Current Topics in Biochemical Research
Vol. 20, 2019

Original Article

Association with serotonin transporter enables the phosphorylation of insulin receptor in placenta

Fusun Kilic[1,*], Imane Moutkine[2] and Luc Maroteaux[2]

[1]Department of Biology, Merced College, Merced, California, USA.
[2]UMR-S1270 INSERM, Sorbonne Université, Institut du Fer à Moulin, Paris, France.

ABSTRACT

Upon binding to insulin, the β-subunit of insulin receptor (IR) is phosphorylated and instantly activates intracellular signaling. A defect in this process causes the development of several metabolic disorders including non-insulin-dependent diabetes, such as type 2 and gestational diabetes mellitus (GDM). Under diabetic conditions the phosphorylation of IR in placenta, but not in platelets, is impaired. Interestingly the cellular distribution of the serotonin transporter (SERT), which utilizes the insulin signaling for posttranslational modification, shows tissue-type-dependent variation: SERT function is impaired in GDM-associated placenta, but not in platelets. In order to understand the correlation between IR, SERT and their tissue-type-dependent features, we tested an association between SERT and IR and whether this association affects the phosphorylation of IR. Using various approaches, we demonstrated a physical association between the Carboxyl terminal of SERT and the β-subunit of IR. This association was found on the plasma membrane of the placenta and the platelets. Next, the contribution of the SERT-IR association to the phosphorylation of IR was analyzed in heterologous and endogenous expression systems following insulin-treatment. The *in vivo* impact of SERT-IR association on the phosphorylation of IR was explored in placenta and platelets of SERT gene knockout (KO) mice. The IR phosphorylation was significantly downregulated only in the placenta, but not in platelets of SERT-KO mice. These findings are supported by time course experiments, which demonstrate that the phosphorylation of IR occurs vis-a-vis IR-SERT association, and at least one of the IR binding domains is identified as the carboxyl-terminus of SERT. These findings suggest an important role for IR-SERT association in maintaining the phosphorylation of IR and regulating the insulin signaling in placenta.

KEYWORDS: platelet versus placenta, tissue-type-dependent phosphorylation of insulin receptor, cellular distribution of serotonin transporter.

INTRODUCTION

Insulin receptor (IR) is a heterotetrameric membrane protein with 2α- and 2β-subunits, connected through disulfide bridges [1, 2]. The α-subunit is located at the extracellular surface and binds insulin; the β-subunit spans the plasma membrane and has cytoplasmic kinase binding domain. Homo- and hetero-oligomerization between the α- and β-subunits confer the right positions to expose tyrosine residues of the β subunit to the kinase domain for phosphorylation [3, 4].

Binding of insulin to α-subunit stimulates the phosphorylation of tyrosine residues on the β-subunit and simultaneously activates the tyrosine kinase to phosphorylate the IR leading to the insulin signaling. This stimulates a cascade of cellular processes and promotes membrane trafficking of glucose transporter to the cell

*Corresponding author: fusun.kilic@mccd.edu

surface in order to uptake glucose in cells. Various factors are involved in triggering the insulin signal transduction pathways including proteins associated with IR as well as by fed versus fasting states, stress levels, and a variety of other hormones.

Insulin signaling is balanced between the phosphorylation and dephosphorylation of IR by tyrosine kinases and phosphatases. Interestingly, while the phosphorylation of IR on tyrosine residues stimulates downstream insulin signaling, the phosphorylation on serine/threonine residues decreases the insulin signaling. Studies demonstrated that, in addition to self-association between α- and β-subunits, IR also associates with other proteins, which stabilize IR at the cell surface and alter the structure of the receptor [5-7].

A non-responsive IR to insulin signaling is the most common metabolic complication of obesity and non-insulin-dependent diabetes mellitus, such as type 2 diabetes and gestational diabetes mellitus (GDM), the most common metabolic complication of pregnancy, affecting up to 10-15% of all pregnancies [8, 9].

Diabetes-associated defects in IR are tissue-type-dependent; in placental cells the phosphorylation level of IR is low, while it is unaffected in platelets of the maternal and cord blood of the same subject with diabetes [10]. Interestingly, the cellular distribution of serotonin (5-HT) transporter (SERT) molecules was found different in placenta and platelet; the level of SERT molecules on the placental cell surface was downregulated, but it is not changed on the surface of platelets from diabetic subjects [10]. Furthermore, SERT knockout (KO) mice were reported as developing insulin resistance with pathologies of obesity and diabetes [11-15]. Based on these published studies, we hypothesized that SERT molecules on the surface of placental cells, trophoblasts should play an important role in developing insulin signaling. Therefore, here our hypothesis is explored by studying the association between the phosphorylation of insulin β-subunit and SERT in placental cells, trophoblasts.

The involvement of SERT in regulating the body fat storage and glucose homeostasis have been reported in mice [15, 16], but the mechanism by which SERT deficiency promotes obesity, and the glucose intolerance with similar symptoms of type 2 diabetes remains unknown. Here, initially, immunoprecipitation assays followed by BRET analyses were used to investigate a possible physical association between SERT and IR in placenta and platelet. Then, the functional implication of SERT-IR association was analyzed with insulin. We found that IR-SERT association was altered by insulin treatment as well as by diabetic conditions. Therefore, a functional association between SERT-IR was tested next, to understand if their association promotes the development of insulin signaling through assisting the phosphorylation of IR. Using various approaches, our hypothesis was tested in endogenous and heterologous expression systems, and importantly, in SERT gene-silenced, SERT-KO mouse placenta. Our findings demonstrate that total IR expression and cell surface levels were similar in SERT-KO as in wild type (WT) mouse placenta, yet the phosphorylation of IR was found significantly reduced in SERT-KO placenta. Further time course experiments evaluated phosphorylation of IR vis-a-vis IR-SERT associations. These findings clearly demonstrate that SERT-IR association plays very important role in insulin signaling *via* facilitating the phosphorylation of IR in placental cells.

METHODS

Twelve weeks old adult male C57BL/6J mice WT, or SERT-KO [17] mice were engineered on a C57BL6 genetic background. SERT-KO mice were provided by The Jackson Laboratory (Bar Harbor, Maine). Homozygous *SERT*$^{-/-}$ male and female mice (12 weeks old) were bred together and litters were analyzed. These mice were genotyped by polymerase chain reaction (PCR) amplification of genomic DNA prepared from the tails of *SERT*$^{-/-}$ mice [17]. Procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Plasmids, constructs, and cell line expression systems

Mutant transporters were constructed utilizing a Stratagene Quickchange XL site-directed mutagenesis

kit. All synthetic constructs were verified *via* DNA sequencing [18, 19].

JAR, human placental choriocarcinoma, cells were maintained in RPMI. Media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. For all studies, cells were transiently transfected with SERT constructs using a 1:2.5 ratio of Lipofectamine 2000 reagent to DNA in OPTIMEM. SERT expression was determined with quantitative Western blot (WB) analysis on a VersaDoc 1000 analysis system [18].

