Estrogen and Serotonin – old dogs, new tricks, Implications for pancreatic beta-cell function

Balhuizen, Alexander

2013

Link to publication

Citation for published version (APA):
Estrogen and Serotonin – old dogs, new tricks

Implications for pancreatic beta-cell function

Alexander Balhuizen

DOCTORAL DISSERTATION
With approval by the Faculty of Medicine, Lund University, Sweden, this thesis will be defended at ‘Lilla aulan’, Jan Waldenströms gata 5 Malmö, on the 15th of November 2013 at 9 a.m.

Faculty opponent
Professor Jens Høiriis Nielsen
University of Copenhagen, Denmark
Islet hormone secretion is tightly regulated by metabolic status as well as local and circulating factors. These factors can activate different receptors on the pancreatic islet cells, for instance G-protein coupled receptors (GPCRs). When activated, these receptors are able to fine-tune islet hormone secretion and regulate overall β-cell function.

Estrogen and serotonin are circulating factors that bind to GPCRs. First, we studied the activation of GPER-1 in pancreatic islets using two agonists G-1 and Estrogen (E2). Both G-1 and E2 displayed a similar response in mouse and human islets even in the presence of estrogen receptor blockers, ICI 182, 720 and EM652. G-1 and E2 potentiated insulin secretion and inhibited glucagon and somatostatin secretion. G-1 induced cAMP generation, suggesting positive coupling to adenylate cyclase and a subsequent rise in insulin release. Furthermore both agonists protected pancreatic islets from cytokine-induced apoptosis via activation of anti-apoptotic signals, CREB, ERK1/2 and AKT and reduced phosphorylation of the pro-apoptotic signals SAPK/JNK and p38.

Second, we studied serotonin (5-HT) receptors in human islets and INS (832/13) cells. We detected 15 different 5-HT receptors and the 5-HT producing enzymes, TPH1 and TPH2 as well as DDC. Cellular localization for 5-HT1A, 5-HT1D and 5-HT2A were observed in both β- and α-cells; while 5-HT2B was only present in β-cells. Agonists targeting these four receptors were able to either inhibit or stimulate insulin secretion from human islets and INS (832/13) cells. In addition, 5-HT was quantified using GC/MS in INS (832/13) cells, rat islets and detected in human α and β-cells with immunohistochemistry.

Third, we investigated the peripheral role of a 5-HT2 receptor agonist, α-methyl serotonin maleate salt (AMS) in insulin resistance and β cell function. Long-term treatment with AMS in a high fat diet fed mouse model resulted in increased insulin sensitivity in vivo in high fat fed AMS treated mice. Moreover, insulin secretion from AMS treated control fed mice in vitro was decreased while plasma glucose levels were similar in vivo between AMS treated and untreated controls. In addition, AMS mediated protection from lipotoxicity in INS-1(832/13) cells.

In conclusion, this thesis contributes to increased understanding of how estrogen and peripheral 5-HT mediate their effects on islet function and overall glucose homeostasis.

Key words: Diabetes, Estrogen, Serotonin, Pancreatic islets, Insulin resistance, β-cell function, GPCR
Estrogen and Serotonin – old dogs, new tricks

Implications for pancreatic beta-cell function

Alexander Balhuizen
Cover illustration: Confocal picture of a human islet immunolabeled for GPER-1 and insulin. Superimposed with chemical structures of the following substances, from the left and downwards; 5-HT, TCB-2, AMS, buspirone, PNU142633, ICI182, 786, EM 652, G-1 and E2. The structures were generated with PyMol 1.6.
To Sandra and my parents

I don’t know which one I like more
– Original or Type II!

Futurama – Saturday fun morning pit
# Table of Contents

LIST OF PUBLICATIONS ........................................................................................................... I

PAPERS INCLUDED IN THE THESIS ............................................................................... I
PAPERS NOT INCLUDED IN THE THESIS ........................................................................ II

ABBREVIATIONS .................................................................................................................. III

INTRODUCTION ...................................................................................................................... 1

*Type 2 diabetes mellitus* ........................................................................................................ 1
*Insulin resistance* .................................................................................................................. 2
*β-cell mass* .......................................................................................................................... 4
*The islets of Langerhans* ...................................................................................................... 5
*Glucose sensing and insulin secretion* .................................................................................. 6
*Modulation of insulin secretion* ............................................................................................ 8

**ESTROGEN** ..................................................................................................................... 11

*E2 synthesis* .......................................................................................................................... 11
*Estrogen receptors* ............................................................................................................... 12
*Pharmacological modulation of estrogen receptors* ............................................................. 13
*Estrogen and glucose homeostasis* ...................................................................................... 14

**SEROTONIN** .................................................................................................................... 16

*Biosynthesis of 5-HT* .......................................................................................................... 16
*5-HT receptors* ..................................................................................................................... 17
*5-HT and energy homeostasis* .............................................................................................. 19

AIMS OF THE THESIS .......................................................................................................... 23

**METHODOLOGY** .............................................................................................................. 24

*Animals and glucose tolerance test* .................................................................................... 24
*Human pancreatic islets* ....................................................................................................... 24
*Measurement of pancreatic hormone release* .................................................................... 25
*Cell culturing and insulin secretion* .................................................................................... 25
*qPCR* .................................................................................................................................... 26
*Western blot* ........................................................................................................................ 26
*Immunohistochemistry* ........................................................................................................ 27
*Flow cytometry* .................................................................................................................... 27

**PRESENT INVESTIGATIONS** .......................................................................................... 29

PAPER I ............................................................................................................................... 29
PAPER II ............................................................................................................................. 31
PAPER III ........................................................................................................................... 33
PAPER IV .................................................................................................................................................. 34
GENERAL DISCUSSION .......................................................................................................................... 35
MAJOR CONCLUSIONS AND FUTURE PERSPECTIVES .................................................................. 38
POPULÄRVENTENSKAPLIG SAMMANFATTNING .............................................................................. 40
ACKNOWLEDGEMENTS ...................................................................................................................... 42
REFERENCES ......................................................................................................................................... 44
List of Publications

Papers included in the thesis

I. *Activation of G protein-coupled receptor 30 modulates hormone secretion and counteracts cytokine-induced apoptosis in pancreatic islets of female mice.*  

II. *Insulinotropic and antidiabetic effects of 17β-estradiol and the GPR30 agonist G-1 on human pancreatic islets.*  

III. Serotonin (5-HT) Receptors Modulate Insulin Secretion *In Vitro* in Human Islets of Langerhans.  

IV. Agonists of the 5-HT2-family affect insulin secretion and glucose tolerance *in vivo* and *in vitro.*  
Balhuizen, A., Bennet, H., and Fex, M. Manuscript.

* Published articles are reproduced with the permission from the publisher.
Papers not included in the thesis

I. A common variant in TFB1M is associated with reduced insulin secretion and increased future risk of type 2 diabetes.

II. Pleiotropic effects of GIP on islet function involve osteopontin.

III. Autoimmunity against INS-IGF2 expressed in human pancreatic islets.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AMS</td>
<td>α-methyl-5-HT maleate salt</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DDC</td>
<td>Amino acid decarboxylase</td>
</tr>
<tr>
<td>E2</td>
<td>Estrogen, 17β-estradiol</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GL/FFA</td>
<td>Glycerolipid/Free fatty acid</td>
</tr>
<tr>
<td>GPER</td>
<td>G protein-coupled estrogen receptor</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose Stimulated Insulin Secretion</td>
</tr>
<tr>
<td>HTR</td>
<td>Serotonin receptor</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol (1,4,5) trisphosphate</td>
</tr>
<tr>
<td>IVGTT</td>
<td>Intravenous glucose tolerance test</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>nER</td>
<td>Nucleic Estrogen receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB/Akt</td>
<td>Protein kinase B/ serine/threonine-specific protein kinase</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>RRP</td>
<td>Ready releasable pool</td>
</tr>
<tr>
<td>SAPK/JNK</td>
<td>Stress-activated protein kinase/c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>SERD</td>
<td>Selective estrogen receptor down-regulator</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor down-modulator</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>T2DM</td>
<td>Diabetes mellitus type 2</td>
</tr>
<tr>
<td>TPH</td>
<td>Tryptophan hydroxylase</td>
</tr>
</tbody>
</table>
Introduction

Diabetes mellitus is a disease taking on pandemic proportions. Today, 350 million cases are reported, with the prediction of over a 50% increase until 2030 (Danaei et al. 2011). Until 2030, developing countries are projected to have a greater rise in prevalence of diabetes than developed countries (Shaw et al. 2010). This increase is most likely due to an adaptation to the westernized culture (excessive food intake and lack of exercise); and will result in an increase of diabetes, overweight and obesity (Nolan et al. 2011).

Type 2 diabetes mellitus

Heritability and genetic background are strong risk factors for the development of metabolic disorders including type 2 diabetes mellitus (T2DM) (Florez et al. 2003). Major risk genes for T2DM are genes that affect overall energy balance and β-cell function (Lyssenko et al. 2008, McCarthy 2010). Gender differences are also known to increase the prevalence of T2DM and men are more prone to develop diabetes than women, although women of course are susceptible for gestational diabetes (Wild et al. 2004). Even though susceptibility to T2DM is increased with a genetic predisposition, major predictors also are; a sedentary lifestyle, excessive food intake, obesity as well as fetal programming (Nolan et al. 2011).

Euglycemia is maintained as insulin is released from the pancreatic β-cells in response to postprandial elevation of blood glucose. Insulin mediated glucose uptake in target tissues (skeletal muscle, liver and adipose tissue) facilitates either glucose utilization or energy storage such as glycogen (in liver) or triglycerides (in adipose tissue) (Nolan et al. 2011) (Figure 1A). Excessive energy intake skews energy homeostasis and saturates glycogen and triglycerides stores. This unbalance can in genetically susceptibility individuals progress to insulin resistance in target tissues (Kahn et al. 2006). In insulin resistant states, impaired insulin signaling is compensated with insulin hyper-secretion, either by increased β-cell mass or increased β-cell efficiency (Bonner-Weir 2000) (Figure 1B). By long-term over-nutrition, β-cells are no longer able to compensate and loss of β-cells and/or β-cell function will occur (Figure 1C). This will result in insufficient insulin release with hyperglycemia and impaired glucose tolerance (IGT) with a subsequent transition into T2DM (Yudkin et al. 1990, Weir et al. 2004). If untreated, chronic hyperglycemia will result in microvascular complications (retinopathy, neuropathy and nephropathy) and
macrovacular complications (stroke, myocardial infarction and atherosclerosis) which significantly shortens life expectancy (Nathan 1993).

**Insulin resistance**

Insulin resistance is a hallmark of T2DM and is often observed in obesity. Insulin resistance is the consequence of an incapability of insulin to adequately stimulate glucose uptake in peripheral target tissues (i.e. liver, adipose tissue and skeletal muscle).

Normally, in adipocytes, liver and skeletal muscle, insulin stimulates glucose uptake and storage by binding to insulin receptor (IR) eliciting IR auto
phosphorylation and binding of insulin receptor substrates. The key substrates are insulin receptor substrate-1 (IRS-1), in skeletal muscle and adipocytes, and insulin receptor substrate-2, (IRS-2), in liver (Cohen 2006, Taniguchi et al. 2006). Phosphorylation of IRS-1 and IRS-2 associates the substrates with phosphatidylinositol 3-kinase (PI3K) which activates serine/threonine-specific protein kinase (AKT) (Stokoe et al. 1997). AKT (Kohn et al. 1996) facilitates phosphorylation of glycogen synthase kinase-3 (GSK-3) and AS160 Rab GTPase-activating protein (Kane et al. 2002, Sano et al. 2003). When AS160 is bound to GDP it interacts with Rab10 and additional Rab proteins to facilitate translocation of glucose transporter-containing vesicles (GLUT) to the cell surface to enable glucose uptake (Sano et al. 2007).

During progression of insulin resistance the peripheral tissues are under constant strain to cope with the increased fatty acid supply. Increased fatty acids cause lipid accumulation when converted to cytosolic diacylglycerides (DAGs), ceramides and triglycerides by long chain-Coenzyme A (LC-CoA) (Kahn et al. 2006). These accumulations of lipid are believed to be involved in the development of insulin resistance through a cascade of serine/threonine phosphorylation phosphorylate IRS-1 and IRS-2, thus reducing their ability to activate PI3K, which decreases downstream events and translocation of GLUT-4 (Shulman 2000). This phosphorylation is further increased by circulating FFAs (Boden 1997) and adipocytes-derived inflammatory cytokines especially tumor necrosis factor α (TNFα) (Hotamisligil et al. 1996).

