DOCTORAL THESIS

The roles of somatostatin and ghrelin in pancreatic islet and beta cell function in type 2 diabetes

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The roles of somatostatin and ghrelin in pancreatic islet and beta cell function in type 2 diabetes

by

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Department of Life Sciences

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To my loving parents

&

In loving memory of Mrs P.W. Fernando -

grandma, may you obtain the supreme

bliss of nibbana!

ABSTRACT

Beta cell dysfunction and loss play central roles in the pathogenesis of type 2 diabetes. Strategies to overcome the detrimental effects of cellular stress factors is fundamental for the protection of beta cell mass and function. In this study two hormones, somatostatin (SST) and ghrelin, were assessed for their roles in regulating 1) beta cell and/or islet survival in response to cellular stressors typical of type 2 diabetes and 2) acute insulin release. In addition, the mechanisms underlying their actions were explored and it was tested whether any effects of ghrelin on beta cells was mediated via somatostatin receptor 3 (SSTR3) to identify a potential interaction between ghrelin and SST in islet function.

The rodent pancreatic beta cell lines MIN6 and INS-1 and primary pancreatic mouse islets were used as research models in this study. Cell viability was assessed by detecting metabolic activity of viable cells and apoptosis by measurement of caspase 3/7 activity. Static incubation experiments and radioimmunoassays were performed to detect hormone secretion. mRNA expression of genes of interest was detected by quantitative PCR (qPCR) and protein expression by Western blot.

Treatment with SST reduced apoptosis in MIN6 cells in response to lipotoxicity. SSTR3 was detected in beta cells and the receptor partly mediated an inhibitory effect of SST on insulin secretion, but not the observed protective effect of SST on MIN6 cell survival. Incubation with ghrelin did not modify the clonal beta cell response to apoptosis, despite the ghrelin receptor (GHSR1a) being expressed in these cells. In contrast, treatment with ghrelin reduced apoptosis in islets from female mice in a sex-dependent manner, but this effect

did not appear to be mediated by the ghrelin receptor, GHSR1a. Antagonising GHSR1a by liver-expressed antimicrobial peptide 2 resulted in a sex-specific increase in insulin secretion in islets from male mice. Finally, SSTR3 did not appear to mediate any potential effects of ghrelin on islet insulin secretion.

In conclusion, the results presented in this thesis suggest that both SST and ghrelin play important roles in protecting the pancreatic beta cells and/or islets against cellular stresses leading to cell death. The underlying mechanisms are complex and may rely on direct interaction between SST and ghrelin. In addition, a novel, sex-dimorphic role for ghrelin in islet function was identified, that highlights a potential need to develop sex-specific treatments for type 2 diabetes.

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AUTHOR'S DECLARATION

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- Hewawasam, N.V., Patterson, M., Reeves, S. and Hauge-Evans, A.C. (2018). Characterising the role of ghrelin and the growth hormone secretagogue receptor Type 1a (GHSR1a) antagonist, YIL781, in the regulation of beta cell survival. *Diabetic Medicine*, 35 (suppl. 1), pp. 50. Poster presented: Diabetes UK professional conference 2018, London, UK.
- Damsteegt, E.L., Hassan, Z., Hewawasam, N.V., Sarnsamak, K., Jones, P.M., and Hauge-Evans, A.C. (2019). A Novel Role for Somatostatin in the Survival of Mouse Pancreatic Beta Cells. *Cellular Physiology and Biochemistry*, 52(3), pp.486-502.
- Hewawasam, N.V., Austin, A.L., King, A., Patterson, M., Reeves, S. and Hauge-Evans, A.C. (2019). Exploring gender differences in islet survival in response to ghrelin treatment. *Diabetic Medicine*, 36 (suppl. 1), pp. 40 . Poster presented: Diabetes UK professional conference 2019, Liverpool, UK.
- Hewawasam, N.V., Austin, A.L., King, A., Patterson, M., Reeves, S. and Hauge-Evans, A.C. (2019). Gender differences in the effect of ghrelin on pancreatic islet survival. Poster presented: European Association for the Study of Diabetes 55th annual meeting 2019, Barcelona, Spain.
- Hewawasam, N.V., Austin, A.L., King, A., Patterson, M., Reeves, S. and Hauge-Evans, A.C. (2019). Gender differences in the effect of ghrelin on pancreatic islet survival. Poster presented: Future Physiology 2019, Liverpool, UK.
- Hewawasam, N.V., Austin, A.L., King, A., Patterson, M., Reeves, S. and Hauge-Evans, A.C. (2020). Pancreatic islets display sexual dimorphism in insulin secretion in response to liver-expressed antimicrobial peptide-2. Poster abstract (online): Diabetes UK Professional Conference 2020, Glasgow, UK.

LIST OF ABBREVIATIONS

2-ME - 2-mercaptoethanol

Ab - Antibody

Acyl-CoA - Acyl-Coenzyme A

ADP - Adenosine diphosphate

Ag - Unlabelled antigen

Ag* - Radiolabelled antigen

Akt/PKB _ Protein kinase B

ANOVA - Analysis of variance

APS - Ammonium persulfate

ATF 4 - Activating factor 4

ATF6 - Activating factor 6

ATP - Adenosine triphosphate

Bip - Heat shock protein binding immunoglobulin protein

BSA - Bovine serum albumin

C peptide - Connecting peptide

cDNA - complementary DNA

CAM - Cell adhesion molecules

cAMP - 3',5'-cyclic adenosine monophosphate

Caspase - Cysteine aspartic acid-specific protease

cGMP - cyclic guanine monophosphate

CHOP - CCAAT-enhancer-binding protein homologous protein

CRE - cAMP Response Element

Ct value – Cycle threshold value

CX36 - Connexin 36

DAG - Diacylglycerol

MCF7 - Michigan Cancer Foundation-7

Ddit3 - DNA damage-inducible transcript 3

DHAP - Dihydroxyacetone phosphate

DMEM - Dulbecco's Modified Eagle's Medium

DNA - Deoxyribonucleic acid

E2 - Oestradiol

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-linked immunosorbent assay

Epac - Exchange protein directly Activated by cAMP

ER - Endoplasmic Reticulum

ERα or ESR1- Oestrogen receptor alpha

ERβ or ESR2 - Oestrogen receptor beta

ERK 1/2 - Extracellular Signal-Regulated Kinases 1/2

FBS - Foetal bovine serum

FFA - free fatty acids

FSK - Forskolin

GABA - Gamma aminobutyric acid

G&G buffer - Gey and Gey buffer

GH - Growth hormone

GOAT - Ghrelin-*O*-acyltransferase

GHRH- Growth Hormone Releasing Hormone

GHSR1a - Growth hormone secretagogue receptor type 1A

GHSR1b - Growth hormone secretagogue receptor type 1b

GI tract - Gastro-intestinal tract

Gly-3P - Glycerol-3-phosphate

GLP-1 - Glucagon-like peptide 1

GLUT - Glucose transporter

GSIS - Glucose-stimulated insulin secretion

GPCR - G-protein coupled receptors

iGluRs - Ionotropic glutamate receptors

IL6 - Interleukin 6

H&E - Haematoxylin and eosin

HbA1c - Glycated haemoglobin A1c

HEK293 - Human embryonic kidney 293

Hhex - Haematopoietically expressed homeobox

HIT-T15 - Hamster islet transformed-tioguanine resistant clone-15

HLA - Human leukocyte antigen

HPRT - Hypoxanthine phosphoribosyltransferase

hTERT - Human telomerase reverse transcriptase

HUVEC - Human umbilical vein endothelial cells

IBMX - 3-isobutyl-1-methylxanthine

IDF - International Diabetes Federation

IFN-γ- Interferon gamma

IL-1β - Interleukin-1 β

IP3 - Inositol 1,4,5 triphosphate

IR - Insulin receptor

IRE1α - Inositol-Requiring Enzyme-1 alpha

IRS-1 - Insulin receptor substrate 1

INS-1 - Insulinoma 1

JNK - c-Jun N-terminal kinase

K_{ATP} channel - Voltage-gated ATP-sensitive potassium ion channels

KCL - King's College London

KCNJ11- Potassium channel inwardly rectifying subfamily J member 11

LEAP2 - Liver-expressed antimicrobial peptide 2

LN2 - Liquid nitrogen

MB - Maximum binding

MEM - Minimal Essential Medium

MIN6 - Mouse insulinoma 6

MODY - Monogenic diabetes of the young

mRNA - messenger RNA

MTT - ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

NCS - New-born calf serum

NSB - Non-specific binding

NFκB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NO - Nitric oxide

NPY - Neuropeptide Y

OGTT - Oral glucose tolerance tests

OVX - Ovariectomised

PBS - Phosphate buffered saline

PC - Prohormone convertases

PEG - Polyethylene glycol

PERK - Protein kinase-R-like ER kinase

PGE1 - Prostaglandin E1

PI3K- Phosphoinositide-3 kinase

PLC - Phospholipase C

PKA - Protein kinase A

PKC - protein kinase C

Pnx - Pannexin

PP cells - Pancreatic polypeptide releasing cells

PPARγ - Peroxisome proliferator-activated receptor-gamma

PPIA - Peptidylprolyl isomerase A

PTX - Pertussis toxin

qPCR -quantitative polymerase chain reaction

RER - Rough endoplasmic reticulum

RIA - Radioimmunoassay

RIPA buffer - Radioimmunoassay precipitation buffer

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RNA - Ribonucleic acid

RPMI 1640 - Roswell Park Memorial Institute 1640

RT - Room temperature

SAP - Severe acute pancreatitis

SDS-PAGE - Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

SERCA - Sarco-endoplasmic reticulum Ca^{2+}-ATPase

SGLT2 - Sodium-glucose co-transporter-2

siRNA - Small interfering RNA

SNAP - Soluble N-ethylmaleimide-sensitive factor attachment protein

SNARE - Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SRE - Serum response element

SRIF - Somatotropin-releasing factor

SST - Somatostatin

SSTR - Somatostatin receptor

STZ - Streptozotocin

SV40 T antigen - Simian virus 40 T antigen

T - Tracer

TBS - Tris buffered saline

TCA - Tricarboxylic acid cycle

TEMED - *N*,*N*,*N′*,*N′*-Tetramethylethylenediamine

Tg - Thapsigargin

TNF-α - Tumour necrosis factor alpha

TSH - Thyroid stimulating hormone

TTBS - Tween tris buffered saline

UAG - Unacylated ghrelin

UPR - Unfolded protein response

VEGF-A - Vascular endothelial growth factor A

wt - Wildtype

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CHAPTER 1 - General introduction

1.1 Pancreatic islets of Langerhans

1.1.1 The structure and function of islets

Pancreatic islets, initially described by Paul Langerhans in 1869, are small spherical organs that constitute the endocrine section of the pancreas (Arrojo e Drigo *et al.*, 2015; Lefèbvre, 2011) (Fig. 1.1). They correspond to 2% of the entire pancreatic tissue with the remainder being exocrine tissue. The number of islets in a healthy human pancreas has been estimated to be approximately 1-14.8 million (Dolenšek *et al.*, 2015; Lefèbvre, 2011) whereas about 1000-5000 are found in a rodent pancreas (Dolenšek *et al.*, 2015; Lefebvre *et al.*, 2010) indicating that islet number is proportional to the size of the organism and metabolic requirements (Steiner *et al.*, 2010). However in all species islet diameter varies between 100-700 μm (Dolenšek *et al.*, 2015; Ionescu-Tirgoviste *et al.*, 2015).

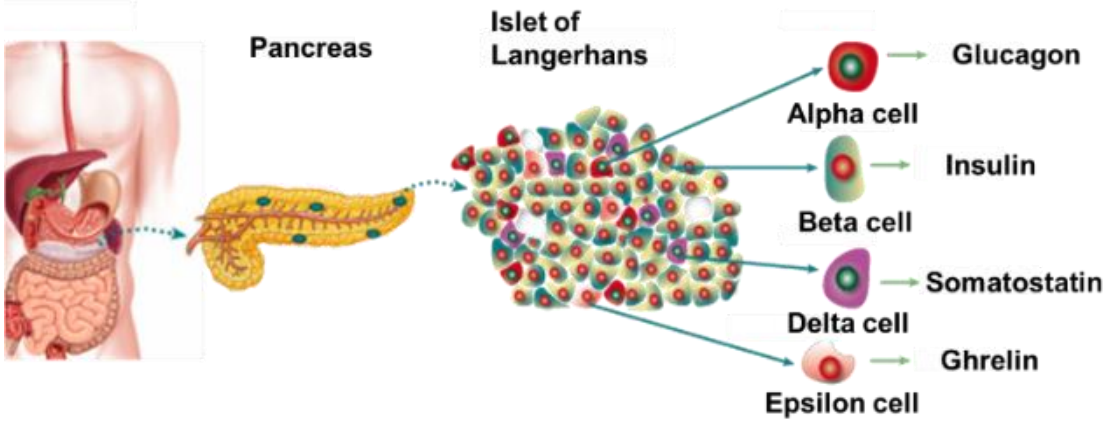

Figure 1.1 Pancreatic islets of Langerhans. Islets of Langerhans are spherical organs found within the pancreas that are clusters of alpha, beta, delta, epsilon and pancreatic-polypeptide (PP) cells. Comprised of about 1000-2000 cells, their tight coordination is important for correct function to maintain glucose homeostasis (Biocrine 2012; Lefèbvre, 2011).

Islets are composed of five endocrine cell types: alpha, beta, delta, epsilon and pancreatic polypeptide releasing cells (PP cells) as well as immune cells, vasculature and innervations (Arrojo e Drigo *et al.*, 2015) (Fig. 1.1). Alpha cells are the glucagon producing cells of the pancreas and constitute about 15-20% of rodent islet cells (Steiner *et al.*, 2010). They are found in close proximity to beta cells and capillaries (Brereton *et al.*, 2015). Beta cells produce insulin and correspond to about 60-80% of the rodent islet cells (Steiner *et al.*, 2010). Delta cells are neurone-like elongated cells that secrete somatostatin (SST) and are found towards the outer layer of islets in rodents (Cabrera *et al.*, 2006). They constitute approximately 5-10% of the rodent islet cell population. Their dendrite-like protrusions extend towards the centre of the islet and allow paracrine communication with other islet cell types (Brereton *et al.*, 2015). The remaining epsilon cells and PP cells correspond to less than 1% of rodent islet cells. Epsilon cells are a more recently identified cell type that produce ghrelin (Wierup *et al.*, 2002). Despite the presence of the same types of cells, human islets contain slightly different proportions of the three main cells types: ~50% beta cells, ~40% alpha cells and ~10% delta cells (Steiner *et al.*, 2010). In addition, numerous studies have highlighted the differences in islet architecture between species. In rodents, beta cells form the central core of islets and non-beta cells form the periphery (Cabrera *et al.*, 2006). In islets of larger species, non-human primates and human islets, the different cell types are more scattered throughout the islet (Fig. 1.2) (Cabrera *et al.*, 2006). Even in these islets beta cells have been shown to form small clusters highlighting the importance of cell-to-cell contact for their function (Bonner-Weir *et al.*, 2015).

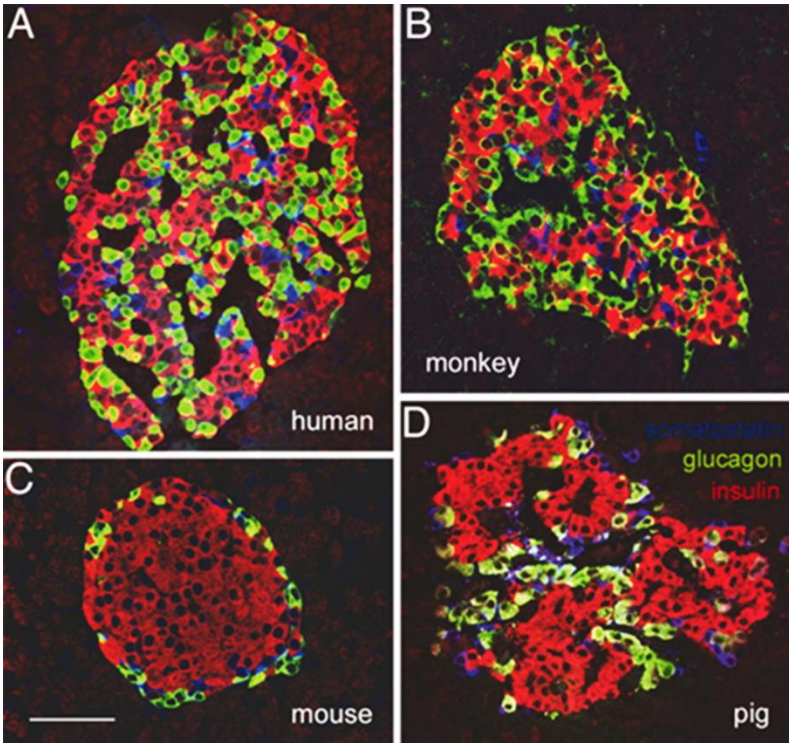

Figure 1.2 Species-specific differences in pancreatic islet structure. Pancreatic sections labelled by immunofluorescence for insulin (red), glucagon (green) and somatostatin (blue). Beta cells are randomly distributed in the islets of human (A) monkey (B) and pig (D) pancreata whereas in mice (A) beta cells constitute the central core. Scale bar 50 μm. (Cabrera *et al.*, 2008:2235).

Islets are highly vascularised organs (Fig. 1.3) which is important for adequate supply of oxygen and other stimuli including nutrients (In't Veld and Smeets, 2014). They consist of large and fenestrated capillaries that develop during embryogenesis stimulated by vascular endothelial growth factor A (VEGF-A) (Arrojo e Drigo *et al.*, 2015). Despite only constituting about 2% of pancreatic mass, islets receive approximately 10% of pancreatic blood flow that is further elevated to 15% with hyperglycaemia (Janssons and Hellerström, 1983).

