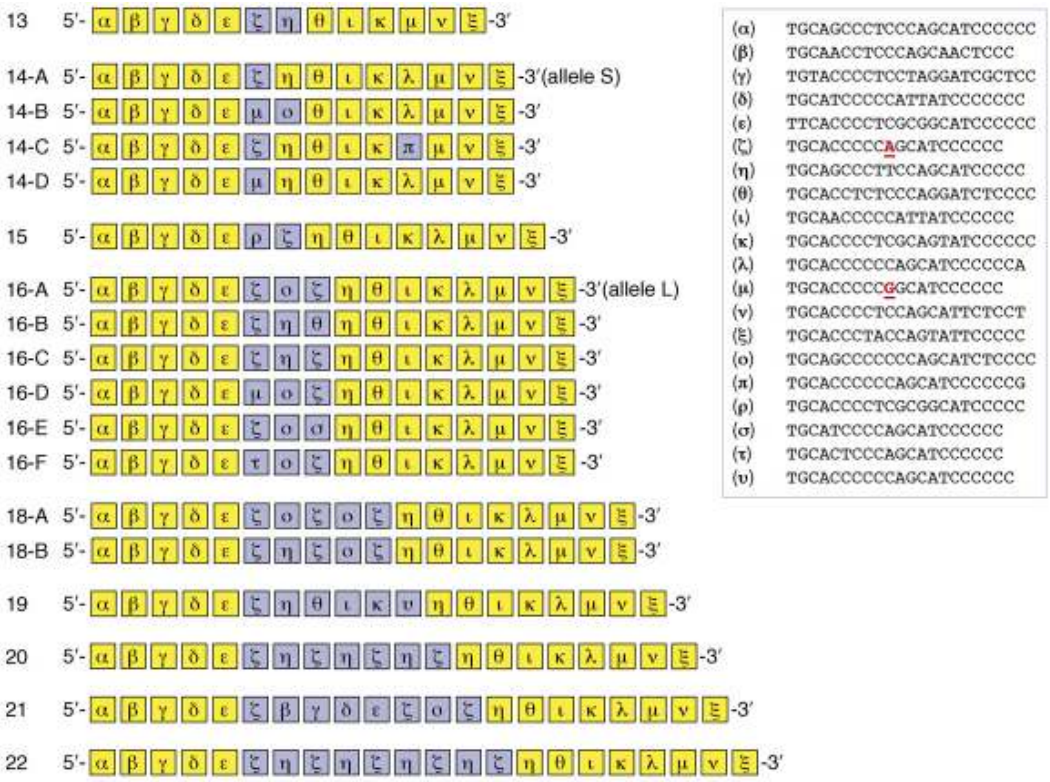

Fig 4 Schematic representation of the allelic variants resulting from the serotonin transporter length polymorphic region (5-HTTLPR)

1.6 SERT is regulated

1.6.1 Rapid Reductions in SERT Activity

Initial observations of rapid (2–30 min) downregulation of SERT Vmax following phorbol ester treatments provided an initial mechanistic framework that supported loss of SERT surface expression in parallel with SERT phosphorylation (Blakely RD. Et al. 1998). Because extracellular 5-HT acting through the transporter (as SSRIs block 5-HT effects) could prevent phorbol ester-triggered SERT internalization as well as phosphorylation, Seversl studies advanced an 'use it or lose it' model for SERT regulation. In that study, the presence of extracellular 5-HT was proposed to help establish the appropriate level of surface expression of SERT proteins needed for amine clearance, presumably through reduced endocytosis (Ramamoorthy S. et al. 1999; Blakely RD. et al. 2000).

1.6.1.1 Trafficking-dependent modulation

The membrane compartments that harbor SERT are distinguished by one or more proteins that contribute structural and/or regulatory properties. The first SERT associated protein defined was the catalytic subunit of the serine/threonine phosphatase PP2A (PP2Ac). Bauman et al. (36) established that okadaic acid-dependent phosphatise activity is enriched in SERT immunoprecipitations, consistent with the formation of SERT complexes with either protein phosphatase 1 or PP2A. Subsequent immunoblotting of SERT immunoprecipitations from transfected cells and brain membranes revealed the presence of PP2Ac. Interestingly, the authors found that PP2Ac associations could be eliminated by pretreatment of cells with okadaic acid but were stabilized by extracellular 5-HT incubation, thus providing one explanation for reduced b-phorbol 12-myristate 13-acetate (PMA)-induced SERT phosphorylation that occurs with coincident 5-HT treatments (Bauman AL et al.. et al. 2000). The simplest scenario (Figure 5) consistent with the existing data thus involves SERT/PP2Ac associations that are enriched in plasma membrane domains containing active SERT, whereas less active SERT pools and inactivated (and possibly recycling) SERTs being relatively deficient in SERT/PP2Ac complexes.

The plasma membrane SNARE protein syntaxin (Syn1A) has been a well-documented associate of a number of neurotransmitter transporters in the SLC6 family including GAT-

1, the GLYT2 glycine transporter and NET (Blakely et al. 2000). Haase et al. provided evidence that Syn1A also associates with SERT (Haase J. et al. 2001). Sung et al. described analogous associations of NET with Syn1A that can be disrupted by PKC activators in parallel with loss of surface NET expression (Sung U. et al. 2003). The latter studies also demonstrated that Syn1A restricts the catalytic function of NET. Comparable evidence for catalytic modulation of SERT by Syn1A has not been advanced. Quick (Quick MW. 2003) showed that Syn1A associations with SERT dictate whether the transporter moves 5-HT in an electroneutral mode, tightly coupled with co-transported ions. When SERT lacks Syn1A associations, 5-HT transport occurs in an electrogenic mode, characterized by nonstoichiometric ion flux. Possibly, as Sung et al. have proposed for NET, Syn1A associations with SERT dictate whether the transporter contributes to membrane excitability and/or whether the transporter enters a higher capacity 5-HT transport mode (Figure 5).

Through yeast two-hybrid screening with the carboxy terminus of DAT, Carneiro et al. identified the focal adhesion protein Hic-5 as a transporter-binding partner that also binds SERT (Carneiro AM. et al. 2002). Hic-5 is a Lin 11, Isl 1, Mec 3 (LIM) domain scaffolding protein that binds the carboxy terminus of SERT, similarly to DAT. Hic-5 is found in association with SERT in platelets where the majority of Hic-5/SERT associations are localized to the CS fraction.

Because, like SERT, the bulk of Hic-5 is found in the TS fraction, unassociated with the transporter, rather than in the CS domain, Carneiro and Blakely advanced the hypothesis that PKC activation recruits Hic-5 to SERT. Hic-5 could support or stabilize catalytic inactivation of the transporter as well as translocation of SERT to the CS compartment, followed by SERT internalization and dissociation of Hic-5. The degree to which this model supports SERT function at synapses is currently being investigated.

Using the yeast two-hybrid assay, Muller et al. identified secretory carrier membrane protein 2 (SCAMP2) as a protein that interacts with the amino terminus of SERT (Muller HK. et al. 2006).

After confirmation of a native SCAMP2/SERT complex in glutathione S-transferase pull-down assays, these investigators documented a decreased Vmax of 5-HT transport when SCAMP2 and SERT were coexpressed in transfected cells. This change was paralleled by reduced transporter surface expression. SCAMP2 localizes to lipid rafts where, as noted above, SERT is also localized. Possibly, SCAMP2 binding could facilitate exit of SERT

from lipid rafts to achieve endocytosis of SERT and a reduction in SERT activity. How SCAMP2 associations are established or are modulated and how this interaction relates to other SERT amino-terminal associations, such as that of Syn1A, are unknown.

Chanrion et al. (Chanrion B. et al 2007) recently identified a carboxy-terminal association of SERT with neuronal nitric oxide synthase (nNOS or NOS1). Cotransfection of yellow fluorescent protein-tagged SERT and nNOS leads to reduced 5-HT transport ascribed to lower surface expression. Evidence of a functional nNOS/SERT association is evident in brain, and synaptosomal 5-HT transport assays using nNOS_/_ mice display increased SERT activity and plasma membrane expression. These studies are particularly intriguing given evidence, noted below, that a NOS-dependent pathway supports rapid, A3 adenosine receptor (A3AR)-dependent elevations of SERT activity, both in mast cells and in serotonergic terminals. Possibly, nNOS associations may contribute to entry of SERT into an intracellular, regulated pathway. In the absence of nNOS, SERT may remain at the cell surface or target the plasma membrane through a constitutive pathway and thus exhibit the observed elevations in basal 5-HT transport activity. One expectation arising from this hypothesis may be achieved at the expense of regulation of SERT through cyclic GMP (cGMP)- and protein kinase G (PKG)-linked pathways.

Another cell surface receptor whose activation has been linked to acute SERT downregulation is the a2 adrenergic receptor (a2AR) (Ansah TA, et al. 2003). Treatment of forebrain synaptosomes with 5-bromo-N-[4,5-dihydro-1H-imidazol-2-yl]-6-quinoxalinamine (UK14304), an a2AR agonist, reduces 5-HT transport, an effect derived from a reduced sensitivity to 5-HT (increased Km). UK14304 reductions in 5-HT transport were found to be dependent on the activity of voltage-sensitive Caþþ channels, suggesting that Caþþ-mediated signaling events downstream of a2AR activation downregulate SERT. Chronoamperometry studies documented an ability of acute UK14304 injection to diminish 5-HT clearance in vivo. These findings support the idea that the well-established adrenergic regulation of 5-HT release is accompanied by modulation of 5-HT clearance and suggests further consideration of a2AR-directed medications in the pharmacotherapy of mood disorders.

1.6.1.2 Trafficking-independent modulation

Ramamoorthy and colleagues made a striking discovery that SERT down regulation by PKC activators in platelets is actually biphasic, with both trafficking-dependent and trafficking-independent phases of downregulation evident (Jayanthi LD et al 2005). Short treatment of rat platelets with PMA leads to rapid inhibition of SERT activity through decreased Vmax and increased Km, yet no change in surface expression of the transporter is observed. In contrast, longer incubations of platelets with PMA produce a further reduction in 5-HT transport Vmax that coincides with a reduction in SERT cell surface abundance. With short PMA treatments, phosphoamino acid analyses indicate phosphorylation of SERT only at Ser residues, whereas longer treatments lead to both Ser and Thr phosphorylation.