Bioluminescence resonance energy transfer (BRET) assays

BRET assays were performed according to published methods [20]. Briefly, CHO cells (5 x 10^5 per well of a 6-well plates) were transfected with 30-100 ng plasmid DNA coding for the BRET donor (IR-Luc) and increasing amounts of BRET acceptor plasmids (SERT-YFP; 100-4000 ng per well). Twenty-four hours after transfection, cells were washed in phosphate buffer saline (PBS), detached using 10 mM EDTA (Ethylenediaminetetraacetic acid) in PBS, centrifuged (1,400 *g* for 5 minutes), resuspended in Hank-balanced salt solution and distributed in 96-well plates (PerkinElmer plates; 1.2×10^6 cells per assay). After addition of the luciferase substrate, coelenterazine-h (5 μM final concentration), luminescence and fluorescence were measured simultaneously (at 485 and 530 nm, respectively) in a Mithras LB940 plate reader. The BRET ratio was calculated as: ([emission at 530 nm/emission at 485 nm] – [background at 530 nm/background at 485 nm]), where background corresponds to signals in cells expressing the Rluc fusion protein alone under the same experimental conditions. For better readability, results were expressed in milli-BRET units (mBRET), 1 mBRET corresponding to the BRET ratio multiplied by 1000. BRET ratios were plotted as a function of ([YFP-YFP0]/YFP0)/(Rluc/Rluc0), where YFP is the fluorescence signal at 530 nm after excitation at 485 nm, and Rluc the signal at 485 nm after addition of coelenterazine-h. YFP0 and Rluc0 correspond to the same values in cells expressing the Rluc fusion protein alone.

Immunoprecipitation (IP) and Western blotting (WB) analysis

JAR cells (1.2×10^6 cells per IP assay) were lysed in IP buffer (55 mm triethylamine (pH 7.5), 111 mm NaCl, 2.2 mm EDTA, 0.44% SDS (Sodium dodecyl sulfate), 1% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (PIM) as previously described [18]. Initially, cell lysate was incubated with protein A sepharose beads to eliminate non-specific interaction (preclear). Anti-SERT monoclonal (Mab technology, Stone Mountain, GA) Ab, was conjugated to protein A bead for 2 hours prior to incubating together with pre-cleared cell lysate overnight at 4 °C.

WB analysis was done the next day using anti-IR Ab (Santa Cruz Biotech, Santa Cruz, CA) phospho-AKT (T308) (Cell Signaling, Danvers, MA) or monoclonal Phospho-tyrosine for primary Ab (eBioscience, S. Diego, CA). Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse was used as the secondary Ab. VersaDoc 1000 gel visualization and analysis system was applied to determine the densitometry of individual bands.

Cell surface biotinylation

Cell surface expression of SERT was detected after biotinylation with membrane-impermeant NHS-SS-biotin as described previously [18, 19]. Briefly, upon the biotinylation reaction, the cells were treated with 100 mM glycine to quench unreacted NHS-SS-biotin and lysed in TBS containing 1% SDS, 1% TX100, and PIM/PMSF. The biotinylated proteins were recovered with an excess of streptavidin-agarose beads during overnight incubation. Biotinylated proteins were eluted in WB sample buffer, resolved by SDS-PAGE and transferred to nitrocellulose, and were detected as described previously [18, 19].

Data analysis

Densitometric analyses were performed with Origin software using nonlinear regression equation. Each experiment was performed at least three times. All data were statistically analyzed and standard deviation (SD) and means were calculated. Data are presented as mean ± S.D. unless otherwise noted. Statistical significance was considered at $P < 0.05$.

RESULTS

Insulin-mediated physical association between IR-SERT in JAR cells

An association between IR and SERT was tested by immunoprecipitation (IP) and BRET assay.

IP assays were performed in 24-hour serum-starved JAR cells (1.2×10^6 cells per assay), which expressed SERT and IR endogenously. Following starvation, JAR cells were treated with insulin at different concentrations (0-500 nM) for 10 min. At each concentration, a group of JAR cells was harvested, lyzed and the lysate incubated with IR-Ab coated protein A Sepharose beads. The proteins associated with the IR-Ab were eluted and analyzed by Western blotting (WB) for SERT as well as for the phosphorylation of IR (Figure 1A). Insulin treatment at various concentration resulted in phosphorylation of IR at different levels but 10 and 100 nM insulin increased the level of phosphotyrosine (pTyr) on IR to the highest level. However, as reported previously, treatment with 500 nM insulin decreased the cellular IR level significantly [21].

Insulin binding to IR leads to the activation of the insulin signaling pathway, by phosphorylation of

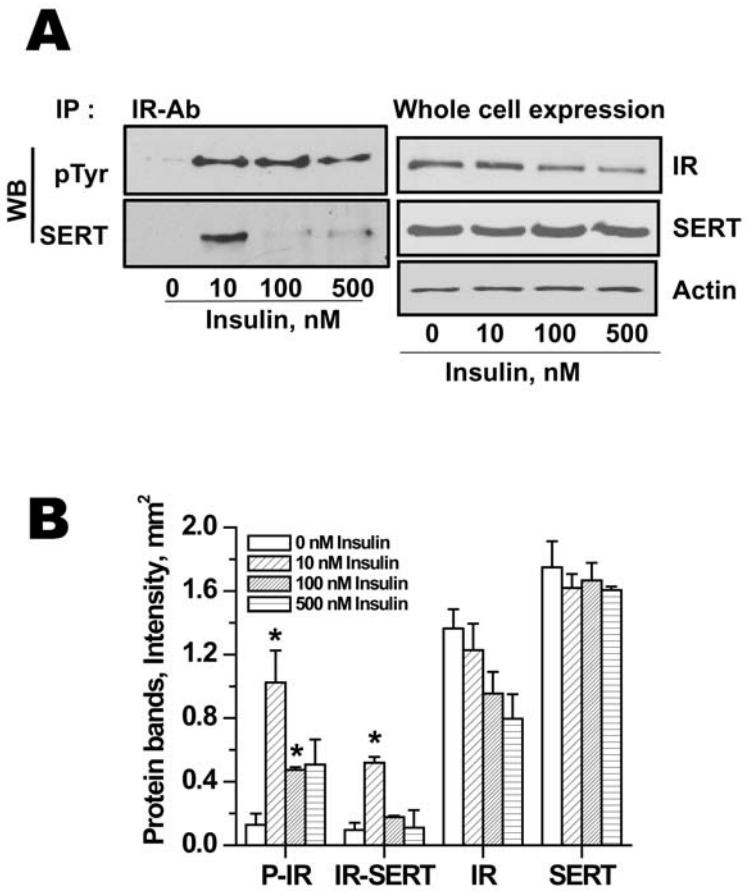

Figure 1. Insulin-mediated physical association between IR-SERT. (**A**) JAR cells endogenously express IR and SERT. JAR cells (1.2×10^6 cells per assay) were first starved overnight in serum-free medium, then treated with 0, 10, 100, 500 nM insulin for 10 min. After the incubation time, IP assay was performed with polyclonal IR-Ab and WB with pTyr-Ab to determine the level of phosphorylation in each group of cells. The levels of p-IR reached to the highest level with 10 nM insulin although at that concentration insulin treatment did not change the total IR expression. At higher concentrations (100 and 500 nM) insulin treatment lower the total IR levels [31]. In serum-starved JAR cells IP analysis determined IR-SERT association only in 10 nM insulin-treated cells. (**B**) The results of WB analysis are the summaries of combined data from four densitometric scans of pTyr. Rate of p-IR is expressed as the means and SD values of triplicate determinations from three independent samples in each group. The (*) represents the results of a two-tailed Student's t-test with $p < 0.05$, compared with 0 nM insulin-treated, control group.