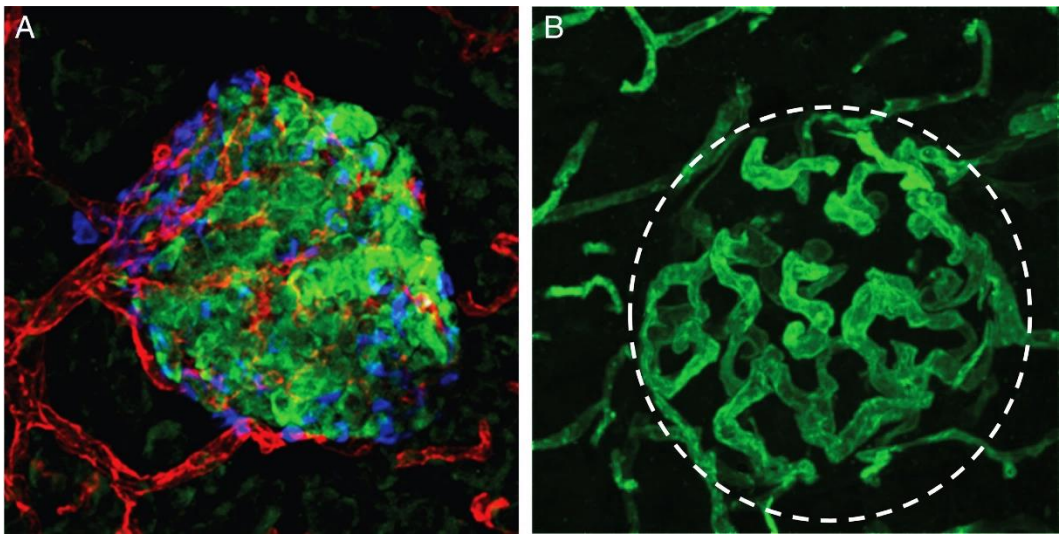

Figure 1.3 Mouse islet vasculature. Mouse pancreatic islet immunolabelled for insulin (green), glucagon (blue) and endothelial cell marker, CD31 (red) (A). Mouse islet from an animal infused with fluorescein isothiocyanate-conjugated tomato lectin (green) to label the functional vasculature (B). Islet capillaries (within dashed line) are thicker, denser and more tortuous than vessels in the surrounding exocrine tissue (Aamodt and Powers, 2017:127).

Three patterns of blood flow have been described in mouse islets: outer to inner, inner to outer and top to bottom (i.e. a polarised pattern). The outer to inner model suggests that blood flows from outer cells into the inner cells therefore suggesting that peripheral alpha and delta cell secretions influence beta cell activity (Fujita and Murakami, 1973). In the inner to outer model beta cells are first perfused followed by non-beta cells suggesting that insulin secreted by beta cells affect the alpha and delta cell function (Bonner-Weir and Orci, 1982; Samols *et al.*, 1988). Finally, the top to bottom pattern implies that islet blood flows from one pole to the other of the islet regardless of the cell types (Liu *et al.*, 1993). More recent *in vivo* imaging showed 60% of blood flow

to be inner to outer and 35% being polarised (Nyman *et al.*, 2008). In contrast, blood flow directionality in human islets remains largely unknown (Jansson *et al.*, 2016). Some have reported that in human islets cellular interactions may not depend on islet blood flow, since different islet cell types are randomly associated with blood vessels (Cabrera *et al.* 2006). This suggestion was confirmed by Cohrs *et al.* (2017) who reported that alpha and beta cell paracrine signalling was not influenced by islet blood flow or size in their study. However, neither of these studies employed direct measurement of blood flow, instead relied on the distribution patterns of islet cells. Others, using non-human primate islets have found that islet blood flow is dynamic and results in rapid changes in directionality due to metabolic demands (Diez *et al.*, 2017). Taken together these studies indicate that rodent islets display distinctive directionality in islet blood flow that is yet to be confirmed in human islets. However, the differences in cytoarchitecture between rodent and human islets imply that the islet blood flow pattern in human islets may differ from that in rodents.

In addition to interactions via microcirculation, islets cells communicate via interstitial space by autocrine and paracrine secretions, as well as through direct cell-to-cell contact, for example via gap junctions. These interactions occur between the same types of cells as well as between different cell types in the islets (reviewed by Arrojo e Drigo *et al.*, 2015; reviewed by Koh *et al.*, 2012; reviewed by Meda, 2013) (Fig. 1.4).

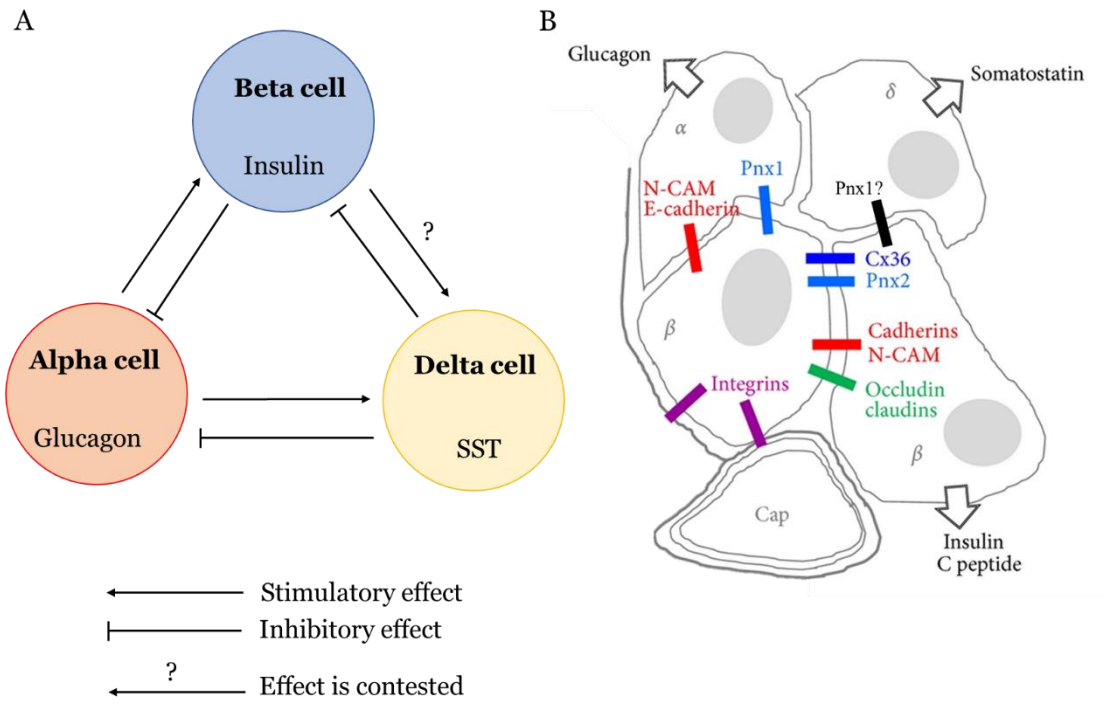

Figure 1.4 Paracrine and direct cell-cell interactions in mammalian islets of Langerhans. The primary islet hormones and their paracrine signalling (A). Islets cells also communicate via direct cell-cell contact, e.g.: via gap junction proteins such as connexin 36 (Cx36) and pannexin 1 (Pnx1) (B) (modified from Meda, 2013:7). The tight coordination between pancreatic islet cells is fundamental for their correct function in order to maintain normoglycaemia. Cadherins and N-CAM are cell adhesion molecules, integrins allow adhesion to extracellular matrix and occludin and claudins form tight junctions.

Paracrine and autocrine signalling between islet cells

As mentioned above paracrine and autocrine interactions can modulate the function of different islet cell types. These interactions occur via secreted hormones, neurotransmitters and ions.

Insulin receptors (IRs) are present on alpha, beta and delta cell membranes (Hauge-Evans *et al.*, 2012; reviewed by Koh *et al.*, 2012). Insulin secreted by beta cells suppress glucagon secretion from isolated rat alpha cells and intact

islets (Franklin *et al.*, 2005). It was suggested that activation of voltage-gated potassium ion (K_{ATP}) channels by insulin, and consequent membrane hyperpolarisation resulted in inhibition of vesicle exocytosis (Franklin *et al.*, 2005). Others have shown that alpha-cell specific IR knockout mice exhibited increased glucagon secretion under both normal and low glucose conditions, confirming the negative regulation of glucagon secretion by insulin (Kawamori *et al.*, 2010). Furthermore, it has been found that in type 1 diabetes patients elevations in plasma insulin inhibited glucagon secretion (Cooperberg and Cryer, 2010), confirming the inhibitory effect of insulin on glucagon in humans. In addition to insulin, beta cells secrete the neurotransmitter gamma aminobutyric acid (GABA). It has been shown that binding of GABA to its receptor subtype $GABA_A$ present on alpha cells resulted in inhibition of glucagon secretion in mouse alpha cell lines (Xu *et al.*, 2006). Similarly, in rat pancreatic islets antagonising GABA receptors specific to alpha cells resulted in increased glucagon secretion (Wendt *et al.*, 2004). Furthermore, contrasting evidence exists on the role of Zn^{2+} ions, released during insulin exocytosis, on glucagon secretion. Some reported that only glucose or insulin are responsible for inhibition of glucagon secretion from clonal or isolated primary mouse alpha cells, but not Zn^{2+} (Ravier and Rutter, 2005), whereas others have shown that Zn^{2+} enhanced glucagon secretion in streptozotocin-induced diabetic rats (Zhou *et al.*, 2007), therefore suggesting a role for Zn^{2+} in islet paracrine regulations.

The effect of beta cell secretions on delta cells, however, remains disputed. Previously, it has been suggested that insulin does not mediate the release of somatostatin from delta cells in the presence of insulin secretagogues (Hauge-

Evans *et al.*, 2012). Although insulin receptor mRNA expression was detected in clonal delta cell line TGP52, treatment with exogenous insulin did not affect SST secretion. In the same study, insulin receptor blockade either pharmacologically or with an antibody reportedly did not alter glucose-induced SST secretion in TGP52 cells (Hauge-Evans *et al.*, 2012). More recently however, it has been reported that insulin stimulates SST secretion in a glucose-dependent manner in mouse islets (Vergari *et al.*, 2019). Here antagonism of the insulin receptor was found to significantly reduce SST secretion (Vergari *et al.*, 2019).

Two isoforms of insulin receptor (IR) A and B are present in the beta cell membrane and insulin acting via these can influence beta cell function in an autocrine manner. Some have shown that insulin mRNA synthesis is upregulated by the action of insulin on beta cells (Muller *et al.*, 2006; Leibiger *et al.*, 2001). Others, however, have reported that insulin's autocrine action via the phosphoinositide-3 kinase (PI3K)-dependent pathway causes downregulation of insulin secretion in human islets (Persaud *et al.*, 2002).

Glucagon receptors are expressed in both alpha and beta cells (Kieffer *et al.*, 1996). Binding of glucagon to its receptor activates adenylate cyclase, and increases 3',5'-cyclic adenosine monophosphate (cAMP). This enhances Ca^{2+}-dependent exocytosis of both glucagon and insulin respectively (reviewed by Gromada *et al.*, 2007; Ma *et al.*, 2005). Glutamate is a neurotransmitter that is secreted simultaneously with glucagon. It has been found to modulate islet cell secretory responses by binding to ionotropic glutamate receptors (iGluRs) that are expressed on alpha and delta cells, although its expression in beta cells remains contested (reviewed by Koh *et al.*, 2012).

However, in islets from humans, monkeys and mice activation of iGluR subtypes enhanced glucagon release (Cabrera *et al.*, 2008). This was suggested to be related to an increase in intracellular Ca^{2+} concentration that promoted Ca^{2+}-dependent exocytosis, hence resulting in autocrine regulation of glucagon secretion (Cabrera *et al.*, 2008). Furthermore, delta cells were found to express a splice variant of an iGluR subtype that reportedly promoted SST release from delta cells in rat islets (Muroyama *et al.*, 2004).

Somatostatin receptors are present in all islet cell types in rodents (Ludvigsen *et al.*, 2004) and humans (Kumar *et al.*, 1999) as described in 1.3.1.2. In humans, administration of SST inhibits glucose-induced insulin release (Efendic *et al.*, 1978). The same effect was observed when stimulated with insulinogogues glucagon and arginine (Efendic *et al.*, 1978). Somatostatin secreted by delta cells inhibits both insulin and glucagon secretion in mice (Hauge-Evans *et al.*, 2009). Here mice lacking *Sst* globally had enhanced insulin and glucagon secretion. Furthermore, neonatal mice lacking delta cells displayed hyperinsulinaemia resulting in hypoglycaemia (Li *et al.*, 2018), highlighting the importance of delta cell paracrine regulation in the maintenance of normoglycaemia.

As mentioned before, delta cells are elongated and contain filopodia-like structures and these have been shown to form the structural basis for communication with other islet cells (Arrojo e Drigo *et al.*, 2019). It was found that under conditions of pre-diabetes delta cell filopodia undergo structural remodelling as a potential adaptation to changes in islet secretions (Arrojo e Drigo *et al.*, 2019). A recent study found that beta cell activity on delta cells via gap-junctions resulted in enhanced delta cell activity and suppressed alpha cell

activity. It was suggested that the modulation of delta cells by beta cells caused SST release and therefore suppression of glucagon secretion from alpha cells (Briant *et al.*, 2017). Others have also reported similar findings that glucose-induced secretion of both insulin and SST were necessary to inhibit alpha cell glucagon secretion in human and mouse alpha cells (Elliott *et al.*, 2015).

In addition to the primary islet hormones insulin, glucagon and somatostatin, ghrelin has been found to modulate islet cell secretions (reviewed by Koh *et al.*, 2012). However, the paracrine actions of islet-specific ghrelin are not well understood, and the role of ghrelin on islet function is further elaborated in section 1.3.2 of this thesis.

Taken together the above reports indicate that the tight coordination among islet cells is important for islet cell secretory responses to changes in circulating blood glucose levels.

1.1.2 Glucose metabolism

Glucose is the main type of fuel in mammalian cells (Nelson and Cox, 2008) and the maintenance of normoglycaemia is fundamental for maintenance of biological function of an organism (In't Veld and Smeets, 2014). The pancreatic hormones insulin and glucagon play central roles in maintaining blood glucose levels between 4-6 mM via their actions on the liver, central nervous system, muscle, gut and adipose tissue (Röder *et al.*, 2016, Fig. 1.5). The liver plays an integral role in glucose homeostasis by promoting storage (glycogen synthesis) or release (gluconeogenesis and glycogenolysis) of glucose via signalling from either insulin or glucagon, respectively (Meshkani and Adeli, 2009).

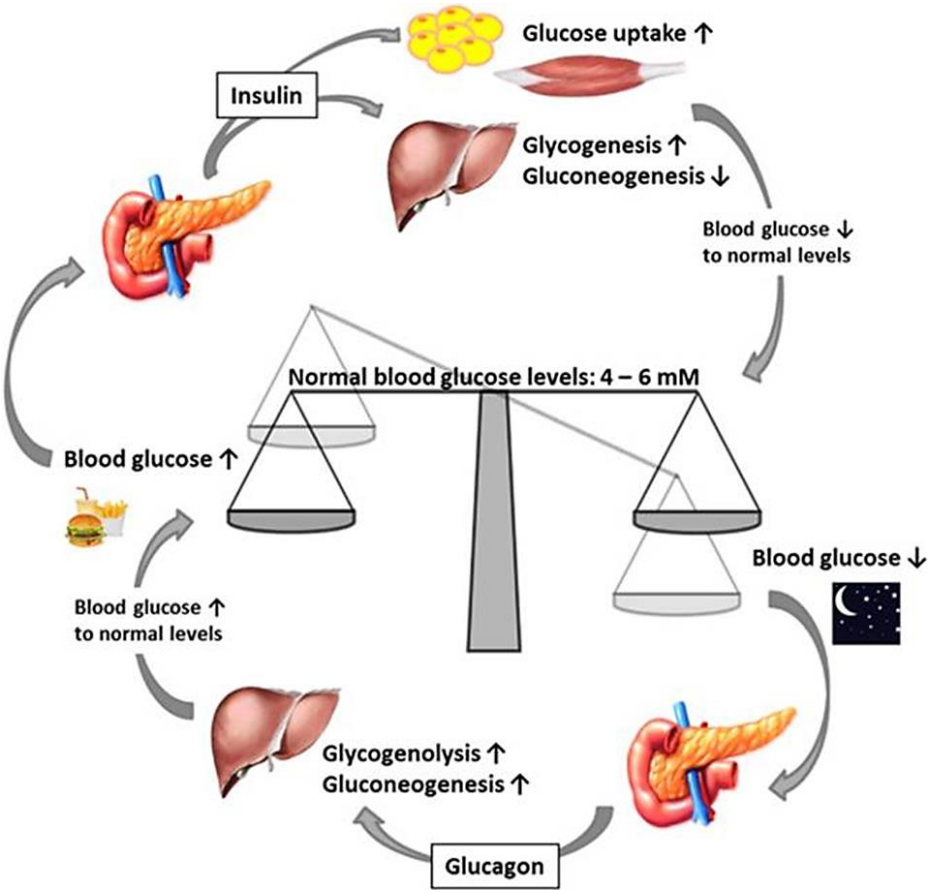

Figure 1.5 The regulation of glucose homeostasis. Combined action of insulin and glucagon contribute to maintenance of normoglycaemia by either storage or release of glucose (reviewed by Röder et al., 2016:3).

Cell membranes of hepatocytes express glucose transporter GLUT2 that facilitates movement of glucose both inwards and outwards of the cell depending on blood glucose levels (Leturque *et al.*, 2005).

In muscles and adipocytes insulin promotes glucose uptake via the glucose transporter GLUT4 which is stored in the internalised vesicles in the cytoplasm (reviewed by Navale and Paranjape, 2016). Binding of insulin to its receptor activates receptor tyrosine kinase that phosphorylates insulin receptor substrate 1 (IRS-1). IRS-1 activates the PI3K pathway that promotes

glucose transport, protein synthesis and glycogen synthesis (reviewed by Olson, 2012). Upon activation of PI3 kinase and protein kinase B (Akt), vesicles containing GLUT4 are translocated to the cell membrane. Their fusion with the plasma membrane increases GLUT4 expression. Glucose internalised by active transport is either utilised by myocytes to generate adenosine triphosphate (ATP) or stored as glycogen (reviewed by Huang and Czech, 2007; reviewed by Olson, 2012).

1.1.3 Insulin biosynthesis and secretion

1.1.3.1 Insulin biosynthesis

Insulin secreted by pancreatic beta cells is a 51-amino acid hormone consisting of two chains (A and B) connected by two disulphide bridges (Nicol and Smith, 1960). The insulin gene that contains 1563 base pairs encodes the inactive protein preproinsulin that includes proinsulin and a signal peptide of 24 amino acids (Chan *et al.*, 1976). The signal peptide is indicative of the molecule being a secretory hormone (Patzelt *et al.*, 1978). Preproinsulin is processed in the rough endoplasmic reticulum (RER) where the signal peptide is cleaved in the lumen by a signal peptidase. The resulting peptide proinsulin is folded, and disulphide bonds are created to produce the tertiary structure consisting of chains A, B and connecting peptide (C peptide) (Huang and Arvan, 1995). Proinsulin is rapidly transported via the Golgi apparatus into immature secretory granules where it is converted to mature insulin and C-peptide (Steiner, 2008). Two types of enzymes are responsible for the processing of proinsulin: trypsin-like endoproteases and an exopeptide. The former peptides have been identified to be prohormone convertases PC2 and

PC 1/3 that cleave the ends of the C domain. First, PC 1/3 cleaves at the B chain-C peptide junction. The resulting intermediate product is then cleaved by PC2 (Davidson *et al.*, 1988; Smeekens and Steiner, 1990; Smeekens *et al.*, 1992). The exopeptidase carboxypeptidase E (also known as H) is responsible for the removal of the basic residues remaining following the activity of endoproteases (Davidson and Hutton, 1987; Steiner, 2008).