Taken together, these studies indicate that PKC activation can influence two sequential modes of SERT regulation, one linked to catalytic downregulation and a second to transporter endocytosis. Although specific sites of PKC-dependent SERT phosphorylation are as yet undefined, Ser and Thr residues in human NET intracellular loop 2 – residues conserved in SERT – were identified as necessary for phorbol ester-triggered phosphorylation (Jayanthi LD et al. 2005).

1.6.2 Rapid Elevations in SERT Activity

The examples of acute SERT regulation discussed up to this point involve a decrease in SERT catalytic activity or diminished cell surface expression. Below, are describe examples of rapid upregulation of 5-HT transport and consider how supporting mechanisms lend credence to a bidirectional SERT regulatory cycle that is poised to respond to needs for both elevated and diminished 5-HT clearance.

1.6.2.1 Trafficking-dependent modulation

Miller and Hoffman were the first to identify a rapid, receptor-dependent mechanism of increasing SERT function in cultured cells (Miller KJ, Hoffman BJ 1994), research corroborated and extended to the CNS by Zhu et al. (Zhu CB. et al 2007). When rat basophilic leukemia (RBL-2H3) cells, derived from serotonergic mast cells, are treated with a broad-spectrum adenosine receptor agonist, 50-N-ethylcarboxamido-adenosine, 5-HT transport capacity (Vmax) increases. Zhu et al. utilized specific A3AR agonists and antagonists and transporter-receptor-cotransfected cells to validate the role of A3ARs in this effect. SERT stimulation by A3ARs requires activation of PKG by a phospholipase C, Caþþ, NOS and cGMP-dependent mechanism (Zhu CB. et al. 2004a). Miller and Hoffman initially ascribed A3AR regulation of SERT to a change in catalytic function because of no detectible changes in SERT antagonist binding. Zhu et al. recognized that the latter measure might have failed to discriminate SERT trafficking from changes in total SERT protein. These investigators opted for membrane impermeant binding conditions using 5-HT displacement at 48C and the cocaine analog methyl 3b-(4-iodophenyl) tropane-2b-carboxic acid methyl ester (RTI-55) (as well as biotinylation measures in transfected cells) to define subcellular pools of SERT.

These approaches revealed an increase in SERT surface expression following treatment with A3AR agonists, 8-BrcGMP or the endogenous cGMP potentiator sildenafil (Zhu CB, et al. 2004 a) comparable to the elevations in 5-HT uptake. Use of membrane impermeant [2-(trimethylammonium) ethyl]-methanethiosulfonate bromide to inactivate surface SERTs prior to A3AR agonist treatments offers evidence that activation of the A3AR–PKG pathway results in the recruitment of intracellular SERTs rather than in retention of surface transporters (Blakely et al. 2004).

Using mouse brain synaptosomes derived from A3AR_/_ mice and chronoamperometry in rat brain, Zhu and colleagues further established that A3ARs regulate SERT in the CNS.

PKG antagonists, including the PKG1 isoformspecific peptide inhibitor DT-2 (YGRKKRRQRRRPPLRK5H), block stimulation of SERT by the A3AR agonist N6-(3-iodobenzyl)-N-methyl-50 carbamoyladenosine (IB-MECA) in these native models, as in RBL-2H3 cells (Zhu CB et al. 2004b). Although these studies with native preparations document the presence of a SERT regulatory cycle in serotonergic terminals, other preparations are needed to advance our understanding of the specific mechanisms that support synaptic transporter localization and function. McDonald et al. (2007) recently demonstrated how direct visualization of Caenorhabditis elegans synaptic DAT proteins can be achieved using enhanced green fluorescent proteinlabeled transporters expressed in transgenic nematodes, taking advantage of the transparency and identified synapses of this model system. Studies are currently underway to adapt this framework to investigation of the regulation of the C. elegans SERT, MOD-5 (Ranganathan R et al. 2001).

Stimulation of SERT through a PKG-linked pathway raises the question as to whether SERTs are phosphorylated as a consequence of PKG activation. Using metabolic labeling, immunoprecipitations and phosphoamino acid analysis of rat midbrain synaptosomes, Ramamoorthy et al. documented SERT incorporation of [32P] after 8-Br-cGMP treatments specifically on Thr residues (Ramamoorthy S et al 2007). After comprehensive mutagenesis of cytosolic Thr residues, Thr276 of human SERT was found to be the only Thr residue essential for 8-Br-cGMP stimulation of phosphorylation. Importantly, mutation of this site to Ala blocked 8-Br-cGMP stimulation of 5-HT uptake. Future studies are needed to define whether PKG directly phosphorylates SERT at Thr276. In contrast to the studies of Zhu et al. who had defined a trafficking-dependent mode of regulation sustained by PKG activation, Ramamoorthy et al. suggested that PKG activation leads to catalytic activation of SERT. Thus, although treatment of synaptosomes with 8-Br-cGMP led to increased 5-HT transport by an elevation in 5-HT transport capacity (Vmax), no increase in surface SERT was detected using cell surface biotinylation approaches. Three differences between the works of these two groups may help clarify this issue. First, Zhu et al. predominantly used stimulation of SERT by A3ARs, stimulation likely to activate only a limited pool or specific isoforms of cellular PKG.

Use of 8-Br-cGMP as in the Ramamoorthy's studies to regulate SERT may activate additional pools of PKG whose actions could lead to other effects. Second, Zhu et al. employed much lower concentrations of 8-Br-cGMP (10 mM) versus that of Ramamoorthy et al. (250 mM). Third, although both groups utilize transfected cell models, levels of

expression of SERT in the Zhu studies are kept low to match those originally encountered in RBL-2H3 cells that express rat SERT from the native SERT promoter. With a biochemical end-point in mind, (the isolation of SERT phosphorylation sites) Ramamoorthy et al. were compelled to work with SERT in culture models at much higher SERT protein levels.

1.6.2.2 Trafficking-independent modulation

Based on the ability of p38 MAPK to support trafficking independent regulation of NET and of PKG to phosphorylate and activate p38 MAPK, Zhu et al. asked whether p38 MAPK was involved in SERT upregulation that follows A3AR activation. Remarkably, the p38 MAPK inhibitor SB203580 blocked fully the increase in SERT activity seen with IB-MECA, although it did not prevent elevations in SERT surface expression (Blakely RD et al 2005). These findings suggest that PKG enhances both surface trafficking of SERT and SERT catalytic activity through two distinct pathways. Indeed, it is possible to bypass PKG altogether by using direct activators of p38 MAPK, such as anisomycin, which predictably increase 5-HT transport. Whereas PKG activation alters SERT transport capacity, p38 MAPK activation increases 5-HT affinity (Zhu CB et al 2005). One clue to the mechanism of this effect may be found in the recent study of Zhang et al. who show that phosphorylation at Thr276 after PKG activation produces an altered conformation of TM5 (Zhang YW et al 2007). This finding leads naturally to the idea that PKC-induced SERT catalytic downregulation may derive from phosphorylation of a nearby residue whose modification stabilizes a conformation of TM5 that is associated with a low 5-HT transport rate and/or prevents PKG-induced phosphorylation at Thr276. Consistent with this idea, PKC activation triggers dissociation of PP2Ac from SERT.

Because p38MAPK activation triggers catalytic activation of SERT and this effect is dependent on PP2A activity, PKG may phosphorylate Thr276 of SERT as well as other proteins that control SERT trafficking. PKG could then generate a conformation of SERT of higher 5-HT affinity as well as activate p38 MAPK, leading to PP2A activation that sustains the inhibitory Ser in a dephosphorylated state so that SERT activation is unopposed. Conversely, with p38 MAPK inhibited, PP2Ac activation may be insufficient to oppose basal inhibitory phosphorylation at this Ser. Such a model could explain the actions of inflammatory cytokines such as interleukin-1b and tumor necrosis factora that activate p38 MAPK in a PKG-independent manner leading to enhanced SERT activity,

without inducing SERT trafficking (Zhu CB et al 2006). By this model, the removal of tonic PKC inhibition is all that is needed to elevate SERT catalytic activity. Further research is needed to understand how transit of SERT between distinct protein complexes or membrane subdomains places SERT in a context for catalytic modulation.

Samuvel et al. have also suggested that basal p38 MAPK activity supports SERT trafficking. Rat brain synaptosomes treated with the p38 MAPK inhibitors SB203580 or PD169316 display decreased basal 5-HT transport that coincides with reduced SERT cell surface levels. Additionally, p38 MAPK antagonists reduce basal SERT phosphorylation. Again, it is likely that p38 MAPK-supported basal trafficking and p38 MAPK-supported SERT catalytic activity can be reconciled if one considers the differences in pharmacology (activator versus inhibitor), the alternate serotonergic preparations and the timing of drug application utilized in various p38 MAPK studies. As Zhu et al. have shown, acute p38 MAPK activation leading to enhanced catalytic regulation is monitored when p38 MAPK is acutely activated, whereas the studies of Samuvel et al. derive from studies where basal p38 MAPK is inhibited. Possibly, changes in basal SERT phosphorylation observed with p38 MAPK inhibitors reflect an indirect consequence of reductions in basal surface expression; for example, alterations in SERT phosphorylation may lead to PP2Ac association in compartments that prepare SERT to recycle back to the plasma membrane.

Recently, Carneiro et al. described a novel platelet SERT interactor that supports a bidirectional regulation of the transporter (Carneiro AM et al 2008). Using platelets derived from mice lacking integrin b3 (Itgb3_/_), the authors determined that 5-HT transport is severely reduced, although total and plasma membrane SERT expression is unchanged. Conversely, aIIb3 activation by immobilized fibrinogen leads to an increase in 5-HT uptake without affecting SERT surface expression. These phenomena appear to be supported, in part, through direct physical interactions of the SERT carboxy terminus with integrin b3. Remarkably, cotransfection of human SERT and a hyperserotonemia- and thrombosisassociated integrin b3 variant, Leu33Pro, leads to increased 5-HT uptake as well as to enhanced SERT surface expression. These effects may well relate to some of the same mechanisms of SERT regulation described above as the impact of the Leu33Pro variant on SERT requires active PP2A and p38 MAPK. Because integrin proteins help establish focal adhesions, these studies draw additional attention not only to the trafficking pathways between cytosolic and plasma membrane for SERTs but also to the movements between plasma membrane subdomains as a critical component of SERT regulation.