IR on cytoplasmic tyrosine residues. In JAR cells, the basal (non-stimulated) level of the phosphorylated IR was 0.13 ± 0.07, which was increased by 8-fold (1.02 ± 0.2, $P < 0.001$) by 10 nM insulin treatment (Figure 1B). Therefore, all subsequent analyses involving insulin treatments were performed with 10 nM insulin.

Next, the association between SERT-IR was tested under the same experimental conditions and determined as 5-fold higher ($P < 0.001$) in 10 nM insulin-treated JAR cells than the untreated counterparts. The cell lysate of each sample was blotted for total SERT and actin as the loading control.

Bioluminescence resonance energy transfer (BRET) analysis of SERT-IR association in JAR cells

The putative formation of heterodimers between SERT and IR was investigated using BRET experiments (Figure 2). The coding region of Renilla luciferase (Rluc, BRET donor) or the yellow variant of the green fluorescent protein

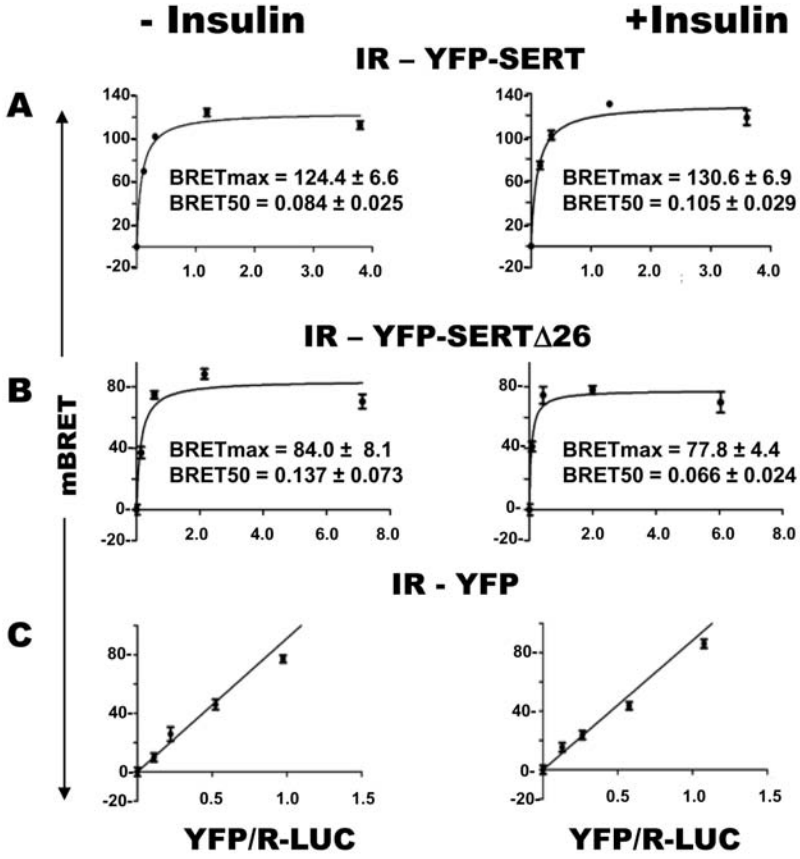

Figure 2. Bioluminescence resonance energy transfer (BRET) analysis on YFP-SERT and IR confirms their association in the presence of insulin treatment. BRET proximity assays between heterodimers of SERT and IR. CHO cells (5×10^5 cells/well) were co-transfected with plasmids coding for a constant amount of RLuc-tagged IR, (IR-RLuc, BRET donor) and increasing concentrations of the corresponding YFP-tagged SERT (SERT-YFP, BRET acceptor). Energy transfer was measured after addition of membrane-permeable luciferase substrate coelenterazine-h. (**A**) SERT and IR BRET pairs tested for association in the presence of Insulin (right) or not (Left); (**B**) The 25 amino acids from the C-terminal of SERT (SERTΔ26) and IR pairs; (**C**) IR and YFP BRET pairs. The BRET signal was determined by calculating the ratio of light emitted at 530 nm and that emitted at 485 nm, as described in "experimental procedures". Error bars indicate SEM of specific BRET-ratio values obtained from duplicates' determination. These values, including BRET values (BRETmax, $BRET_{50}$), are representative of 4 independent experiments. Plots were established using Graphpad software.

(YFP, BRET acceptor), was inserted at the cytoplasmic C-terminal tail of IR and SERT, respectively, in phase with the coding region. Saturation BRET experiments were conducted in CHO cells co-transfected with constant amounts of BRET donor plasmids and increasing amounts of BRET acceptor plasmids. In case of a close proximity between the investigated partners, hyperbolic saturation of the BRET signal is expected (*see methods*) [20]. Hyperbolic curves were obtained when SERT and IR were tested for association in the presence of insulin (Figure 2A). Noteworthy, the same experiment with IR and YFP BRET pairs led to a linear plot, consistent with a bystander (non-specific) BRET and thus with the absence of association with YFP alone (Figure 2C). Hyperbolic curves were also obtained with IR and SERTΔC, the 25 amino acids from the C-terminus of SERT truncated form. (Figure 2B), suggesting that SERTΔC and IR can form heterodimers. $BRET_{50}$ values (values of YFP/Rluc for half-maximal BRET) reflect the propensity of association between the investigated proteins (Figure 2B).

Time course analysis of IR phosphorylation and IR-SERT association

Serum-starved JAR cells (1.2×10^6 cells per WB assay) were first treated with 10 nM insulin and at various time (0-30 min), harvested and analyzed for the phosphorylation of IR (p-IR) and IR-SERT association (Figure 3A). WB analysis of proteins eluted from the IR-Ab-coated protein A beads showed that the levels of phosphorylated IR and the time of the insulin treatment on the starved JAR cells are in a biphasic relationship. Specifically, in the first 5 min of insulin treatment, the phosphorylated forms of IR were elevated 3.7-fold ($P < 0.001$), but then fall below normal as the incubation time continues to 10 min. The whole cell expression of IR or SERT appeared unchanged with 10 nM insulin treatment in between 0-30 min.

Tyr-phosphorylation of IR in placentas and platelets of SERT-KO mice

Placentas were dissected from E.18-day gestational SERT-KO and WT mice, weighed and prepared for analyses for p-IR level. An equal amount of placental tissues was homogenized in enzyme-cleavage buffer on ice and the homogenized tissue was filtered on cell strainer. Following centrifugation, the pellet was washed, and the intact cells were observed under the microscope. Then, the cells were incubated in insulin-containing buffer for 10 min and harvested for analysis.

WB analysis of the proteins bound to pTyr-Ab detected p-IR only in insulin-treated WT, but not in SERT-KO placental cells, although the total IR expressions in all placental cells were not changed (Figure 4A). Surprisingly, this was not the case for the platelets of the same mice (SERT-KO and WT), although insulin treatment phosphorylated the IR in the absence of an association between IR and SERT (Figure 4B).