Inflammatory mediators and FFAs are released when adipocytes are enlarged due to triglyceride overload, which in turn attract macrophages (Guilherme et al. 2008). Macrophages release pro-inflammatory mediators such as TNFα and IL-1β, resulting in inflammation (Kanda et al. 2006). TNFα impairs triglyceride storing and increases lipolysis via downregulation of peroxisome proliferator-activated receptor γ (PPARγ) (Zhang et al. 1996), resulting in increasing circulating FFA (Guilherme et al. 2008). TNFα also inhibits adiponectin; an adipokine known to positively affect insulin sensitivity via increased GLUT4 translocation and stimulation of fatty acid oxidation through 5’ AMP-activated protein kinase (AMPK) (Yamauchi et al. 2002, Lafontan et al. 2006).

The increase in circulating fatty acids alters the liver metabolic function as triglycerides and intermediates of DAGs and ceramides build up inside, via an inhibition of the FFA β-oxidation (McGarry 2002, Li et al. 2007). This will result in inhibition of LC-CoA translocation to mitochondria, which cause lipid species accumulation in the endoplasmic reticulum (ER) (Chavez et al. 2003, Holland et al. 2007). This can initiate ER stress and provoke protein misfolding which is suggested to interfere with the insulin suppressing effect on gluconeogenesis. Subsequently, hepatic glucose output will increase (Muoio et al. 2008), thus increasing hyperglycemia.

In skeletal muscle, the excess in cytosolic fatty acids initiates metabolic reprogramming which can cause mitochondrial overload by a misbalance between the
fatty acid oxidation and the TCA-cycle flux. As a result, metabolic side products are accumulated by incomplete fat oxidation. These products can cause excessive stress on the mitochondria which may activate, serine kinases, via DAG, PKC, thus increasing phosphorylation of IRS-1 resulting in less GLUT-4 translocation (Kahn et al. 2006, Muoio et al. 2008) and decreased glucose uptake.

**β-cell mass**

β-cell mass is dynamic and can be increased by β-cell neogenesis, proliferation, and α-cell conversion (Habener et al. 2012, Lysy et al. 2013). Neogenesis is suggested to occur from multipotent precursor cells, in the pancreatic duct. The rate of neogenesis is believed to be highest during the neonatal stages and/or in response to injury when β-cells are depleted (Weir et al. 2013).

Proliferation of existing β-cells exists in human pancreas but decreases with age (Reers et al. 2009). Overall proliferation is very low since β-cells have a long life span (Cnop et al. 2011). However, proliferation of β-cells is increased in response to a higher metabolic demand, such as during pregnancy and in insulin resistant states (Rahier et al. 2008, Hanley et al. 2010). For instance, rodents fed with high fat diet increase their β-cell volume by proliferation of β-cells, with no formation of new β-cells (Hull et al. 2005). Similar observations are made during pregnancy, when overall metabolic demand is increased (Butler et al. 2010, Rieck et al. 2010). During pregnancy, in rodents, proliferation of existing β-cells appears to be the mechanism by which β-cell mass is increased (Parsons et al. 1995), while in humans, neogenesis is more pronounced as the number of small β-cells is increased in islets of Langerhans (Bonner-Weir et al. 2010, Butler et al. 2010). Recently, an increased production of serotonin and serotonergic signaling in rodent islet cells during pregnancy was suggested to drive β-cell proliferation (Kim et al. 2010, Schraenen et al. 2010).

Another source for new β-cells is the neighboring pancreatic α-cell. For instance, injured β-cells can be rescued by α-cells in response to the released cytokines from the injured cells, such as stromal cell-derived factor-1 (SDF-1) and interleukin-6 (IL-6) (Habener et al. 2012). α-cells respond by releasing glucagon-like peptide-1 (GLP-1) (Buteau et al. 2003), thereby protecting β-cells from undergoing apoptosis and/or stimulating β-cell proliferation. In addition, heavily damaged β-cells can be replaced by α-cells that revert to pro-α-cells and differentiate into new β-cells (Habener et al. 2012).

In insulin resistance, β-cells are only able to temporarily sustain the increased production of insulin due to exposure to prolonged high glucose and FFA levels. Increased glucose and FFA levels are toxic and induce apoptosis which decrease β-cell mass. This is generally termed – glucotoxicity, lipotoxicity and glucolipotoxicity. β-cells will be exhausted and can no longer replenish the ready releasable pool (RRP) of insulin granules (Sako et al. 1990). The replenishment cannot be sustained due to
increased ROS production from mitochondria, deregulated TG/FFA cycling, ER stress, inflammation and finally apoptosis is induced (Prentki et al. 2006).

Apoptosis is initiated as members of the Bcl-2 family fail to control the mitochondria fission machinery due to overexpression of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2. Membrane permeabilization occurs and cytochrome C is released into the cytosol, binding to apaf-1 (Jiang et al. 2000) which in turn activates the caspase cascade (Martinou et al. 2011). The initiation of caspases can also be induced by ER stress. ER stress is suggested to induce apoptosis by the unfolded protein response (UPR) in the ER. UPR, a protein class that is Ca\(^{2+}\)-dependent and detects misfolded protein, e.g. proinsulin, in the ER. This response induces Bax expression and releases Ca\(^{2+}\) and via inositol requiring 1 (IRE-1) which phosphorylate JNK, c-jun NH2-terminal kinase and p38, an inhibitor of Akt/PKB which inhibits apoptosis and the caspase cascade (Bensellam et al. 2012). The caspase cascade can also be initiated by circulating cytokines; interleukin 1\(\beta\) (IL-1\(\beta\)), TNF\(\alpha\) and interferon-\(\gamma\), (IFN\(\gamma\)). IL-1\(\beta\) and TNF-\(\alpha\) inhibit NF-\(\kappa\)B and activate JNK/p38 while IFN\(\gamma\) mediates its apoptotic signal via the JNK/STAT pathway. When activated, the balance between JNK/p38 and the anti-apoptotic signal, extracellular signal-regulated kinases (ERKs) and cAMP regulated binding element (CREB), is changed and apoptosis is initiated (Mandrup-Poulsen 2001, Gysemans et al. 2008). The final stage of apoptosis is the assembly of the apoptosome which consists of the initiator caspases-7, -8, -10, the effector caspases,-3,-6, -9 and later of cytochrome C, which cleaves the effector caspases. The apoptosome activates DNases and DNA is fragmented (Riedl et al. 2007).

The islets of Langerhans

The islets of Langerhans are embedded in the exocrine tissue of the pancreas. These cell clusters are the main tissue regulating blood glucose levels. The islets contain several different cell types, which respond to changes in nutritional status by secreting the hormones; glucagon, insulin and somatostatin. These hormones are released from \(\alpha\)-, \(\beta\)- and \(\delta\)-cells. In addition, islets of Langerhans also contain, in smaller quantities, pancreatic polypeptide producing (PP) cells (Ahnfelt-Ronne et al. 2007) and ghrelin producing cells (Wierup et al. 2002) (Figure 2).

Rodent islets have a rich vascular supply (Jansson et al. 1986) and are extensively innervated while the human islets are less (Rodriguez-Diaz et al. 2011). In rodents, the islets consist of a core of \(\beta\)-cells with surrounding \(\alpha\)-, \(\delta\)- and PP-cells (Orci et al. 1975). Smaller human islets have a similar architecture while larger islets have a more heterologous arrangement of \(\alpha\)-, \(\beta\)- and \(\delta\)- cells along the blood vessels in the core, larger human islets also have a higher ratio of \(\alpha\) to \(\beta\)-cells (Cabrera et al. 2006, Bosco et al. 2010). This unique architecture is suggested to play a part in regulating intra-islet hormonal secretion via autocrine and paracrine signaling (Bosco et al. 2010). The pancreatic islet hormones regulate each other; secreted insulin
inhibits glucagon secretion while secreted glucagon stimulates insulin secretion (Samols et al. 1966), whereas somatostatin inhibits both insulin and glucagon secretion (Hauge-Evans et al. 2009).

**Glucose sensing and insulin secretion**

After food intake glucose is absorbed in the intestine into the blood stream. To regulate blood glucose, insulin is secreted in a biphasic manner; the acute and initiating 1st phase followed by a sustained steady-state 2nd phase (Henquin et al. 2006, Rorsman et al. 2013). Glucose is the major initiator of insulin secretion. The secretory machinery in the β-cells is activated by the uptake of glucose via the membrane glucose transporters (GLUT); GLUT1 and GLUT3 in human β-cells and GLUT2 in murine β-cells (De Vos et al. 1995, McCulloch et al. 2011).

During the initiating 1st phase of insulin release, glucose undergoes glycolysis where pyruvate is generated, controlled by the rate limiting enzyme glucokinase (Doliba et al. 2012) (Figure 3). The intracellular ATP:ADP ratio rises and as pyruvate enters the mitochondria, it is metabolized in the tricarboxylic (TCA) cycle and mitochondrial oxidative metabolism (Henquin et al. 2006), thus increasing the ATP:ADP ratio even further. The rise in ATP:ADP ratio closes the ATP sensitive K⁺-channels and the membrane potential is increased (Rorsman et al. 1985). As a consequence, voltage-gated T-type-Ca²⁺-channels opens and depolarize the membrane.
Figure 3. Consensus model of insulin secretion in β-cells. Insulin secretion is initiated when glucose is taken up into the β-cells by GLUTs. Glucose is metabolized in glycolysis to pyruvate. Pyruvate then enters the mitochondria and is funneled into the tricarboxylic cycle (TCA) via the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA (acCoA). As acCoA is metabolized in the tricarboxylic (TCA), thus increasing overall mitochondrial metabolism, the ATP/ADP ratio is increased in the cytoplasm. The increase in ATP causes closure of the ATP sensitive K⁺-channels and depolarizes the membrane, which in turn opens voltage dependent calcium channels (VDCC). The VDCCs causes an influx of Ca²⁺ and insulin granules release their content into the circulation. Insulin secretion is sustained by replenishment of the TCA-cycle and generation ATP. The TCA intermediates citrate, isocitrate and malate are converted into pyruvate in the cytosol, and enters the mitochondria via specific shuttles to replenish the TCA-cycle. Reducing equivalents such as NADH and FADH₂ are also generated and drive the proton motive force in the mitochondria which further increases ATP production and thus insulin secretion. Cycling of citrate generates acCoA which is converted into malonyl-CoA (malCoA). MalCoA inhibits fatty acid-CoA (FA-CoA) uptake in the mitochondria thus increases the rate of FA-CoA entering the GL/FFA cycle, which increases the generation of lipid signals essential for adequate insulin release. Moreover, insulin secretion can be modulated and fine-tuned by G-protein coupled receptors (GPCR). GPCRs increase insulin secretion via Gs stimulation of adenylate cyclase (AC) or inhibited by Gi. Gq-coupled receptors potentiate insulin secretion by activating phospholipase C (PLC) and Ca²⁺ is released from the endoplasmic reticulum (ER) which raises the [Ca²⁺]i.

which in turn activates L-type-Ca²⁺-channels and Na⁺-channels (Arkhammar et al. 1987). As a result, the action potential peaks and P/Q-type-Ca²⁺-channels opens and rises the [Ca²⁺]i by influx of [Ca²⁺] which trigger exocytosis of insulin granules (Rorsman et al. 2013). The initial surge of insulin release comes from the ready releasable pool (RRP) close to the P/Q-type-Ca²⁺-channels (Barg et al. 2001). The RRP is replenished from the resting pool (RP) through priming and docking of insulin granules with the plasma membrane and/or the P/Q-type-Ca²⁺-channels by SNARE-
complexes, syntaxin-1, munc-13 and are ATP-dependent (Barg et al. 2008, Seino et al. 2011).

Besides the initiating phase, the sustained steady-state 2nd phase is slowly building up (Rorsman et al. 2013) which amplifies the response to glucose, as glucokinase limits glucose stimulated insulin secretion (GSIS) (Matschinsky 1996), and recruits granules and replenish the RRP (Seino et al. 2011). This phase is believed to be dependent on mitochondrial metabolism of nutrients; such as glucose, FFA and amino acids. This provides metabolic coupling factors to maintain and enhance mitochondrial metabolism via ATP, NADH, FADH2 and stimulation of electron transport chain (Prentki et al. 2013). This generates even more ATP, closure of KATP-channels and increases [Ca2+]e influx (Rorsman et al. 1985).