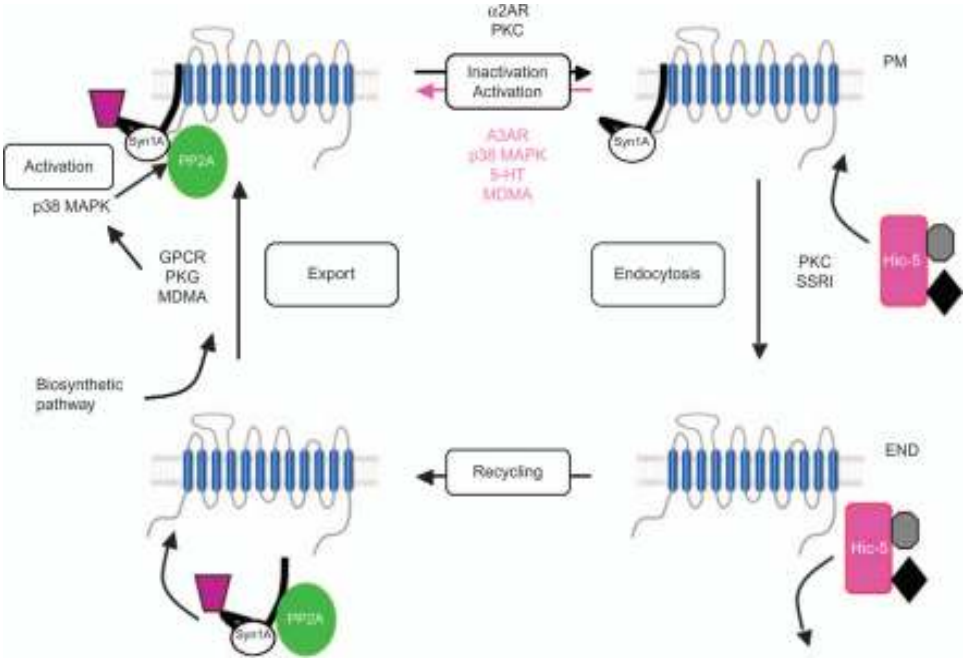

Fig.5: Schematic illustration of SERT regulation. SERT traffics in and out of the plasma membrane (PM) where it resides in distinct membrane compartments and recycles through endosomal (END) compartments. Various SERT-associated proteins, including Syn1A, PP2A and Hic-5 (among others), differentially associate with SERT in distinct compartments where they can influence transporter trafficking and/or catalytic function. MDMA, 3,4-methylenedioxymethamphetamine.

Chapter 2

2.1 Obesity

The epidemic of obesity has become pandemic, defined as an epidemic occurring over a wide geographic area and affecting an exceptionally high proportion of the population. The rise in obesity rates was first noted in the US, but has spread to other industrialized nations and it is even now being documented in developing countries.

Indeed, the global extent of the obesity pandemic was formally recognized by the World Health Organization (WHO) in 1997, and worldwide obesity rates are increasing dramatically (Caballero B. 2007). Estimates from the WHO indicate that as of 2005, at least 400 million adults (9.8%) were obese. Unlike most global health concerns, the obesity crisis is even more severe in developed nations. From 1960 to 2004, the prevalence of obesity in the US has more than doubled among adults from 13.3% to 32.1%, while the percentage of Americans overweight during the same period has increased from 44.8% to 66%, with most of this rise occurring since 1980. Obesity is clinically identified based on measurements of body mass index (Mei Z. et al. 2002), but can be generally defined as the physiological condition in which excess body fat has accumulated to an extent that can negatively affect health. This definition is based on the dramatically enhanced risk for a myriad of disease conditions with obesity, including type 2 diabetes, cardiovascular disease, gastrointestinal and respiratory difficulties, and many types of cancer (Haslam D.W. 2005). Furthermore, obesity is very closely associated with metabolic syndrome, which is characterized by a group of metabolic disorders that can include abdominal obesity, insulin resistance or glucose intolerance, atherogenic dyslipidemia, elevated blood pressure, and increased expression of prothrombotic and proinfiammatory markers (Olufadi R. et al. 2008).

Based on the large body of evidence that strongly indicates that obesity accelerates the onset and exaggerates the severity of a myriad of age-related disorders diseases, including hypertension (Hubert H.B. et al. 1983) myocardial infarction (Piegas L.S. et al.2003) and stroke (Walker S.P.et al. 1996) one could theorize that obesity accelerates age and age-related pathologies. Thus, it is possible that obesity might synergistically interact with the aging process to significantly accelerate the development of age-related disease and speed functional declines in large proportions of the US and global

population. The clinical significance of this possibility is amplified by observations that obesity rates among the elderly may be even higher than in the general population, as estimates indicate that 72% of Americans aged 60 and over are overweight with 32.4% obese (Wang Y. et al. 2007). Thus, to address this ominous potential public health crisis, it is necessary that clinicians and investigators work together to better understand the etiology of obesity-induced alterations to overall health in both clinical and experimental settings.

One especially costly and debilitating deficit of aging is the loss of cognitive function and the onset of dementia. All cognitive disorders, including dementia, become more common with age. Indeed, it has been estimated that dementia affects as many as 6–10% of people in industrialized nations aged 65 or older, 40–60% of whom may have Alzheimer's disease [Riedel-Heller S.G. et al. 2001; Wimo A. et al. 2003; Ferri C.P. et al. 2005). Despite it's strong association with age, dementia has been proposed as a mainly preventable condition with a large number of modifiable risk factors, including obesity, metabolic syndrome, and cardiovascular disease (Haan M.N. et al. 2004). In light of the 1 billion overweight and 300 million obese individuals worldwide (James P.T.et al.2001) a better understanding of the degree to which, and the mechanisms by which, obesity affects the brain could not only result in significant advances in public health, but could also unmask the pathophysiologic processes that might underlie age-related cognitive dysfunction and dementia.

2.2 Food intake and Serotonin

It is now clear that monoaminergic neurotransmitters act in conjunction with neuropeptides and peripheral hormones to control physiologic states such as hunger, satiation, and satiety. Serotonin has a suppressive effect on food intake and body weight Blundell JE. Et al. 1984; Leibowitz SF. Et al. 1988). This has been observed in studies of serotonin receptor antagonists and other drugs that decrease serotonin's activity. Injection of serotoninergic agents into the PVN (Currie PJ. Et al. 1996; Currie PJ. Et al. 1996; Leibowitz SF. Et al. 1989). VMH, anterior hypothalamic area (suprachiasmatic nucleus), and DMN decreases food intake (Leibowitz SF. et al. 1994). In addition to decreasing food intake, serotoninergic serotoninergic stimulation of the PVN enhances energy metabolism (Sakaguchi T. et al. 1989).

Pharmacologic evidence suggests that central serotonin-1B receptor (5-HT1B) mediates the feeding-suppressive action of serotoninergic stimulation in the medial hypothalamus (Curzon G. et al. 1997) by mediating the satiety-inducing effect of serotonin (Curzon G. 1990). In rats, 5-HT1B and possibly 5-HT1C/2C are specifically involved in hypophagia, in contrast to 5-HT1A, which may mediate hyperphagia (Hutson PH. Et al. 1988; Kennet GA et al . 1988; Dourish CT. Et al. 1986). Acute injection of the 5-HT1A agonist flesinoxan stimulates eating and increases NPY concentrations in the PVN and ARC (Dryden S. et al 1996). Further, in autoradiographic analyses of brain sections, dense concentrations of 5-HT1B and 5-HT1C receptor sites occur in the hypothalamus, in contrast with low concentrations of sites for the hyperphagic 5-HT1A receptor (Razos A.et al 1985). Neurons expressing 5-HT1B receptor are found in the PVN, ARC, supraoptic nucleus, and retrochiasmatic nucleus. Prominent groups of large and medial neurons with different 5-HT1B expressions are located in the LHA and are distributed in a specific rostrocaudal direction. Medium and light staining is observed in neurons of the PVN, VMN, and DMN (Makarenko IG et al. 2002).

As outlined in the previous sections, several experimental studies have consistently shown that abnormal hypothalamic serotonin activity contributes to hyperphagia and body weight gain. These data suggest that brain neurotransmission may represent a common step at which different appetite-related messengers converge. From the clinician's perspective, this hypothesis might yield interesting approaches to effective therapy for disturbed eating

behavior. Specifically, the inhibitory effect on food intake of hypothalamic serotonin may be exploited to decrease food intake and achieve weight loss in hyperphagic obese patients. Although serotoninergic agents have been used to treat hyperphagia, it must be emphasized that their side effects may limit their use (Jung RT. 1998). Sibutramine, a molecule that enhances serotoninergic and dopaminergic activities, is one pharmacologic therapeutic approach to the management of hyperphagia and obesity (Pederson KJ. Et al. 2003). Serotoninergic drugs that act on specific serotonin receptors (5-HT1B or 5-HT2C) decrease food intake and specifically fat intake. Several drugs that influence serotonin release (e.g., fenfluramine and dexfen- fluramine) or serotonin reuptake (e.g., fluoxetine and sertraline) have been used in obese patients.

D-fenfluramine was used at an average dose of 15 mg twice daily and had been shown to increase adherence to weight-lowering programs, to double the number of patients losing 10 kg or more when compared with a fairly efficient placebo plus dietary counseling, and to prevent weight regain when continued over a 1-y period. Moreover, significant improvement in metabolic risk factors and blood pressure were clearly demonstrated (Guy-Grand B.1995). However, severe side effects (in particular pulmonary hypertension and valvular heart disease) were reported with these antiobesity drugs, leading to the withdrawal of the licensed fenfluramine and D-fenfluramine in 1997 (Scheen AJ.et al. 1999).

Sibutramine is a combined 5-HT and noradrenaline re-uptake inhibitor and induces marked weight loss by affecting food intake and energy expenditure. There is a doserelated decrease in body weight, with weight loss up to 11% when it is associated with a low caloric diet, and patients lose 15% of weight over a 1-y period. Further, biochemical risk factors associated with obesity ameliorate with sibutramine therapy (Nisoli E. et al. 2000). However, the most commonly reported adverse effects of sibutramine are headache, constipation, and nausea. Adverse effects related to the nervous system include dizziness, dry mouth, and insomnia and are reported by more than 5% of patients. Increases in blood pressure and heart rate are possible adverse effects that require regular monitoring especially in obese hypertensive patients (Nisoli E. et al. 2003).