The impact of 5-HT signaling on phosphorylation of IR

The impact of 5-HT on insulin-dependent phosphorylation of IR was analyzed in serum-starved JAR cells (1.2×10^6 cells per WB assay). In the absence of insulin, 5-HT, regardless of its concentration, showed no effect on IR phosphorylation. However, a 5-HT concentration of 50 μM, but not 20 μM, reduced the phosphorylation rate of IR, 68% ($P < 0.01$), compared to insulin-treated cells (Figure 5A). Although 5-HT treatment did not alter the expression level of IR up to the 20 μM concentration, at 50 μM, 5-HT reduced the phosphorylation rate of IR even in the presence of insulin (Figure 5A).

The 5-HT-dependent p-IR was evaluated further by elevating the extracellular 5-HT through reducing the 5-HT uptake rates of SERT by paroxetine (PAR), a specific SERT reuptake inhibitor (Figure 5B). Serum-starved JAR cells (1.2×10^6 cells per WB assay) were treated with 10 nM insulin together with 1 μM PAR. The insulin-dependent phosphorylation of IR was reduced by PAR by 64% only in insulin-treated group, almost to the level of an IR blocker, AGL (Figure 5B). However, blocking the 5-HT2 receptor with an antagonist, Sarpogrelate, in insulin-treated JAR cells did not change the phosphorylation rate of IR (data not presented). Therefore, we next tested the impact of 5-HT on p-IR by analyzing the surface level of SERT on 5-HT-treated JAR cells.

5-HT-treated JAR cells were labeled with NH-SS-biotin and the biotinylated surface proteins were analyzed by WB with SERT-Ab (Figure 6A). The

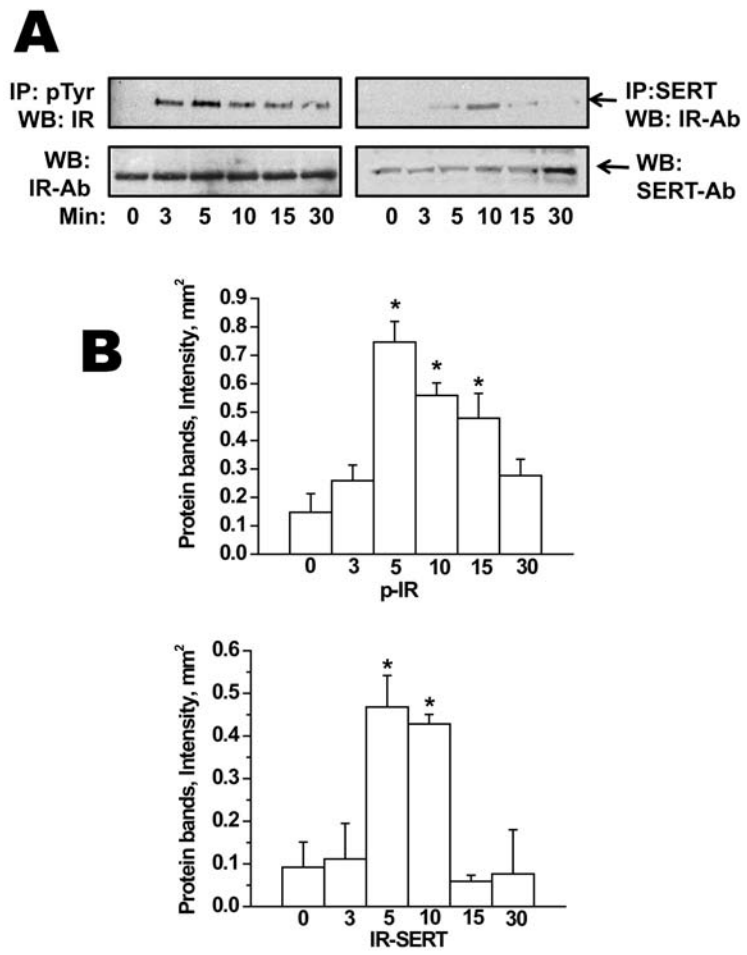

Figure 3. The time-course analysis of p-IR and IR-SERT association. (**A**) JAR cells (1.2×10^6 cells per assay) were first starved for insulin and then treated with 10 nM insulin for various time, 0-30 min. At the end of each incubation time, the cell lysates were either analyzed in WB with IR- or SERT-Ab, or prepared for IP with pTyr-Ab or SERT-Ab-coated protein A beads. The following WB analysis of pTyr- pulled down proteins with IR Ab showed a decrease in the level of phophorylated IR in longer insulin treatment. The highest level of p-IR was found at the end of 5 min insulin treatment. The WB analysis of SERT-pulled down proteins with IR-Ab showed an association between IR and SERT at the end of 10 min of insulin treatment. (**B**) The band densities were calculated as the ratio of each band to the level of IR or SERT. Averaged data from three independent experiments are presented ± SD. The values are statistically different ($p < 0.001$, Student's *t* test). Asterisks indicate statistical difference between control (0 min) and insulin-treated groups at various time (*).

densitometry analysis of the protein bands probed with SERT-Ab shows a 30% increase in SERT level at the surface of 20 μM 5-HT-treated JAR cells, whereas 50 μM 5-HT decreased the surface level of SERT by 60%. Reciprocal changes were also observed in SERT-Ab-depleted cell lysate, while the whole cell expressions of SERT in 5-HT treatment were not changed (Figure 6A). No effect of 5-HT treatment on cells was found on the surface expression of IR. Regardless of the concentrations, 5-HT treatment did not alter the cellular localization of IR (Figure 6B).

The cellular location of the IR-SERT complex

Here, the 26 amino acids truncated YFP-SERT (YFP-Δ26) or full length YFP-SERT-transfected JAR cells (1.2×10^6 cells per assay) were treated with 10 nM insulin and harvested for IP using GFP-binding protein (GBP)-conjugated coated Sepharose A beads.