Secretory granules mature in the cytosol of beta cells. Within the acidic environment of granules (pH 5.0-5.5) (Orci *et al.*, 1986) insulin molecules form crystals with Zn^{+} to stabilise the structure. The insulin-zinc complexes are stored within the dense core of the granules with the C peptide residing in the periphery (Dodson and Steiner, 1988; Frank and Veros, 1970).

1.1.3.2 Insulin secretion

Upon stimulation by a number of factors such as glucose, nutrients as well as other hormones, beta cells secrete insulin (reviewed by Fu *et al.*, 2014). This process involves fusion of mature secretory granules with the plasma membrane resulting in the release of insulin and C peptide (reviewed by Steiner, 2008). Glucose remains the main stimulus of insulin secretion. In response to elevations in plasma glucose beta cells secrete insulin to achieve normoglycaemia (4-6 mM). Islet vasculature plays an important role in this process by allowing rapid exchange of molecules via fenestrated blood vessels that are abundant within the islets (reviewed by Fu *et al.*, 2014). Insulin secretion follows a biphasic pattern consisting of a short term first phase followed by a more prolonged second phase (reviewed by Röder *et al.*, 2016). Insulin granules are stored in the cytoplasm of the beta cell, however only <5% of these are ready to be released immediately called the readily releasable pool

(RRP). The remaining >95% called the reserve pool need to undergo modifications and translocation towards the plasma membrane. It is reported that the first phase of insulin secretion primarily results in the release of RRP and second phase of the reserve pool (Eliasson *et al.*, 1997; Rorsman *et al.*, 2000; Rorsman and Renström, 2003).

1.1.3.3 Glucose-induced insulin secretion

Glucose transporters (GLUTs) are constitutively expressed on beta cells, with GLUT2 being expressed in rodents, and GLUT1 and 3 being the primarily expressed types in human islets (Pingitore *et al.*, 2017). These transporters act as glucose sensors and allow facilitated diffusion of glucose into the cytoplasm (Fig. 1.6, reviewed by Fu *et al.*, 2014; reviewed by Navale and Paranjape, 2016). The main glucose sensor found within the cytoplasm is glucokinase, a subtype of hexokinase that displays lower binding affinity for glucose than hexokinase and is not negatively regulated by increases in glucose concentration (Fu *et al.*, 2014; Nelson and Cox, 2008). Upon entering the cell glucose is phosphorylated by glucokinase to produce glucose-6-phophate that undergoes glycolysis resulting in the production of pyruvate. In the mitochondria pyruvate is further oxidised via the tricarboxylic acid cycle (TCA cycle) to generate ATP molecules (Nelson and Cox, 2008). Increases in the cytoplasmic ATP to adenine diphosphate (ADP) ratio causes closure of voltage-gated ATP-sensitive potassium channels (K_{ATP}) resulting in cell membrane depolarisation. At low glucose K_{ATP} channels are open and K^+ ions flow outwardly maintaining a membrane potential of -70 mV (MacDonald and Rorsman, 2006). As a consequence of K_{ATP} channel closure, voltage-gated calcium channels are opened causing influx of Ca^{2+} ions into the cytoplasm

(Komatsu *et al.*, 2013). Exocytosis is a Ca^{2+}-dependent process that is regulated by several proteins as described below.

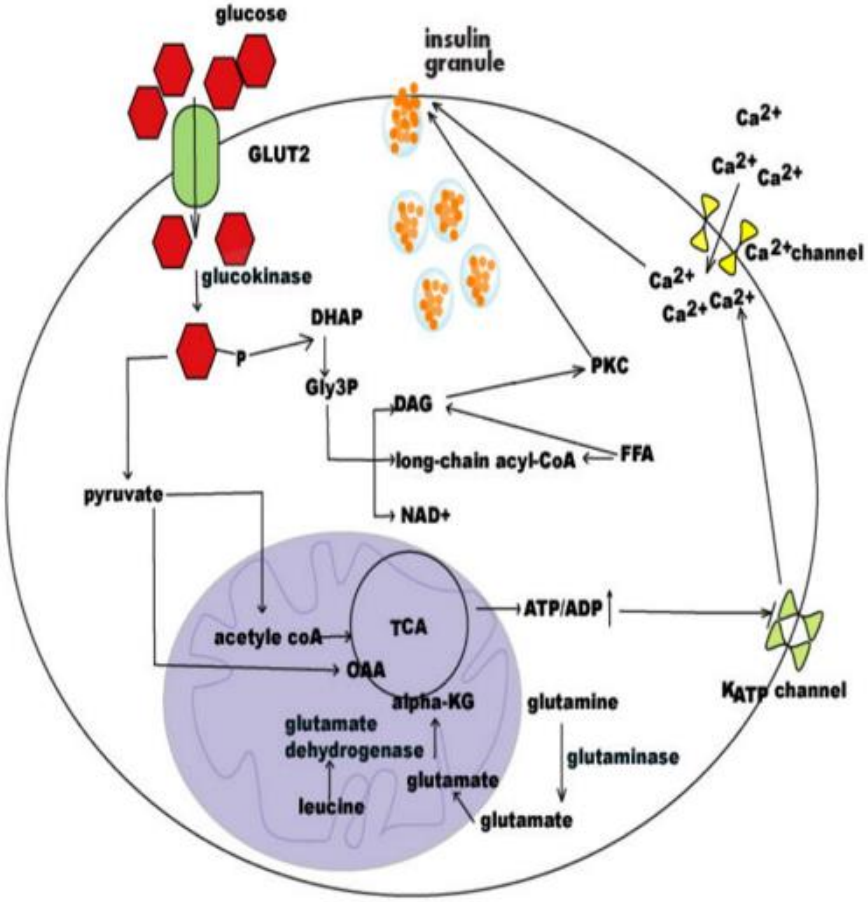

Figure 1.6 Illustration of glucose-stimulated insulin secretion. Glucose is transported into beta cells via GLUT2 and undergoes glycolysis to generate ATP. The resulting closure of K_{ATP} channels causes opening of voltage-gated calcium channels and influx of Ca^{2+} ions. Alternatively, during glycolysis glucose-6-phosphate can be metabolised into dihydroxyacetone phosphate (DHAP) resulting in the production of diacylglycerol (DAG) or acyl-Coenzyme A (acyl-CoA). Both pathways lead to insulin exocytosis via fusion of insulin containing granules with the plasma membrane (Fu et al., 2014). ATP= adenosine triphosphate; ADP= adenosine diphosphate; Gly-3P= glycerol-3-phosphate; FFA= free fatty acids; NAD+= nicotinamide adenine dinucleotide; PKC= protein kinase C; TCA= tricarboxylic acid cycle; alpha-KG= alpha-Ketoglutaric acid.

In addition, in the mitochondria anaplerotic reactions occur to replenish components of the TCA cycle such as glutamate that can also signal insulin secretion by amplifying K_{ATP} channel-dependent insulin secretion (Maechler and Wollheim, 1999).

Alternatively, glucose-6-phosphate can be metabolised into dihydroxyacetone phosphate (DHAP) that produces glycerol-3-phosphate (Gly3P) (Bender *et al*, 2006). Acyl-Coenzyme A (acyl-coA) and diacylglycerol (DAG) are generated by Gly3P and can also result in increased insulin secretion (reviewed by Fu *et al*., 2014).

Insulin secretion is regulated by a number of proteins such as the soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE) that facilitates fusion of vesicles with the plasma membrane (Wang and Thurmond, 2009). Upon docking of insulin containing vesicles three SNARE proteins form a complex that simultaneously binds to the membrane of the granule, the beta cell membrane and the membrane associated protein SNAP-25 to promote vesicle fusion and exocytosis of insulin (Wang and Thurmond, 2009). Membrane fusion requires the calcium sensor synaptotagmin, which is found on the membranes of the vesicles (Martens *et al*., 2007). Binding of synaptotagmin to Ca^{2+} triggers membrane fusion of vesicles via mechanisms that are not clearly understood (Jahn and Fasshauer, 2015; Trexler and Tarasaka, 2017).

Other stimuli such as nutrients and incretins can activate cAMP, cyclic guanine monophosphate (cGMP), inositol 1,4,5 triphosphate (IP3) and DAG that result in the initiation of cell signalling cascades to promote exocytosis, with cAMP playing a key role (reviewed by Fu *et al*., 2014; Henquin, 1985). cAMP

activates protein kinase A (PKA) that potentiates stimulus-induced insulin secretion from beta cells (Thams *et al.*, 2005). cAMP-mediated potentiation of insulin secretion can also occur via a PKA-independent pathway by activation of exchange protein directly activated by cAMP (Epac) (Seino and Shibasaki, 2005). Both pathways result in an increase in cytosolic Ca^{2+} ions and exocytosis of insulin granules (Henquin and Nenquin, 2014).

1.2 Diabetes mellitus

1.2.1 Diagnosis and classification of diabetes mellitus

According to the International Diabetes Federation (IDF) there are currently 463 million people affected by diabetes mellitus and this number is predicted to reach 700 million by 2045 (IDF, 2019). Diabetes mellitus is a chronic condition characterised by hyperglycaemia that over extended periods results in damage to various organs including heart, eyes, nerves, kidneys and blood vessels. The condition is diagnosed if one or more of the criteria indicated below are met: fasting plasma glucose of ≥ 7.0 mmol/L, glycated haemoglobin A1c (HbA1c) ≥ 48 mmol/mol (equivalent to 6.5%) and plasma glucose ≥ 11.1 mmol/L two hours following 75 g oral glucose load or taken at random (IDF, 2019). The most common types of diabetes are type 1, type 2 and gestational (pregnancy related) diabetes (Kharroubi and Darwish, 2015), although other forms have been reported (Schwitzgebel, 2014).

Type 1 diabetes

Type 1 diabetes accounts for about 5-10% of patients with diabetes and generally manifests between 0-14 years of age with increasing incidence

between 5-7 years and at puberty. However, this condition can also arise at a later stage (Atkinson *et al.*, 2014; Bluestone *et al.*, 2010). Type 1 diabetes develops due to immune cell-mediated destruction of islet beta cells or insulitis resulting in reduced or no production of insulin. This is caused by beta cells being infiltrated by T cells (CD8+ and CD4+), macrophages (CD68+) and CD20+ B cells (Atkinson *et al.*, 2014). Two distinct disease phenotypes have been identified based on the rate of B cell infiltration namely hyper-immune CD20Hi and pauci-immune CD20Lo (Arif *et al.*, 2014). The former was found to be characterised by high level of autoantibodies and interferon gamma and identified in patients at age of five or less, whereas the latter was characterised by none or few autoantibodies and a higher level of interleukin-10, and observed in older patients (Arif *et al.*, 2014). More recently, it has been reported that patients diagnosed before the age of seven displayed CD20Hi insulitic profile with impaired insulin processing (endotype 1), whereas those diagnosed later than 12-13 years of age are always of CD20Lo profile with better insulin processing than the former (endotype 2) (Leete *et al.*, 2016; Leete *et al.*, 2020).

The causes of the immune response are not fully understood, however genetic factors and environmental factors such as viral infections have been found to trigger the immune response in type 1 diabetes (reviewed by Richardson and Morgan, 2018; reviewed by Robertson and Rich, 2018). For example, genome-wide association studies and meta-analyses have found about 40 loci contributing to type 1 diabetes risk, including human leukocyte antigen (HLA) (Barrett *et al.*, 2009). A number of HLA class II haplotypes were found to be associated with autoantibody formation, such as HLA-DR4-DQ8 associated

with insulin autoantibodies (Krischer *et al.*, 2015). Beta cells are highly susceptible to human enteroviral infections and these viruses can activate chronic low-level infection in some cases (reviewed by Richardson and Morgan, 2018). One such virus is Coxsackie B that was detected in pancreatic tissues from patients with type 1 diabetes (Busse *et al.*, 2017). Type 1 diabetes is managed by daily insulin injections where regular monitoring of blood glucose levels is imperative. In a small number of cases pancreas or islet transplantation can be an option (reviewed by Atkinson *et al.*, 2014).

Type 2 diabetes

Type 2 diabetes is a chronic condition associated with metabolism of carbohydrates, lipids and proteins. It constitutes about 90-95% of cases of patients with diabetes and is generally diagnosed later in life (40-59 years). Recently, younger people in their 30s have been diagnosed (reviewed by Wu *et al.*, 2014). Type 2 diabetes is characterised by insulin resistance in target tissues in conjunction with relative insulin insufficiency, resulting from beta cell dysfunction and loss that may arise due to oxidative stress and endoplasmic reticulum stress as further described in the next section (reviewed by Kharroubi and Darwish, 2015; Wu *et al.*, 2014).

The risks of developing type 2 diabetes are associated with genetics and environmental factors. The condition is considered polygenic, therefore influenced by polymorphisms at multiple loci associated with reduction in peripheral insulin sensitivity and impaired beta cell function (reviewed by Ali, 2013). For example, polymorphisms at peroxisome proliferator-activated receptor-gamma (*PPARγ*) locus, that are involved in adipose tissue differentiation and insulin sensitivity, are associated with type 2 diabetes risk

(Ruchat *et al.* 2009). Similarly, polymorphisms at loci involved in beta cell function such as potassium channel inwardly rectifying subfamily J member 11 (*KCNJ11*), that encodes K_{ATP} channels found in beta cells have been identified as risk factors of the condition (reviewed by Ali, 2013; Hani *et al.*, 1998). Genetic polymorphisms can not only directly influence beta cell function and peripheral insulin sensitivity, but also alter physiological responses to environmental risk factors (reviewed by Ali, 2013). Numerous environmental factors are considered as mediators of type 2 diabetes risk including inadequate physical activity, pollution, unhealthy diets and stress (reviewed by Dendup *et al.*, 2018). These life-style changes result in high cholesterol, obesity, increased blood pressure and prediabetes that in the long term can develop into type 2 diabetes (reviewed by Dendup *et al.*, 2018).

The management of type 2 diabetes can be achieved by healthy diet, physical activity and pharmacological agents that lower blood glucose levels (Reusch and Manson, 2017). Some of these compounds include biguanides (metformin), which act by increasing insulin sensitivity in peripheral tissues and suppressing hepatic gluconeogenesis (reviewed by Rena *et al.*, 2017); and more recently developed sodium-glucose co-transporter-2 (SGLT2) inhibitors such as empagliflozin, which prevent glucose reabsorption in kidneys and promote renal glucose excretion (Ndefo *et al.*, 2015). In patients with poorly controlled blood glucose levels, i.e.: displaying frequent fluctuations in glycaemic levels, administration of subcutaneous insulin is recommended (Inzucchi *et al.*, 2015; reviewed by Wu *et al.*, 2014).

1.2.2 Factors affecting beta cell dysfunction and loss in type 2 diabetes

1.2.2.1 Insulin resistance in target tissues

Insulin resistance refers to the inefficiency of insulin to increase glucose uptake and utilisation in target tissues compared to the extent in a healthy individual (reviewed by Lebovitz, 2001). It therefore develops in liver, skeletal muscle and adipose tissue. Many mechanisms of insulin resistance exist due to multiple pathways activated by insulin upon binding to its receptor as described in section 1.1.2. Potential causes of insulin resistance are suggested to be, but not limited to genetic mutations in the signalling pathways and adiposity (reviewed by Lebovitz, 2001). Obesity constitutes a risk factor in developing insulin resistance due to incidence of chronic systemic inflammation (reviewed by Kwon and Pessin, 2013; reviewed by Stinkens *et al*., 2015). Adipose tissue is involved in the regulation of feeding behaviours and inflammation. It is suggested that increased subcutaneous adipocyte size results in impaired glycaemic control and insulin responses (Hoffstetd *et al*., 2010). In addition, adipocyte hypertrophy can cause oxidative and endoplasmic reticulum (ER) stress, described below, resulting in adipocyte dysfunction. This causes excessive secretion of pro-inflammatory cytokines that triggers local and systemic inflammation (reviewed by Kwon and Pessin, 2013). Furthermore, adipocyte dysfunction increases circulating free fatty acids (FFAs) that infiltrate other tissues such as skeletal muscle and cause muscle insulin resistance (reviewed by Boden, 2011). As mentioned in 1.1.2, skeletal muscle is a main target of glucose where it is taken up, stored and utilised. It has been shown that elevated circulating FFAs cause intramuscular lipid accumulation and insulin resistance in healthy humans (Boden *et al*.,

2001). This has been suggested to be associated with lipid metabolite production and subsequent interference with insulin signalling via impaired translocation of GLUT4 (Boden, 1997; Cline *et al.*, 1997; Garvey *et al.*, 1998). FFAs can cause muscle cell insulin resistance in a number of ways including triggering of ER stress, and via secretion of pro-inflammatory cytokines (reviewed by Rachek *et al* , 2014).

Mitochondrial dysfunction due to oxidative stress can also impair FFA oxidisation resulting in accumulation of FFAs in the cells, which is a feature of high-fat diet induced insulin resistance (Bonnard *et al.*, 2008). Similarly, in the liver, chronic inflammation by FFAs interfere with insulin signalling resulting in impaired glycogen synthesis and increased hyperglycaemia (Meshkani and Adeli, 2009).

Insulin resistance can lead to type 2 diabetes in some individuals, but not all due to compensatory mechanisms maintaining normoglycaemia. However, over long-term hypersecretion of insulin can result in beta cell dysfunction that progresses the condition to type 2 diabetes mellitus (reviewed by Fu *et al.*, 2014).

1.2.2.2 Beta cell dysfunction and loss

The secretory dysfunction of beta cells and the consequent beta cell loss are causative factors in developing diabetes. Analysis of pancreata from patients with type 2 diabetes showed a marked depletion in beta cell mass compared to patients without diabetes (Cho *et al.*, 2011; Matveyenko and Butler, 2008). Although it is known that in type 1 diabetes beta cell loss occurs as a result of

an autoimmune response, in type 2 diabetes the mechanism is not very well understood.

Beta cell dysfunction is a determining factor in developing type 2 diabetes (reviewed by Kahn, 2000). In order to manage hyperglycaemia due to insulin resistance beta cells hypersecrete insulin. To compensate for this beta cells undergo hypertrophy (Ackermann and Ganon, 2006). The two phases of insulin secretion (pg. 14) are altered in type 2 diabetes. In people with impaired glucose tolerance the first phase of insulin secretion was reported to be absent (Gerich, 2002). In another study investigating the effects of acute hyperglycaemia it was found that short-term modest hyperglycemia inhibited first phase of insulin secretion, whereas second phase was found to increase linearly with hyperglycaemia (Toschi *et al.*, 2002). Over time, however, sustained severe hyperglycaemia results in complete inefficiency in glucose-stimulated insulin secretion (GSIS) including second phase of insulin secretion. At this stage beta cells display morphological changes such as degranulation of the cell indicating lack of insulin production including downregulation of insulin mRNA expression (Weir *et al.*, 2001).