Metabolism of glucose generates NAD+ and pyruvate, which is funneled into the TCA cycle via conversion to acetyl-CoA and oxaloacetate, in the mitochondria (Figure 3). The complete oxidation of acetyl-CoA provides CO2, ATP and the reducing equivalents NADH and FADH2 which supply electrons to the respiratory chain, and the electrochemical gradient drives the generation of ATP by ATP synthase. Moreover, amino acids; glutamine, leucine and arginine produce α-ketoglutarate in the mitochondrial matrix which enters and replenish the TCA cycle (Ishiyama et al. 2006). Arginine is believed to affect membrane potential directly, via an influx of [Ca2+]e by opening of Ca2+-channels (Sener et al. 2000). TCA intermediates (citrate, isocitrate, α-ketoglutarate and malate) are shuttled to the cytoplasm and participate in pyruvate cycling; pyruvate/citrate, pyruvate/isocitrate and pyruvate/malate cycles, these cycles generate cytosolic NADPH and pyruvate (Prentki et al. 2013). The pyruvate/citrate cycle generates acetyl-CoA, malonyl-CoA and NAD+ in the cytoplasm (Jitrapakdee et al. 2010). α-ketoglutarate can also be converted into glutamate which stimulates insulin secretion (Maechler et al. 2002). Generation of cytosolic NADPH increases the NADPH/NADP ratio and NADPH, via glutaredoxin, and is suggested to facilitate insulin exocytosis (Ronnebaum et al. 2006). NADPH can bind to a voltage dependent K+-channel which repolarizes the membrane after the glucose induced action potential and stops the influx of Ca2+, this enhances and allows a more sustained insulin secretion (MacDonald et al. 2003).

Acetyl-CoA is further carboxylated in the cytosol to malonyl-CoA. Malonyl-CoA and FFA enter the glycerolipid/FFA cycle (GL/FFA) to produce lipid signals that facilitate insulin exocytosis; FA-CoA, mono- and di-acylglycerols (MAG and DAG). FA-CoA and DAG, via PKC, raise the cytosolic [Ca2+] from intracellular Ca2+-storage in the endoplasmic reticulum (Prentki et al. 2002, Prentki et al. 2013).

Modulation of insulin secretion

To keep the glycemic level within range, insulin secretion is modulated by exogenous and endogenous signals targeting the pancreatic islets. These agents target a group of receptors termed G-protein coupled receptors (GPCRs). The signal from GPCRs can
for instance modulate cytosolic Ca\textsuperscript{2+} or even effect the docking of insulin granules (Sundler et al. 1980, Ahren 2009, Ahrén 2012).

The GPCR family consists of a seven trans-membrane α-helix region with an attached heterotrimeric α-, βγ-subunit; the guanine nucleotide-binding (G) proteins (Figure 4). The G-proteins are activated upon ligand binding and the receptor couples to the G-proteins (Cabrera-Vera et al. 2003, Luttrell 2008). This coupling induces exchange of GDP/GTP (GDP release is the limiting step of activation) on the Gα-unit, which dissociates, and Gα and Gβγ are free to activate secondary signals (Hamm 1998). This signal is terminated by the dephosphorylation of the bound GTP molecule by GTPases and the heterotrimeric unit reforms (Cabrera-Vera et al. 2003, Luttrell 2008). The G-protein activation is tightly regulated by several small proteins (GPCR-interacting proteins (GIPs)), interacting with the C-terminal or the third loop of the GPCR. The GIPs attach to the GPCR and can either alter the signal or initiate internalization of the GPCR (Bockaert et al. 2004).

The GPCR receptors are attached to different Gα-subunits. Gα\textsubscript{s} stimulates the formation of cyclic adenosine 3’,5’-monophosphosphate (cAMP) by adenylate cyclase, while Gα\textsubscript{i/o} inhibits cAMP production. Gα\textsubscript{q/11} induce the formation of phospholipase Cβ (PLCβ) and Gα\textsubscript{12/13} increases iNOS, promotes cell proliferation and cytoskeletal changes (Cabrera-Vera et al. 2003).

Activation of Gα\textsubscript{s} potentiates insulin secretion via cAMP activation of PKA that mediates influx of Ca\textsuperscript{2+} from voltage-dependent L-type Ca\textsuperscript{2+}-channels. PKA also synergizes with cAMP to activate Epac, which increases the docking of insulin secretory vesicles to the plasma membrane (Ammala et al. 1993, Cheng et al. 2008).
cAMP can also mediate cell proliferation by activating ERK in the mitogen activated MAP kinase pathway, and CREB which is also targeted by the anti-apoptotic Akt (Stork et al. 2002, Kim et al. 2008). Additional effects can be mediated by PKA e.g. inhibit the interferon stimulated JNK/STAT pathway (David et al. 1996), thereby protecting the cells from cytokine mediated cell destruction.

Activation of Gαq potentiates insulin secretion by hydrolysis of phosphatidylinositol bisphosphate PIP2 into DAG and inositol triphosphate IP3. IP3 releases Ca2+ from the ER by binding to the IP3 sensitive Ca2+ channel. The release of Ca2+ increases the cytosolic Ca2+ and potentiates insulin secretion. The membrane bound DAG activates PKC. PKC is also activated by the raise in Ca2+ from IP3 in a feed forward loop (Tengholm et al. 2009). PKC is, among other things also suggested to exert similar functions as PKA by increasing the RRP (Gillis et al. 1996).

Signals targeting GPCRs in the islets of Langerhans could for instance be GIP, GLP1, acetylcholine, adrenalin and FFAs. GLP1 and GIP are incretins released postprandially from the L- and K-cells in the intestine. GLP-1 and GIP potentiate insulin secretion by binding to the Gαs connected GLP-1 receptor and GIP receptors located in the plasma membrane of the pancreatic β-cell. In addition, the hypothalamus innervates and affects insulin secretion by parasympathetic and sympathetic neurons (Rodriguez-Diaz et al. 2012) where the parasympathetic neurons by acetylcholine increase insulin secretion via the M3-receptor. Innervation of sympathetic neurons in pancreas inhibit insulin secretion via α2-receptor, and promote insulin secretion via β2-receptor on β-cells (Ahren 2009). Circulating short and long-chain FFAs can also potentiate insulin secretion via the Gαq GPR40 (Itoh et al. 2003), GPR41, GPR43, GPR84 (Stoddart et al. 2008) and GPR120 (Oh et al. 2010). Additional signals are the female sex hormone estrogen and the neurotransmitter serotonin (Sundler et al. 1980). All these signals are important as the level of insulin resistance and amount required insulin varies throughout life, during puberty (Moran et al. 1999), pregnancy (Dahlgren 2006) and aging (Broughton et al. 1991).
Estrogen

Estrogen (E2) is a steroid hormone functioning as the primary female sex hormone but is also present in males, albeit at lower concentrations. In females, the amount of estrogen fluctuates during life; with increased levels during puberty and pregnancy and a decreased level at menopause. Previous studies show that decreased estrogen in women after menopause increases the risk of developing obesity, insulin resistance and diabetes mellitus (Godsland 2005, Prossnitz et al. 2011).

E2 synthesis

E2 is a hormone synthesized from cholesterol and 3-β-hydroxysteroid dehydrogenase (3β-HSD) yielding the intermediates androstenedione and testosterone which are aromatized by the enzyme estrogen aromatase (rate-limiting step) into estrone (E1), estradiol, 17β-estradiol, (E2) and estriol (E3) (Figure 5). E2 is mainly synthesized in the ovaries and during pregnancy by the placenta. E2 is also synthesized in mammary tissue, brain, adipose tissue, liver and a small quantity is also produced by the Leyding cells in the testis (Prossnitz et al. 2011) (Nelson et al. 2001). In the circulation, E2 is mainly bound to the sex hormone-binding globulin and albumin, only a small fraction (2.2 %) is free and biological active (Wu et al. 1976).

![Figure 5. Estrogen (E2) synthesis](image)

E2 is synthesized from cholesterol via androstenedione and testosterone by 3-β-hydroxysteroid dehydrogenase (3β-HSD), 17β-HSD and aromatase. In addition to E2, estrone and estriol are also synthesized by aromatase during menopause or pregnancy.
**Estrogen receptors**

E2 binds to two types of receptors: the nuclear estrogen receptors (nER) which mediate the E2 signal mainly by genomic mechanisms, and the membrane bound GPCR; the GPER-1/GPR30 (G-protein Estrogen receptor-1) which rapidly activates downstream second messenger systems and cause multiple cellular effects (Prossnitz et al. 2011).

**Nuclear estrogen receptors**

There are two nuclear estrogen receptors, ERα (Jensen et al. 1973) and ERβ (Kuiper et al. 1996) and they are at the inactive state bound to Heat shock protein 90 (Hsp90) in the cytoplasm (Figure 6). When E2 binds to these receptors Hsp90 dissociates, the receptors form either homodimers or heterodimers and the functional sites are exposed, activation function-1 and 2 (AF-1 and AF-2). The receptors translocate to the nucleus and AF-1 and AF-2 connect the receptors to the estrogen responsive element (ERE), situated in the promoter region upstream of estrogen regulated genes and transcriptional activity is initiated (Beato et al. 2000) (Figure 6).

Several splice variants of the estrogen receptors exist, which are capable to modulate the estrogen signal; wild type ERα-66, ERα-46 and ERα-36. ERα-46 and ERα-36 are missing the AF-1 site and ERα-36, located in the plasma membrane, contains a unique C-terminal amino acid sequence which induces non-genomic estrogenic actions; phosphorylation of ERK and increasing Ca²⁺-levels (Kang et al. 2010, Kampa et al. 2013). In addition, there are also reports on novel yet unidentified receptors, the ERx, which mediates extra-nuclear signaling in breast cancer cells (Kampa et al. 2012) and mER-Gαq in neurons (Qiu et al. 2003). Furthermore, ERα and ERβ are capable of relocalization to cytoplasmic membranes by palmitolyation (Kampa et al. 2013) (Figure 6).

**Membrane bound estrogen receptor (GPER-1)**

The diverse estrogen receptor system also includes GPER-1, which induces rapid intracellular signaling events. GPER-1 was classified as an estrogen receptor in 2005 as endogenous E2 binds to GPER-1/GPR30 in SKBr3 and HEK (293) cells lacking ERα and ERβ (Revankar et al. 2005, Thomas et al. 2005) (Figure 6). GPER-1 signals primarily via Gαs to stimulate cAMP formation (Filardo et al. 2002) but there are also reports that GPER-1 is capable of increasing Ca²⁺-levels (Revankar et al. 2005, Brailoiu et al. 2007) via PLC and IP₃ (Revankar et al. 2005) and the Gβγ-subunit (Filardo et al. 2012). Via an increase of [Ca²⁺], subsequent activation of proto-oncogene tyrosine-protein kinase (Src) and matrix metalloproteinases (MMPs), GPER-1 is also involved in the release of the epidermal growth factor (EGF)-like polypeptide which induces ERK1/2 activation in MCF-7 and SKBr3 cells (Filardo et al. 2000, Filardo et al. 2012). In addition, GPER-1 can regulate expression of Bcl-2 (Kanda et al. 2003), cyclin D2 and CREB (Kanda et al. 2004) as well as c-Fos
Maggiolini et al. 2004. Intercellular, GPER-1 has been visualized at the plasma membrane (Filardo et al. 2007), Golgi (Matsuda et al. 2008) and ER membranes (Revankar et al. 2005) (Figure 6). Pharmacological modulation of estrogen receptors

To untangle the GPER-1 effect among the other E2 effects, several pharmacological tools have been developed. A specific agonist, G-1 for the GPER-1 was developed (Bologa et al. 2006) to study the activation of the receptor. It was later found that G-1 also activates ERα-36 (Kang et al. 2010). G15 (Dennis et al. 2009) and G36 (Dennis et
al. 2011) are two antagonists of GPER-1. Both G15 and G36 inhibited the G-1 and E2 induced Ca^{2+}-mobilization and MAPK activation. Beside this inhibition, G15 exerted a low inhibition of nERs and the ERE in SkBR3 and MCF7-cells, while G36 displayed no inhibition of nERs and the ERE in the same cell strains (Dennis et al. 2009).

Another approach is to block the nERs by either antagonists; selective estrogen down regulator (SERD), fulvestrant (ICI-182,781) which inhibits the dimerization of nERs or by selective estrogen receptor modulator (SERM) e.g. tamoxifen (Johnston 2005). These blockers could also target GPER-1, where tamoxifen acts as an antagonist and ICI-182,781 acts as a partial agonist on GPER-1 (Thomas et al. 2005). EM652 is also a SERM, has higher affinity than the previous mentioned blockers to nERs and blocks the AF1 and AF2 sites of the nERs with no reported agonistic effects (Labrie et al. 1999, Johnston 2005).

**Estrogen and glucose homeostasis**

E2 is reported to have both positive and negative effects on glucose homeostasis and this dual effect is concentration-dependent. The physiological range of circulating E2 is pico- to nanomolar concentrations and up to micromolar levels in ovarian follicles (Albanito et al. 2007). When E2 remains low, after ovariectomy in mice or after menopause in humans, the prevalence of T2DM is increased. Glucose induced insulin secretion is reduced and insulin resistance is progressing. This can be reversed by estrogen replacement therapy (Walton C 1993, Godsland 2005, Vogel et al. 2013). On the other hand, excess E2 for instance during high doses of oral contraception in humans is associated with deficient insulin secretion and insulin resistance (Walton C 1993, Godsland 2005). This concentration-dependent effect by E2 is also reflected on insulin secretion as E2 both decreases and increases insulin secretion (Tesone et al. 1979, Etchegoyen et al. 1998, Nadal et al. 1998) and (paper I). In addition, E2 is also reported to mediate protective effects on pancreatic islets (Eckhoff et al. 2004).