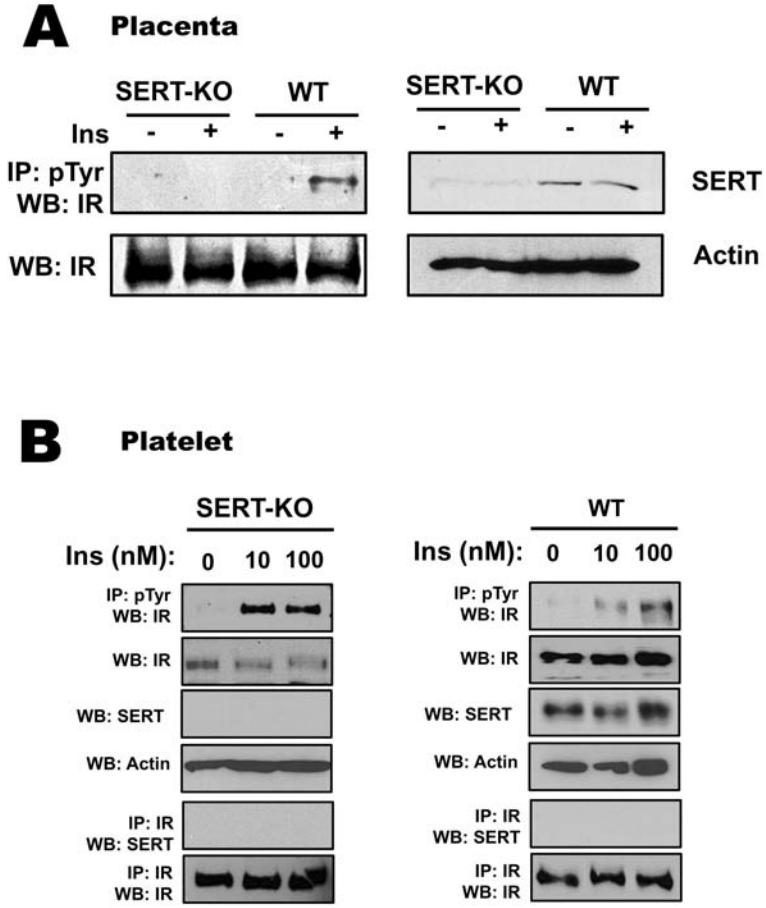

Figure 4. Differences in IR phosphorylation in platelet and placenta. (**A**) In the absence of SERT, placental IR phosphorylation is impaired. Placentas were harvested from pregnant WT or SERT-KO mice at E18. Placental tissue was minced and incubated with or without 10 nM insulin for 10 minutes. Cells were lysed and IP was performed to analyze the phosphorylation of IR. Phosphorylated IR bands were detected in insulin-treated WT placenta, but not in SERT-KO placenta. Experiments were performed in triplicate. (**B**) In platelet, insulin treatment facilitates the phosphorylation of IR but does not facilitate an association between IR-SERT, indicating that IR-SERT association is specific for placenta and insulin treatment. Platelets were isolated *via* centrifugation of whole blood collected from WT or SERT-KO mice. After washing with Tyrode HEPES, platelets were incubated for 10 minutes in the absence or presence of 10 nM or 100 nM insulin for 10 minutes. Co-IP was performed which demonstrated similar IR phosphorylation levels in WT and SERT-KO platelets. Analysis also showed no association between IR and SERT in platelets. Experiments were performed in duplicate.

IR interaction with YFP-tagged SERT was mainly found in insulin-treated JAR cell transfected with YFP-SERT or YFP-Δ26 (Figure 7). However, IR interaction was noticeably reduced in cells transfected with YFP-Δ26, compared to the YFP-SERT, suggesting that the C-terminus of SERT is at least one of the binding domains for IR. An antibody recognizing the N-terminus of SERT was used as a loading control for these co-IP experiments.

The whole cell lysate was analyzed using a specific Ab generated against the C-terminus of SERT which did not recognize Δ26 in these cells.

In insulin-treated JAR cells either non-transfected, SERT or Δ26-transfected cells gave rise to phosphorylated IR (Figure 7), suggesting IR could be activated independently of SERT. However, the decreased IR phosphorylation in Δ26-transfected cells suggests that the interaction between SERT and IR contributes to the regulation of IR phosphorylation.

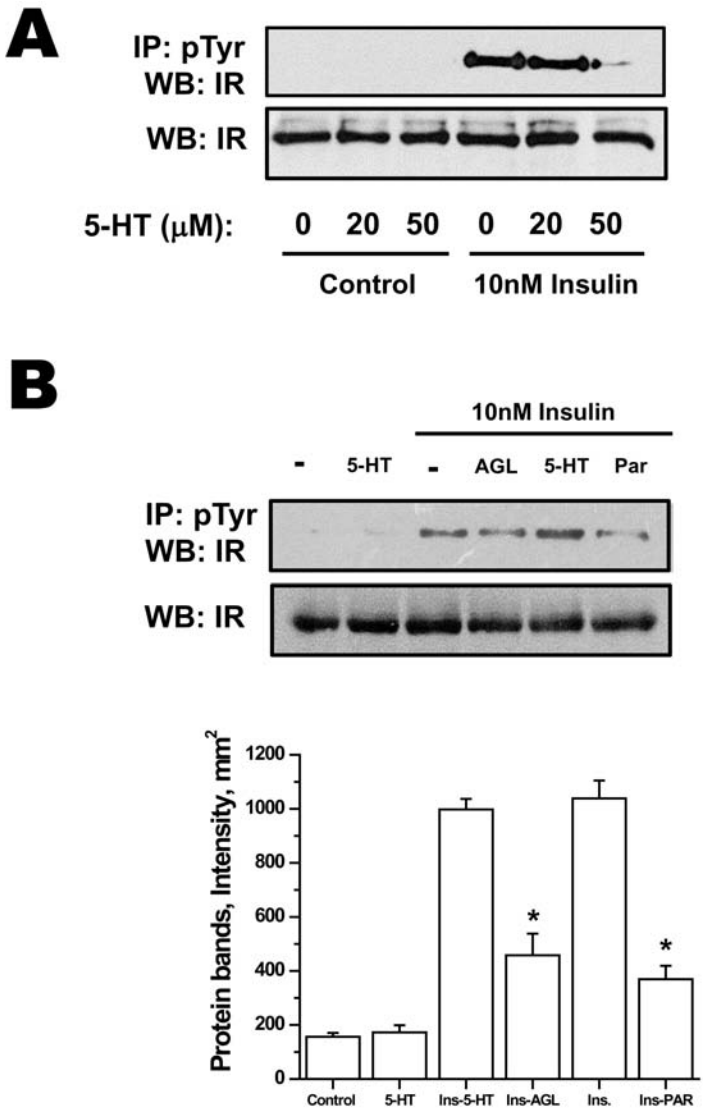

Figure 5. Effects of 5-HT signaling on IR phosphorylation. (**A**) Serum-starved JAR cells (1.2×10^6 cells/assay) were pre-treated with increasing concentrations of 5-HT for 20 minutes and then treated with 10 nM insulin or untreated (controls). Subsequently, IR phosphorylation was analyzed by IP. 5-HT treatment alone does not activate phosphorylation of IR. However, 50 μM 5-HT treatment decreases the phosphorylation of IR significantly. Interestingly at 20 μM, 5-HT had no significant effect on IR phosphorylation. (**B**) Under similar conditions, IR phosphorylation was also investigated in the presence of insulin receptor antagonist, AGL and SERT-blocker, paroxetine. Pre-treatment with AGL or PAR decreased IR phosphorylation. 5-HT (20 μM) had no effect on IR phosphorylation. Densitometry graph shown represents average band intensity with error bars indicating SD. Experiments were performed in triplicate. *P < 0.05 compared to no treatment controls.