As described in 1.1.1 islets are highly vascularised and in type 2 diabetes islet vasculature has been found to adapt to high metabolic activity due to hyperglycaemia (Dai *et al.*, 2013). In addition, it has been found that the islet architecture is altered in animal models of type 2 diabetes and that beta cells can differentiate into alpha cells (Talchai *et al.*, 2012). These changes therefore result not only in islet cell secretory dysfunctions but also in the disruption of intra-islet regulation in people affected by diabetes (Brereton *et al.*, 2015).

The drivers of beta cell dysfunction are described as cellular stress signals generated from prolonged exposure to high glucose (glucotoxicity), high fat (lipotoxicity), a combination of both (glucolipotoxicity) as well as pro-inflammatory cytokines resulting is inflammation (reviewed by Hasnain *et al.*, 2016). These factors cause beta cell oxidative stress and endoplasmic reticulum (ER) stress that are characteristic of beta cells in patients with type 2 diabetes (reviewed by Hasnain *et al.*, 2016). High metabolic activity of beta cells leads to excessive production of reactive oxygen (ROS) and reactive nitrogen (RNS) species. These include nitric oxide (NO), superoxide, hydrogen peroxide and peroxynitrite (Nelson and Cox, 2008). Usually, beta cells are capable of coping with their production, however due to high demand for oxygen during insulin resistant hyperglycaemia, the overproduction of ROS and RNS result in beta cell oxidative stress (Meares *et al.*, 2013). The RNS and ROS species can also react and form more potent stress factors that can induce ER stress. One such example is peroxynitrite that is generated by nitric oxide reacting with superoxide (Nelson and Cox, 2008). In addition, prolonged exposure to elevated concentrations of fatty acids causes downregulation of insulin gene expression, impaired glucose-stimulated insulin secretion and apoptosis (Hagman *et al.*, 2005; Johnston *et al.*, 2018). For example, rat pancreatic islets cultured in medium containing a high concentration of fatty acids for 7 days displayed a number of apoptotic features including DNA fragmentation and ceramide formation (Shimabukuro *et al.*, 1998). The presence of FFAs has been found to increase production of ROS and RNS via mitochondrial metabolism that upregulates expression of nitric oxide synthase (iNOS) resulting in the generation of NO (Rachek *et al.,* 2006).

Oxidative stress and ER stress are interconnected, and the detrimental effects of each process are difficult to distinguish. Instead this indicates that beta cell dysfunction is connected to beta cell apoptosis. Protein misfolding is an inevitable occurrence in the ER, however exceeding the threshold that cannot be counter regulated triggers the unfolded protein response (UPR), leading to ER stress (reviewed by Hasnain *et al.*, 2016). Prolonged ER stress can cause inflammation, autophagy and apoptosis. UPR is activated by heat shock protein binding immunoglobulin protein (Bip). Dissociation of Bip from UPR initiating molecules inositol-requiring enzyme-1 (IRE1α), protein kinase-R-like ER kinase (PERK) and activating factor 6 (ATF6) triggers their activation and consequently UPR. The primary function of these molecules is to reduce ER workload and recovery of ER function which is achieved by reducing protein translation, restoration of correct protein folding and ER associated degradation of misfolded proteins (reviewed by Bravo *et al.*, 2013). However, in this process downstream molecules of PERK and IRE1α can trigger apoptosis. PERK phosphorylates transcription factors activating factor 4 (ATF4) and CCAAT-enhancer-binding protein homologous protein (CHOP) that can trigger ER stress induced apoptosis in beta cells and other cell types (Marciniak *et al.*, 2004). IRE1α is mainly involved in degradation of misfolded proteins, however it can also activate c-Jun N-terminal kinase (JNK) signalling resulting in cell death (Urano *et al.*, 2000).

Oxidative stress and ER stress due to lipotoxicity can also activate nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) that is a regulator of inflammation. NFκB can trigger release of cytokines such as interleukin-1 β (IL-1β) from resident leukocytes of the pancreatic islets

(Igoillo-Esteve *et al.*, 2010). Pro-inflammatory cytokines in turn exacerbate oxidative stress and ER stress and production of detrimental nitric oxide (Kacheva *et al.*, 2011).

Since early stages of type 2 diabetes is characterised by beta cell dysfunction rather than loss, the former can be considered the main driver of this condition. This process is potentially reversible and therefore identifying target candidates modifying the progression is of utmost relevance (reviewed by Hasnain *et al.*, 2016).

1.3 The roles of somatostatin and ghrelin in maintenance of glucose homeostasis and type 2 diabetes.

1.3.1 Somatostatin

1.3.1.1 The structure and function of somatostatin

Somatostatin (SST), also known as somatotropin-releasing factor (SRIF), is primarily an inhibitory peptide that acts on several tissues including brain, gut, retina and pancreas (reviewed by Günther *et al.*, 2018).

There are two main forms of the hormone: a 14 amino acid peptide (SST-14) that was initially identified in the sheep's hypothalamus (Brazeau *et al.*, 1973) and a 28 amino acid peptide isolated later (Pradayrol *et al.*, 1980). Both forms of SST have a cyclic structure with a disulphide bridge (Fig. 1.7).

The precursor of both peptides is preprosomatostatin. It is encoded by the gene SST1 and consists of 116 amino acids that includes a signal peptide at the N terminus (Goodman *et al.*, 1983). Preprosomatostatin undergoes rapid cleavage into prosomatostatin consisting of 92 amino acids which is then

processed by specific protein convertases such as PC2 to generate the mature forms (Goodman *et al.*, 1983; Marcinkiewicz *et al.*, 1994). Somatostatin is widely expressed in the body. For example, in rats 65% of total SST is found in the gut, 25% in the brain, 5% in pancreas and 5% in other tissues such as thyroid, liver, kidney, heart, muscle and blood vessels (Kumar and Grant, 2009; reviewed by Martinez, 2013). SST-14 is mainly found in the central nervous system and pancreatic islets, whereas SST-28 is produced in the gastro-intestinal (GI) tract and corresponds to the majority of circulating SST (Kumar and Grant, 2009; Mani and Zigman, 2015).

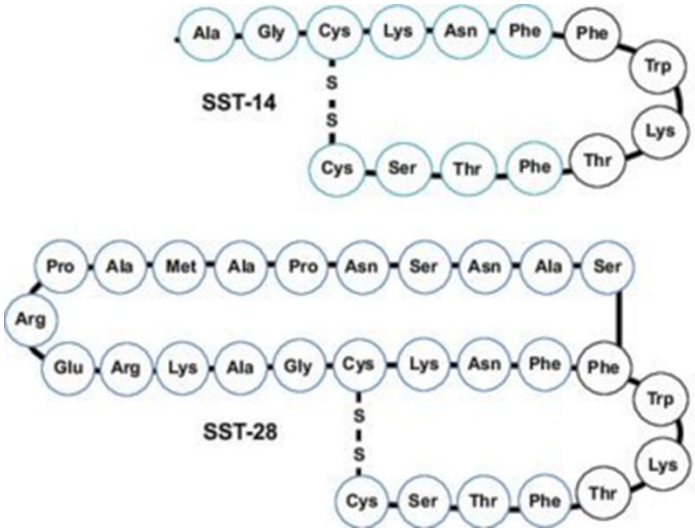

Figure 1.7 The structure of the two somatostatin isoforms in humans. Somatostatin (SST) is a cyclical peptide of 14 (SST-14) or 28 (SST-28) amino acids linked together by a disulphide bridge (Cuevas-Ramos and Fleseriu, 2016:229).

As mentioned before SST mainly acts as an inhibitor of hormone secretion (Fig. 1.8). Indeed, SST-14 has been well characterised as an inhibitor of growth hormone (GH) secretion in the brain. Rats injected with somatostatin had reduced GH concentration compared to saline injected controls (Brazeau *et al.*, 1973).

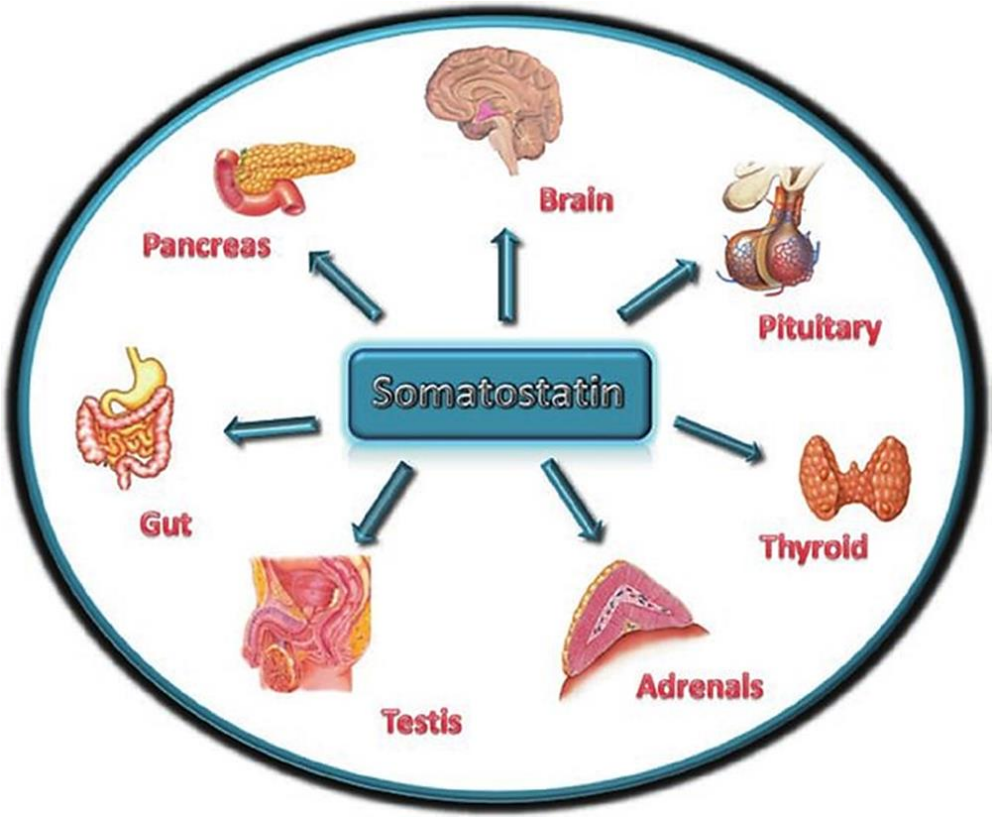

Figure 1.8 The hormonal targets of somatostatin. Somatostatin mainly acts as an inhibitory hormone in central nervous system, gut and pancreatic islets. (Kumar and Grant, 2010:142).

GH release is stimulated by growth hormone releasing hormone (GHRH) and SST-14 was found to inhibit GHRH (Barinaga *et al.*, 1983). Others have shown that SST-14 acts directly on the pituitary to inhibit GH secretion and indirectly suppresses GHRH (Katakami *et al.*, 1988). These results were further confirmed in *Sst* null mice where basal GH levels were higher compared to wild type controls (Low *et* al., 2001). In addition, the action of SST-14 on the anterior pituitary reduced secretion of thyroid stimulating hormone (TSH) (Ridgway *et al.*, 1983). As a result of reduced TSH levels the production of calcitonin from parafollicular cells in the thyroid is hindered (Kumar and Grant, 2009). Furthermore, in patients with hypothyroidism SST-14 directly suppresses the TSH-induced release of thyroxine (T4) and

triiodothyronine (T3), and therefore is considered to be a causative factor of hypothyroidism (Loos *et al.*, 1978).

In the GI tract SST-28 suppresses gastric acid secretion, release of gut hormones such as gastrin and motilin, bile and digestive enzymes. It also impairs gallbladder contractility, gastric emptying and intestinal peristalsis (reviewed by Günther *et al.*, 2018). For example SST infusion into the duodenum of dogs prevented motilin-induced contractility and therefore digestive process (Ormsbee *et al.*, 1978).

SST-14 is produced by pancreatic islet delta cells (Orci and Unger, 1975) and influences both insulin and glucagon secretion. For example, in healthy humans administration of SST decreased plasma insulin levels (Alberti *et al.*, 1973). In the same study, infusion of SST into perfused canine pancreata resulted in impaired glucose-mediated insulin secretion that was recovered following termination of SST infusion (Alberti *et al.*, 1973). Indeed, it has been shown that SST-14 inhibits insulin and glucagon secretion in a paracrine manner (Hauge-Evans *et al.*, 2009). This finding was confirmed by the discovery that the haematopoietically expressed homeobox (*Hhex*) gene, a gene important for developmental regulation of several organs, is primarily expressed on pancreatic delta cells, but not alpha and beta cells. Its islet-specific knockout resulted in ~75% reduction in delta cell area. Consequently, decreased SST release and concomitant paracrine regulation of beta and alpha cells' secretory responses were observed (Zhang *et al.*, 2014). As described in 1.3.1.2, somatostatin acts via five receptor subtypes (SSTRs), with SSTR2 being primarily expressed in alpha cells. Targeting of this receptor by a SSTR2 agonist caused increase in arginine-induced glucagon secretion in wildtype

mice, but not in *Sst*$^{-/-}$ mice (Strowski *et al.*, 2006). Similarly, in a recent study tolbutamide-induced glucagon secretion was elevated in islets from *Sst*$^{-/-}$mice (Cheng-Xue *et al.*, 2013). Taken together these studies further confirm the role of SST as paracrine regulator of islet secretions.

In addition to its role as a regulator of secretory functions, SST has been studied in the context of cell survival and apoptosis as further described in section 3.1. Briefly, a number of studies exploring the role of somatostatin in various tissues have found that it exerts an anti-proliferative effect in some cancers including pancreatic, breast, small-cell lung and colorectal (reviewed by Pyronnet *et al.*, 2008; Schally *et al.*, 1988). In primary pancreatic neuroendocrine tumour cells treatment with somatostatin analogues octreotide and pasireotide reduced cell viability and induced apoptosis (Mohamed *et al.*, 2014). In contrast, in other tissues such as the retina and nerve fibres incubation with SST has been reported to prevent cell apoptosis and reduce inflammation (Hernandez *et al.*, 2013; Pintér *et al.* 2006). In accordance with the latter studies, work conducted in our lab showed that SST-14 protects beta cells and islet cells from apoptosis induced by palmitate and/or a combination of pro-inflammatory cytokines (Damsteegt *et al.*, 2019). However, the receptor subtype mediating the pro-survival effect of SST in beta cells and islets remains to be elucidated.

1.3.1.2 Somatostatin receptors

Somatostatin receptors are classified as G-protein coupled receptors (GPCR) that consist of seven transmembrane spanning alpha helices, an extracellular C terminal and an intracellular N terminal (reviewed by Günther *et al.*, 2018; reviewed by Martinez, 2013). There are five SST receptor subtypes identified:

SSTR1-5. These receptors are coupled to G proteins $G_{\alpha i}/G_0$ and G_q and adenylate cyclase. Therefore, the activation of SSTR and the second messenger systems can trigger numerous downstream signalling events leading to regulation of hormone secretions, vesicle exocytosis, and cell survival and proliferation (reviewed by Ferjoux *et al.*, 2000; reviewed by Kumar and Grant, 2009).

Similar to somatostatin its receptor subtypes are also widely expressed in the body including in the brain, kidneys, thyroid, pancreas and GI tract (reviewed by Günther *et al.*, 2018). SSTR2 was preferentially expressed in the stomach both at mRNA and protein level confirmed by immunohistochemistry (reviewed by Martinez, 2013). In the small intestine and colon all five receptor subtypes were detected with SSTR2 being expressed at the highest level (reviewed by Martinez, 2013). All five SST receptors are present in the brain (Bruno *et al.*, 1993) and SSTR1 predominates particularly in the arcuate nucleus together with SSTR2 (Tannenbaum *et al.*, 1998).

In the pancreatic islets of both rodents and humans all five SSTR subtypes have been detected. Using fluorescence immunohistochemistry Kumar *et al.* (1999) have found that human islet beta cells expressed SSTR1, SSTR5 and to a lesser extent SSTR2. They have also reported that SSTR2 was the dominant subtype in alpha cells and SSTR5 in delta cells. In accordance with these findings SST was found to inhibit glucagon secretion via SSTR2 and insulin secretion via SSTR5 in rodents (Strowski *et al.*, 2000). However, in human islets SSTR2 activation exerted the most potent inhibition of insulin and glucagon secretion (Singh *et al.*, 2007). The expression patterns of SSTRs in the rodent islets was elucidated in a number of studies (Adriaenssens *et al.*,

2016; Ludvigsen *et al.* 2004; Somvanshi *et al.*, 2018) Ludvigsen *et al.* (2004) showed that mouse beta cells express SSTR1,2 and 5 at the highest level (85-95%) and SSTR3 and 4 at lower level. In rats no difference in the levels of SSTRs was detected in beta cells in this study. SSTR2 and 5 were detected in 80% of alpha cells with the remaining subtypes detected at much lower level in islets of mice and rats. Finally, similar levels of SSTR1-4 were detected in delta cells with SSTR5 being the lowest. This pattern was observed in both mice and rats (Ludvigsen *et al.*, 2004). These findings were mostly confirmed in a recent study by Somvanshi *et al.* (2018). Here beta cells were found to mainly express SSTR1 and 5 with lower expression of SSTR3 and 4, alpha cell expressed SSTR2, 3 and 5, and delta cells expressed SSTR3 at a low level (Somvanshi *et al.*, 2018). Despite many studies reporting the detection of SSTR1, 2 and 5 in beta cells, recent single-cell transcriptomic analyses have reported only *Sstr3* to be present in beta cells and *Sstr2* and *3* in alpha cells of mouse islets (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016) as described in section 3.1 of this thesis.

A number of SSTR antagonists have been developed including the pan antagonist PRL2915 (Hocart *et al.*, 1999) and SSTR5 antagonist PRL3195 (Rajeswaran *et al.*, 2001). However commercially available SSTR3-selective antagonists are limited. MK4256 is a recently identified SSTR3 competitive antagonist (He *et al.*, 2012) (Fig. 1.9). In oral glucose tolerance tests (OGTT) performed in mice, increasing doses of MK4256 resulted in reduced blood glucose levels. This was confirmed in *Sstr3*$^{-/-}$ mice where the reduction in blood glucose following administration of MK4256 was not significant (He *et al.*, 2012).