The E2 mediated affect in pancreatic islets is a result of a wide range of signals and receptors. Which of the estrogen receptors that dominate these effects is currently under debate, due to an overall impact on islet hormone secretion by all estrogen receptors (Nadal et al. 2011). The classical estrogen receptors and E2 have been studied for their role in β-cell function. ERα is reported to increase insulin content upon E2 stimulation (Alonso-Magdalena et al. 2008) and to mediate pancreatic β-cell survival in streptozotocin treated mice (Le May et al. 2006). ERβ is also involved in insulin secretion by closure of K_{ATP}-channels, enhancing [Ca^{2+}]_i and GSIS (Soriano et al. 2009). This effect is replicated in studies using an ERβ agonist (Alonso-Magdalena et al. 2013). Moreover, ERβ deficient mice display delayed first phase insulin secretion and islet hypertrophy (Barros et al. 2009).

The non-classical receptor GPER-1 is also reported to be involved in the E2 effect on pancreatic islets. Evidence from GPER-1 knockout mice show that mice deficient of GPER-1 are more prone to develop obesity and insulin resistance (Haas et
al. 2009, Martensson et al. 2009). Mårtensson et al. also show that GPER-1 knockout mice exhibit, glucose intolerance and loss of first phase insulin release in females. In addition, islets from both genders show decreased GSIS and glucagon secretion when compared to wild-type mice. Moreover, islets from GPER-1 deficient mice failed to release insulin and glucagon in response to E2 (Martensson et al. 2009). Haas et al., describes an increase in body weight and abdominal fat in their GPER-1-deficient mouse line in both genders (Haas et al. 2009). GPER-1 deficiency in 18 month old male mice resulted in insulin resistance, impaired glucose tolerance, dyslipidemia and a pro-inflammatory state with increased circulating cytokines: TNF-α, IL-1β, MCP-1 and IL-6 (Sharma et al. 2013). However, these effects were not replicated in four other independently generated KO strains (Liu et al. 2009, Langer et al. 2010).

GPER-1 has also been evaluated for an insulin potentiating effect, *in vitro*, with the agonist G-1. G-1 generates Ca\(^{2+}\) mobilization in SKBr3 cells (Dennis et al. 2009) as well in neurons (Brailoiu et al. 2007). Both E2 and G-1 stimulate insulin secretion in female mice islets (Ropero et al. 2012) and in MIN6 cells (Sharma et al. 2011). The antagonist G-15 inhibited the stimulatory effect of E2 on GSIS in MIN6 cells (Sharma et al. 2011). In addition, during pregnancy the amount of E3 is increased and E3 is reported to function as an antagonist for GPER-1 in cells lacking ERα and ERβ (Lappano et al. 2010).
Serotonin

Serotonin (5-HT), a bioamine and a neurotransmitter, first discovered in the gut, later in the blood stream and subsequently in the CNS, is mainly known for its involvement in several of the delicate neuronal processes that regulate behavior. Moreover, it regulates overall energy metabolism from the CNS and certain neuronal developmental phases (Gaspar et al. 2003). In the 1960s and 1970s, independent research groups observed the presence of serotonin in pancreatic islets from several species (Falck et al. 1964, Cegrell 1968, Ekholm et al. 1971).

Biosynthesis of 5-HT

5-HT is synthesized from the amino acid L-tryptophan by tryptophan hydroxylase (TPH) (the rate limiting step) into 5-hydroxy-L-tryptophan (5-HTP) (Figure 7). Thereafter 5-HTP is decarboxylated into 5-HT. 5-HT may be further metabolized to melatonin in the presence of the enzymes 5-HT-N-acetyltransferase and hydroxyindol-N-methyltransferase. The catabolic pathway of 5-HT follows cellular uptake and breakdown to 5-hydroxyindoleacetic acid (5-HIAA) by the enzymes monoamine oxidase (primarily by MAO-A but also by MAO-B) and aldehyde dehydrogenase. 5-HIAA is excreted via the urine. Due to the blood brain barrier (BBB), 5-HT exists in

![Figure 7. Biosynthesis of serotonin (5-HT).](image-url)

The biosynthesis of 5-HT begins with L-tryptophan which is hydroxylated by tryptophan hydroxylase (TPH1 and 2) into 5-hydroxytryptophan (5-HTP). 5-HTP is further carboxylated to 5-HT by aromatic L-amino acid decarboxylase (DDC). Subsequently, 5-HT is metabolized either by monoamine oxidase (MAO) and/or aldehyde dehydrogenase into 5-hydroxyindoleacetic acid (5-HIAA) or into melatonin.
two distinct pools; the central pool (CNS) and the peripheral pool (outside CNS) (Lam et al. 2007). Central 5-HT is synthesized in raphé nuclei, from the BBB passable L-tryptophan and 5-HTP. In the brain, 5-HT act as a synaptic signal which is terminated by the 5-HT transporter (SERT) (Lam et al. 2007). Peripheral 5-HT on the other hand is synthesized in enterochromaffin cells of the intestine and released into the intestinal mucosal layer. Excess 5-HT reaches the circulating system and is absorbed by circulating platelets where it regulates blood pressure and targets peripheral tissues (Pletscher 1987). The expression of TPH differs between the central and peripheral tissues and there are two isoforms of TPH; TPH 1 and 2 (Lam et al. 2007). The expression of TPH2 is expressed in the CNS, while TPH1 is expressed in peripheral tissues (Walther et al. 2003, Sakowski et al. 2006), with the exception of pancreatic islets, which express both isoforms (Schraenen et al. 2010, Ohta et al. 2011).

5-HT receptors

5-HT receptors were classified in the 1950s when serotonergic effects were blocked by morphine (M) and dibenzyline (D), and the receptors were subsequently termed 5-HT M and 5-HT D receptors (Gaddum et al. 1957). Later, 5-HT receptors were classified according to NC-IUPHAR (Hoyer et al. 1994) into seven groups of receptors, all except one group are GPCRs. 5-HT1 signals via Gαi, 5-HT2 via Gαq, 5-HT3 is an unspecific cation channel, 5-HT4 via Gαs, 5-HT4 can reportedly also activate a T-type Ca2+-channel (Contesse et al. 1996) and 5-HT5 via Gαi or Gαq and can as well activate a G-protein coupled rectifying inward K+ channel (GIRK) (Figure 8). There are, according to NC-IUPHAR, 14 different 5-HT receptors divided into seven 5-HT families depending on their amino sequence homology, pharmacological function and gene expression, with the exception of splice variants from a single gene.

Table 1: 5-HT receptor nomenclature.

<table>
<thead>
<tr>
<th>5-HT1</th>
<th>5-HT2</th>
<th>5-HT3</th>
<th>5-HT4</th>
<th>5-HT5</th>
<th>5-HT6</th>
<th>5-HT7</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A</td>
<td>5-HT2A</td>
<td>5-HT3A</td>
<td>5-HT3A</td>
<td>5-HT5A</td>
<td>5-HT6</td>
<td>5-HT7</td>
</tr>
<tr>
<td>5-HT1B</td>
<td>5-HT2B</td>
<td>5-HT3Ba</td>
<td>5-HT3Bb</td>
<td>5-HT5Bd</td>
<td>5-HT6e</td>
<td>5-HT7</td>
</tr>
<tr>
<td>5-HT1D</td>
<td>5-HT2C</td>
<td>5-HT3Ca</td>
<td>5-HT3Ca</td>
<td>5-HT5Be</td>
<td>5-HT6ed</td>
<td>5-HT7ed</td>
</tr>
<tr>
<td>5-HT1E</td>
<td>5-HT3D</td>
<td>5-HT3D</td>
<td>5-HT3D</td>
<td>5-HT5Be</td>
<td>5-HT6ed</td>
<td>5-HT7ed</td>
</tr>
<tr>
<td>5-HT1F</td>
<td>5-HT2C</td>
<td>5-HT3Ea</td>
<td>5-HT3Ea</td>
<td>5-HT5Be</td>
<td>5-HT6ed</td>
<td>5-HT7ed</td>
</tr>
<tr>
<td>5-HT1Pb</td>
<td>5-HT3Ea</td>
<td>5-HT3Ea</td>
<td>5-HT3Ea</td>
<td>5-HT5Be</td>
<td>5-HT6ed</td>
<td>5-HT7ed</td>
</tr>
</tbody>
</table>

a Not functional as homodimers
b Could be due to heterodimerization
c RNA editing yields multiple isoforms
d Not functional in humans.
e Several splice variants have been described with the same function.
Lower case denotes that the receptor has not yet achieved full receptor status.
Receptors in bold are classified by NC-IUPHAR.
(Hoyer 2001, Hannon et al. 2008, Nichols et al. 2008) (Table 1). In addition, there is one additional putative 5-HT\(_1\) receptor expressed in the ENS, 5-HT\(_{1P}\) (Gershon et al. 1991). There are more than 20 functional sub-variants generated from splice variants of the 14 classified receptors (Pytliak et al. 2011) (Table 1). The 5-HT\(_{2C}\) undergoes mRNA editing which yields 24 different proteins (Burns et al. 1997). Splice variants of the 5-HT\(_4\) gene produce 10 functional receptors (Bockaert et al. 2008) and the 5-HT\(_7\) gene is spliced into 4 variants with the same functional characteristics (Heidmann et al. 1997, Krob et al. 2002).

The 5-HT\(_2\) receptor group is implicated in regulation of energy homeostasis. This group consists of 5-HT\(_{2A}\), 5-HT\(_{2B}\) and 5-HT\(_{2C}\). 5-HT\(_{2A}\) signals predominantly via G\(\alpha_q\) but there are also reports of signaling via; PLA\(_2\) and AA (Berg et al. 1998) and G\(\alpha_{12/13}\) which induce cytoskeletal rearrangements. 5-HT\(_{2A}\) is expressed both in CNS and the periphery where it for instance is detected in gastrointestinal tract and in pancreatic islets from rodents. In the periphery it has an overall contractive effect on vascular smooth muscles (Hannon et al. 2008, Kim et al. 2010). Constitutive activity of 5-HT\(_{2A}\) has been reported in vivo (Aloyo et al. 2009) and in vitro from mutated 5-HT\(_{2A}\) (Muntasir et al. 2006) or by overexpression (Weiner et al. 2001) but it appears that wild type in vitro 5-HT\(_{2A}\) is not constitutive active (Aloyo et al. 2009). In the CNS, 5-HT\(_{2A}\) is located on nerve endings where it elicits excitatory effects via Ca\(^{2+}\) increase, and is involved in regulation of behavioral diseases and hallucinations. Knockdown of the receptor in mice suppress feeding, reduce body weight, disrupt sleeping patterns and increase anxiety (Popa et al. 2005, Weisstaub et al. 2006, Salomon et al. 2007).

5-HT\(_{2B}\) is mainly expressed in periphery, with restricted expression in the brain (cerebellum, lateral septum, dorsal hypothalamus and medial amygdala). mRNA is found in fundus, gut, heart, lung, the cardiovascular system and in pancreatic islets (Hannon et al. 2008, Kim et al. 2010). 5-HT\(_{2B}\) mediates fundus contraction, induces proliferation via MAP kinases and, when overexpressed, causes fibrosis and pulmonary hypertension (Hannon et al. 2008). 5-HT\(_{2B}\) also mediates mitochondrial protective mechanisms in cardiomyocytes (Nebigil et al. 2003). The 5-HT\(_{2B}\) knockout (KO) mice are lethal and produce severe embryonic defects, whereas overexpression of 5-HT\(_{2B}\) has detrimental effects on the cardiovascular system (Nebigil et al. 2000, Nebigil et al. 2001).

5-HT\(_{2C}\) is almost exclusively expressed in the CNS, and has similar pharmacological properties as the 5-HT\(_{2A}\) receptor. Dysfunction of 5-HT\(_{2C}\) is involved in several psychiatric disorders and is constitutively active depending on neuronal cell types and brain regions (Aloyo et al. 2009). The receptor expression is regulated either by two non-functional splice variants or by mRNA editing, which yields 24 different proteins and generates 5-HT\(_{2C}\) receptors with differences in activity (Burns et al. 1997). 5-HT\(_{2C}\) KO mice have marked hyperphagia, increased body weight as well as increased locomotor activity (Tecott et al. 1995). In addition, a global KO of the 5-
HT$_{2C}$ receptor displays impaired glucose tolerance (Xu et al. 2011, Papazoglou et al. 2012).