DISCUSSION

Binding of insulin to IR stimulates a cascade of cellular processes that promote the usage or the storage of glucose [1, 2, 8]. Various factors are identified as triggering the insulin signal transduction pathways including proteins associated with IR, as well as fasting versus non-fasting states, stress levels, and a variety of other hormones. Studies reported a significant, 33%, increase in the incidence of type 2 diabetes in the United States over the past 20 years, which has been associated with a parallel increase in obesity [9]. Like other forms

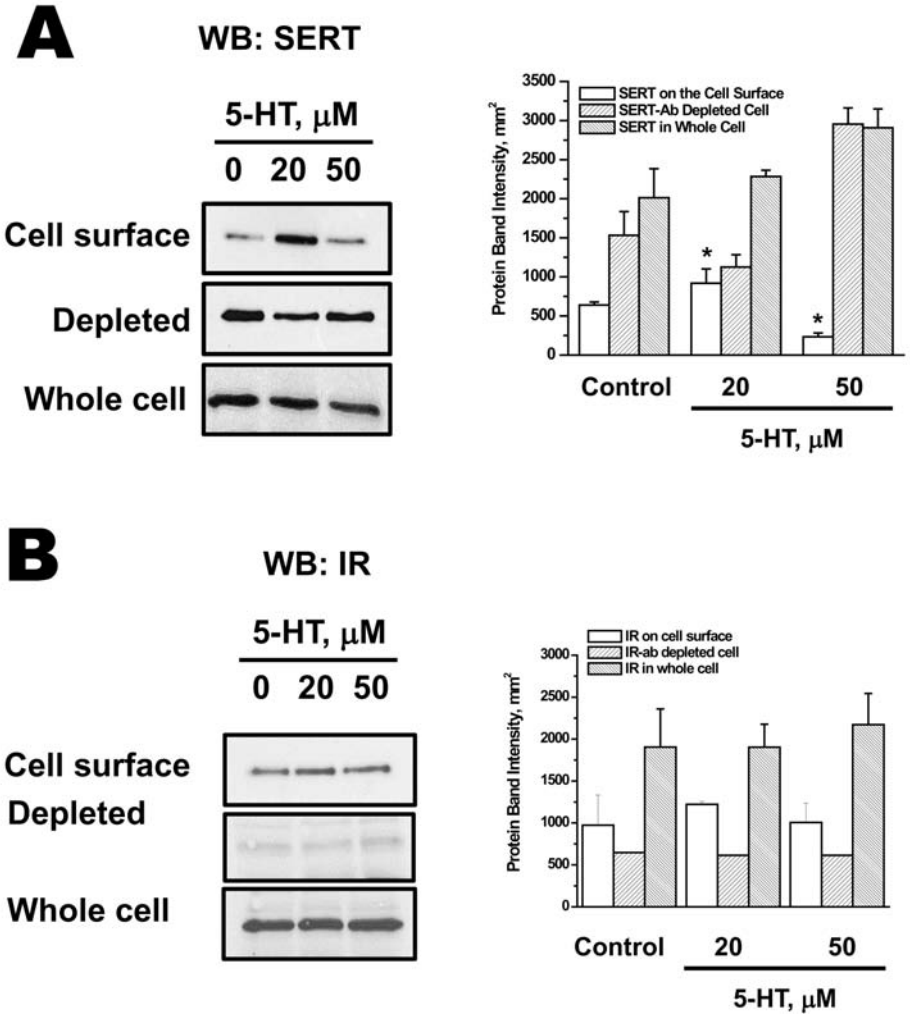

Figure 6. 5-HT regulates plasma membrane localization of SERT but not IR. JAR cells (1.2×10^6 cells/assay) were treated with 0 μM, 20 μM or 50 μM 5-HT and plasma membrane proteins were biotinylated with NHS-SS-biotin. Biotinylated proteins were captured with streptavidin agarose beads and analyzed by WB. (**A**) Cell surface SERT levels are dependent on extracellular 5-HT concentration. Plasma membrane SERT increased significantly when cells were treated with 20 μM, but not 50 μM. Whole cell SERT was used as an input control. Experiments were completed in triplicate. Densitometry graph represents average band intensity with error bars indicating SD. *P < 0.05 compared to no treatment controls. (**B**) Plasma membrane IR levels are not affected by extracellular 5-HT levels. Cell surface IR levels are similar in untreated and 5-HT-treated cells. Whole cell IR was used as an input control. Experiment was performed in duplicate.

of hyperglycemia, decreased insulin sensitivity is coupled with an inadequate insulin response *via* impairment in insulin signaling mechanism [9], leading to glucose uptake or production that cannot be stimulated or suppressed, respectively, although they normally change reciprocally [22, 23]. During pregnancy, while glucose in maternal blood freely crosses to the placenta, insulin does not; therefore, in a diabetic intrauterine environment such as GDM or type 2 diabetic pregnancy, the fetus produces an excess amount of insulin, which in turn acts as a growth hormone and promotes over growth and adiposity in the fetus. Growing in a diabetic intrauterine environment presents a high risk for the offspring to develop chronic obesity and metabolic health problems such as diabetes and cardiovascular disease, later in life [22-24].

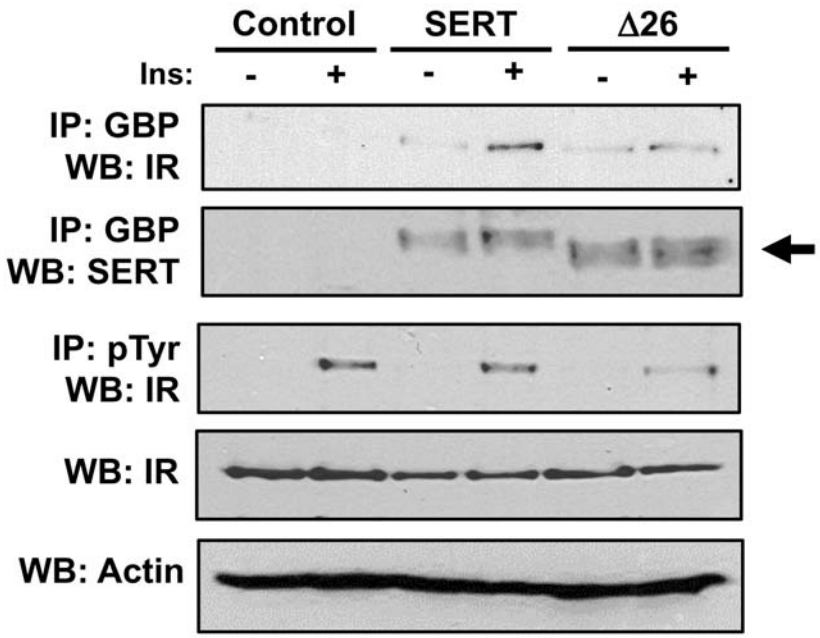

Figure 7. One of the IR–SERT association domains is the last 26 amino acids from the C-terminus of SERT. JAR cells (5×10^5 cells/well) were transfected with 4 μg of either YFP-SERT or YFP-Δ26 plasmids. 72-hour post-transfection, cells were starved overnight and treated with 10 nM insulin for 10 minutes. Cell lysates were prepared, and co-IPs were performed to analyze YFP-IR association or IR phosphorylation. Insulin treatment initiated IR association with YFP-SERT but decreased association occurred with YFP-Δ26. Additionally, IR phosphorylation was decreased in cells transfected with YFP-Δ26, compared to YFP-SERT or mock-transfected controls. GBP: GFP-binding protein.