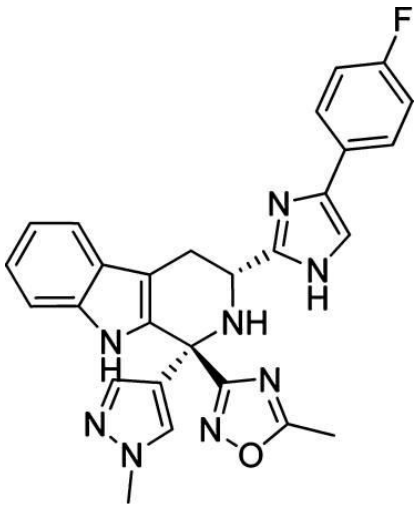

Figure 1.9 The structure of MK4256 (He *et al.*, 2012).

Furthermore, the compound showed synergistic effects on glucose levels when given in adjunct with sitagliptin which is an antidiabetic medication that prevents degradation of incretins resulting in increased insulin secretion and reduced glucagon secretion (Thornberry and Gallwitz, 2009). Therefore, MK4256 was used in the present study to assess the role of SSTR3 in modulating clonal beta cell and mouse islet function.

1.3.1.3 The role of Somatostatin in diabetes

Being a paracrine regulator of insulin and glucagon secretion, somatostatin is inevitably associated with diabetes mellitus. By inhibiting glucagon secretion, it has been suggested that SST-14 may help to prevent hyperglycaemia in patients with both type 1 and 2 diabetes (reviewed by Rutter, 2009; reviewed by Strowski and Blake, 2007). In type 1 diabetes, adjunct therapy of SST with insulin helped to reduce the amount of exogenous insulin required to obtain normoglycaemia. It was thought that suppression in glucagon release

following administration of SST may have contributed to this effect, although other effects of SST on GI tract and islet exocrine secretions could not be dismissed (Gerich *et al.*, 1977). Similarly, in patients with type 2 diabetes co-administration of SST analogue octreotide with insulin resulted in reduced postprandial blood glucose (Giustina *et al.*, 1991). Some have reported that the number of somatostatin producing delta cells is increased in both type 1 and 2 diabetes as reviewed by Strowski and Blake (2007) whereas Lawlor *et al.* have reported this to be lower using transcriptomic analysis (Lawlor *et al.*, 2017). Furthermore, the expression patterns of SSTRs have been shown to be altered in the islet cells of patients with diabetes (Portela-Gomes *et al.*, 2010). Beta cell SSTR3 level was significantly higher in isolated mouse islets cultured in 28.8 mM glucose, but not in alpha cells (Ludvigsen *et al.*, 2011). In another study, expression of SSTR1 and 4 was reduced in alpha cells whereas SSTR1, 3 and 4 expression was reduced in delta cells (Portela-Gomes *et al.*, 2010). Taken together these studies indicate that somatostatin plays a role in the management of diabetes mellitus.

In summary, these studies highlight that islet cell type specific SST receptor expression patterns are disputed and require further elucidation to understand the mechanism of action of SST in these islets.

1.3.2 Ghrelin

1.3.2.1 The structure and function of ghrelin

Ghrelin is a 28-amino acid orexigenic hormone that is mainly produced in X/A-like cells in the oxyntic glands of the stomach, but also found to be expressed in pancreatic islets, brain, heart and upper intestines (Gnanapavan,

2002; Sato *et al.*, 2011; Wierup *et al.*, 2002; Wierup *et al.*, 2014). The ghrelin gene encodes a precursor molecule of 117 amino acids called preproghrelin that is cleaved into ghrelin and a 23-amino acid peptide named obestatin of unclear physiological function (Granata *et al.*, 2010). Ghrelin exists in two forms: the acylated form (acyl-ghrelin) and the unacylated form (des-acyl-ghrelin). The acylation is catalysed by the enzyme ghrelin-*O*-acyltransferase (GOAT) that transfers an octanoate group on to the third serine residue of the peptide. This modification is essential for the binding of the peptide to its known receptor. Ghrelin is highly conserved between species where rat and mouse ghrelin only differ from human ghrelin by two amino acids (Fig.1.10; Castañeda *et al.*, 2010; Sivertsen *et al.*, 2013). The term ghrelin will be used to refer to the acylated form of the peptide in the subsequent sections unless otherwise specified.

Numerous studies have found that both in humans and rodents ghrelin plays a role in regulating appetite, food intake, energy metabolism, weight gain and fat mass via its activity on the hypothalamus, where it signals via the arcuate nucleus (reviewed by Delporte, 2013; Kojima *et al.*, 1999; reviewed by Pradhan *et al.*, 2013).

In both obese and lean participants, infusion of ghrelin resulted in increased food intake (Druce *et al.*, 2005). Similarly, it was stimulated by administration of ghrelin in rats (Wren *et al.*, 2001). Chronic administration of ghrelin causes adiposity in rodents (Tschöp *et al*, 2000). In particular, treatment lasting one-week increased food intake in satiated rats, resulting in hyperphagia and higher body weights. These rats had elevated levels of white adipose tissue deposits (Wren *et al.*, 2001). It is known that ghrelin regulates body weight in

the long term and that ghrelin levels are lower with weight gain, whereas caloric restriction can increase ghrelin levels (reviewed by Delporte, 2013). Ghrelin is also known to act via the pituitary and promote growth hormone secretion (Kojima *et al.*, 1999). In addition to its orexigenic effects a number of studies have reported that ghrelin modulates cardiac functions, muscle atrophy, bone metabolism and cancer (Fig 1.11; reviewed by Delporte, 2013; reviewed by Pradhan *et al.*, 2013).

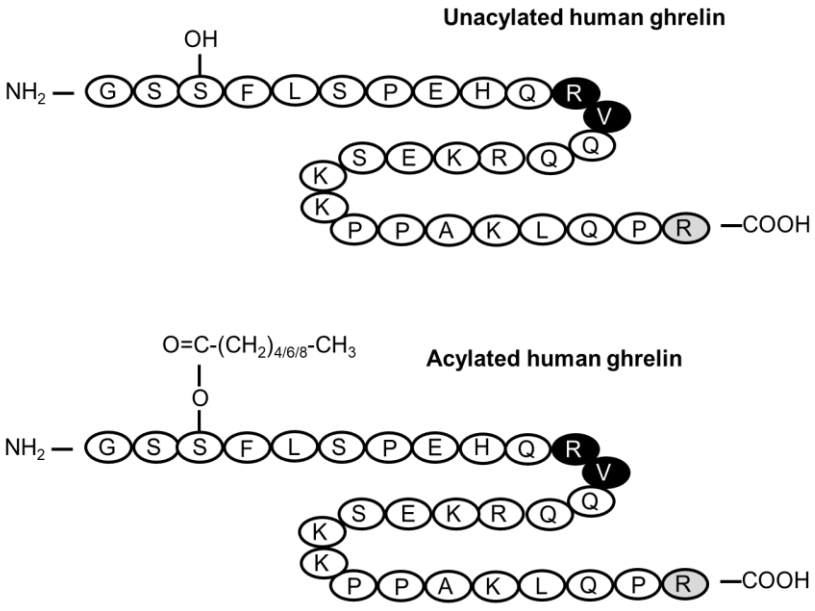

Figure 1.10 The structures of human unacylated (des-acyl) ghrelin and acylated (acyl) ghrelin. Octanoate group O=C-$(CH_2)_{4/6/8}$-CH_3 is transferred by GOAT to des-acyl ghrelin to generate acyl-ghrelin.

Cleaved amino acid

Amino acids differing between human and rodent ghrelin. Arginine11 – valine12 are found in human ghrelin whereas lysine11 – alanine12 are present in rodents. (Modified from Castañeda *et al.*, 2010:45).

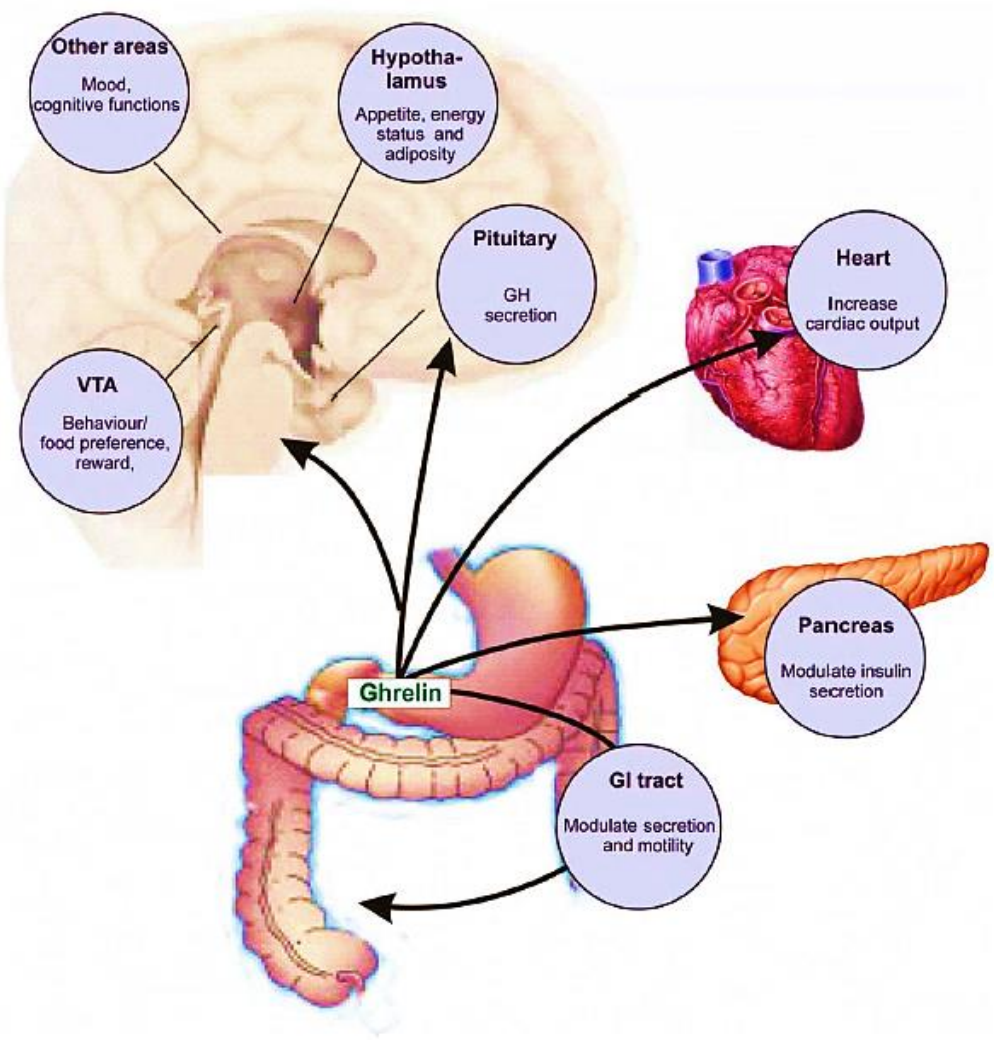

Figure 1.11 Image representing well-known functions of ghrelin. Ghrelin has been shown to have multiple physiological functions in brain, gut, cardiovascular system, pancreas, liver and bone. (Sivertsen *et al.*, 2013:1350). VTA = Ventral tegmental area.

The functions of ghrelin in the cardiovascular system vary from decreasing mean arterial pressure to improving chronic heart failure. For example, administration of ghrelin improved cardiac output, left ventricular function, increased muscle strength and decreased systemic vascular resistance (Nagaya *et al.*, 2004). In rats with myocardial infarction, frequency of arrhythmias was reduced by acute ghrelin treatment thereby improving survival (Schwenke *et*

al., 2008) Ghrelin has also been shown to activate extracellular signal-regulated kinases (ERK 1/2) and Akt in cardiomyocytes and endothelial cells resulting in inhibition of apoptosis (Baldanzi *et al*., 2002).

In the gastrointestinal tract peripheral administration of ghrelin increased gastric acid secretion (reviewed by Delporte, 2013). Similarly, it was reported that the central administration of ghrelin induced gastric acid secretion in fasted rats (Date *et al*. 2001), in contrast gastric acid secretion was found to be reduced in fasted mice in another study (Sibilia *et al*. 2002).

Some studies have indicated an anti-inflammatory function of ghrelin in a number of conditions including inflammatory bowel disease, sepsis, pancreatitis and arthritis (reviewed by Delporte, 2013). For example, pre-treatment with ghrelin prior to establishing experimental pancreatitis, showed improved blood flow to the pancreas, reduced IL-1β levels and stimulation of pancreatic cell proliferation (Warzecha *et al*., 2010). Furthermore, ghrelin mRNA was detected in a number of pituitary tumours such as prolactinomas suggesting a role for ghrelin in cancer growth and progression as reviewed by Pradhan *et al*. (2013). In the context of bone metabolism, ghrelin was found to increase proliferation and differentiation of osteoblasts *in vitro* and bone mineral density *in vivo* (reviewed by Delhanty *et al*., 2013).

Furthermore, ghrelin also regulates glucose homeostasis (reviewed by Alamri *et al*., 2016). For example, administration of ghrelin resulted in increased plasma glucose and reduced insulin levels in healthy humans (Broglio *et al*., 2001). Acute administration of ghrelin alone and in combination with arginine caused elevated glycaemia in young males with concomitant reduction in insulin secretion (Broglio *et al*., 2003). Similarly, in healthy humans, infusion

of ghrelin caused decreased acute insulin response to glucose suggesting that ghrelin modulates glucose tolerance in humans (Tong *et al*., 2010). In a study conducted in mice, inhibition of GOAT, enzyme responsible for acylation of ghrelin resulted in elevated plasma insulin and reduced blood glucose levels compared to control treated mice (Barnett *et al*., 2010). Here inhibition of GOAT in isolated pancreatic islets caused increased glucose-induced insulin secretion compared to control, suggesting a direct role for ghrelin in islet insulin secretion and therefore glucose homeostasis (Barnett *et al*., 2010).

In the pancreas ghrelin has been found to be produced in pancreatic epsilon cells by Wierup *et al*., (2002). However, Volante *et al*., (2002), showed that ghrelin is also expressed in beta cells by double insulin and ghrelin staining. Others reported ghrelin to be expressed in both alpha and beta cells (Kageyama *et al*., 2005) and in alpha cells only (Date *et al*., 2002). The role of ghrelin in the pancreas has been mainly focused on the regulation of insulin secretion from beta cells. In the clonal beta cell line INS-1 (832/13) glucose-induced insulin secretion was suppressed by ghrelin in a dose-dependent manner (Wierup *et* al., 2004). Simultaneous injection of ghrelin and glucose in oral glucose tolerance tests (OGTT) resulted in reduced insulin response and increased glucose responses in mice compared to controls without ghrelin. Here the use of a ghrelin receptor (GHSR1a) antagonist reversed the effects of ghrelin (Dezaki *et al*., 2004). Others however have found that 100 nM ghrelin enhanced insulin secretion in response to 20 mM glucose in isolated mouse islets (Salehi *et al*., 2004) suggesting that the function of ghrelin in the islets remains controversial.

As mentioned above, ghrelin has been reported to promote cell survival, proliferation and angiogenesis in a number of organs as well as in regulating insulin secretion in the pancreas, however its function in promoting beta cell and islet survival is not as well understood. Some studies have shown that ghrelin promoted survival in the INS-1E and HIT-T15 beta cell lines (Diaz-Ganete *et al.*, 2015; Zhang *et al.*, 2007). It has also been shown that ghrelin promoted beta cell and human islet proliferation and inhibited apoptosis in HIT-T15 cells and isolated human islets (Granata *et al.*, 2007) as further described in section 4.1.

Unacylated ghrelin (UAG) does not bind the known ghrelin receptor named growth hormone secretagogue receptor type 1A (GHSR1a), therefore initially it was considered to be inactive. However, subsequent studies have found that UAG prevents doxorubicin-induced apoptosis in cardiomyocytes (Baldanzi *et al.*, 2002) and to influences food intake signalling. In the hypothalamus UAG was found to induce food intake via a mechanism independent of GHSR1a/ neuropeptide Y (NPY) related signalling (Toshinai *et al.*, 2006). When administered peripherally, however, UAG decreased food intake in rats (Chen *et al.*, 2005). Others have found that co-administration of UAG with acyl-ghrelin opposed the increase in blood glucose seen when treated with acyl-ghrelin in humans (Broglio *et al.*, 2004). These studies suggest that unacylated ghrelin exerts effects that are common, opposite or independent of acyl-ghrelin. Since UAG does not bind GHSR1a, it is believed that its effects are mediated by an as-yet-unknown receptor (reviewed by Granata *et al.*, 2010).

1.3.2.2 Ghrelin receptor

Ghrelin is known to act via the GPCR GHSR1a (Fig. 1.12). Initially thought to be important for growth hormone release, GHSR1a is mainly known and characterised for promoting orexigenic effects via NPY/agouti-related peptide neurons in arcuate nucleus of hypothalamus (Sivertsen *et al.*, 2013). Alternative splicing of the *Ghsr* gene produces GHSR1b, a truncated receptor that shows opposing actions to GHSR1a (reviewed by Albarrán-Zeckler and Smith, 2013).

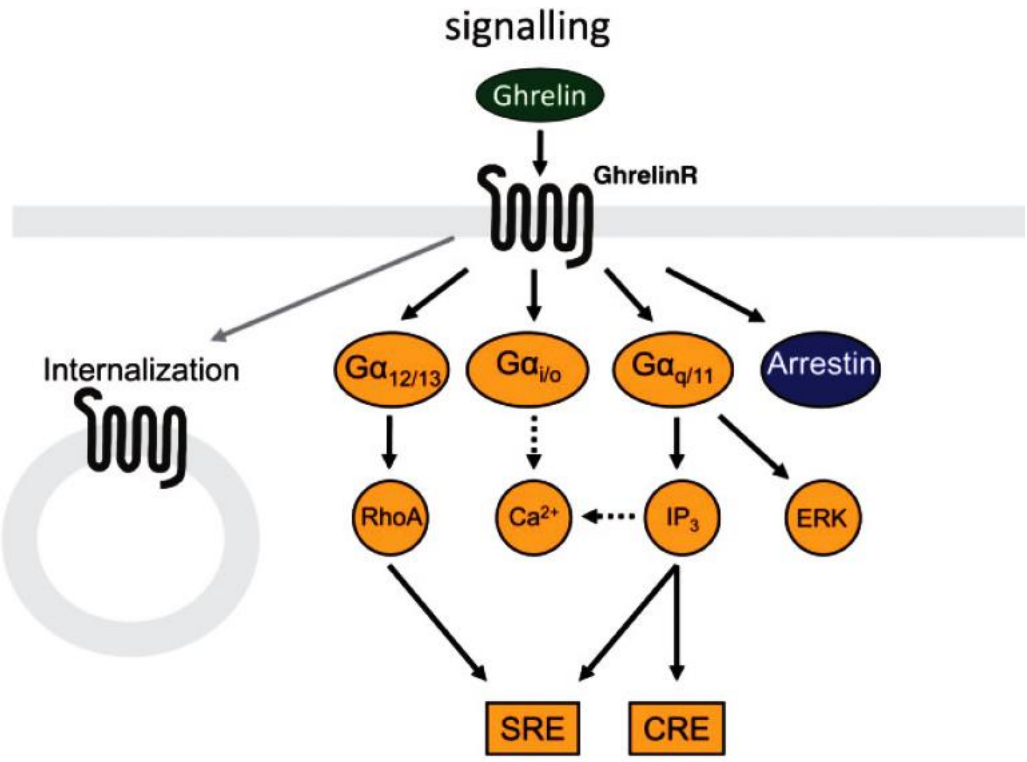

Figure 1.12 GHSR1a activates multiple signalling pathways. GHSR1a has been shown to signal via G-protein subunits: $G_{\alpha q}$, $G_{\alpha i}$ and $G_{\alpha 12/13}$ as well as via arrestin coupling. $G_{\alpha q}$ activates phospholipase C (PLC) and leads to increased inositol triphosphate (IP3) and diacylglycerol (DAG) thereby increasing Ca^{2+} signalling. This pathway can also activate ERK. $G_{\alpha 12/13}$ acts via RhoA leading to Serum response element (SRE) transcription. $G_{\alpha i}$ activation inhibits adenylate cyclase thereby reducing cAMP activation and reduction in Ca^{2+} signalling. (Sivertsen *et al.*, 2013:1352; Dezaki, 2013).