**Figure 8. Hypothesis of 5-HT action on pancreatic β-cell.** Extracellular 5-HT, released from islet cells, can stimulate the membrane bound 5-HT-receptors which consist of 7 families, 5-HT$_1$, G$_i$; 5-HT$_2$, G$_q$; 5-HT$_{4,6,7}$, G$_q$; and 5-HT$_5$, G$_i$; all except 5-HT$_3$ are G-protein coupled receptors, the 5-HT$_3$ is a cation channel. These receptors can increase insulin secretion via G$_s$ stimulation of adenylate cyclase (AC) or inhibit insulin secretion by G$_i$, modulating the level of cAMP. G$_q$-coupled receptors potentiate insulin secretion by activating phospholipase C (PLC) which converts phospholipids (PL) into diacylglycerol (DAG) and inositol phosphate 3 (IP$_3$). IP$_3$ binds to the IP$_3$R and Ca$^{2+}$ is released from the endoplasmic reticulum (ER) which raises the [Ca$^{2+}$]. Besides insulin in the insulin granules, 5-HT is also loaded by the vesicular monoamine transporter 2 (VMAT2). Another pathway to stimulate insulin secretion is by intracellular 5-HT, which could serotonylate Rabs on the granules. This promotes replenishment of the ready releasable pool of granules. The released 5-HT is recycled by 5-HT transporter (SERT), reused or broken down by MAO.

### 5-HT and energy homeostasis

5-HT affects glycemic levels *in vivo* and causes hyperglycemia in rodents when 5-HT and some 5-HTR agonists are infused (Chaouloff et al. 1987, Chaouloff et al. 1990, Yamada et al. 1995, Uvnäs-Moberg et al. 1996, Yamada et al. 1997) as well as hypoglycemia (Gagliardino et al. 1971, Yamada et al. 1989). Whether these effects are centrally or peripherally dependent remains unclear. Hyperglycemic and hyperinsulinemic effects were also reported by 5-HTP injections (Jacoby et al. 1979).
Central 5-HT
Synaptic 5-HT in the hypothalamus is linked to satiety, regulation of food intake and regulation of energy expenditure. Increasing synaptic 5-HT induces hypophagia, reported by 5-HTP injections, blockage of SERT with SSRIs, e.g. fenfluramine, or with inhibition of MAO-A (Lam et al. 2007). The hypophagic effect is suggested to be regulated by 5-HT₁B and 5-HT₂C, but is also linked to the leptin system as there are leptin receptors on serotonergic neurons. In addition, postprandial increase of insulin increases tryptophan access to the brain by increasing the utilization of amino acids, thus increasing central 5-HT levels (Lyons et al. 1988, Donovan et al. 2013). As the bioavailable 5-HT rises or by direct activation of 5-HT₂C receptors on pro-opiomelanocortin (POMC)/cocaine and amphetamine regulated transcript (CART) neurons, POMC/CART neurons release α-melanocyte-stimulating hormone (α-MSH). α-MSH activates the melanocortin 4 receptor expressing neurons which in turn induces central satiety stimulating signals (Marston et al. 2011, Xu et al. 2011) and improves peripheral insulin signaling by phosphorylation of PKB in skeletal muscle and liver (Zhou et al. 2007). Satiety can also be influenced by 5-HT₁B receptors, which suppress the release of gamma-aminobutyric acid (GABA), neuropeptide Y and by inhibiting POMC neurons via the agouti-related peptide (AgRP) (Marston et al. 2011, Donovan et al. 2013).

Peripheral 5-HT
Platelet loaded 5-HT affects glucose homeostasis and insulin resistance by acting not only via the vagal afferent serotonergic neurons but also through serotonergic receptors in the peripheral tissues. Circulating 5-HT might be capable to induce satiety signals via 5-HT₂ receptors (Edwards et al. 1991) possibly via 5-HT₂B mediated fundus contraction and/or by influencing gastric emptying (Beattie et al. 2004). While, Simansky et al. reports that peripheral 5-HT is not enough to induce satiety without postingestive signals (Simansky et al. 1992). Moreover, circulating 5-HT is reported to improve insulin sensitivity by increasing the GLUT4 vesicles translocation to the plasma membrane via the 5-HT₂A receptor in rat skeletal muscle (Hajduch et al. 1999).

On the other hand, the agonist α-methyl-5-HT (AMS) induced insulin resistance in rat muscle (Rattigan et al. 1999). The agonist AMS has been used in several reports to study the peripheral serotonergic effects and AMS displayed stimulatory effects on hepatic glycogenesis via 5-HT₁, 5-HT₂A, ckδ/p35 and glycogen synthase, while 5-HT₂BC counter-regulated this effect and stimulated gluconeogenesis (Tudhope et al. 2012). This is also shown during fasting, where 5-HT inhibits hypoglycemia via the 5-HT₂B receptor in mice, by increasing gluconeogenesis as well as inhibiting glucose uptake by GLUT2 in hepatocytes and increasing lipolysis in adipocytes (Sumara et al. 2012). Moreover, in insulin and glucagon clamped dogs at hyperglycemic and hyperinsulinemic conditions, intraportal infused 5-HT enhances the net hepatic glucose uptake (Moore et al. 2004). Furthermore, peripheral 5-HT increases bile acid production and lipid metabolism (Watanabe et al. 2010). 5-HT is also increased in
mice fed a high fat diet, indicating that 5-HT signaling is involved in the overall peripheral response to a metabolic challenge (Kim et al. 2011).

**Pancreatic 5-HT**

Local production and/or circulating 5-HT affect pancreatic hormone secretion and pancreatic islets express both the 5-HT transporter (SERT) (Ohta et al. 2011), which mediates 5-HT uptake into islet-cells, and the vesicular monoamine transporter 2 (VMAT2) (Anlauf et al. 2003), which loads 5-HT into vesicles. As the islets express both these transporters, 5-HT can serve as an autocrine and a paracrine signal to regulate islet hormone secretion (Sundler et al. 1980) (Figure 8).

Different strategies have been used to determine the role of 5-HT in pancreatic islets. These strategies have generated several reports but the overall context of 5-HT is still controversial. For instance, the exposure of selective 5-HT reuptake inhibitors (SSRIs) blocks extracellular intake of 5-HT and decreases insulin secretion in mouse islets and MIN-6 cells (Isaac et al. 2013). Moreover, inhibition of 5-HT catabolism with MAO-inhibitors can, at low concentrations, potentiate insulin secretion while at higher concentrations these compounds inhibit insulin secretion (Aleyassine et al. 1975) or at 1000x IC_{50} MAO-inhibitors stimulate insulin secretion (Lenzen et al. 1983). Both compounds increase the extra- and intracellular levels of 5-HT within and surrounding the islets. Moreover, exogenously added 5-HT reportedly inhibits insulin secretion; from golden hamster islets (Feldman et al. 1970, Feldman et al. 1970) and from mouse islets (Lernmark 1971). Large amounts of exogenous 5-HT acts as a weak inhibitor on perfused pancreas from rabbit (Feldman et al. 1972) and 5-HT increases insulin secretion from sectioned rabbit pancreas (Telib et al. 1968) and in vivo in humans (Federspil et al. 1974). Dual actions of 5-HT is observed in isolated mouse islets (both inhibitory and stimulatory actions on insulin release) (Gagliardino et al. 1974). In addition to these reports in clonal β-cells; 5-HT inhibits insulin secretion at 5.6 mM glucose from INS-1 cells (Heimes et al. 2009). Furthermore, high extracellular 5-HT concentration can inhibit insulin secretion via the 5-HT_{1A} receptor, while low extracellular and high intracellular concentrations of 5-HT stimulates insulin secretion via serotonylation (covalent binding of 5-HT) of GTPases Rab3a and Rab27 (Paulmann et al. 2009) (Figure 8). 5-HT is suggested to be co-released together with insulin secretion as preloaded β-cells, with either 5-HTP or 5-HT, secrete the amine by exocytosis upon stimulation of insulin release (Gylfe 1978). In addition, glucagon secretion is inhibited by 5-HT stimulation of mouse pancreatic islets (Marco et al. 1977).

Pharmacological regulation of 5-HT receptors in vitro has mixed effect on insulin secretion. 5-HT receptor antagonists of the 5-HT_{2} family potentiate glucose induced insulin secretion from rabbit pancreas (Feldman et al. 1972) and atypical antipsychotics (broad antagonists of 5-HT_{2} family) are reported to have either a potentiating effect on insulin release from INS-1 cells (Melkersson 2004) or no effect at all (Melkersson et al. 2005). This variation is most likely related to the
pharmacological profile of the atypical antipsychotics, as the compounds also target the M₁, M₃, H₁, the dopaminergic receptors and α₁- and α₂-adrenergic receptors (Starrenburg et al. 2009). In addition, the broad 5-HT₂C agonist mCPP diminishes insulin secretion from MIN-6 cells (Zhang et al. 2013).
Aims of the thesis

The overall aim of this thesis was to study the involvement of the estrogen receptor GPER-1 and the 5-HT activated 5-HT receptor family on β-cell function. More specifically, the aims are:

- Evaluate GPER-1 expression and G-1 mediated effect on insulin secretion and β-cell viability in murine and human pancreatic islets.

- *In vitro* evaluation of 5-HT receptors; expression, cell type localization and involvement on insulin secretion in human pancreatic islets.

- Study the effect of two 5-HT₂ receptor agonists (AMS and TCB-2) on insulin secretion and β-cell viability *in vitro* as well as effects on glucose homeostasis and insulin resistance *in vivo*. 

Methodology

Animals and glucose tolerance test

In paper I, we used both male and female mice from the Swiss type outbred NMRI strain developed by Lynch and Poiley (Scanbur B&K Sollentuna, Sweden). In paper IV, we used the female inbred C57Bl/6J strain (Surwit et al. 1988) (Taconic, Skensved, Denmark). Kept on a high caloric (fat) diet this strain develops insulin resistance and glucose intolerance with compensatory hyperinsulinemia (Ahren et al. 1998).

For intravenous glucose tolerance tests (IVGTTs) (Paper IV), mice were fasted overnight and anesthetized with hypnorm (fluanisone 0.02 mg/kg and fentanyl 0.8 µg/kg) and midazolam (0.01 mg/kg). D-glucose (1 g/kg in 10 µl/g 0.9 % NaCl) was injected intravenously in a tail vein. Blood was collected from the retrobulbar intraorbital capillary plexus for determination of plasma glucose and insulin levels with Infinity Glucose Oxidase (Thermo Scientific, Sweden) and Insulin ELISA (Mercodia, Uppsala, Sweden). Glucose and insulin were displayed as incremental area under the curve (iAUC) and the acute insulin response (iAIR) was calculated with iAUC, 1 and 5 min. Insulinogenic Index (IGI), Insulin/Glucose. The glucose elimination constant (K_G) was calculated as the slope of the logarithmically transformed circulating glucose concentration between 5 min and 20 min after administration of glucose bolus (Pacini et al. 2009, Omar et al. 2012).

Human pancreatic islets

In paper II, isolated human pancreatic islets were used from non-diabetic males and females [glycosylated hemoglobin, 4.3–6.2; body mass index (BMI), 20.1–30.2 kg/m^2; age, 26–71 yrs; n = 19] and diabetic females (glycosylated hemoglobin, 6.8–7.0; BMI, 29.4–33.1 kg/m^2; age, 43–55 yrs; n = 7), while in paper III were n = 77 donors used and divided into 67 controls and 10 T2DM donors, the T2DM donors were clinically diagnosed with T2DM. The human islets were provided by the Nordic network for clinical islet transplantation (O. Korsgren, Uppsala University, Uppsala, Sweden). The human islets had been cultured at 37 °C (5 % CO_2) for 1–5 days before the experiments in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA) supplemented with 10 mM HEPES, 2 mM L-glutamine, 50 µg/ml gentamicin, 0.25 µg/ml fungizone (Life Technologies, Inc., BRL, Gaithersburg, MD, USA), 20 µg/ml ciprofloxacin (Bayer
Healthcare, Leverkusen, Germany), and 10 mM nicotinamide. The islets had 70–90% purity when they arrived; the islets were then handpicked under stereomicroscope before use. All procedures were approved by the ethical committees at Uppsala and Lund Universities.