2.3 Obesity and Inflammation

C Reactive Protein (CRP) is an ancient, highly conserved molecule and a member of the pentraxin family of proteins (Du Clos TW. et al. 2000). CRP, secreted by the liver in response to a variety of inflammatory cytokines, increases rapidly in response to trauma, inflammation, and infection and decreases just as rapidly with the resolution of the condition. Thus, the measurement of CRP can be used to monitor inflammatory states.

CRP has a role in the function of the innate immune system. CRP activates complement, binds to Fc receptors, and acts as an opsonin for various pathogens. Binding of CRP to Fc receptors leads to the generation of proinflammatory cytokines. CRP can recognize altered self- and foreign molecules based on pattern recognition. Thus, enhanced levels of CRP can be used as a marker of inflammation.

Cook et al. (Cook DG. Et al. 2000) observed that higher adiposity indicates higher CRP levels in children. A strong relation between elevated CRP levels and cardiovascular risk factors, fibrinogen, and high-density lipoprotein (HPL) cholesterol has been noted, suggesting a role for inflammation throughout life in the development of atherosclerosis and cardiovascular disease. Visser et al. (Visser M. et al. 1999) demonstrated that higher BMI is associated with higher CRP concentrations in young adults aged 17 to 39 y, thus confirming a state of low-grade systemic inflammation in overweight and obese persons.

The elevated CRP concentrations can be ascribed to the increased expression of interleukin-6 (IL-6) in adipose tissue (Crichton MB. et al. 1999; Mohamed-Ali V. et al. 1997) and its release into the circulation (Fried Sk et al. 1998). IL-6 is a proinflammatory cytokine that stimulates the production of CRP in the liver (Banks RE. et al. 1995).

Higher adipose tissue content of IL-6 has been associated with higher serum CRP levels in obese subjects (Bastard JP. et al. 1999). This association is strengthened by the observation that in transgenic mice IL-6 is absolutely required for the induced expression of CRP (Szalai AJ. et al. 1998). CRP induced a three-fold increase in soluble IL-6 receptor (sIL-6R) production and promoted a loss of membrane-bound IL-6R, (Jones SA. et al. 1999) indicating that CRP may affect IL-6-mediated inflammatory events. In several diseases, CRP levels correlated with those of sIL-6R (Kyriakou D. et al. 1997; Desgeorges A. et al. 1997). CRP binds to specific receptors on human neutrophils and diminishes neutrophil chemotaxis,(Szalai AJ. et al. 1997) generation of superoxide anion and degranulation by

chemoattractants (Kew RR. et al. 1990) CRP prevents neutrophil adhesion to endothelial cells (Zouki C. et al. 1997) and inhibits neutrophil recruitment in models of inflammation, (Ahmed N. et al. 1996) suggesting that CRP also performs an anti-inflammatory function.

In overweight and obese subjects, serum levels of tumor necrosis factor-α (TNF-α), serum-soluble TNF receptor p55 (sTNFRI), and serum-soluble TNF receptor p75 (sTNF-RII) were significantly higher than those in lean subjects.

Both studies (Tsukui S. et al 2000; Zahorska-Markiewicz B. et al. 2000) reported that weight reduction or moderate-intensity regular exercise decreases the serum concentrations of TNF-α. Tsukui et al. observed that TNF-α, sTNF-RI, and sTNF-RII were negatively correlated with HDL cholesterol. Regular exercise decreased BMI, percentage body fat, glycosylated hemoglobin, serum TNF-α, sTNF-RI, and sTNF-RII and increased HDL cholesterol levels. Exercise-induced change in serum TNF-α was independently correlated with changes in glycosylated hemoglobin and serum insulin concentrations. Chu et al. (Chu N-F. et al. 2000) observed that men in the highest quintile of plasma leptin weighed more, were less physically active, and had higher circulating insulin, C peptide, sTNF-RI, and sTNF-RII concentrations than men in the lowest quintile. On further analysis, only glycosylated hemoglobin and sTNF-RI were independently and positively associated with plasma leptin in men with BMIs of at least 25 kg/m2, suggesting that glucose homeostasis and the activity of the TNF system can modulate leptin secretion among overweight men.

2.4 Cytokines, Neurotransmitters and Obesity

Since plasma concentrations of IL-6, TNF-α, and CRP are increased in obesity and several neurotransmitters can influence food intake, is it possible that there is an interaction between cytokines and neurotransmitters? Infection, injury, and inflammation are associated with negative energy balance characterized by reduced food intake, weight loss, increased thermogenesis, and fever. Administration of bacterial lipopolysaccharide (LPS), a potent inducer of cytokines, to animals upregulates leptin gene expression and serum protein levels (Sarraf P. et al. 1997; Grunfeld C. et al 1996). IL-1 and TNF-α increase ob mRNA expression and serum leptin concentration in rodents, whereas LPS does not increase leptin levels in mice lacking IL-1 (Faggioni R. et al. 1998). Conversely, exogenous leptin upregulates phagocytosis and the production of IL-6, IL-12, and TNF-α (Loffreda S. et al. 1998). Leptin-deficient mice (ob/ob) and rats that possess a defective leptin receptor (fa/fa) exhibited attenuated levels of TNF-α and IL-6 in response to LPS (Loffreda S. et al. 1998). Luheshi et al. (Luheshi GN. Et al. 1999) showed that leptin directly induces the release of IL-1 within the brains of normal rats and mimics the action of IL-1 in the central nervous system (CNS), and that its effects on food intake and body temperature are mediated by IL-1. IL-1 and IL-6 act as excitatory neuromodulators of gastrointestinal motility and caused a presynaptic inhibition of ACh release from cholinergic nerve terminals (Kelles A. et al. 2000). These results are interesting because vagus nerve stimulation in vivo inhibits TNF synthesis and ACh, the principle vagal neurotransmitter, attenuates the release of proinflammatory cytokines TNF, IL-1, IL-6, and IL-18 but not the antiinflammatory cytokine IL-10 in LPS-stimulated macrophages (Borovikova LV et al. 2000). Thus, serotonin, dopamine, NPY, and leptin can influence the synthesis and release of proinflammatory cytokines by their ability to alter ACh release in the brain. Hence, ACh may be a neurotransmitter and acts as an antiinflammatory molecule in the brain. NO participates in inflammation. The inducible type of NO produced by macrophages has proinflammatory actions (which is stimulated by TNF-α, IL-1, and IL-6), whereas eNO and neuronal NO show antiinflammatory actions. Leptin, by enhancing IL-1 concentrations, may possess proinflammatory actions, whereas neurotransmitters such as serotonin, dopamine, and NPY show antiinflammatory actions by inducing ACh release, which in turn

suppresses the production of proinflammatory cytokines (Borovikova LV et al. 2000). This is supported by the observation that dopamine attenuates the chemoattractant effect of IL-8 (Sookhai S. et al. 2000). This interaction between neurotransmitters and cytokines is supported by the work of Cho et al. (Cho L. et al 1999) who showed that endotoxin and TNF can alter the metabolism of norepinephrine and the concentrations of serotonin and dopamine in the hypothalamus.

Chapter 3

3.1 Introduction

Several studies have shown the involvement of the serotonergic transmission and of the serotonin in the eating behaviour and body weight in humans (Leibowitz S.F. et al 1998; McElroy S.L. et al. 2000; Wolfe B.E. et al. 2000). In general, treatments and procedures believed to activate 5-HT receptors reduce food intake consumption, on the contrary procedures which, either directly or indirectly, decrease 5-HT receptor activation bring about the opposite effect. Patients with bulimia nervosa have been shown to have lower cerebrospinal fluid concentrations of the 5-HT and their metabolites than healthy controls (Jimerson D.C. et al 1992). Serotonergic receptor responsiveness seems also to be impaired in bulimic patients (Brewerton T.D 1995). In addiction, selective serotonin re-uptake inhibitors have been shown to be effective in the treatment of bulimia nervosa (Rissanen A. et al. 1998). Moreover, alterations in the 5-HT system has been demonstrated in binge eating disorder (BED) (Kaye W. et al. 1998). It is recent the ability to label "in vivo" 5-HT transporter in BED (Kuikka J.T. et al. 2001). Single-photon emission tomography (SPECT) imaging with a ligand specific for SERT, shows a significantly decreased SERT binding in the mid-brain of obese BED women, thus providing evidence that diminished serotonergic activity could be involved in this eating behaviours. The contribution of the 5-HT system to obesity induced in normal rats by feeding a palatable diet has been investigated by measuring the density of binding to the main 5-HT receptor subtypes implicated in feeding (5-HT1A, 5-HT1B, 5-HT2A, 5-HT2C) and to SERT using quantitative autoradiography of midbrain and hypothalamic sections (Park S. et al 1999). It has been speculated that sustained changes in 5-HT neuronal activity would lead to reciprocal alterations in binding to the specific 5-HT receptor subtypes involved, consistent with observations that increased or decreased 5-HT release leads to fewer or greater 5-HT binding sites, respectively (Casanovas J.M. et al. 1999). Recently, a serotonergic marker of night eating syndrome (NES) has been implicated with the recently discovered therapeutic response to the selective serotonin reuptake inhibitor, sertraline. A SPECT study compared the serotonin transporter (SERT) uptake ratios, using the radiopharmaceutical (123)I-ADAM, in midbrain, basal ganglia, and temporal lobes of night eaters with those of healthy controls. Night eaters had significantly greater SERT uptake ratios in the midbrain than

healthy controls. These findings, in conjunction with the therapeutic response of NES to sertraline, indicate that the serotonin system is involved in the pathophysiology of NES (Lundgren JD. Et al. 2008). Moreover, Bruce KR et al. (Bruce KR et al. 2006) showed that women with anorexia nervosa (AN) had significantly lower levels of platelet paroxetine binding compared with control group. Simple correlation analyses showed that, within AN but not within controls, paroxetine binding was inversely related to dieting preoccupations, affective instability, anxiousness, compulsivity, restricted expression and social avoidance but independent of age, body mass index, depression, and other eating symptoms. Findings suggest that reduced peripheral serotonin transporter density in AN relates to increased dieting preoccupations, affective instability and anxiousness–fearfulness.

It appeared interesting to evaluate SERT expression in obese patients and in normalweight subjects. Since it is difficult to directly study the 5-HT function in the brain of living human, we used circulating cells. Platelets are terminally differentiated cells that exhibiting SERT presence, rapid phosphorilation upon agonist-induced activation and represent a good model system to study signal transduction events (Aharanovitz O. et al. 1999; Lesch K.P. et al. 1993). Moreover, they have been widely used in biological studies on psychiatric disorders, for the investigation of neuronal serotonergic system (Marazziti D. et al. 1998; Stahl S.M. 1997).