With the use of human embryonic kidney 293 (HEK293) cells over-expressing both receptor subtypes, it was found that GHSR1b dimerises with GHSR1a at the endoplasmic reticulum to regulate GHSR1a expression on the cell membrane (Chow *et al.*, 2012).

GHSR1a belongs to a small group of GPCRs that present constitutive activity including melanocortin receptor (MC4, Holst *et al.*, 2007) and the ghrelin receptor has been shown to present about 50% intrinsic constitutive activity (Damian *et al.*, 2011; Veldhuis and Bowers, 2010).

GHSR1a has been reported to be expressed in stomach, brain, pancreas, blood vessels, reproductive organs (testis and ovaries), intestines and heart (Gnanapavan *et al.*, 2002; Katugampola *et al.*, 2001; Ueberberg *et al.*, 2009). However, the localisation of GHSR1a within specific islet cell types remains disputed with some indicating that it is exclusively expressed on delta cells (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016) and others reporting its detection in alpha (Chuang *et al.*, 2011) and beta cells (Kageyama *et al.*, 2005) as further described in section 5.1 of this thesis.

There are a number of GHSR1a antagonists that can be used to investigate if the functional effects of ghrelin are mediated by the known ghrelin receptor, These include the synthetic compounds YIL781, JMV2959, GSK1614343 and a more recently identified endogenous compound named liver-expressed antimicrobial peptide 2 (LEAP2).

YIL781 or 6-[(4-fluorophenyl) oxy]-2-methyl-3-{[(3S)-1-(1-methylethyl)-3-piperidinyl]methyl}-4(3H)-quinazolinone was synthesised by Esler *et al.* (2007) and was reported to be a competitive antagonist of GHSR1a (Fig. 1.13).

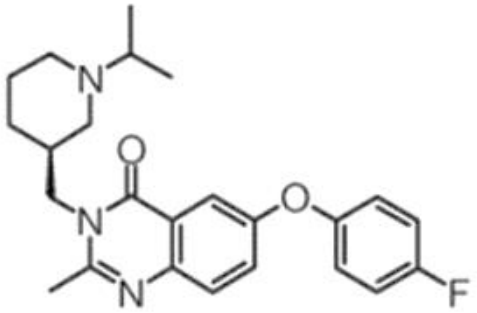

Figure 1.13 The structure of YIL781. A piperidine-substituted quinazolinone derivative, YIL781, was identified as a novel GHSR1a antagonist (Esler *et al.*, 2007).

In addition, the compound was selective to GHSR1a as it did not bind the closest homolog of GHSR1a – the motilin receptor. In the same study further analysis conducted using dispersed rat islets found that inhibition of insulin secretion by ghrelin was completely reversed by this compound. *In vivo* analysis showed that pre-administration of YIL781 to fasted rats caused a 23% decrease in blood glucose levels immediately after administration of glucose via stimulation of insulin secretion. This effect was similar to an incretin mimetic used as control (Esler *et al.*, 2007).

In a study testing the efficacy of a newly synthesized GHSR1a antagonist, YIL781 was used as a control and similar to observations by Esler *et al.*, YIL781 was shown to bind GHSR1a (Perdonà *et al.*, 2011). Others investigating the effect of a peptide encoded by the ghrelin gene known as obestatin on insulin secretion, found that obestatin increased insulin secretion in INS-1 cells that was reversed in the presence of YIL781 suggesting that obestatin acts via GHSR1a (Pradhan *et al.*, 2017).

A recent publication (Ge *et al.*, 2018) identified the peptide LEAP2 to be an endogenous irreversible antagonist of GHSR1a. LEAP2 is a 40-amino acid

peptide containing two disulphide bridges (Henriques *et al.*, 2010). The mature form of the peptide is identical in humans and mice. *In vivo* testing showed that LEAP2 antagonised the ghrelin-induced increase in growth hormone release and food intake (Ge *et al.*, 2018).

When LEAP2 was expressed in a mouse model of caloric restriction, mice lost approximately 28% of their body weight and their blood glucose levels dropped to below 30 mg/dL, suggesting hypoglycaemia, however, the effect on insulin secretion and regulation of beta cell mass was not investigated (Ge *et al.*, 2018). Being an endogenous compound, and as the number of commercially available GHSR1a antagonists is limited this discovery was of great interest for the current project as it was found to bind GHSR1a in an irreversible manner. Therefore, the GHSR1a antagonists YIL781 and LEAP2 were used in this study to identify the role of GHSR1a in mediating the effects of ghrelin on function and survival of clonal beta cells and primary mouse islets.

1.3.2.3 The role of ghrelin in diabetes

Due its association with insulin secretion and glucose homeostasis ghrelin has been reported to play a role in type 2 diabetes, however its role in obesity and beta cell function remain contested. A study looking at *Ghsr* null mice found that the mice showed resistance to diet induced obesity (Zigman *et al.*, 2005). In another study, however, ablation of ghrelin gene expression in leptin-deficient *ob/ob* mice did not revert their obese phenotype. Despite a lack of effect on obese phenotype, *Ghrelin*$^{-/-}$ mice displayed improved insulin secretion and peripheral insulin sensitivity resulting in reduced hyperglycaemia (Sun *et al.*, 2006). Others have shown that antagonising the action of ghrelin improved glycaemic control and prevented weight gain in rats

(reviewed by Poher *et al.*, 2018). Furthermore, transgenic mice overexpressing *Ghrelin* exhibited increased food intake, reduced glucose-stimulated insulin secretion and increased insulin tolerance (Bewick *et al.*, 2009). In contrast, in streptozotocin-induced diabetic rats treatment with ghrelin significantly elevated basal insulin secretion in isolated pancreatic fragments (Adeghate and Ponery, 2002). These studies confirm that alteration of ghrelin expression in mouse models of obesity and diabetes impacts parameters, which are important in diabetes, such as insulin secretion, insulin sensitivity and hyperglycaemia, however, findings are at times conflicting.

Some have reported that ghrelin inhibited apoptosis in beta cell lines and human islets in the presence of pro-inflammatory cytokines typical of type 1 diabetes (Diaz-Ganete *et al.*, 2015; Granata *et al.*, 2007). Since some pro-inflammatory cytokines are also causative factors of beta cell dysfunction and death in type 2 diabetes as described in 1.2.2, it is therefore possible that ghrelin could be employed to prevent this.

1.3.3 Interaction between somatostatin and ghrelin

As mentioned in 1.1.1 somatostatin inhibits both insulin and glucagon release in a paracrine manner in pancreatic islets, and ghrelin has also been shown to influence beta cell secretory function. Both ghrelin and somatostatin act on several common target tissues including the central nervous system and pancreatic islets (Barkan *et al.*, 2003), and a number of studies have pointed towards an interaction between the two hormones both centrally and in peripheral physiological functions. It has been reported that SST-14 and its analogue octreotide reduced plasma ghrelin levels in humans (Norrelund *et al.*, 2002). In another study continuous infusion of octreotide decreased

plasma ghrelin level in healthy men compared to saline infusion (Barkan *et al.*, 2003). Furthermore, in rats subcutaneous injection of octreotide decreased fasting plasma ghrelin levels, and in a dose-dependent manner in *ad libitum* fed rats (Silva *et al.*, 2005). In the same study treatment with octreotide for two weeks caused weight loss compared to saline treated controls. Shimada *et al.* (2003) also reported reduction in plasma ghrelin by SST. Here treatment with octreotide dose-dependently reduced ghrelin secretion in perfused rat stomach further suggesting the regulatory action of SST on ghrelin secretion in the stomach. These studies suggest that SST negatively regulates plasma ghrelin levels and stomach ghrelin secretion.

As described in 1.3.1 and 1.3.2 both SST and ghrelin regulate pancreatic islet function, however the role of ghrelin on SST secretion remains controversial. It has been reported that ghrelin suppressed SST secretion in perfused rat pancreata (Egido *et al.*, 2002). In contrast, findings by recent single-cell transcriptomic analyses showed that ghrelin stimulated SST release in islets, suggesting that the action of ghrelin is mediated by SST released by pancreatic delta cells (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). In both studies treatment with ghrelin stimulated SST secretion from delta cells, resulting in consequent reduction in insulin secretion in either perfused mouse pancreata (Adriaenssens *et al.*, 2016) or in isolated mouse and human islets (DiGruccio *et al.*, 2016). It is, therefore, possible that ghrelin and somatostatin may interact in promoting pancreatic islet survival. Having already identified a pro-survival role for somatostatin in pancreatic beta cells and islets under conditions typical of type 2 diabetes (Damsteegt *et al.*, 2019), the next step is to assess whether ghrelin may also modulate islet function under those

conditions, prior to addressing a potential interaction between the two hormones.

1.4 Cell models for the study of beta cell and islet function

Many factors limit the utilisation of primary pancreatic beta cells in biological research. These include the reduced availability of pancreatic tissue as well as technically challenging islet isolation and purification procedures and maintenance. In addition, conforming to the principles of the 3Rs (replacement, reduction and refinement) as set out by Russell and Burch (1959) is fundamental for ethical research. This has led to the development of cell lines derived from primary explants or dispersed cell suspensions (Skelin *et al.*, 2010). Others have developed pseudoislet models to study cell-cell interactions (Persaud *et al.*, 2010). Nonetheless primary islets still remain the gold standard for the study of their function (Balboa *et al.*, 2019) and are highly used in the study of diabetes mellitus. Some clonal beta cell lines and pseudoislet models are described below including the experimental model used in the current study.

1.4.1 Clonal beta cell lines

1.4.1.1 Mouse insulinoma-6 (MIN6 cells)

Mouse insulinoma 6 or MIN6 cell line was derived from transgenic mice expressing simian virus 40 (SV40)-T antigen/insulin promoter construct where primary beta cells were transformed into immortalised insulinomas (Miyazaki *et al.*, 1990). MIN6 cells express glucokinase and GLUT2 and respond to glucose at physiological levels similar to primary islets (Ishihara *et*

al., 1993). It has however been reported that their glucose responsiveness is reduced in later subculture passages (35-40) (Kayo *et al.*, 1996).

1.4.1.2 Insulinoma -1 (INS-1 cells)

The Insulinoma 1 or INS-1 cell line was developed by X-ray irradiation-induced formation of rat insulinomas. INS-1 cells express GLUT2 and glucokinase, have high insulin content (about 20% of primary beta cells) and have high insulin responsiveness to glucose at physiological levels (2.8-20 mM) (Asfari *et al.*, 1992). The main disadvantage of these cells is the use of 2-mercaptoethanol for culture that can result in protein denaturation, however it is important for INS-1 cell proliferation and function (Skelin *et al.*, 2010). In addition, similar to MIN6 cells, glucose responsiveness of INS-1 cells has been reported to be decreased in later passages (Hohmeier *et al.*, 2000).

1.4.1.3 Hamster islet transformed-tioguanine resistant clone-15 (HIT-T15)

HIT-T15 cells were produced by lentiviral expression of SV40-T antigen *in vitro* in isolated hamster pancreatic beta cells (Santerre *et al.*, 1981). These cells have membrane bound insulin granules that is a feature of native hamster beta cells. However, they contain 2.5-20 times less insulin content than native cells. HIT-T15 cells secrete insulin in response to numerous stimuli including glucose, glucagon and pharmacological agents. With increase in passage number from 41 to 88 insulin secretion decreased by 30% (Santerre *et al.*, 1981).

1.4.1.4 EndoC-βH1 cells

EndoC-βH1 cells are a transformed human beta cell line derived from transduction of human pancreata and islets with a lentiviral expressing SV40 large T antigen (SV40LT) (Ravassard *et al.*, 2011). The transduced tissues were transplanted under the kidney capsule of severe combined immunodeficient (SCID) mice to allow differentiation of islet tissue. The resulting highly vascularised and differentiated tissue was isolated and following dissociation into clusters, were re-transduced with a lentiviral vector expressing human telomerase reverse transcriptase (hTERT) to achieve immortalisation of cells. After a second round of transplantation for further tissue expansion secondary insulinomas were isolated (Ravassard *et al.*, 2011). The expression of insulin mRNA is about 40% and insulin content is less than 10% in EndoC-βH1 cells compared to native islets. These cells are responsive to glucose and other insulin secretagogues such as sulfonylurea drugs and exendin-4 (Ravassard *et al.*, 2011; Weir and Bonner-Weir, 2011). Although other human pancreatic beta cell lines have been developed before, namely CM, TRM1 and βlox5, these cells were either non-responsive to glucose or had low insulin content (Skelin *et al.*, 2010).

1.4.2 Pseudoislets

Pseudoislets are aggregates of one or more types of islet cells that constitute islet-like structures in culture (Kelly *et al.*, 2011). They were first reported in 1980 when culture of single islet cells obtained following digestion of a dog pancreas resulted in the formation of cell aggregates after 4-8 days (Scharp *et al.*, 1980). Usually pseudoislets are used to study structure-function relationships between islet cells. Using a pseudoislet model consisting of

clusters of beta cells only, it has for example been shown that beta to beta cell interaction resulted in enhanced secretory responses to nutrient stimuli compared to a model using a single layer of beta cells (Hauge-Evans *et al.*, 1999). Similarly, heterotypic pseudoislets comprised of three islet cell types (beta, delta and alpha) have been found to display a better secretory response to glucose and other insulin secretagogues compared to monolayer cells (Kelly *et al.*, 2011). In addition, pseudoislets constitute an especially attractive approach to increase islet availability for transplantation in patients with type 1 diabetes mellitus since this process is greatly hindered by the lack of adequate donor islets (Kojima *et al.*, 2014). However, their use has clear limitations in this context.

The research model adopted in the current investigation is based on the experimental use of rodent pancreatic beta cell lines and primary pancreatic mouse islets and aimed to assess pancreatic islet function and potential targets to promote beta cell and islet survival.

1.5 Aims of the thesis

This study aims to explore the roles and the molecular mechanisms underlying the action of somatostatin and ghrelin in clonal beta cell and pancreatic islet function, with particular focus on apoptosis induced by stressors typical of type 2 diabetes. Therefore, the following aims were investigated as part of this thesis.

1. To investigate the role of somatostatin and SST receptor 3 in the modulation of clonal beta cell function in response to cellular stressors typical of type 2 diabetes.
2. To explore the role of ghrelin in clonal beta cell and primary mouse islet function in the presence of cellular stressors typical of type 2 diabetes.
3. To evaluate the role of GHSR1a in mediating the functional effects of ghrelin in clonal beta cells and mouse islets.
4. To assess the roles of GHSR1a-independent factors in mediating the functional effects of ghrelin in clonal beta cells and mouse islets.

CHAPTER 2 - Methods

The research model used in this study aimed to investigate pancreatic islet function and survival to identify and assess potential targets to promote beta cell and islet survival. It was based on the experimental usage of rodent pancreatic beta cell lines and primary pancreatic mouse islets. Cell lines were maintained in culture at facilities available at the University of Roehampton, whereas the primary mouse islets were obtained through collaboration with Dr Aileen King, King's College London (KCL). Laboratory mice of the CD-1® strain were supplied by Charles River Laboratories International Inc, UK and were housed at the animal facilities of KCL.

2.1. Cell culture of pancreatic beta cell lines

As mentioned in 1.4 due to limitations in usage of primary pancreatic islets in biological research, cell lines have been developed as models. Cell lines constitute a model with numerous advantages such as the ready availability of cells, reduced cost, no requirement of an animal house, tissue consisting of one cell type only which can be easily manipulated genetically (Kaur and Dufour, 2012; Skelin *et al*., 2010; Ulrich *et al*., 2002). Most importantly, in this study cell lines were used for screening and optimisation purposes to reduce animal usage. However, cell lines have the disadvantage of loss of phenotype/function over time and one cell type does not constitute an ideal physiological model for pancreatic islets. Therefore, it was imperative to repeat the results of cell line work using primary islets (Kaur and Dufour, 2012; Skelin *et al*., 2010; Ulrich *et al*., 2002).

2.1.1 MIN6 cell culture

The MIN6 cells used in this study were kindly provided by Prof. J-I Miyazaki (University of Tokyo, Japan). Cells were maintained in Dulbecco's modified

eagle's medium (DMEM) containing 4500mg/L glucose and 4 mM L-glutamine, additionally supplemented with 10% v/v foetal bovine serum (FBS), 2 mM L-glutamine and penicillin (100 U/ml)/ streptomycin (0.1 mg/ml). The cells were maintained in culture in T75 flasks in a humidified incubator at 37 ºC and 5% CO_2, and when 70-80% confluent were spilt using 0.05% w/v trypsin- ethylenediaminetetraacetic acid (EDTA) and re-cultured every week up to passage number 40 or cryopreserved.

2.1.2 INS-1 cell culture

The INS-1 cell line used in this study was kindly provided by Dr Natasha Hill (Kingston University London, UK). The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 11.1 mM D-glucose and 2.1 mM L-glutamine. The medium was additionally supplemented with 10% v/v FBS, 2 mM L-glutamine, 10 mM HEPES buffer (pH 7.2-7.5), 1 mM sodium pyruvate, penicillin (100 U/ml)/ streptomycin (0.1 mg/ml) and 0.05 mM 2-mercaptoethanol (2-ME). The cells were grown in T75 flasks in a humidified incubator at 37 °C and 5% CO_2. Cell were passaged at about 80% confluency using 0.05% w/v trypsin-EDTA for a maximum of 10-12 passages or cryopreserved.

2.1.3 Splitting clonal beta cells and counting

Following removal of old media, cells were washed in 1x phosphate buffered saline (PBS, pH 7.4) not containing $CaCl_2$ and $MgCl_2$ and incubated with 0.05% w/v trypsin-EDTA for 3 min to dislodge the cells from the flask. Trypsin was neutralised by adding media containing serum and the cells were sub-cultured as necessary.