There is a dynamic relationship between pregnancy and 5-HT that acts as a mitogen-promoting cell division, and a developmental signal early in embryogenesis [25-28]. Clinical studies suggest that the blood 5-HT concentration is significantly higher in type 2 diabetes or GDM than in the blood of healthy/control groups [29]. A separate study measured the 5-HT levels in maternal blood and compared them with non-pregnant blood samples. They reported 15.6% elevation in blood 5-HT levels during pregnancy [30, 31]. Preclinical studies supported these findings by demonstrating the involvement of 5-HT in insulin and glucose metabolism [32-37]. 5-HT acts as an appetite regulator and SERT gene is related with obesity [37, 38]. However, two independent studies with SERT-KO [11, 37] and TPH1 (the rate limiting enzyme in peripheral 5-HT biosynthesis)-KO mice [39] reported that obesity associated with SERT gene is not related to appetite control by 5-HT. Due to the deletion of SERT gene, SERT-KO mice have elevated 5-HT level in peripheral system and exhibit reduced food intake [11], yet they develop an obese phenotype under normal diet [16], suggesting an involvement of other factors in the development of obesity rather than appetite control by 5-HT. In support to this hypothesis, TPH1-KO mice fed a high-fat diet did not develop obesity or insulin resistance [40] suggesting that peripheral 5-HT functions may not be directly involved in the development of obesity and glucose intolerance in SERT-KO mice. Furthermore, a low expression of SERT was correlated with the increased risk of weight gain and type 2 diabetes [12, 14, 40, 41].

Defect in phosphorylation of IR is tissue-type dependent: the phosphorylation of IR is impaired in the GDM-placenta but is unaffected in GDM-platelets [10]. Interestingly, this was also observed on the cellular distribution of SERT molecules; downregulation of cell surface SERT was observed in placental cells, but not in platelets of GDM subjects [31]. Furthermore, SERT-KO mice were reported as developing insulin resistance [11].

Therefore, in the current study, we investigated the phosphorylation rate of IR in SERT-KO placenta, and found a significant reduction in KO compared to WT. Additional studies demonstrated a functional role for the insulin-dependent physical association between SERT and IR, including BRET experiments. These findings were strongly supported by the findings with time course experiments, which demonstrated the phosphorylation of IR in parallel to IR-SERT association. These additional findings suggest an important role for IR-SERT association in maintaining the phosphorylation of IR and regulating the insulin signaling in placental cells. Although the BRET analysis did not reveal insulin-dependence of their association, we believe that this may be related to the heterologous expression of SERT and IR in COS cells.

Extracellular 5-HT level is regulated by SERT, which is expressed on the surface of the placental-brush border membranes of trophoblast cells [10]. SERT prevents vasoconstriction in the placental vascular bed thereby securing stable blood flow to the embryo *via* regulating the plasma 5-HT level [29, 30]. Furthermore, an earlier study from our laboratory demonstrated that in the presence of high glucose and a lack of insulin, at diabetic-like conditions, the 5-HT uptake rates of placental JAR cells were downregulated *via* altering the surface level of SERT on these cells [42].

Under diabetic pregnancy, specifically in GDM, IR phosphorylation is impaired and 5-HT levels are significantly elevated [31]. If a lower concentration of 5-HT was used in insulin-treated JAR cells, the phosphorylation rate of IR was not changed, though the p-IR levels were significantly decreased at 50 μM 5-HT treatment. The series of studies from our laboratory summarize the biphasic effect of 5-HT on the cell surface level of SERT *via* interfering the rate of its membrane trafficking [18, 19, 43-46]. Furthermore, IR phosphorylation was also partially inhibited by treatment with paroxetine, a SERT blocker. These data suggest a role for SERT 5-HT uptake activity in IR phosphorylation. We have previously shown a 5-HT concentration-dependent biphasic effect on 5-HT uptake via SERT – 5-HT uptake increases as 5-HT concentration reaches up to a maximum; further increasing 5-HT causes a reduction in 5-HT uptake [18, 19]. Perhaps reduced 5-HT uptake rates through the SERT inhibitors, such as paroxetine, causes the reduction in IR phosphorylation due to high extracellular 5-HT concentration.

CONCLUSION

In summary, this study adds to the growing literature on the significant contribution of SERT in diabetes and obesity by presenting an important role for SERT in the regulation of insulin signaling. Therefore, we propose that interaction between IR and SERT facilitates and/or maintains the phosphorylation of placental IR. These findings need to be investigated further to clarify if the phosphorylation of C-terminus of SERT facilitates the phosphorylation of IR β-subunits and/or binding to the SERT C-terminus exposes the phosphorylation sites of the IR β-subunits and maintains insulin signaling in placenta.

ACKNOWLEDGEMENTS

The authors thank Anthonya Cooper, PhD for producing some of these data which was a part of her dissertation project while she was in Dr. Kilic's Laboratories at University of Arkansas for Medical Sciences, Little Rock AR.

CONTRIBUTIONS

BRET analysis was performed in LM laboratories by IM. FK and LM designed and directed the project. FK analyzed the data. FK and LM participated in manuscript writing and scientific discussions, giving detailed feedback in all areas of the project.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests that might be perceived to influence the results and/or discussion reported in this paper.

REFERENCES

1. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y. C. and Tsubokawa, M. 1985, Nature, 313, 756.
2. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J. H., Masiarz, F.,