**Measurement of pancreatic hormone release**

Mouse islets were isolated by a retrograde injection of ice-cold collagenase via the bile pancreatic duct (Gotoh et al. 1985) and hand-picked under stereo-microscope. Thereafter islets were pre-incubated in 2.8 mM glucose in Krebs Ringer solution and 0.1% Fatty acid free BSA for 1 h at 37 °C, before they were subjected to either high or low glucose in combination with secretagogues for an additional hour. In paper I and paper II, 12 islets/tube with 4-8 replicates for each condition were incubated with secretagogues in KRB and 0.1% Fatty acid free BSA in either 1 mM or 12 mM glucose. Aliquots were taken and the amount of insulin, glucagon and somatostatin were measured with radioimmunoassay (RIA) (Millipore, Malmö, Sweden). In paper III and IV we used either 3 mouse islets or 5 human islets/well with 6-8 replicates per condition. Incubations were performed in 2.8 or 16.7 mM glucose, with a combination of secretagogues and agonists for 5-HT receptors. Insulin concentrations were measured with Insulin ELISA (Mercodia, Uppsala, Sweden).

**Cell culturing and insulin secretion**

In paper III and IV we used INS-1 (832/13) cells which were cultured as previously described (Hohmeier et al. 2004). These cells are a clonally derived β-cell line originated from rat with transfected human insulin gene, and is used as a model to test β-cell function. The cells were cultured in RPMI1640 cell culture medium containing 11.1 mM glucose supplemented with 10% fetal bovine serum, (100 units/ml penicillin, 100 µg/ml streptomycin), 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol at 37 °C in 95% air and 5% CO₂. Insulin secretion was performed in 24-well plates where cells were seeded the day before. The cells were washed and pre-incubated during 2 h in secretion assay buffer (SAB), containing 2.8 mM glucose, 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 25.5 mM NaHCO₃, 20 mM HEPES, 2.5 mM CaCl₂, and 0.2% BSA. For 1 h incubation the cells were incubated in SAB with either 2.8 mM or 16.7 mM glucose with or without with secretagogues and 5-HT receptor agonists. Subsequently, cells were washed with PBS and homogenized. Protein concentration was measured to normalize the insulin secretion from the cells with bicinchoninic acid (BCA) kit according to manufacturer’s instructions (Pierce Biotechnology, Rockford, IL, USA). Secreted insulin was measured with Insulin ELISA (Mercodia, Uppsala, Sweden).
qPCR

qPCR was performed to determine the mRNA level of genes of interest in islets and cells. In paper I and II we used the TRIzol method (Invitrogen, Carlsbad, CA, USA) and in paper III and IV we used the mRNA easy kit (Qiagen, Valencia, CA, USA) to extract mRNA from islets and cells. mRNA concentrations were determined with NanoDrop (Nanodrop Technologies, Wilmington, DE, USA). Thereafter, cDNA was obtained by reverse transcription and qPCR was performed with either SYBR Green/ROX or Taqman Probe/ROX. An ABI PRISM 7900 (Applied Biosystems, Foster City, CA, USA) was used for quantification of mRNA. In paper I and II we employed the ddCt calculations (Pfaffl 2001) and glyceraldehyde 3-phosphate dehydrogenase, Gapdh as a reference gene, while in paper III and IV we used the $2^{(\text{minCt}-\text{Ct})}$ formula with applied error of propagation rule for normalization with the geometric mean of reference genes, cyclophilin A (Ppia), polymerase 2 (Polr2a), hypoxanthine guanine phosphoribosyl transferase (Hprt) (Applied Biosystems, Foster City, CA, USA).

Western blot

Western blot was used to detect proteins in extracts from 400 islets (mouse or human) or INS (832/13) and MIN6c4 cells. Proteins were extracted with; SDS-buffer (50 mM Tris-HCl and 1 mM EDTA) (paper I) or in paper II with PBS supplemented with phosphatase and protease inhibitors (Roche, Basel, Switzerland) and sonicated at the day of analysis, in paper IV INS (832/13) cells were lysed in urea buffer (9 M Urea, 100 mM HEPES, 1 % TritonX-100, 0.25 % IcoPAL-630, 0.25 % DDM, pH 7.2). Extracted proteins were loaded at equal concentrations on a 10 % SDS-polyacrylamide gel (paper I and II from Biorad, Hercules, CA, USA or in paper IV from Pierce Biotechnology, Rockford, IL, USA), at 100V for 1 h. Proteins were transferred with tank-blotting for 1 h, 40-100V, on to a membrane, either nitrocellulose (paper I) or Immobilion-FL polyvinylidene difluoride (PVDF) (paper II and IV) (Millipore, Billerca, MA, USA). The membranes were subsequently blocked with blocking buffer (Li-Core, Lincoln, NE, USA) and were either single- or dual-labeled with primary antibodies overnight; GPER-1 (Acromed Invest AB, Lund, Sweden) (paper I-II), pp-38, pSAPK/JNK pERK1/2, pAKT, pCREB, all in paper II (Cell signaling, USA), 5-HT$_{2A}$ (Becton Dickinsson, NJ, USA) and 5-HT$_{2B}$ (Santa Cruz Biotech, Dallas, TX, USA) (paper IV) and with either β-actin (paper IV) or α-tubulin (both from Sigma-Aldrich, Stockholm, Sweden) (paper I-II). On the second day the membranes were incubated with secondary antibodies; in paper I with horseradish peroxidase and in paper II and IV with far infra-red conjugated antibodies for either 680 nm or 800 nm wavelength. The membranes were evaluated on an Odyssey (Li-Core, Lincoln, NE, USA) and the protein density was related to endogenous controls; β-actin or α-tubulin.
**Immunohistochemistry**

To investigate the presence of a specific receptor/protein, we used confocal (paper I and II) and fluorescent (paper III) microscopy. For confocal microscopy, isolated islets were washed with PBS, fixed with 4 % formaldehyde, and permeabilized with 5 % Triton X-100, and unspecific sites were blocked with 5 % normal donkey serum (Jackson Immunoresearch Laboratories Inc., West Grove, PA). Whole islets were stained with primary antibodies and double labeled for insulin, glucagon and somatostatin, (guinea pig anti-insulin (Eurodiagnostica, Malmö, Sweden), guinea pig anti-glucagon and rat anti-somatostatin (Jackson Immunoresearch Laboratories, West Grove, PA, USA), followed by staining with fluorescent-conjugated secondary antibodies (1:150) from Jackson Immunoresearch. The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505–530 nm (Cy2), 543/ 570 (Cy3), and 633/ 650 nm (Cy5). The co-localization of the different hormones and GPER-1 was quantified pixel by pixel with Manders’ overlap coefficient; noise and cross talk were corrected, using Zen 2008 (Carl Zeiss, Oberkochen, Germany) software.

In paper III, paraffined human pancreas sections were labeled with antibodies detecting 5-HT_1A, 5-HT_1D, 5-HT_2B (Santa Cruz Biotech, Dallas, TX, USA) and 5-HT_2A (MBL International Corporation, Woburn, MA, USA), proinsulin and glucagon (Eurodiagnostica, Malmö, Sweden). Epi-fluorescence microscopy (Olympus, BX60) was used to detect the localization of 5-HT receptors in pancreatic islet cells. The localization was determined by double staining with secondary antibodies and by changing filters. Images were captured with a digital camera (Nikon DS-2Mv).

**Flow cytometry**

Evaluation of the number of apoptotic cells were performed with annexin-V conjugated antibody and propidium Iodide (PI) data acquisition was performed by flow cytometry. Annexin-V in cellular apoptosis binds to the exposed phosphatidylserine (PS) on the cell membrane while PI is an intercalating dye and binds to nucleic acids.

In paper I and II; isolated islets from mice and female donors were dispersed into single cells using Ca^{2+}-free medium. The cells were incubated during 24 h in RPMI 1640 medium with 5 mM glucose, supplemented with 10 % fetal bovine serum with and without apoptotic stimulants. In paper III and IV, INS (832/13) cells were cultured with and without apoptotic stimuli. Cells were detached with Accutase (Sigma-Aldrich, Stockholm, Sweden) and pooled with the culture media. Thereafter, cells were washed with ice cold PBS and stained according to manufacturer’s instructions with Annexin-V/488 and Propidium Iodide (PI) (Life Technologies Ltd, UK). The data is shown as PI-/Annexin V488+ % of total apoptotic events. Data acquisition was performed with FACSCalibur and CellQuest Pro (Becton Dickinson,
NJ, USA) compensated with single color compensation controls, and analyzed with FlowJo 7.6.5 (Tree star, CA, USA).
Present investigations

Paper I

Activation of G protein-coupled receptor 30 modulates hormone secretion and counteracts cytokine-induced apoptosis in pancreatic islets of female mice.

E2 modulates insulin secretion (Tesone et al. 1979, Godsland 2005) and estrogen receptors, ERα, and ERβ are previously described in β-cells (Tesone et al. 1979, Nadal et al. 1998). The GPER-1 receptor is documented in cancer cells (Filardo et al. 2000) and GPER-1 KO mice display perturbed glucose tolerance with abolished E2 potentiation of insulin secretion (Martensson et al. 2009). In this paper the objectives were to evaluate the role of GPER-1 in islet hormone secretion and to investigate possible anti-apoptotic effects of GPER-1 activation using the agonist G-1.

Gper1 was detected in male and female mouse islets and in MIN6c4 cells, with increased expression in islets from female mice. The classical estrogen receptors, Esr1 (ERα) and Esr2 (ERβ), were also expressed in both male and female mouse pancreatic islet with no differences in expression due to gender. The GPER-1 mRNA expression was also verified by western blot and GPER-1 displayed same pattern at the protein level.

To determine in which cell types GPER-1 were localized, female mouse islets were immunohistochemically stained for insulin, glucagon and somatostatin. GPER-1 was present in insulin, glucagon and somatostatin positive cells.

As GPER-1 was present in α-, β- and δ-cells, the GPER-1 agonist G-1 was evaluated along E2 for effects on islet hormone secretion using islets from female mice. Both E2 and G-1 potentiated glucose induced insulin secretion at 12 mM glucose. Moreover at 1 mM glucose both E2 and G-1 inhibited glucagon secretion while at stimulatory concentrations of glucose (12 mM) they inhibited somatostatin secretion. G-1 mimicked the E2 effect on pancreatic hormone secretion, except in the case of insulin secretion where G-1 displayed a 10 times lower potency than E2.

The potentiation of insulin secretion by G-1 (100 nM) and E2 (100 nM and 5 μM) was accompanied by an increased cAMP concentration in female mouse islets, which was sustained even in presence of the nER blockers; ICI 182,720 (100 nM) and EM-652 (100 nM).
Anti-apoptotic effect of E2 in absence of ERα has previously been reported (Le May et al. 2006). Consequently, G-1 (100 nM) and E2 (100 nM and 5 µM) was evaluated for their anti-apoptotic effects during a 24 h incubation in dispersed female mouse islet cells with a cocktail of pro-apoptotic cytokines (IL-1β, 100 ng/ml, TNFα, 125 ng/ml and IFNγ, 125 ng/ml). Incubation with G-1 (100 nM) and E2 (100 nM and 5 µM) decreased the number of apoptotic cells, even in the presence of nER blockers; ICI 182,720 (100 nM) and EM-652 (100 nM).

In conclusion, our results show that GPER-1 activation in mouse islets participates in regulation of islet hormone secretion via an increase of cAMP. Moreover, GPER-1 activation protects islet cells against inflammatory insults.
Men have higher prevalence to develop T2DM than premenopausal women and lower E2 levels increase the prevalence of T2DM in postmenopausal females (Godsland 2005). As shown in (paper I), GPER-1 is involved in the E2 response in female mice and here we further evaluated GPER-1 and G-1 in human pancreatic islets. In this paper, we investigated the expression of ESR1 (ERα), ESR2 (ERβ) and GPER1 in human islets from female and male subjects. ESR1 and GPER1 expression was higher in islets from female subjects than in islets from male subjects. Microarray from human islets displayed that GPER1 expression in female islets were positively correlated to BMI. In addition, visualized by confocal microscopy, GPER-1 was localized to α-, β- and δ-cells in female islets. Both G-1 and E2 potentiated glucose-induced insulin secretion at 12 mM glucose and inhibited glucagon secretion at 1 mM glucose as well as reduced somatostatin secretion at 12 mM glucose, in female human non-diabetic and diabetic islets.

G-1 (100 nM) and E2 (100 nM) induced cAMP production and PI-hydrolysis in a concentration-dependent pattern. The induction of cAMP was parallel with the potentiation of insulin secretion, even in the presence of nER blockers ICI 182,780 and EM-652. Inhibition of PLC and the formation of IP3 and DAG by RHC80267 resulted in only a slight reduction of the stimulatory effects on insulin release by G-1 and E2. However, inhibition of PKA with H89 decreased G-1 and E2 potentiation of insulin secretion, thereby concluding that the insulinotropic effects of G-1 and E2 is mediated via a cAMP and subsequent activation of PKA.