When plating for experiments, cells were counted prior to adding to the plates or dishes. For this the cell suspension was centrifuged at 1500 rpm (MIN6) and 1000 rpm (INS-1) for 3-4 min respectively once trypsin was neutralised. The pellet was re-suspended in 1 ml of media and 10 µl of cell suspension was added to 190 µl of PBS in a 1.5 ml tube at 1:20 dilution. Cells were counted manually using a Neubauer haemocytometer (Hawksley, UK) where only live cells that appeared bright and intact were included in the count. The total cell number was estimated using the following equation:

$$\frac{Cells}{ml} = Average\ cell\ count \times 10^4 \times dilution\ factor$$

2.1.4 Cryopreservation and thawing of beta cell lines

Long-term storage of cells was obtained by preservation of cells in liquid nitrogen (LN2) at -196 °C. Cells were trypsinised at ~80% confluency, resuspended in respective fully supplemented media and pelleted at 1000 rpm for 3 min. Cell pellets were re-suspended in media with dimethyl sulfoxide (DMSO) added as cryoprotectant (MIN6: 95% complete DMEM and 5% DMSO and INS-1: 90% complete RPMI and 10% DMSO) at a seeding density of approximately 3 x10^6 cells/ml. 1.5ml of cell suspension was added to 2 ml cryovials and placed in a Mr Frosty freezing container (Nalgene, ThermoFisher Scientific, UK) containing 250 ml 98-99% isopropyl-alcohol for 24 h at -80 °C. The presence of DMSO prevents formation of ice crystals whereas isopropyl alcohol in Mr Frosty ensures that the cells are frozen down gradually at a rate of 1 °C/min to maintain cell viability.

Cryopreserved cells were defrosted by quickly thawing in a water bath at 37 °C and transferring gradually to a 15 ml tube containing 5 ml of complete media. The cells were then pelleted at 1000 rpm for 3 min, re-suspended in fresh fully supplemented media and transferred to a T75 flask. Cells were allowed to grow for 10 days with media refreshed every three days prior to splitting.

2.2 Islet isolation from mice

The islet isolation procedure followed in this study was granted approval by the University of Roehampton ethics committee (Ref: LSC 17/ 195). Male or female CD-1® mice aged 6-10 weeks supplied by Charles River, UK, were humanely killed by cervical dislocation in accordance with UK Home Office regulations (Schedule 1, section 6 of Guidance on the Operation of the Animals Act, 1986). The Schedule 1 killing and islet isolation procedures were conducted at a designated licenced animal facility at King's College London. Isolated islets were transported to Whitelands College, University of Roehampton, in a tightly sealed container. The transportation process was assessed as non-hazardous to the general public (Risk Assessment no. RISK_LSC_00365).

2.2.1 Surgical procedure

Prior to commencing surgery collagenase from *Clostridium histolyticum* was prepared at 1 mg/ml in Eagle minimal essential medium (MEM) and placed on ice. Mice were sacrificed by cervical dislocation and the abdominal cavity was surgically exposed. The sternum was clipped, liver was flipped over and covered with a delicate task wipe to expose the common bile duct and the mouse was placed under a dissecting microscope. Vater's ampulla was

clamped (Fig. 2.2.1) and 2.5 ml of 1 mg/ml collagenase was injected at a steady pace into the common bile duct. The pancreas was removed surgically and placed in a 50 ml tube on ice. Up to three pancreata were added per tube.

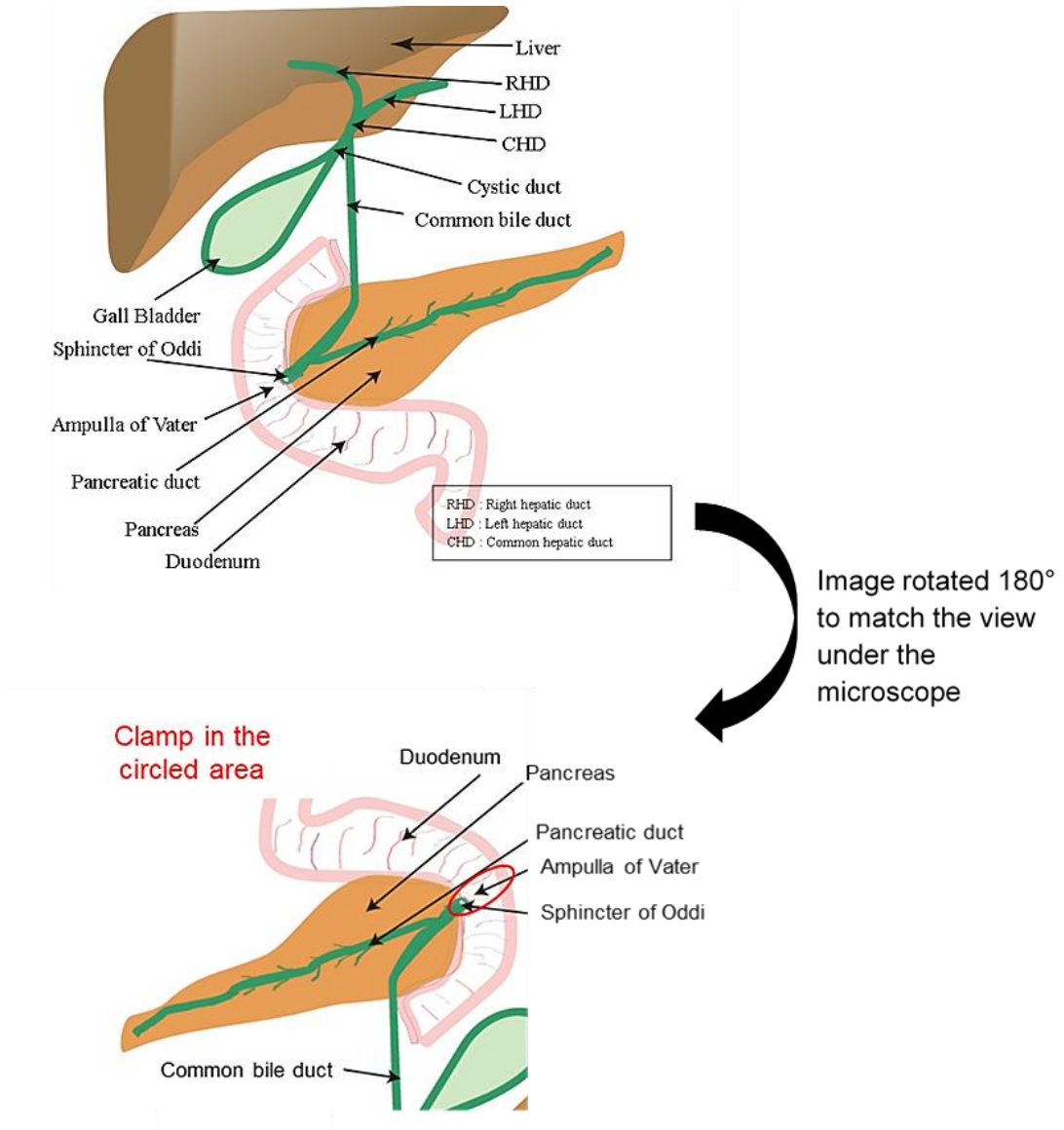

Figure 2.2.1 Clamping of Vater's ampulla during islet isolation. Prior to injection of collagenase the pancreatic duct is clamped at Vater's ampulla to prevent the collagenase from leaking into the duodenum and to ensure maximum distension of the pancreas. (Modified from Hundt and Young, 2019).

2.2.2 Pancreas digestion and purification of islets

Tubes containing isolated pancreata were incubated for 10 min at 37 °C in a water bath to allow digestion of exocrine tissue by collagenase. During incubation MEM media was supplemented with 10% v/v new-born calf serum (NCS), penicillin (100 units/ml) and streptomycin (100 µg/ml). Upon completion of incubation, tubes containing digested pancreata were transferred onto ice and 25 ml of supplemented MEM was added to stop any further digestion. Then the tubes were vigorously shaken by hand to separate digested pancreatic tissue and centrifuged at 1400 rpm for 1.15 min at 10 °C. Following careful removal of supernatant each pellet was re-suspended in 25 ml of supplemented MEM, tubes were vortexed to disrupt the pellet and were centrifuged. This step was repeated two more times. Following final wash, the newly re-suspended pellets were sieved through a 425 mm sieve and a funnel into new 50 ml tubes. The tubes were centrifuged for 1.5 min at 1500 rpm at 10 °C. Next the supernatant was removed, tubes were kept inverted and any medium left on the wall of the tubes was removed with a delicate task wipe without disturbing the pellet. Then the pellet was re-suspended in Histopaque-1077 by vortexing and 10 ml of fresh supplemented MEM was added very gently using the G setting of the auto-pipettor in order to allow formation of density gradient interface between cell suspension in histopaque and media. The tubes were centrifuged at 3510 rpm for 24 min and without breaking at 10 °C. Following centrifugation islets were removed from the interface and placed in a new 50 ml tube and topped up with MEM up to 50 ml mark and centrifuged at 1500 rpm for 1.5 min at 10 °C. The centrifuged tubes were placed on ice for 4-5 min to allow sedimentation of islets, 25 ml media was removed from the top, fresh media was added up to 50 ml mark and

centrifuged. This step was repeated a further three times and pelleted islets in 20 ml of media were transferred to a non-tissue culture treated 90 mm petri dish. Islets were handpicked into a new petri dish and washed 3-4 times with 10 ml fresh RPMI supplemented with 10% v/v FBS, penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Following purification, isolated tissue was transported in supplemented RPMI in 50 ml tubes placed in a wet ice box and upon arrival at the University of Roehampton, cultured in RPMI (Gibco, UK) containing 11 mM glucose and 2.1 mM L-glutamine, additionally supplemented with 10% v/v FBS, 2 mM L-glutamine and penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Mouse islets were maintained in culture at 37 ºC and 5% CO_2 in a designated humidified incubator.

2.3 Haematoxylin and Eosin staining

Haematoxylin and eosin staining of vaginal smears were performed to determine the oestrus cycle stage of the female mice used in the current study. This was conducted in collaboration with Drs Natasha Hill and Amanda Dandagama at Kingston University London.

Prior to islet isolation procedures female mice were housed for two weeks to allow synchronisation of their oestrus cycle. Following removal of the pancreas a vaginal smear was taken from female mice and placed on a glass slide. The smears were fixed in 3.7% formaldehyde in PBS for 15 min then washed twice with PBS and left to dry. The fixed smears were first hydrated in a series of industrial methylated spirit (IMS) washes (2x 100% IMS and 2x 70% IMS) each for 3 min. The hydrated slides were washed three times in dH_2O for two minutes each and stained with haematoxylin, Gill no 2 (Sigma cat no. GHS232) for 8 min. The slides were washed three times in running

dH_2O and stained with eosin (Sigma cat no. HT110116) for 2-3 min and washed again three times in running dH_2O. The stained slides were dehydrated in a series of IMS washes (2x 70% IMS and 2x 100% IMS) each for 3 min. Coverslips were added on the stained samples using one drop of mountant (Vectamount H5000, Vector Labs) and secured with nail varnish. The slides were imaged using a Nikon Eclipse NI-E microscope (Nikon, Japan; model no. 920529) and NIS-Elements imaging software.

The Oestrus cycle stages were determined according to (Caligioni, 2009) as follows:

- Pro-oestrus: predominantly consists of single or clustered nucleated epithelial cells. Occasionally, cornified epithelial cells can also be detected. Here the mice are at pre-ovulatory stage.
- Oestrus: This stage is identified by the presence of distinctive clusters of cornified epithelial cells. The irregular cytoplasm is granular with no apparent nucleus. Oestrogen (E2) levels are elevated during the morning and then decrease to basal levels.
- Metestrus: This stage is characterised by a mixture of leukocytes and a small number of epithelial cells that can be either nucleated or cornified. E2 concentration in plasma is low.
- Dioestrus: Leukocytes are predominant in dioestrus stage where E2 levels begin to increase.

2.4 Measurement of hormone secretion

Insulin secretion from beta cell lines and islets and ghrelin secretion from islets was determined by static incubation experiments. For this clonal beta cells or islets were continuously incubated in a set volume of buffer containing treatments as indicated in the respective results chapters for a set period of time during which the buffer was neither removed nor replaced.

2.4.1 Static incubation experiments using MIN6 cells

During static incubation experiments, clonal beta cells were incubated in 1x Gey and Gey (G&G) buffer, pH 7.4, with either 2 mM or 20 mM glucose with or without any insulin secretagogues and/or compounds of interest. G&G buffer was prepared as a 2x solution, pH 8.4 (Table 2.4.5.1) and stored at 4 °C. For static secretion experiments 1x G&G buffer was first prepared by diluting 2x buffer 1:1 with distilled water and pH was adjusted to pH 7.4 with CO_2. The buffer was then supplemented with 2 mM $CaCl_2$ and 0.5 mg/ml bovine serum albumin (BSA).

MIN6 cells were plated at 20,000 cells/wells in clear 96 well plates in complete DMEM and incubated in a humidified incubator at 37 °C and 5% CO_2 for 48 h to allow the cells to adhere. Following incubation the cells were washed twice with 200 µl of 2 mM glucose G&G buffer per well and incubated with 2 mM glucose G&G for 2 h at 37 °C to lower the insulin secretion to basal levels. At the end of the incubation G&G buffer was aspirated and treatments were added at 200 µl/well and the plates were incubated for 1 h at 37 °C. Finally, 160 µl of supernatant from each well was transferred to a new 96 well plate and stored at -20 °C or diluted for determination of insulin content by radioimmunoassay.

2.4.2 Static incubation experiments using mouse islets

2.4.2.1 Insulin secretion

Isolated mouse islets cultured for two days in fully supplemented RPMI were washed three times with 2 mM glucose G&G buffer and incubated for 1 h at 37 °C in a humidified incubator with 5% CO_2. Following incubation 3-5 islets were picked and transferred into 1.5 ml tubes placed on ice containing 400 µlof agents of interest prepared in G&G buffer containing either 2 or 20 mM glucose. Islets were then incubated in a water bath at 37 °C for 1 h after which the tubes were transferred on to ice and centrifuged at 4°C for 1 min at 2000 rpm. Up to 350 µl of supernatant was transferred to new 1.5 ml tubes and either stored at -20 °C or diluted as required for assessment of insulin content by radioimmunoassay.

2.4.2.2 Ghrelin secretion

Isolated mouse islets cultured for two days in fully supplemented RPMI were washed three times with 11 mM glucose G&G buffer and incubated for 1 h at 37 °C. Following incubation 25-30 islets were picked into 1.5 ml tubes placed on ice containing 300 µl of treatment prepared in G&G buffer containing either 2 or 11 mM glucose. Islets were then incubated in a water bath as described above. Following centrifugation up to 260 µl of supernatant was transferred to new 1.5 ml tubes and either stored at -80 °C or diluted as required for assessment of ghrelin content by radioimmunoassay.

2.4.3 Extraction of, MIN6 cell, islet and stomach tissue contents for measurement of ghrelin

Following static incubation and removal of supernatants, islets were pelleted and washed three times in ice cold PBS. Islet pellets were frozen at -80 °C until

extraction of hormone contents. For MIN6 cells, $5x10^6$ cells were transferred into a 1.5 ml tube following trypsinisation and counting. The cells were pelleted by centrifugation at 1500 rpm for 3 min, the supernatant removed and the cells were re-suspended in ice cold PBS. This step was repeated a further two times to wash the cells, and pellets were frozen at -80 °C until content extraction.

Following isolation of the pancreas, stomach was removed and cut around the area delineated by a dashed line indicating the fundus (Fig. 2.4.1). The tissue was cleaned by removing any solid contents, washed with ice cold PBS and kept frozen at -80 °C. Approximately 3-4 mg of fundus was cut on the day of content extraction.

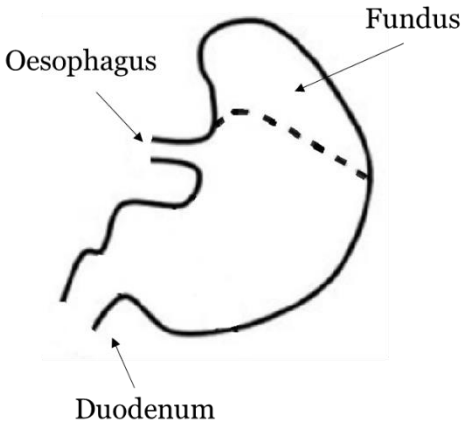

Figure 2.4.1 Diagram of mouse stomach with fundus delineated. Stomach was cut around the area indicated by the dashed line to isolate the fundus (modified from Rolig *et al.*, 2012: 3714).

Since the cell/tissue contents were tested for the presence of ghrelin, 0.5 M acetic acid was used for hormone extraction. Briefly, 0.5 M acetic acid in dH_2O was warmed for 5 min at 100 °C and added to cell/tissue pellets. Samples were

boiled at 100 °C for 15 min and transferred onto ice for further 10 min. Samples were centrifuged for 5 min at 5000 rpm at 4 °C and the supernatants were transferred into new 1.5 ml tubes. Samples were kept frozen at -80 °C until assessment of hormone content by radioimmunoassay.

2.4.4 Radioimmunoassay

Radioimmunoassay (RIA) is a competitive binding assay that relies on the binding of radiolabelled (Ag*) and unlabelled (Ag) antigen to an antibody (Ab) and allows the detection of low concentrations of antigen in a sample (Goldsmith, 1975). The assay is performed by incubating fixed amounts of Ag* and Ab with samples containing Ag (standards or unknown samples). These complexes are allowed to reach equilibrium for 48-72 h at 4 °C.

$$Ab + Ag + Ag^* \quad \longleftrightarrow \quad Ab{:}Ag + Ab{:}Ag^* + Ag + Ag^*$$

2.4.4.1 Insulin radioimmunoassay

Insulin content of the supernatants was assayed using an in-house radioimmunoassay (Jones *et al.*, 1988). Insulin was radiolabelled with radioactive iodine (^{125}I) to make Ag* by Dr Astrid Hauge Evans as previously reported (Salacinski *et al.*, 1981). Insulin antibody (Guinea pig polyclonal to insulin) was kindly provided by Prof. Peter Jones (King's College London). Insulin from bovine pancreas (Sigma, UK) was prepared as a 10 ng/ml stock. Borate buffer, pH 8.0 (Table 2.4.5.2) was used in this procedure.