Obtained binding data of all subjects were statistically correlated with relative clinical parameters (as glucose, leptinemia, TNF-α, insulinemia).

3.2 Materials and Methods

3.2.1 Subjects

174 individuals, matched for age and sex, whose had no history of psychological disturbance or behavioral disorder of eating or drinking were recruited from Department of Endocrinology and Metabolic disorders of University Division, Cisanello Hospital, Pisa. The degree of obesity was estimated by body mass index (BMI) and all subjects were classified using the following values:

- Obesity class III° (BMI > 40 kg/m^2)
- Obesity class II° (BMI > 35 kg/ m^2)
- Obesity class I° (BMI between 30 and 35 kg/ m^2)
- Overweight (BMI between 25 and 29,9 kg/ m^2)
- Normal weight (BMI between 18,5 and 25 kg/ m^2)

Each class was comparable for number of subjects.
Prior screening verified the absence of co-morbid disease, including infection, inflammatory disease, underlining neuroendocrine abnormality, or cancer. Subjects with hypertension, cardiac dysrhythmias, hypercholesterolemia, hypertriglyceridemia, and diabetes mellitus were excluded.

The inclusion criteria for the study groups comprised a negative history for psychoactive drug treatment and other neurological disorders. None of the subjects had co-morbid psychiatric disorders or had received treatment with antidepressant drugs. No patient was under pharmacological treatment.

To exclude major psychiatric disorders, all patients were evaluated by means of a diagnostic interview consisting of the administration of the Structured Clinical Interview for DSM-IV axis-I disorder (SCID-I/P). This assessment was conducted by psychiatrists who were trained and certified in the use of the study instruments at our department.

All subjects gave their informed consent to participation in the study which was approved by the Ethics Committee of Pisa University.

3.2.2 Clinical and anthropometric parameters evaluation

All subjects were submitted to clinical (blood levels of leptin, insulin, glucose, cytokines, cholesterol) and anthropometrics parameters evaluation.

3.2.3 Platelet separation

Venous blood (30ml) was drawn from fasting subjects between 8 and 10 a.m., collected into plastic tubes containing 5 ml of anticoagulant (2.2% sodium citrate and 1.2% citric acid) and centrifuged at 150 xg for 15 min at 23 °C. Platelet-rich-plasma was collected and centrifuged at 1500 xg for 15 min at 23 °C. Platelets were counted automatically with a flux cytometer (Cell-dyn 3500 system; Abbott, Milano, Italy).

For measurement of [3H]serotonin reuptake, platelets were used immediately, whereas for [3H]paroxetine binding, platelets were precipitated by centrifugation at 10,000g for 10 minutes at 4°C and the pellets were then stored at -80°C until the assay.

3.2.4 Membrane preparation for binding assay

Platelet pellets were suspended in buffer (5 mM Tris–HCl, pH 7.4) containing protease inhibitors Benzamidine 160μg/ml, Bacitracine 200μg/ml and Tripsine inhibitor 20μg/ml, homogenized using an Ultraturrax homogenizer and centrifuged at 48 000x*g* for 15 min at 4 °C. The ensuing pellet was washed with buffer (50 mM Tris–HCl, pH 7.4) and treated as described above. The resulting pellets were suspended in assay buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) and homogenized using Ultraturrax. Protein content was determined by Bredford method (Bio-rad), using γ-globuline as standard.

3.2.5 [^{3}H]Paroxetine binding assay

The [^{3}H]-paroxetine (PerkinElmer, A.S. 21.3Ci/mmol) binding was carried out incubating 100 µl of membranes (50–100 µg proteins/tube) mixture consisted with 50 µl of [^{3}H]-paroxetine at five concentrations ranging from 0.08 nM to 1.5 nM, and 1850 µl of assay buffer. Specific binding was obtained as the binding remaining in the presence of 10 µM fluoxetine as a displacer. All samples were assayed in duplicate and incubated at 22 °C for 1 h. The incubation was halted while adding 5 ml of cold assay buffer and simples were treated as previously described.

3.2.6 [^{3}H]5-HT re-uptake

Platelet 5-HT uptake assays were carried out on intact platelets from all subjects. Platelets were maintained under physiological conditions, such as isotonic buffer, presence of NaCl and glucose, 37°C assay temperature.

After separation, platelets in PRP were counted automatically through a cytofluorimeter.

Serotonin uptake was carried out by incubating for 10 min. at 37°C aliquots of a cellular suspension ($2x10^6$ platelets) with crescent concentrations of [^{3}H]-5-HT (range: 40nM-1200nM) in a Krebs assay buffer, containing: 118mM NaCl; 4.7mM KCl; 1.07mM $Mg_2SO_4 \cdot 7H_2O$; 1.17mM KH_2PO_4; 25mM $NaHCO_3$; 11.6mM glucose; pH=7.4. The final assay volume was 0.5ml.

For each concentration of [^{3}H]-5-HT, non specific uptake was measured by adding 10µM unlabelled fluoxetine. After incubation, the radioactivity present inside cells was separated from the free amount by means of a rapid filtration on Whatman GF/C filters by Brandel, and three washes were subsequently carried out (5ml each). Filters were incubated for 5 min in vials containing 200 µl 0.2N NaOH to break platelets and after neutralized by adding 200 µl 0.2N glacial acetic acid. Finally, radioactivity was counted through a liquid phase scintillation β-counter Packard 1600 TR and taken as dpm. The specific uptake was calculated from the difference between total and non specific uptake.

3.2.7 Data analysis

Equilibrium-saturation binding data, the maximum binding capacity (Bmax, fmol/mg of protein) and the dissociation constant (Kd, nM) were analysed by means of the iterative curve-fitting computer programs EBDA and LIGAND (Kell for Windows, v. 6.0). For 5-HT uptake experiments, the specific uptake obtained at the different radiolabeled neurotransmitter concentrations were converted into pmol (picomoles) of 5-HT/10^9 platelets/min and data elaborated by means of the Michaelis-Menten equation.
Statistical analysis was conducted using Graph-Pad Prism 3.

3.3 Results

3.3.1 [3H]Paroxetine binding assay

Saturation and Scatchard specific binding analysis of [^{3}H]-paroxetine revealed a single population of high affinity sites in platelet membranes from all subjects under investigation. The specific binding represented about the 90% of total binding at the K_d concentration.

Table 1 shows the mean of Bmax values for the density, and Kd values for the affinity, of [3H]paroxetine binding on platelet SERT in all subjects including in this study.

Subjects	Bmax (fmol/mg proteins) (mean±S. D.)	Kd (nM) (mean± S. D.)
Normalweight	1443,8±242,55	0,088±0,032
Overweight	1340,2±203,42	0,076±0,049
Obesity Class I	891,315±133,72*§	0,047±0,006
Obesity Class II	925,233±362,52*§	0,068±0,022
Obesity Class III	926,860±261,53*§	0,071±0,039

Table 1: Kinetic parameters (B_{max} and K_d) of [3H]paroxetine binding in platelets from the populations under study. Data are presented as mean ± SEM of experiments each performed in duplicate. (*p<0,05 vs normalweight; § p<0,05 vs overweight)

Data showed a reduction of SERT density with respect to increasing body weight. By mean of t-test of Student for unpaired data, a significant reduction of platelet [^{3}H]-paroxetine B_{max} in obesity classes vs. subjects with normal BMI is revealed. Moreover a significant reduction of Bmax values was showed between obesity classes vs overweight subjects.
Age, sex and smoke did not seem to influence the binding parameters of platelet SERT in either normalweight and overweight or obese subjects.

In figure 1, the linear regression analysis between BMI and SERT B_{max} considering all subjects introduced into the study is shown: there is an inverse significant correlation between BMI and [^{3}H]-paroxetine B_{max} as fmol/mg protein (***significant, p <0,0001).

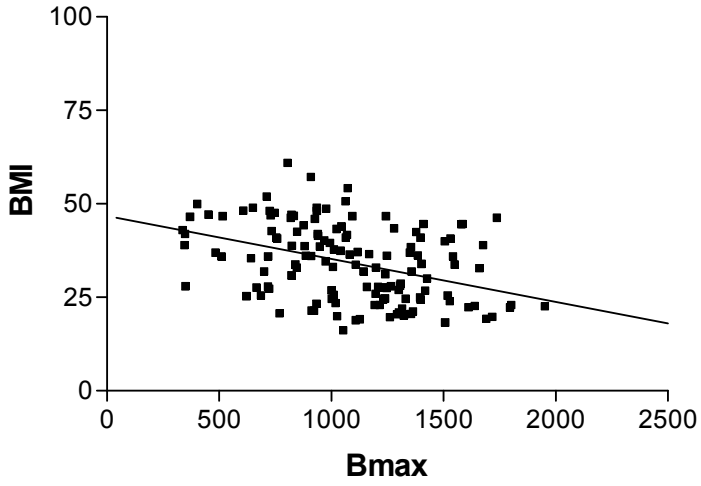

Fig.1 Linear regression between BMI and Bmax values (p < 0,0001)

Moreover, several important positive correlations was evidenced between BMI and several clinical serum parameters. Consequently negative correlations was evidenced between Bmax and the same clinical data (Table 2).

	TNF-α	**Aptoglobin**	**Leptin**	**PAS**	**Glicemia**	**Insulin**	**Triglicerids**
BMI (positive correlations)	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0088	P=0.0003	p=0.0125
Bmax (negative vorrelations)	p<0.001	p=0.0277	p=0.0003	p=0.065	p=0.00034	p<0.001	p=0.0147

Table 2: Statistical correlations between clinical parameters and BMI or Bmax values

3.3.2 [3H]5-HT up-take assay

Table 3 displays the mean ± S.E.M of maximal velocity, V_{max}, expressed as pmol/10^9plt/min, and the Michaelis-Menten, K_m, expressed in nM, of the [^{3}H]-5-HT uptake site in platelets from the sub-groups of subjects under investigation.

Neither a significant reduction of 5-HT uptake V_{max}, was observed in any group, nor a significant correlation between BMI and V_{max} was reported. Similarly to K_d, K_m did not significantly differ with respect to body weight.