G-1 and E2 protected dispersed female pancreatic islets from apoptosis in a 24 h cytokine-induced (IL-1β, 100 ng/ml, TNFα, 125 ng/ml and IFNγ, 125 ng/ml) apoptosis experiment observed as reduced caspase-3 activity. This protective effect was counter-acted with H89, a PKA inhibitor. Moreover, we could show that the anti-apoptotic effect was mediated by a phosphorylation of CREB, ERK1/2 and AKT and by an inhibition of p-38 and JNK/STAT phosphorylation (Figure 9).

In conclusion, G-1 decreases the apoptotic rate of pancreatic islet cells and displays a potentiating effect on the glucose-induced insulin secretion which was mediated via the cAMP-PKA dependent pathway. Effects mediated by GPER-1 are beneficial for β-cells, but whether this effect is mediate solely by GPER-1 or not, remains to be determined.
Figure 9. **GPER-1 induced cell protective signals.** Mechanisms involved in β-cell survival via GPER-1 induced by G-1 and E2. For details see text.
Serotonin (5-HT) Receptors Modulate Insulin Secretion *In Vitro* in Human Islets of Langerhans.

5-HT affects β-cell function and the presence of 5-HT receptors are reported in rodent pancreatic islets (Kim et al. 2010), but not yet in human pancreatic islets. Thus we set out to evaluate the expression profile of 5-HT receptors in human islets of Langerhans as well as investigating effects of specific 5-HT receptors in regulation of insulin release.

In the present study, we observed the expression of 15 different 5-HT receptors in human islets of Langerhans, including the enzymes *TPH1*, *TPH2* and *DDC*. Expression of *Htr1a*, *Htr1d*, *Htr2a* and *Htr2b* was also confirmed in INS (832/13) cells. Moreover, the *HTR1D* expression was increased in islets from diabetic subjects compared to non-diabetic control subjects. We continued to investigate cellular localization for receptors using immunohistochemistry and found receptors 5-HT$_{1A}$, 5-HT$_{1D}$, 5-HT$_{2A}$ and 5-HT to be present in both α- and β-cells, while 5-HT$_{2B}$ only were present in β-cells. 5-HT were readily detected in INS-1(832/13) cells and rodent islets.

The amine 5-HT targets all receptors present in the pancreatic islet and INS-1 (832/13) cells. The human islet responded with a decrease in GSIS when challenged with 5-HT. We next stimulated islets with agonists for the following receptors; 5-HT$_{1A}$, 5-HT$_{1D}$, 5-HT$_{2A}$ and 5-HT$_{2B}$. Both buspirone (5-HT$_{1A}$) and PNU142633 (5-HT$_{1D}$) inhibited insulin secretion at 16.7 mM glucose while TCB-2 (5-HT$_{2A}$) and AMS (5-HT$_{2B}$) potentiated insulin secretion from human islets. A similar observation was made in INS (832/13) cells, where TCB-2 and AMS potentiated insulin secretion to a similar extent as carbachol.

The presence and activation of multiple 5-HT receptors could explain the broad actions of 5-HT on insulin secretion. The expression of 5-HT receptors on different islet cell types may explain differences in insulin release between primary and clonal β-cells. Expression levels of these receptors can change in pathophysiological conditions and may therefore be involved in the insulin secretory deficiencies observed in T2DM.
Paper IV

Agonists of the 5-HT₂-family affect insulin secretion and glucose tolerance in vivo and in vitro.

We observed that the agonist α-methyl 5-HT maleate salt (AMS) potentiates glucose stimulated insulin secretion from INS (832/13) cells and human islets of Langerhans (paper III). As AMS mediates, both hypo- and hyperglycemia in vivo (Chaouloff et al. 1990, Tudhope et al. 2012) we evaluated the long-term effects of AMS in vivo in an animal model for insulin resistance.

We tested two agonists for the 5-HT₂ family in vitro in INS (832/13) cells. Expression for the Htr2-family was verified in the cell line. Both Htr2a and Htr2b were present, thereby confirming our previous study (paper III) and others (Kim et al. 2010). TCB-2, an agonist for 5-HT₂A potentiated insulin release, and this rise in GSIS was counteracted with antagonist EMD281014 (5-HT₂A). Both AMS and TCB-2 were evaluated for long-term effects of INS (832/13) cells. Interestingly, AMS decreased the level of apoptosis during lipotoxicity, while TCB-2 increased the number of apoptotic cells during lipotoxicity and glucolipotoxicity.

In vivo AMS treatment of mice fed a high fat diet (HFD) decreased the insulinogenic index at fasting state compared to untreated HFD fed mice. AMS treated control fed animals were normoglycemic and maintained their insulin secretory response when challenged with a bolus injection of glucose during an IVGTT. However, in vitro experiments using islets from AMS treated mice fed a control diet displayed decreased insulin secretion at stimulatory concentrations of glucose (16.7 mM) and upon 1 h stimulation with AMS in vitro, compared to untreated control fed mice. Thus islets appear pre-programmed to release less insulin when treated with AMS while peripheral insulin sensitivity is increased.

Our results suggest that AMS and thus peripheral actions of 5-HT via the 5-HT₂-family may have positive effects on insulin resistance.
General discussion

In this thesis we show that estrogen (E2) and serotonin (5-HT) affect overall glucose homeostasis. E2 and 5-HT mediate their effects through a broad field of receptors and we have only begun to comprehend the complex regulatory pattern that these two hormones exert. Paper I and II in this thesis describe the effect of GPER-1 and G-1 on islets hormone secretion. Paper III and IV cover the involvement of 5-HT receptors and their agonists in islet function and insulin resistance.

In paper I and II, GPER1 was determined to be expressed in human and mouse pancreatic islets. This observation was later confirmed by Liu et al. and Sharma et al., as they detected GPER-1 with western blot in MIN6 cells (Liu et al. 2009, Sharma et al. 2011), in human and mouse islets (Liu et al. 2009), and by qPCR in male, female mouse islets and in INS-1 cells (Ropero et al. 2012).

As all estrogen receptors, ERα, ERβ and GPER-1, are available to mediate the E2 effect on pancreatic islets, we used the following pharmacological tools to determine the role of GPER-1 on cell viability and pancreatic hormone secretion; the GPER-1 agonist G-1 and nER antagonists, ICI 182,780 and EM-652. The agonist G-1 activates GPER-1 whereas G-1 is also reported to bind and activate the Gαq-ERα36 receptor, which mediates similar action as GPER-1 in cancer cells (Kang et al. 2010). However, no reports exist regarding the expression of Gαq-ERα36 receptor in pancreatic islets. Blocking nERs’ activity with ICI 182,760 is controversial as it has partial agonistic properties of GPER-1 and at lower concentration it may only block the genomic actions and not the non-genomic actions of the nERs and ERα36 (Chambliss et al. 2005). In addition to ICI 182,760, we used EM652, a more potent SERM than ICI 182,760 to block nERs’ activity (Tremblay et al. 1998).

The observed effect on insulin secretion by G-1 (paper I and II) is most likely related to GPER-1 and E2 as GPER-1 mice showed impaired insulin secretion upon stimulation with 5 μM E2 (Martensson et al. 2009). The G-1 effect on insulin secretion was later confirmed (Sharma et al. 2011) and by (Ropero et al. 2012). The latter showed only significant effect at 10 nM for both G-1 and E-2 at 12 mM glucose. Besides activating GPER-1, lower E2 concentration could be related to activation of ERβ as it activates KATP-channels in β-cells and E2 potentiates insulin secretion in the range to 100 pM to 50 nM (Ropero et al. 2012), while at higher concentrations E2 signals mainly via activation of GPER-1 (Ropero et al. 2012).

In paper II, E2 and G-1 induced two second messenger systems, cAMP and PLC in human islets. Dual stimulation of second messengers by GPER-1 was previously reported by (Filardo et al. 2002, Revankar et al. 2005). The dual
stimulation by G-1 and E2 could be mediated by GPER-1 as either the receptor could be promiscuous and induce two secondary messengers or the induction of PLC upon G-1 stimulation could be influenced by Gaq36 receptor.

In both paper I and II, G-1 mimics the protective effect of E2 against cytokine-induced apoptosis. The E2 effect is previously described in pancreatic islets but the E2 activated nERs are not fully responsible for mediating the various E2 anti-apoptotic effect; as it is partially reversed by the nER blocker ICI 182,780 (Contreras et al. 2002), E2 partly protects against apoptosis in ERα KO (Le May et al. 2006) and ERα/ERβ KO partly protects against apoptosis (Liu et al. 2009) and protected against H2O2-induced apoptosis via involvement of a non-genomic estrogen receptor (Liu et al. 2009). G-1 mimics the anti-apoptotic effects of E2 in pancreatic islets and these effects were not fully abolished in nER KO mice or in the presence of nER blockers, these findings point toward involvement of GPER-1 receptor.

Our result in paper I and II, and previous result from the GPER-1 mice, displaying impaired insulin secretion, the G-1 potentiating effect on insulin release and G15 blockage of E2 on MIN6 cells conclude that GPER-1 is involved in the effects previously observed in the islets of Langerhans.

In paper III the enzymes TPH1, TPH2 and DDC were expressed in human islets. These enzymes are associated with local 5-HT synthesis in the pancreatic islets (Paulmann et al. 2009, Kim et al. 2010, Schraenen et al. 2010, Ohta et al. 2011). In the same paper we confirmed the expression of 5-HT receptors in human islets, some are previously reported in mouse islets (Kim et al. 2010). However our result differ as we also detected HTR1A, HTR1F. Although, Paulman et al., managed to detect Htr1a in MIN6 cell (Paulmann et al. 2009). The observed increased expression of HTR1D in human T2DM islets may change the 5-HT receptors balance and the effect mediated by HTR1D is larger than during normal conditions. This is similar to the 5-HT1A which has an insulin decreasing effect in MIN6 cells (Paulmann et al. 2009). In addition, increased expression for HTR1D in islets from diabetic subjects may be related to a decline in β-cell mass as 5-HT1D reportedly normalize β-cell mass during the later stages of pregnancy and postpartum in rodents (Kim et al. 2010).

In our study, 5-HT stimulation of human pancreatic islets at 16.7 mM glucose inhibited GSIS which is in line with previous reports as 5-HT in vitro decreases GSIS in human pancreatic islets (Simpson et al. 2012) and in INS (832/13) cells (Heimes et al. 2009). 5-HT stimulates all the 5-HT receptors present, and to differentiate the effect among the general serotonergic stimulation, pancreatic islets were stimulated with buspirone, PNU142633, TCB-2 and AMS. Although we observed inhibitory actions of buspirone on insulin secretion from human islets, the in vitro response can differ from the in vivo response. Buspirone can be metabolized into 1-(2-pyrimidinyl)piperazine (1-PP), an α2-adrenoceptor antagonist, (Berlin et al. 1995) and can thus increase insulin secretion in vivo in rodents (Sugimoto et al. 2003) and decrease plasma glucose in humans (Ojha et al. 2006). In both human islets and INS (832/13) cells potentiated TCB-2 and AMS insulin secretion, an effect most likely
mediated via an increase in $[\text{Ca}^{2+}]_i$ via PLC as previously shown in N1E-115 cells (Niebert et al. 2011) and HIK (293) cells (Lin et al. 2011). Moreover, the antagonist SB204741 (5-HT$_{2B}$) induces glucose intolerance in pregnant mice (Kim et al. 2010), by direct inhibition of insulin secretion or inhibition of glucose uptake. This is in line with our observations in vitro in human islets and INS (832/13) cells.

The detrimental effect of TCB-2 in combination with glucolipotoxic conditions could be due to its ability to induce PLA$_2$ via AA which in the long run generates ceramides and induces apoptosis. (Lei et al. 2008) AMS on the other hand was not toxic for the INS (832/13) cells and as it is a peripheral acting agonist it is suitable for long-term treatment. Long-term treatment with AMS resulted in decreased insulin secretion from in vitro stimulated mouse islets with retained in vivo plasma glucose, thus implying improved insulin sensitivity. During the long-term treatment with AMS, neither the in vitro insulin secreting response, in paper III, nor the in vivo short-term induced hypo-/hyperglycemic effect by DOI, 5-HT and possibly AMS were apparent. Perhaps this in vivo effect is mediated by an increase of plasma adrenaline (Chaouloff et al. 1992, Yamada et al. 1995, Sugimoto et al. 1996). The different outcome could be explained by the long-term treatment with AMS which could affect the target receptors via tachyphylaxis by downregulation, desensitization or by GIPs modulating the G-protein signals. Another possibility is that treatment with AMS in vivo has either a direct effect on the pancreatic islets or mediates an effect on peripheral tissues, such as glucose uptake (Hajduch et al. 1999).