Kan, Y. W. and Goldfine, I. D. 1985, Cell, 40, 747.
3. Hubbard, S. R., Wei, L., Ellis, L. and Hendrickson, W. A. 1994, Nature, 372, 746.
4. Hubbard, S. R. 1997, EMBO J., 16, 5572.
5. Syed, R. S., Reid, S. W., Li, C., Cheetham, J. C., Aoki, K. H., Liu, B., Zhan, H., Osslund, T. D., Chirino, A. J., Zhang, J., Finer-Moore, J., Elliott, S., Sitney, K., Katz, B. A., Matthews, D. J., Wendoloski, J. J., Egrie, J. and Stroud, R. M. 1998, Nature, 395, 511.
6. Livnah, O., Stura, E. A., Johnson, D. L., Middleton, S. A., Mulcahy, L. S., Wrighton, N. C., Dower, W. J., Jolliffe, L. K. and Wilson, I. A. 1996, Science, 273, 464.
7. Livnah, O., Johnson, D. L., Stura, E. A., Farrell, F. X., Barbone, F. P., You, Y., Liu, K. D., Goldsmith, M. A., He, W., Krause, C. D., Pestka, S., Jolliffe, L. K. and Wilson, I. A. 1998, Nat. Struct. Biol., 5, 993.
8. Herrera, R. and Rosen, O. M. 1986, J. Biol. Chem., 261, 11980.
9. Buchanan, T. A., Xiang, A., Kjos, S. L. and Watanabe, R. 2007, Diabetes Care, 30(Suppl. 2), S105.
10. Li, Y., Cooper, A., Odibo, I. N., Ahmed, A., Murphy, P., Koonce, R., Dajani, N. K., Lowery, C. L., Roberts, D. J., Maroteaux, L. and Kilic, F. 2016, J. Biol. Chem., 291, 9657.
11. Chen, X., Margolis, K. J., Gershon, M. D., Schwartz, G. J. and Sze, J. Y. 2012, PLoS One, 7, e32511.
12. Giannaccini, G., Giannaccini, G., Betti, L., Palego, L., Marsili, A., Santini, F., Pelosini, C., Fabbrini, L., Schmid, L., Giusti, L, Maffei, M., Lanza, M., Cristofaro, M., Baroni, S., Mauri, M., Vitti, P., Fierabracci, P. and Lucacchini, A. 2013, BMC Neurosci., 14, 128.
13. Sookoian, S., Gianotti, T. F., Gemma, C., Burgueno, A. and Pirola, C. J. 2008, Obesity (Silver Spring), 16, 488.
14. Iordanidou, M., Tavridou, A., Petridis, I., Arvanitidis, K. I., Christakidis, D., Vargemezis, V. and Manolopoulos, V. G. 2010, Clin. Chim. Acta, 411, 167.
15. Breisch, S. T., Zemlan, F. P. and Hoebel, B. G. 1976, Science, 192, 382.
16. Murphy, D. L. and Lesch, K. P. 2008, Nat. Rev. Neurosci., 9, 85.
17. Hadden, C., Fahmi, T., Cooper, A., Savenka, A. V., Lupashin, V. V., Roberts, D. J., Maroteaux, L., Hauguel-de Mouzon, S. and Kilic, F. 2017, J. Cell Physiol., 232, 3520.
18. Ahmed, B. A., Jeffus, B., Harney, J. T., Bukhari, I. S., Unal, R., Lupashin, V. V., van der Sluijs, P. and Kilic, F. 2008, J. Biol. Chem., 283, 9388.
19. Ahmed, B., Bukhari, I. A., Jeffus, B. C., Harney, J. T., Thyparambil, S., Ziu, E., Fraer, M., Rusch, N. J., Zimniak, P., Lupashin, V., Tang, D. and Kilic, F. 2009, PLoS One, 4, e4730.
20. Achour, L., Kamal, M., Jockers, R. and Marullo, S. 2011, Methods Mol. Biol., 756, 183.
21. Okabayashi, Y., Maddux, B. A., McDonald, A. R., Logsdon, C. D., Williams, J. A. and Goldfine, I. D. 1989, Diabetes, 38, 182.
22. Vrachnis, N., Antonakopoulos, N., Iliodromiti, Z., Dafopoulos, K., Siristatidis, C., Pappa, K. I., Deligeoroglou, E. and Vitoratos, N. 2012, Exp. Diabetes Res., 2012, 538474.
23. Colomiere, M., Colomiere, M., Permezel, M., Riley, C., Desoye, G. and Lappas, M. 2009, European Journal of Endocrinology 160, 567.
24. Balsells, M., García-Patterson, A., Gich, I. and Corcoy, R. 2012, Diabetes Metab. Res. Rev., 28, 252.
25. Lauder, J., Tamir, H. and Sadler, T. W. 1988, Development, 102, 709.
26. Lauder, J. M. 1993, Trends Neurosci., 16, 233.
27. Côté, F., Thévenot, E., Fligny, C., Fromes, Y., Darmon, M., Ripoche, M. A., Bayard, E., Hanoun, N., Saurini, F., Lechat, P., Dandolo, L., Hamon, M., Mallet, J. and Vodjdani, G. 2003, Proc. Natl. Acad. Sci. USA, 100, 13525.
28. Rubenstein, J. L. R. 1998, Biol. Psychiatry, 44, 145.
29. Barradas, M. A., Gill, D. S., Fonseca, V. A., Mikhailidis, D. P. And Dandona, P. 1988, Eur. J. Clin. Invest., 18, 399.
30. Gall, V., Kosec, V., Vranes, H. S., Jukić, M., Filipović-Djaković, I. and Vuković, A. 2011, Coll. Antropol., 35, 715.

31. Li, Y., Hadden, C., Singh, P., Mercado, C., Murphy, P., Dajani, N. K., Lowery, C. L., Roberts, D. J., Maroteaux, L. and Kilic, F. 2014, Proc. Natl. Acad. Sci. USA, 111, 52.
32. Kim, H., Toyofuku, Y., Lynn, F. C., Chak, E., Uchida, T., Mizukami, H., Fujitani, Y., Kawamori, R., Miyatsuka, T., Kosaka, Y., Yang, K., Honig, G., van der Hart, M., Kishimoto, N., Wang, J., Yagihashi, S., Tecott, L. H., Watada, H. and German, M. S. 2010, Nat. Med., 16, 804.
33. Paulmann, N., Grohmann, M., Voigt, J. P., Bert, B., Vowinckel, J., Bader, M., Skelin, M., Jevsek, M., Fink, H., Rupnik, M., Walther, D. J. 2009, PLoS Biol., 7, e1000229.
34. Viau, M., Lafond, J. and Vaillancourt, C. 2009, Reprod. Biomed. Online, 19, 207.
35. Chen, Y., Quick, W. W., Yang, W., Zhang, Y., Baldwin, A. and Moran, J. 2009, Popul. Health Manag., 12, 165.
36. Breisch, S. T., Zemlan, F. P. and Hoebel, B. G. 1976, Science 192, 382.
37. Kim, D. K., Tolliver, T. J., Huang, S. J., Martin, B. J., Andrews, A. M., Wichems, C., Holmes, A., Lesch, K. P. and Murphy, D. L. 2005, Neuropharmacology, 49, 798.
38. Savelieva, K. V., Zhao, S., Pogorelov, V. M., Rajan, I., Yang, Q., Cullinan, E. and Lanthorn, T. H. 2008, PLoS One, 3, e3301.
39. Crane, J. D., Palanivel, R., Mottillo, E. P., Bujak, A. L., Wang, H., Ford, R. J., Collins, A., Blümer, R. M., Fullerton, M. D., Yabut, J. M., Kim, J. J., Ghia, J. E., Hamza, S. M., Morrison, K. M., Schertzer, J. D., Dyck, J. R., Khan, W. I. and Steinberg, G. R. 2015, Nat. Med., 21, 166.
40. Sookoian, S., Gianotti, T. F., Gemma, C., Burgueno, A. and Pirola, C. J. 2008, Obesity (Silver Spring), 16, 488.
41. Coustan, D. R. 1995, Gestational Diabetes, National Institutes of Diabetes and Digestive and Kidney Diseases. Diabetes in America. 2nd Edition. Bethesda (MD): NIDDK. NIH Publication No. 95-1468, 703-717.
42. Unal, R., Ahmed, B. A., Jeffus, B. A., Harney, J. T., Lyle, C. S., Wu, Y. K., Reece, E. A. and Kilic, F. 2007, J. Neurochem., 101, 937.
43. Brenner, B., Harney, J. T., Ahmed, B. A., Jeffus, B. C., Unal, R., Mehta, J. L. and Kilic, F. 2007, J. Neurochem., 102, 206.
44. Kilic, F., Ziu, E. and Freyaldenhoven, S. 2009, Plasma Serotonin and Platelet Serotonin Transporter: Molecular and Cellular Aspects in Cardiovascular Research, J. Brataas and V. Nanstveit, (Eds.): Handbook of Cardiovascular Research. Nova Science Publishers, Inc. Hauppauge, NY. ISBN: 978-1-60741-792-7.
45. Mercado, C. and Kilic, F. 2010, Molecular Interventions, 10, 231.
46. Mercado, C., Ziu, E. and Kilic, F. 2011, Current Opinion in Pharmacology, 11, 23.