Subjects	**V_{max} (pmol/10^9plt/min)**	**K_m (nM)**
Normalweight	134,5±16,86	194,8±46,65
Overweight	156,1±22,56	114,5±14,56
Obesity Class I	152.1±23.20	196,1±17,53
Obesity Class II	150.3±20.12	190.2±15.62
Obesity Class III	107.8±15.45	184,1±40,53

Table 3 Kinetic parameters (V_{max} and K_m) of 5-HT uptake in platelets from the populations under study. Data are presented as mean ± SEM of experiments each performed in duplicate.

3.4 Discussion

Amongst molecules active in modulating 5-HT transmission, SERT exerts a main function by promoting 5-HT clearance and re-uptake into the pre-synaptic terminal, therefore controlling either the duration and extent of the transmitter action at specific targets after release or its "reserve" inside pre-synaptic vesicles. For this reason, SERT expression and function is under the control of several intracellular phosphorylation systems (Ramamoorthy et al. 2007). More recently, a relationship between obesity and inflammatory-immune response has been suggested (Matarese and La Cava, 2004), indicating a cross-talk between CNS and periphery in the control of body weight. In a preliminary attempt to elucidate these networks, the present study was thus designed to investigate the influence of 5-HT on body weight by measuring the equilibrium binding parameters (maximal binding capacity, B_{max} and dissociation constant, K_d) of the high affinity SERT ligand [^{3}H]-paroxetine in platelets from 174 subjects recruited on the basis of their body mass index (BMI), starting from 18 to > 40 kg/m^2. The high affinity ligand [^{3}H]-paroxetine was used as a selective tracer of SERT and subjects recruited to obtain five groups as regards to BMI: 1. normal weight (18-25 kg/ m^2), 2. overweight (26-30 kg/ m^2), 3. obese, I grade, (31-35 kg/ m^2), 4. obese, II grade, (36-40 kg/ m^2); 5. obese, III grade, (>40 kg/ m^2). All subjects were also accurately monitored for clinical-biochemical parameters, including insulinemia and leptinemia blood glucose, insulin, leptin cytokines and other clinical-chemical parameters. We have chosen to study platelets as these non nucleate cells display an identical SERT to the brain one as well as several 5-HT receptor subtypes. For such reasons, platelets are considered "indicators" of 5-HT-regulated CNS-periphery connections and have been extensively studied in psychiatric-affective disorders since many years (Mellerup et al, 1983; Martini C. et al., 2004). Finally, platelets respond to agonist-induced activation by rapid phosphorilation and represent, therefore, a good model to study neuronal signal transduction events in periphery (Aharanovitz and Granot, 1996).

Our study is the first that showed a significant reduced SERT density in platelets of obese subjects ($p<0.05$), providing new insights in the role of 5-HT system and obesity.

Moreover, no BMI-dependent changes in [3H]-paroxetine Kd (nM) values, but a significant inverse correlation ($p<0,0001$) between [3H]-paroxetine Bmax values (fmol/mg protein) and BMI.

Consequently, a negative significant correlation was observed between [^{3}H]-paroxetine B_{max} values and: blood leptin, TNF-α, insulin, glicemia, PAS, aptoblobin and triglycerides.

In conclusion, these findings show that platelet SERT number is reduced in obese subjects and such a decrease could be linked, in part, to the efficiency of the insulin/adipokine-related downstream signaling cascade in periphery. These results could explain that adipose tissue entity are able to modulate SERT expression, but not his functionality, in platelets membrane.

Chapter 4

4.1 Introduction

The serotonergic system is involved in the energetic homeostasis regulation and its activation tends to produce a negative energetic imbalance, mean an inhibition of food intake and an higher caloric expenditure. Moreover, adipocytes produce different factors which are able to regulate appetite, thermogenesis and insulin sensitivity. It is unknown how the serotonergic system is integrated with other pathways involved in energetic metabolism control, and in particular if it is regulated by adipose depots entity.
Our laboratory showed a reverse correlation between the body mass index (BMI) of obese and lean subjects and Bmax (maximal density) values of serotonin transporter (SERT) obtained in [3H]paroxetine binding assays. These results could explain that adipose tissue entity are able to modulate SERT expression in platelets membrane. After that, our results showed a significant correlation between Bmax values and serum concentrations of different factors, which values are greater in obese subjects, as inflammation markers.

Identification of isolated cellular model, capable to express SERT and having biochemical characteristics of platelet, could be useful to study the modulation of serotonin transporter by different factors related with obesity. We identified this model in MEG-01, a human megakaryoblastic leukaemia cell line, which specifically retains the morphological and functional properties of bone marrow megakaryocytes. This cell line is considered to be the most suitable one for evaluating human megakaryocytic maturation and differentiation into platelet-like cells (Isakari Y. et al 2007).
Literature studies reveal that MEG-01 cells mantain the capacity to differentiate morphologically, ultrastructurally and biochemically into more mature megakaryocytes through phorbol esters (β-TPA) stimulation (Yang M. et al 1996).
A first aim of this research project was to evaluate serotonin transporter (SERT) expression in Meg-01 cell line, before and after treatment with β-TPA, using monodimensional electrophoresis, quantitative real-time PCR and immunofluorescence technique. Moreover we evaluated SERT functionality by mean of radiolabeled 5-HT up-take.

4.2 Material and Methods

4.2.1 Cell culture and treatment

The human megakaryoblastic cell line MEG-01 was purchased by LGC Promochem (American Type Culture Collection (ATCC),VA, USA- Milan, Italy).

MEG-01 cells were seeded (2x105 cells) on T-75 ventilated cap flasks (Sarsted,Verona, Italy) in modified RPMI 1640 medium (LGC Promochem), containing 0.3g/liter L-glutamine (LGC Promochem), supplemented with 10% fetal bovine serum, FBS (LGC Promochem), antibiotics (100 U/ml penicylline and 100 mg/ml streptomycine, LGC Promochem). Cells were cultured inside a humidified incubator at a temperature of 37°C under an atmosphere of 5% CO2. Each 3-4 days the spent medium was diluted with fresh medium after centrifugation of cells at 1100 g for 5 min at room temperature. MEG-01 passages and sub-cultures were carried out in 10- cm dishes (Sarsted) for about 10-12 days after seeding. Cell mortality was tested by the Trypan blue dye test. Between passages 3-4, when they were usually in good growth, showing a low cell mortality (2-5%) and low rate of spontaneous differentiation, cells were divided in two different sub-cultures: 1) control MEG-01 cells, allowed to grow in usual RPMI medium; 2) treated MEG-01 cells, cultured in RPMI medium supplemented with 10-7M β-TPA (4-β-12-tetradecanoylphorbol-13-acetate/Phorbol 12-Myristate 13-acetate; Sigma-Aldrich, P1585, Milan, Italy). Since β-TPA is dissolved in ethanol (EtOH), control cells were treated with EtOH vehicle. Control and treated sub-cultures were carried out by changing medium as above described, except for SERT mRNA expression experiments, where cells were maintained in culture for 3 day only: in fact, in pilot experiments, after 3 days of β-TPA exposure, SERT mRNA transcription reached a steady state plateau.

4.2.2 SERT Western Blot analysis

For Western blot analysis, both control and treated MEG-01 cells were harvested and centrifuged at 1000 g for 25 min at room temperature. Pellets were solubilized n lysis buffer: 25mM HEPES, 0.44% sodium-dodecyl sulfate (SDS, Sigma- ldrich), 1% Triton X100, 111 mM NaCl, 2.2mM EDTA, Protease Inhibitor ocktail (Sigma-Aldrich) pH 7.4, sonicated and centrifuged at 12 000 g for 20min.

Total protein amount in soluble fractions was estimated using the Bio-Rad RC/DC rotein assay, using bovine serum albumin (BSA) as the standard. Sample aliquots were mixed to SDS-reducing buffer (Laemmli 2X: 1.25 ml 0.5 m Tris-HCl, pH 6.8, .15 ml 20% SDS, 0.5 ml β-mercaptoethanol (Sigma-Aldrich), 1 ml glycerol, 6.1 ml 2O, 2 mg bromophenol blue (Sigma-Aldrich) and boiled for 3min. 50µg of sample and standard proteins (Precision Plus Protein Standards, Biorad, 161-0373) were then loaded into a SDS-PAGE 10% polyacrylamide gel and separated by electrophoresis (16 mA for the first 15 min; 30 mA for about 1h.30 min) in running buffer (25 mM trizma base, 192 mM glycine, 0,1% SDS) at 4°C. Electrophoresis was carried out in a dual Mini vertical gel system (Biorad): one gel was blue-Comassie stained for band visualization, the other one was electroblotted (25V for 2hours) into a 0.2 µm nitrocellulose paper. The nitrocellulose blotted membrane was then incubated in a PBS-milk solution (10 mM NaH2PO4, 0.9% NaCl pH 7.5, 3% milk, 0.2% Tween) for 60 min under agitation; afterward, nitrocellulose was incubated for 2 hours at 4 °C under agitation in PBS-milk solution containing the anti-SERT rabbit polyclonal first antibody (1:400 diluted) (Chemicon, AB9322).

Nitrocellulose was then washed 4 times in PBS-milk and incubated in PBS-milk with a peroxidase-labelled goat anti-rabbit secondary antibody (1:20 000; Santa Cruz) for 1 hour at room temperature. For specific SERT band visualization, nitrocellulose was incubated with a solution containing Luminol (ECL kit, Amersham) for 1 min and exposed to a Kodak Biomax ML film for 30 min. SERT band signal intensity in control and treated cells was analyzed by a densitometry program (Image J).

4.2.3 Isolation of RNA and PCR of SERT m-RNA

Total RNA was isolated from MEG-01 cells with an extraction kit (Tripure, Roche Molecular Biochemicals) and its integrity was evaluated by formaldehyde agarose gel electrophoresis. RNA was treated with Rnase-free Dnase (Roche Molecular Biochemicals) to remove any contaminating genomic DNA. First-strand cDNA synthesis was performed using Superscript III reverse transcritase (Invitrogen) and random oligo hexamers (Pharmacia), following manufacturer instructions. SERT expression in control and treated MEG-01 cells was evaluated by TaqMan (Applied Biosystems 7700) quantitative Real Time PCR. In detail, 50 ng of cDNA were amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey USA) using specific oligos and probes commercially available from Applied Biosystems (SERT: HS00169010_m1). The relative abundance of mRNA was calculated using the TATA Binding Protein (TBP) mRNA as the invariant control (TBP: HS0042760_m1).