5-HT and E2 affect each other’s pathways and activity, and can mediate a combined effect. High E2 in neurons can increase $Htr2a$ expression (Sumner et al. 1998) decrease $Htr1a$ (Birzniece et al. 2001) and $Htr1b$ expression (Hiroi et al. 2009). E2 is also reported to increase $Tph2$ expression in neurons, thus increasing 5-HT synthesis (Hiroi et al. 2006). As $Tph2$ is expressed in pancreatic islets (Schraenen et al. 2010), E2 could potentially increase 5-HT synthesis in pancreatic islets. In addition, E2 positively affects prolactin production (Ismail et al. 1998) via an inhibition of dopamine which in turn inhibits prolactin production (Ben-Jonathan et al. 2001). Thus, both E2 and 5-HT could interact to regulate both β-cell mass and function during pregnancy.
Major conclusions and future perspectives

Pancreatic islets and insulin secretion are tightly regulated by circulating factors, hormones and innervated by neurons signaling via GPCRs. We studied two GPCR families GPER-1 and 5-HT receptors and their effect on pancreatic islets. The work in this thesis generated the following conclusions:

- G-1 mimics the E2 effect on pancreatic islets by potentiating insulin secretion and protecting against cytokine induced apoptosis, via ERK1/2, AKT and CREB in both rodent and human islets.
- 15 different 5-HT receptors are expressed in human islets and 5-HT1A, 5-HT1D, 5-HT2A and 5-HT2B are localized in β-cells. 5-HT1 receptors negatively regulate insulin release, while 5-HT2 receptors potentiate glucose stimulated insulin secretion (GSIS).
- TCB-2 and AMS both have insulinotropic effect in clonal β-cells and TCB-2 induces apoptosis in clonal β-cells during glucolipotoxic conditions while AMS has anti-apoptotic properties as well as beneficial effects on insulin resistance in vivo.

Further studies of GPER-1 and the estrogen receptors are needed to determine their role on β-cell function. Especially the ER subtype α36, if it is present and involved in the estrogenic response in β-cells. ERα36 has been studied in cancer cells where Kang et al. observed that knockdown or KO of ERα36 also decreased GPER-1 expression (Kang et al. 2010). (Kang et al. 2010), hypothesized that the expression of GPER-1 and ERα36 are connected to each other. This connection will make it hard to determine the GPER-1 and ERα36 differences but evaluation will perhaps be possible with the new available antagonists for GPER-1. These antagonists could also be evaluated in animal models of diabetes to investigate their effects on insulin resistance, glucose tolerance and β-cell function.

Regarding the studies on 5-HT, we will continue to investigate clearance rate of AMS and the duration of action in chronic studies in vivo. In addition, the allocation of injected AMS would be interesting to assess, for instance, which tissue has the highest AMS activity or concentration? Labeled compound may shed some
light in this issue. This could be accompanied with 5-HT receptor expression profile in relevant tissues for glucose homeostasis; liver, skeletal muscles and adipose tissue.

As AMS also targets the 5-HT_{2A} receptor, the peripheral 5-HT_{2A} agonist, the AL-34662 (Sharif et al. 2007), could verify the findings in paper IV and clarify which receptor is more dominant in mediating the *in vivo* effects of AMS on glucose homeostasis and insulin resistance. Further *in vivo* studies of AMS will be continued in a spontaneous diabetic model, Goto-Kakizaki model, where both β-cell function and insulin resistance will be assessed with intravenous glucose tolerance tests and hyperinsulinemic euglycemic clamps, as well as investigate changes in blood pressure as targeted receptors may affect this parameter.

In addition, as 5-HT_{2B} displays pro-proliferative properties during pregnancy (Kim et al. 2010). Proliferative capabilities of 5-HT_{2B} and AMS can be further evaluated in this context and in normal β-cells/islets.

The increase in circulating 5-HT during high fat diet (Kim et al. 2011) is also of high interest. What role does this amine play in compensatory hyperinsulinemia? What tissue does the amine originate from? Does it exert protective effects on β-cells?

Further studies on 5-HT effects on human β-cell function are needed as the range of 5-HT receptors most likely varies in different islet cells, and each islet cell may express different sets of 5-HT receptors. With new pharmacological tools it will be possible to distinguish between the 5-HT effects as it both induces insulin secretion and inhibits as well increases and decreases proliferation. The 5-HT_{3} is also an interesting target to further investigate as the 5-HT_{3} receptor, when assembled, is permeable to sodium, potassium, and calcium ions, and can affect the membrane potential.

The interaction between E-2, G-1 and 5-HT receptors and transporters need to be clarified as E2 increases *Slc6a4, Htr1a* and *Htr2a* expression in rat brain and GPER-1 colocalizes with 5-HT_{1A} in rat hypothalamus (Xu et al. 2009). In addition, the regulation of 5-HT and its receptors may influence the outcome of gestational diabetes. If the beneficial aspects of either E2 and 5-HT or agonists of G-1 and peripheral 5-HT_{2} can be combined as previously shown with E2 and GLP-1 (Dietrich et al. 2012). E2 and 5-HT might increase the central activity such as satiety where G-1 and peripheral 5-HT agonists can be combined to boost the peripheral effects to become a potential anti-diabetic drug.

I vår forskning visar vi att östrogen har en skyddande effekt på de insulinproducerande cellerna samt att insulinproduktionen ökar. Östrogen, som framförallt är ett kvinnligt hormon, finns också i lägre koncentrationer hos män. Östrogen fungerar som ett signalhormon i kroppen och aktiverar sina mottagare (receptorer). I kroppens celler finns det två typer av östrogenreceptorer, de som verkar inuti cellkärnan och de som finns i cellens membran. Vi har i möss studerat den membranbundna, ”yttre” receptorn, som kallas GPER-1. När denna receptor signalerar ser vi positiva effekter av östrogen. Här har vi använt en specifik substans, G-1, som endast aktiverar GPER-1.

Vi kan konstatera att östrogenreceptorerna finns i de hormonproducerande cellerna i bukspottskörteln. G-1:s effekt följer östrogenets effekt på bukspottskörteceller från både möss och människor. Insulinproduktionen ökade och glukagonproduktionen minskade. Vid behandling med G-1 och östrogen, överlevde tre gånger så många celler då vi utsatte dem för ämnen som är involverade i inflammation.

Vi har även studerat serotoninets (5-HT) effekt på bukspottskörtecellerna. Serotonin har man tidigare studerat som en signalsubstans i nervbanor, där serotonin fungerar som stämningsreglerare och finns cirkulerande i blodomloppet där det kan påverka bland annat blodtrycket. Serotonin signalerar genom flera olika sorters serotoninreceptorer. Vi har funnit femton olika receptorer för 5-HT i bukspottskörtelns hormonproducerande celler från människa och att dessa olika receptorer kan antingen påverka frisättningen positivt eller negativt. Vidare visar vi också att ett specifikt läkemedel som aktiverar en 5-HT₂B receptor (AMS) har en positiv effekt på insulinutsöndring, samt att det kan motverka något som kallas för insulinresistens.
Insulinresistens gör att kroppens celler har svårt att ta upp socker, vilket bidrar till ökade blodsocker nivåer och slutligen typ 2 diabetes.

Resultaten i denna avhandling bidrar till att öka den allmänna kunskapen om östrogen och hur förändrade östrogennivåer kan leda till diabetes, till exempel vid graviditet och efter övergångsåldern. Vår forskning bidrar även till ökad kunskap om vilka receptorer för 5-HT som är involverade i reglering av insulinfrisättning och att stämningsreglerande läkemedel, till exempel antidepressiva läkemedel, som primärt verkar genom att öka mängden serotonin i hjärnan, kan ha oönskade effekter på energiomsättningen och ge upphov till diabetes och övervikt. Vi visar också att systemet för 5-HT i bukspottskörteln kan ha positiva effekter beroende på vilka receptorer man väljer att aktivera. Systemen för östrogen och 5-HT är komplexa, men vidare studier av dessa system kan bidra till utvecklingen av nya, mer effektiva läkemedel för typ 2 diabetes.
Acknowledgements

First of all, I like to thank my supervisor Dr. Malin Fex for her great support and supervision, and the positive view you mediate on the ‘roller coaster’ of science. I truly enjoyed learning from you and working with you. You are an ambitious and hardworking scientist - keep up the good work and you will go very far. Furthermore, I appreciate that whenever I popped into your office, you always took the time to answer my questions.

My co-supervisor Professor Leif Groop, thanks for your input and comments on the projects.

Rajesh, my great friend and colleague during my PhD. It’s an honor to have you as my friend. I really appreciate the time we worked together and your comments were always valuable. Your enthusiasm and knowledge about medicine are boundless and I believe that you have a promising future ahead of you. Thanks for your feedback on my thesis.

Hedvig, I really appreciated your friendship and your friendly collaboration. It was always nice to discuss our projects and the care take of our precious and hardworking cells. Thanks for your feedback on my thesis.

Sabina, I will never forget your support; enjoy your stay in the USA.

Zeliha, Caroline, Ida and Rasmus, thanks for the good times and your company during coffee and lunch breaks. Ida, thanks for your feedback on my thesis.

Norio and Hanna, my office mates, thanks for thescientific-, printer- and ventilation-discussions. Norio - Good luck in Japan.

Professor Åke Lernmark and members of the unit of diabetes and celiac disease, thanks for all the help received, and Linda thanks for helping me with the massive amount of insulin ELISAs.

I like to thank present and former colleagues and friends at CRC, especially I like to thank:
Yang, Pawel, Monika, Jonathan, Vini, Thomas, Britt-Marie, Vishal, Professor Lena Eliasson, Professor Erik Renström, Anna, Anna-Maria, Enming, Taman, Sarheed, Arvind, Mia, Liliya, Ann-Helen and Nils.

Friends, my siblings with their families and my family-in-law, thank you for reminding me of the world outside the academic walls, always great time to meet you.

Johan, Tomas, Kim, Kalle, Anna, Ola, Annika, Sara and Jeanette.

Donald and Carolien; Ragnhild, Jörgen, Sophia, Maria, Julia and Simon; Marianne, Peter, Sanna, Pontus and Oscar; Andreas, Lisa and Lukas; Anna, Stefan, Moa, Ella and Jakob.

Per-Erik, Yvonne, Eric, Mikaela, Thea and Johan. Thea, thanks for helping me with the invitation cards.

Mom and dad, thank you for always believing in me and in my pursuit for PhD.

Sandra, my wonderful wife, thank you for your endless support and infinite love.
References


novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain." Journal
of Biological Chemistry 277(25): 22115-22118.
"Involvement of Estrogen Receptor Variant ER-[alpha]36, Not GPR30, in Nongenomic
"Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic
Kim, H., Y. Toyofuku, F. C. Lynn, E. Chak, T. Uchida, H. Mizukami, Y. Fujitani, R.
Kawamori, T. Miyatsuka, Y. Kosaka, K. Yang, G. Honig, M. van der Hart, N.
Kishimoto, J. Wang, S. Yagihashi, L. H. Tecott, H. Watada and M. S. German (2010).
"Serotonin regulates pancreatic beta cell mass during pregnancy." Nat Med 16(7): 804-
808.
and serum from high-fat diet induced obese mice." J Proteome Res 10(2): 722-731.
Insulinotropic Polypeptide-Mediated Up-Regulation of β-Cell Antiapoptotic Bcl-2 Gene
Expression Is Coordinated by Cyclic AMP (cAMP) Response Element Binding Protein
(CREB) and cAMP-Responsive CREB Coactivator 2." Molecular and Cellular Biology
Constitutively Active Akt Ser/Thr Kinase in 3T3-L1 Adipocytes Stimulates Glucose
Uptake and Glucose Transporter 4 Translocation." Journal of Biological Chemistry
271(49): 31372-31378.
variants: constitutive activity and inverse agonist effects." Br J Pharmacol 135(6): 1563-
1571.
"Cloning of a novel receptor expressed in rat prostate and ovary." Proc Natl Acad Sci U S
A 93(12): 5925-5930.
Labrie, F., C. Labrie, A. Belanger, J. Simard, S. Gauthier, V. Luu-The, Y. Merand, V.
Giguere, B. Candas, S. Luo, C. Martel, S. M. Singh, M. Fournier, A. Coquet, V.
Richard, R. Charbonneau, G. Charpenet, A. Tremblay, G. Tremblay, L. Cusan and R.
Veilleux (1999). "EM-652 (SCH 57068), a third generation SERM acting as pure
antiestrogen in the mammary gland and endometrium." J Steroid Biochem Mol Biol
69(1-6): 51-84.
Langer, G., B. Bader, L. Meoli, J. Isensee, M. Delbeck, P. R. Noppping and C. Otto


Phosphatidylinositol-3,4,5-trisphosphate in the Activation of Protein Kinase B. " *Science* 277(5325): 567-570.


Surwit, R. S., C. M. Kuhn, C. Cochrane, J. A. McCubbin and M. N. Feinglos (1988). "Diet-Induced Type II Diabetes in C57BL/6J Mice." *Diabetes* 37(9): 1163-1167.