4.2.4 Immunofluorescence/bis-benzimide labeling

MEG-01 cells ($2x10^5$ cells/well) were seeded in BD-BIOCOAT poly D-lysine cellwares (8 well culture slides: BD BIOSCIENCE). Treated and control Meg-01 cells were rinsed with PBS 0.1 M buffer (81 mM NaH_2PO_4, 19 mM Na_2HPO_4, 0.9 % NaCl, pH=7.4), fixed in paraformaldehyde solution (4% in PBS) at room temperature (RT) for 15 min and then washed three times with PBS for 5 min. Non-specific site blocking and cell permeabilization was performed by incubating cells in a PBS solution containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 at RT for 30 min. Cells were then incubated at 4 °C with the first antibody, specific for the 516-630 amino acidic C-terminal region of SERT (rabbit anti SERT polyclonal antibody, Chemicon, AB9322), 1: 1000 diluted in PBS containing 1% BSA and 0.01% Triton X-100. Cells were then washed three times with PBS and incubated at RT for 2 hr with a 1:200 diluted Alexa Fluor 488 (Goat Anti rabbit IgG (H+L), Molecular Probes, A11008) fluorescein isothiocyanate (FITCH) labeled secondary antibody.

After PBS washing, cells were mounted in Vectashield (Vector Laboratories) medium. To improve signal visualization, cells were also labeled with the nuclear labelling bis-Benzimide (Hoechst 33258, Sigma) 1:10 000 dilution in PBS at RT for 2 min. Cell fluorescent signal (λex.=495 nm and λem.=519 nm) was viewed using a Zeiss Axioskop microscope (Carl Zeiss GmbH, Jena, Germany). Digital images were taken by a Leica DC100 camera (Leica, Wetzlar, Germany). The brightness and contrast of final images were analyzed by means of Adobe Photoshop version 6.00 (Adobe Systems, Mountain View, CA).

4.2.5 Measurement of 5-HT up-take

[^{3}H]-5-HT re-uptake was measured in control and β-TPA-treated MEG-01 cells according to a slightly modified procedure from Arora and Meltzer (1981): aliquots of cells (0.2mg of proteins/assay tube) were incubated for 10 min at 37 °C with [^{3}H]-5-HT (Perkin Elmer Life Science, Milan, Italy; specific radioactivity: 28.1 Ci/mmol) at six concentrations (0.1-10 μM) in 0.5 ml (final volume) of assay buffer (Krebs buffer, KHP) composed by 118 mM NaCl, 4.7 mM KCl, 1.07 mM $MgSO_4.7H_2O$, 1.17 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.6 mM glucose, pH 7.4, in the presence of 0.1 % ascorbate and 100 μM pargyline. Non-specific uptake was measured in the presence of 10 μM fluoxetine. SERT velocity was stopped by ice-cold KHP buffer and rapid filtration through Whatman GF/C filters, using a Brandel cell harvester apparatus. Filters were then washed three times with assay buffer, put into a vial containing 4 ml scintillation cocktail liquid (UltimaGold, Packard), shacked and measured by means of a β-counter (Perkin Elmer Tricarb 2800TR). SERT velocity was calculated as pmol 5-HT /mg protein/10 min, using the Eadie-Hofstee linear regression analysis. Total protein assay was carried out following the procedure of Bradford (Bradford 1976).

4.2.6 Membrane preparation for binding assay

Cells were harvested, precipitated and centrifuged at 1000x*g* for 10 min at RT. Pellets were suspended in buffer (5 mM Tris–HCl, pH 7.4) containing protease inhibitors Benzamidine 160μg/ml, Bacitracine 200μg/ml and Tripsine inhibitor 20μg/ml, homogenized using an Ultraturrax homogenizer and centrifuged at 48 000x*g* for 15 min at 4 °C. The ensuing pellet was washed with buffer (50 mM Tris–HCl, pH 7.4) and treated as described above. The resulting pellets were suspended in assay buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) and homogenized using Ultraturrax. Protein content was determined by Bredford method (Bio-rad), using γ-globuline as standard.

4.2.7 [^{3}H]Paroxetine binding assay

The incubation mixture consisted of 100 µl of membranes (100-200 µg proteins/tube), 50 µl of [^{3}H]-paroxetine at five concentrations ranging from 0.08 nM to 1.5 nM, and 1850 µl of assay buffer. Specific binding was obtained as the binding remaining in the presence of 10 µM fluoxetine (Tocris) as a displacer. All samples were assayed in duplicate and incubated at 22 °C for 1 h. The incubation was halted while adding 5 ml of cold assay buffer. The content of the tubes was immediately filtered under vacuum, through glass fiber filters GF/C (Whatman) 2.5 cm in diameter and washed three times with 5 ml of ice-cold assay buffer. Equilibrium-saturation binding data, the maximum binding capacity (*B*max fmol/mg) and dissociation constant (*K*d, nM), were analysed by means of the iterative curve-fitting computer program (Kell Radlig, version 6)

4.3 RESULTS

4.3.1 SERT Western blot analysis in β–TPA-treated and control MEG-01 cells

As shown in Figure 1A , Western blot produced a single SERT band with an apparent molecular weight of 71 KDa in both treated or untreated cells; the figure also depicts the semi-quantitative comparison of SERT band signals by densitometry, revealing significantly higher mean optical density values (t-test, P=0.0416) in 8 day β-TPAtreated (8743 ± 886, mean ± S.E.M., O.D. arbitrary units) vs. control (5694 ± 527, mean ± S.E.M., O.D. arbitrary units) MEG-01 cells, corresponding to an about 1.5 fold increase of SERT protein (Figure 1B). After 3 day of β-TPA treatment, no difference in SERT band optical density was instead observed between control and treated cells (data not showed).

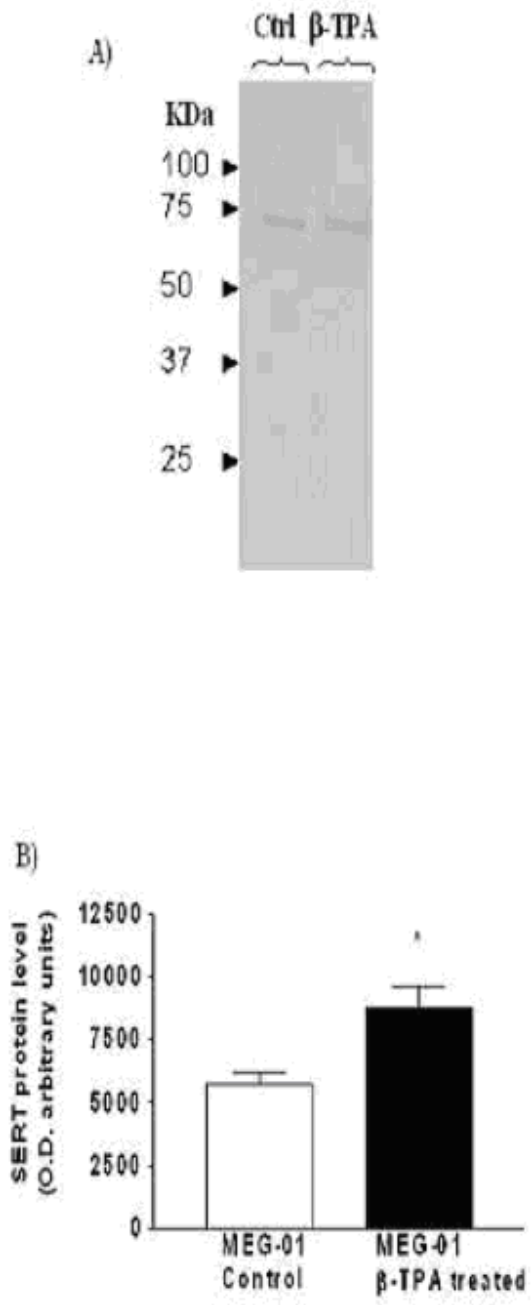

Fig.1 Western Blot (A) and SERT protein levels (B)

4.3.2 SERT mRNA transcription is enhanced in β-TPA-treated MEG-01 cells

After MEG-01 cell treatment by β-TPA for 3 days, real time PCR revealed a threefold increase of SERT mRNA concentration in treated vs. control cells. SERT mRNA levels (n=10, independent experiments) are represented in Fig. 2, expressed as relative measure to the housekeeping gene transcript, TBP. Statistical analysis resulted in a highly significant greater amount (t-test, $p<0.0001$) of SERT mRNA in treated cells (0.911 ± 0.013, mean ± S.E.M., relative SERT mRNA) vs. control (0.346 ± 0.028, mean ± S.E.M., relative SERT mRNA).

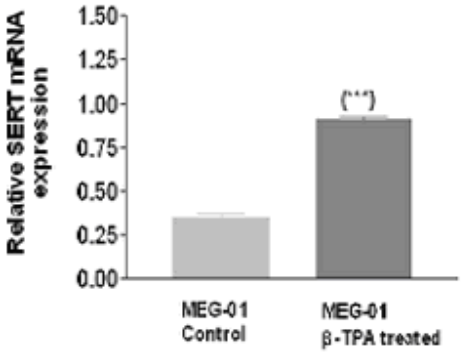

Fig. 2: SERT mRNA levels

4.3.3 β-TPA-treated MEG-01 cells show a different SERT immunofluorescence pattern

After cell exposure to 10-7 M β-TPA in 10% FBS for 8 days, significant MEG-01 morphological changes were observed in culture flasks by inverse microscopy: megakaryoblasts increased in size, became adherent and emitted cytoplasm extrusions (blebs): about 70-80% of total alive MEG-01 cells presented cytoplasm blebs (Figures 3). Figure 4 shows double-labeled (green SERT/blue DNA-specific dye) MEG-01 cells, control (4a) vs. 8 day β-TPA-treated cells (4b,c,d). Fluorescence microscopy observation of undifferentiated cells reveals specific SERT signals, clearly indicating that megakaryoblasts express SERT. A more differentiated megakaryoblast is also visible, showing a greater cytoplasm with a more diffuse fluorescence (see arrow, Figure 4a). After 8 days of culture with β-TPA, morphological changes were paralled by a greater and diffuse fluorescence pattern of SERT protein, distributed in correspondence of either cell bodies or blebs, as shown by arrows in Figure 4b,c,d.

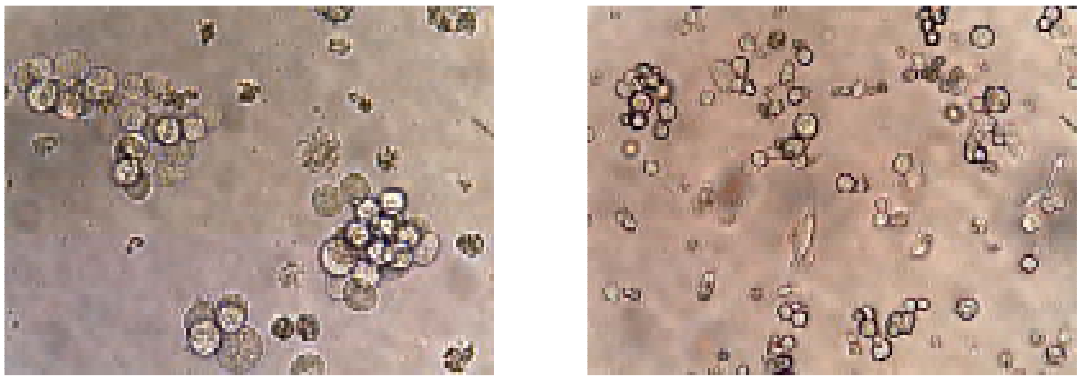

Fig 3: Meg-01 control and Meg-01 after treatment with 10^{-7} M β-TPA

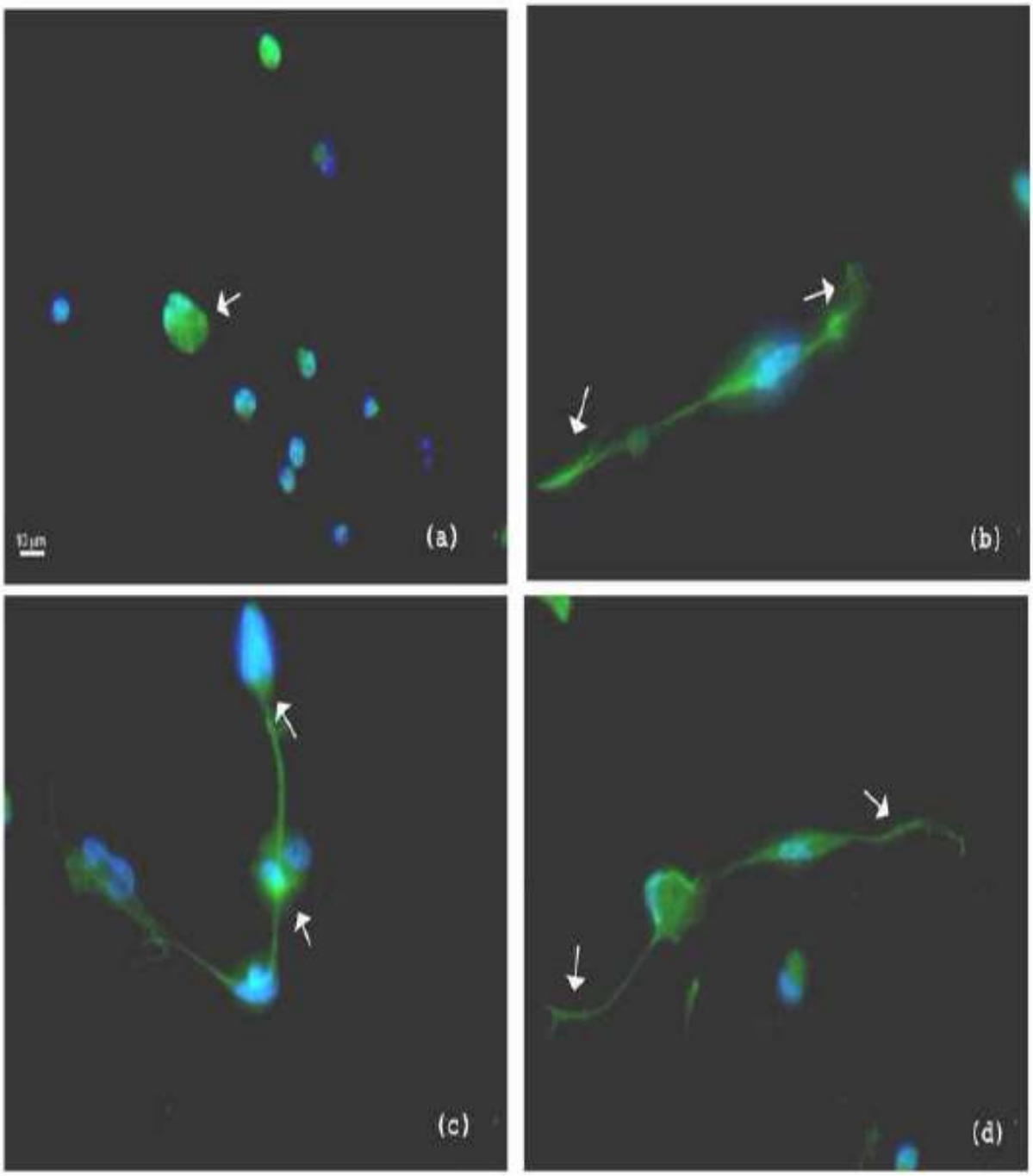

Fig.4 Meg-01 Immunofluorescence

4.3.4 Comparison of [3H]-serotonin uptake in β–TPA-treated and control MEG-01 cells

Table 1 reports SERT functional kinetic parameters, the Michaelis-Menten constant, Km (μM), and the maximal 5-HT uptake velocity, V_{max}, in treated and control MEG-01 cells. The mean SERT V_{max} in 8 day β-TPA exposed cells resulted about 1.37-fold higher than that observed in control cells, without reaching however the statistical (t-test, p=1609). Km uptake values resulted unchanged in β- TPA-treated MEG-01 cells vs. controls.

	Km (μM)	**Vmax (pmol/mg protein/10min)**
Control n=3	0.51 ±0.10	0.30 ±0.05
Treated n=3	0.48 ±0.08	0.41 ±0.03

Table 1: Up-take kinetic parameters

4.3.5 [^{3}H]Paroxetine binding assay

The Scatchard analysis on untreated and β- TPA-treated cell membranes did not showed serotonin transporter expression.

4.4 Discussion

The evidence that SERT is present in megakaryoblasts suggests the physiological relevance of a balanced control of 5-HT levels during the differentiation processes leading to platelet formation (Liu YS et al 2006; Yang M et al 2007). After 8 days of MEG-01 cell culture with β-TPA, significant cell morphological variations were observed, accompanied by changes in SERT immunostaining, from a perinuclear (undifferentiated cells) to a cytoplasm-diffuse (differentiated cells) fluorescence pattern. In β-TPA activated MEG-01 cells a maximal increase of SERT mRNA was observed after 3 day of stimulation, reaching the steady state plateau; a moderate increase in protein expression was noticeable after 8 days of culture in β-TPA, as shown by densitometric analysis of SERT immunoblot band and, even if not significantly, by 5-HT uptake results. 5-HT re-uptake experiments have shown that SERT is present in the plasma membrane of MEG-01 cells. Western blot revealed a single SERT band in control and treated cells, with an apparent size of 71 KDa, a M.W. comparable to that commonly reported for SERT in human brain and platelets (67-75 KDa). These findings demonstrate that megakaryoblastic cells increase SERT expression during megakaryocytopoiesis and that differentiation into megakaryocyte and proplatelet-like formation ensures an adequate SERT reserve and 5-HT storage in circulating platelets. The enhancement of SERT expression in β-TPA treated cells seems in apparent contradiction with β-TPA early events observed in platelets (Marazziti D et al 1999c; Jayanthi LD et al.2005; Carneiro AM et al. 2006). Nevertheless, some authors have reported that 1 μM β-TPA is able either to reduce (after 2h) or to stimulate (after 16 h) SERT uptake velocity in JAR placental cells (Ramamoorthy JD et al 1995). Jiang and coauthors (2002) have observed that both fibronectin and protein kinase C-dependent ERK1/2 MAPK activities are essential to promote megakaryoblastic differentiation. These authors have demonstrated that activation of protein kinase C alone (serum free β-TPA stimulation) is not able to promote a full megakaryocytopoiesis process. Thus, the observed SERT increase might depend from β-TPA-protein kinase C pathways and other signaling cascades. The protein kinase network and downstream signal convergent MAPK pathways could play a key role in SERT modulation. Proteomic analysis and gene expression studies together the use of selective protein kinase (also protein kinase C) or MAPK inhibitors will clarify the signaling pathways involved. On the basis of results we hypothesize that our findings derived from an equilibrium between different SERT regulatory effects:

differentiation events, inducing morphological changes and leading to the formation of cytoskeleton-organized cells with 5-HT storage vesicles (Ogura M et al 1998), increase total SERT protein density (necessity to accumulate 5-HT in dense granules); phosphorylation, down-regulation and trafficking mechanisms (linked to cytoskeleton formation) can start having influence on SERT protein expression and function, especially in late events of megakaryocytopoiesis. This could explain the observed discrepancy between SERT mRNA and protein expression after 8 days of treatment with β-TPA. We cannot, in fact, exclude that our Western blot protein extracts were enriched fractions of cytoplasm and plasma membrane components. These data seem to be in accordance with binding results obtained on cell membranes. Confocal microscopy of MEG-01 cells activated by β–TPA will verify SERT localization during early and late differentiation events. Since diverse protein regulatory patterns are present in immature megakaryoblasts cells as regards to pro-platelets and platelets (Tytgat GAM et al. 2002), the MEG-01 model should be primarily applied as a developmental model, for investigations regarding molecular differentiation events. For instance, it should be mentioned that a SERT regulatory endoproteolytic cleavage has been observed in human platelets, producing fragments of different size after immunoblot analysis, whereas a single SERT band was resolved in MEG-01 cell extracts, using the same primary antibodies, as here reported.

5. Future studies

Megakaryoblastic MEG-01 differentiation is a complex phenomenon leading to up or down-regulation of a variety of genes (Isakari Y et al 2009). Nevertheless, taking into account all limits, the use of MEG-01 cells can be the starting point for many investigations as the evaluation of hormone and transmitter effects on megakaryoblastic differentiation. Moreover, this cell line could be a cellular model to screen those of obesity serum factors are mostly important for long-term SERT down or up-regulation.

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