Static insulin secretion samples were diluted in borate buffer as needed to obtain concentrations within the range of the standard curve and a series of doubling dilutions of insulin stock was prepared (0.0-10 ng/ml) to construct a

standard curve. Tracer only (T), non-specific binding (NSB) and maximum binding (MB) were defined as reference samples. The Insulin antibody stock was further diluted to obtain a final dilution of 1/200,000. Insulin tracer was diluted to obtain 10,000 cpm per 100 ul/tube. Insulin standard was diluted in LP3 tubes with a final volume of 100 µl in triplicates and 100 µl of diluted samples were added in duplicates to LP3 tubes. Diluted antibody (100 µl) was added to all tubes except for T and NSB whereas tracer (100 µl) was added to all tubes with a final volume of 300 µl in all tubes except T that contained 100 µl of tracer only (Table 2.4.1). The tubes were incubated at 4 °C for 48-72 h to allow formation of antigen-antibody complexes. Polyethylene glycol (PEG) was prepared as a 30% w/v solution (Table 2.4.5.3) and 1 ml of precipitation solution (Table 2.4.5.4) was added per tube at a final concentration of 12 % v/v PEG to precipitate the insulin and antibody complexes formed during incubation. The tubes were centrifuged at 2700 rpm for 15 min at 4 ºC. Supernatants were aspirated and radioactive counts per minute of the precipitate were detected using a Perkin Elmer Gamma Counter (2470-0020). The machine is equipped with WorkOut plus® (My Assays) software that generated a standard curve (Fig. 2.4.2) and calculated the concentration in each sample by interpolating from the standard curve according to a pre-set protocol.

Table 2.4.1 Experimental set up of RIA

Condition	Borate buffer	Antibody	Tracer	Standard	Sample
Non-specific binding	200 µl		100 µl		
Maximum binding	100 µl	100 µl	100 µl		
Totals			100 µl		
Standards		100 µl	100 µl	100 µl	
Samples		100 µl	100 µl		100 µl

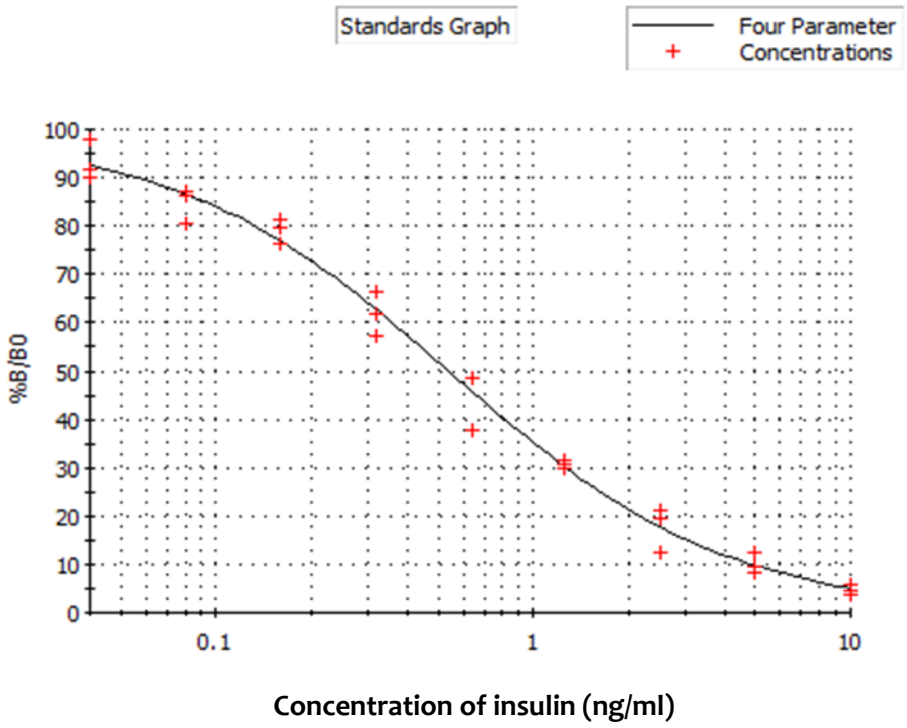

Figure 2.4.2 Example standard curve of insulin RIA.

2.4.4.2 Radioimmunoassay for ghrelin

The ghrelin radioimmunoassay used in this study was developed in-house using the existing protocol for insulin RIA and the method previously used by Dr Michael Patterson with the same antibodies against ghrelin (Patterson *et al.*, 2005). Although others have reported the employment of RIA for detection of ghrelin this was conducted mainly for tissues such as stomach and whole pancreas but not beta cells alone. Only one group reported the detection of islet ghrelin utilising an in-house RIA (Dezaki *et al.*, 2004). However, the method of islet sample preparation was not provided, and the RIA method was based on the use of anti-sera against ghrelin and employed an alternative assay set up and incubation times (Hosoda *et al.*, 2000) to the ones used by us.

Acyl-ghrelin (Tocris) was iodinated by Dr Michael Patterson using the same procedure as for the insulin iodination, but with 0.06 M phosphate buffer, pH 7.2 (0.0001% v/v Tween®20, Table 2.4.5.5). Ghrelin standard was prepared at a concentration of 100 pmol/ml and diluted to a final concentration of 0.0-5.0 pmol/ml for acyl-ghrelin and 0.0-2.5 pmol/ml for total ghrelin. Dilutions of Gey and Gey buffer and acetic acid were added as assay controls to check for non-specific binding. Antibody to acyl-ghrelin rabbit polyclonal (G0-1) was kindly provided by Prof M. Ghatei (Imperial College, London) and antibody to total ghrelin goat polyclonal was obtained from Santacruz (C-18, cat no SC-10368).

As with the insulin RIA, a standard curve was prepared in triplicate and with diluted samples in duplicates. The radiolabelled tracer was diluted to a final radioactivity of 3500-4000 cpm/tube and the antibodies diluted 1:5000 (G-01) and 1:1500 (C-18), respectively.

The tubes were incubated for at least 72 h at 4 °C prior to precipitation as described in 2.4.4.1. Supernatants were aspirated and the radioactivity of samples determined on the Perkin Elmer Gamma Counter with a pre-set protocol generating a standard curve (Fig. 2.4.3) and calculating concentrations in samples.

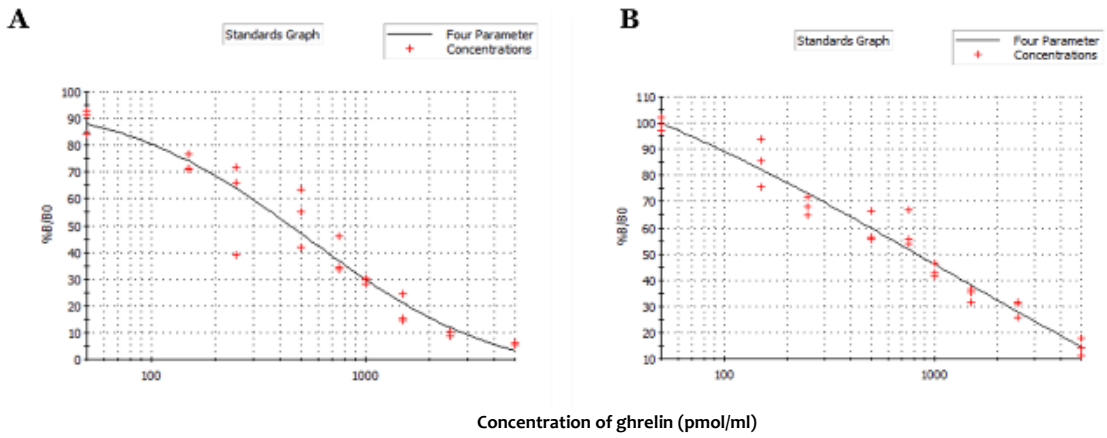

Figure 2.4.3 Example radioimmunoassay standard curves for acyl-ghrelin (A) and total ghrelin (B).

2.4.5 Reagents

Static incubation experiments

Static insulin and ghrelin secretion experiments were conducted in Gey and Gey buffer (Table 2.4.5.1). The indicated reagents were dissolved in dH_2O and pH was adjusted to 8.4 and the buffer stored at 4 °C.

Table 1.4.5.1 2x Gey and Gey buffer, pH 8.4 for 2 L

Reagent	Mass (g) for 2 L	Final concentration (mM)
NaCl	26	111.23
KCl	1.48	4.96
$NaHCO_3$	9.08	27.02
$MgCl_2.6H_2O$	0.84	1.03
KH_2PO_4	0.12	0.22
$MgSO_4.7H_2O$	0.28	0.28

Radioimmunoassay

Borate buffer

Insulin secretion experiment samples were diluted and radioimmunoassay was set up utilising borate buffer (Table 2.4.5.2). First boric acid, NaOH and EDTA were dissolved in dH_2O and the pH of the solution was adjusted to 8.0. Finally, BSA was added to the solution and the buffer subsequently stored at 4 °C.

Table 2.4.5.2 Borate buffer for insulin RIA, pH 8.0 for 2 L

Reagent	Mass (g) for 2 L	Final concentration
Boric acid	16.5	133 mM
NaOH	5.4	67.5 mM
EDTA	7.4	10 mM
BSA	2.0	1 g/L

30% PEG solution

A 30% PEG solution was prepared by gradually dissolving PEG in 1 L of distilled water and then topping up with dH_2O up to 2 L (Table 2.4.5.3). The solution was stored at 4 °C.

Table 2.4.5.3 30% PEG solution

Reagent	Amount
PEG (MW 6000)	600 g
Distilled water	2 L

RIA precipitation solution

To prepare a 12% PEG RIA precipitation solution, gamma globulins were fully dissolved in PBS at 1mg/ml and an equal volume of 30% PEG was added. Finally, Tween 20 was added at 0.5 µl/ml (Table 2.4.5.4). The solution was kept at constant agitating until use.

Table 2.4.5.4 RIA precipitation solution

Reagent	Amount for 200 ml
30% PEG	100 ml
1x PBS	100 ml
Gamma globulins	200 mg
Tween 20®	100 µl

0.06 M Phosphate buffer

Ghrelin secretion experiment samples and extracted hormone contents were diluted and radioimmunoassay was set up in phosphate buffer (Table 2.4.5.5). The reagents were dissolved in dH_2O and the pH of the solution was adjusted to 7.2 and stored at 4 °C.

Table 2.4.5.5 Phosphate buffer, pH 7.2

Reagent	Mass (g) for 1 L	Final concentration
Na_2HPO_4	9.0	63.4 mM
KH_2PO_4	0.826	6.1 mM
$C_{10}H_{14}H_2O_8N_2 \cdot 2H_2O$	3.722	11.34 mM
BSA	3	3 g/L

2.5 Measurement of cell viability

The MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was utilised in this study for assessment of cell viability. This type of colourimetric assay is based on the colour change caused by reduction of tetrazolium salts to formazan by viable cells with active metabolism (Riss *et al.*, 2004). It is a cost-effective assay which has been widely used for testing of cell viability and cytotoxicity for many years (Mosmann, 1983) including studies investigating beta cell and islet survival (Diaz-Ganete *et al.*, 2015; Granata *et al.*, 2007; Inberg and Linial, 2010). In this study the cells were plated in clear 96-well plates, pre-incubated as indicated in chapter 4 and exposed to 0.5 mM palmitate for 20 h. Media was aspirated and MTT prepared in a physiologically balanced solution of 0.5 mg/ml in 1x PBS was added to cells and plates were incubated for 3 h at 37 °C. Formazan crystal formation was confirmed under the microscope and crystals were dissolved using DMSO. Absorbance was recorded at 570 nm using a Multiscan EX plate reader (Thermo Scientific, UK).

The MTT assay does not, however, assess the underlying cause of changes in cell viability (i.e. proliferation and/or programmed and un-programmed cell death). Therefore, CaspaseGlo assay (Promega, UK) that detects changes in the enzymatic activity of caspases 3 and 7 was used as a measurement of apoptosis as described in 2.6.

2.6 Measurement of Apoptosis

Apoptosis is a form of programmed cell death that occurs in response to a number of stimuli such as DNA damage, exposure to irradiation and cytotoxic

drugs. It is an energy-dependent process that is orderly executed via a pathway conserved among species (Elmore, 2007) and predominantly relies on the activation of cysteine aspartic acid-specific protease (caspase) members that are divided into initiators and effectors (Shi, 2004).

Promega Caspase-Glo® 3/7 assay was used to determine cell apoptosis in this study. It measures the activity of caspase -3 and -7 that are key effectors of mammalian cell apoptosis (Shi, 2004). The assay reagent contains a luminogenic tetrapeptide sequence (DEVD-aminoluciferin), a proprietary thermostable luciferase and a lysis agent. Following cell lysis the released caspases -3 and -7 cleave DEVD-aminoluciferin substrate to free aminoluciferin that in the presence of ATP and O_2 reacts with luciferase generating light (Fig. 2.6.1).

The luminescent signal is measured on a luminometer (Promega GloMax Navigator) and is directly proportional to the activity of caspase -3 and -7.

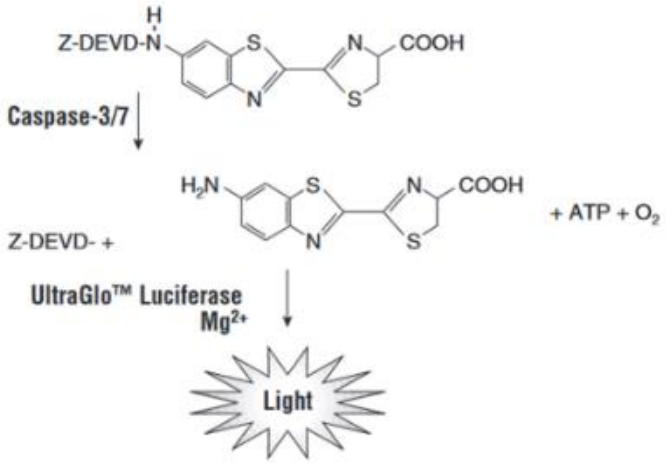

Figure 2.6.1 Caspase-3/7 cleavage of luminogenic substrate containing the DEVD sequence. Following cleavage by caspase, a substrate for luciferase (aminoluciferin) is released. This results in the luciferase reaction and the production of light that is measured using a luminometer (Promega, 2019:2).

2.6.1 Measurement of apoptosis in beta cells

Beta cells were trypsinised and seeded into white-walled 96 well plates. Following treatment, the cells were exposed to cellular stressors as indicated (100 µl medium/well) for 20 h at 37 ° C and 5% CO_2. On the day of the assay, 75 µl of medium was removed and 25 µl of re-constituted assay buffer was added to each well to provide a 1:1 ratio of medium and buffer. The plate was covered with foil, shaken for 5 min at 300 rpm and subsequently incubated for 10 min at room temperature (RT) prior to reading.

2.6.2 Measurement of apoptosis in islets

Islets were pre-treated with agents of interest in 6 cm dishes and picked into a white-walled 96 well plate (5 islets/well) in 40 µl of medium with or without 1000 U/ml tumour necrosis factor alpha (TNF-α) and 50 U/ml IL-1β. The islets were incubated for 20 h at 37° C and 5% CO_2 after which 40 µl of assay reagent was added to each well after which the experimental protocol proceeded as described in 2.6.1.

2.7 Determination of gene expression

2.7.1 Total RNA extraction

2.7.1.1 Cell line and tissue lysis

- MIN6 cells were trypsinised, washed in ice-cold PBS and pelleted. The cell pellets were lysed with 350 µl RLT® buffer containing 10 µl/ml 2-ME.
- Mouse islets (130-150/tube) were picked and washed in ice-cold PBS. Following centrifugation the islet pellets were lysed as indicated for MIN6 cells.

- Hypothalamic tissue was isolated following the removal of pancreas. First the whole brain was removed from the skull and placed on a petri dish with ventral side up. Then it was dissected around the area marked by a dashed square (Fig. 2.7.1) to obtain a piece of tissue of approximately 0.4 cm^3 corresponding to the hypothalamic area. The tissue was kept frozen at -80 °C.

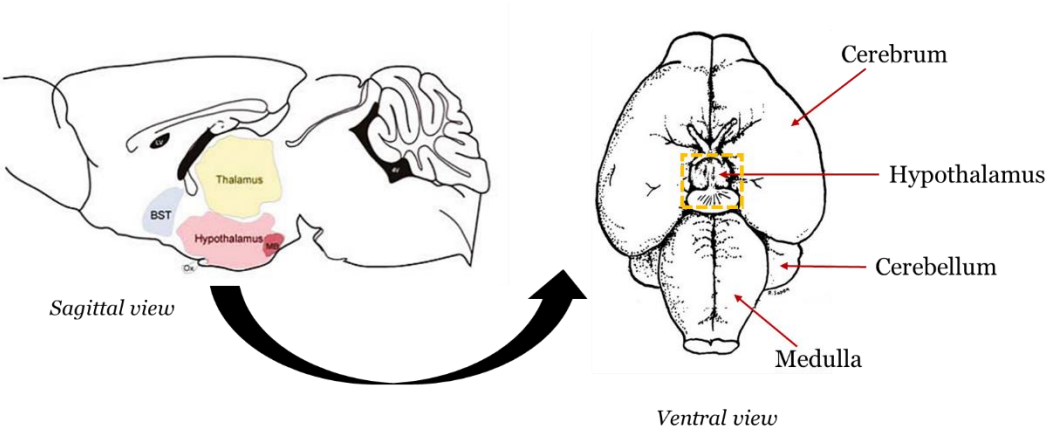

Figure 2.7.1 Diagram showing sagittal and ventral views of mouse brain. Hypothalamus was isolated by cutting around the area delineated by the dashed square. (Modified from Hummel *et al.*, 1966; Zha and Xu, 2015:630).

Ice-cold PBS washed hypothalamus was cut (25-30 mg) and kept frozen in liquid nitrogen. Immediately prior to lysing, 600 µl RLT® buffer containing 10 µl/ml 2-ME was added and the tissue was lysed at 5 A for 5-10 s until completely lysed using a cell disruptor (Microson™ ultrasonic cell disruptor XL, Misonix).

- Stomach fundus washed in ice-cold PBS was cut, 30 mg of tissue transferred to a cold 1.5 ml tube and snap frozen in liquid nitrogen. The frozen tissue was then moved to a mortar with a small volume of LN2 added on top and disrupted using the pestle. Once a powder-like consistency was

obtained the tissue was transferred into a cold 1.5 ml tube. 600 µl of RLT® buffer containing 10 µl/ml 2-ME was added to lyse the tissue.

A Qiagen RNeasy Mini kit (Qiagen) and spin technology were utilised to extract RNA. The lysed samples of cells and tissues were homogenised using QIAshredders by centrifugation at 15,000 rpm for 2 min. 70% ethanol was added to the eluted lysates at a 1:1 ratio and mixed by pipetting. The lysates were transferred to an RNeasy mini spin column placed in a 2 ml collection tube and centrifuged at 12,000 rpm for 30 s at room temperature (RT). The flow through was discarded and the step was repeated if necessary, until the full amount of lysate was processed. Next, wash buffer RW1 (350 µl) was added to each column and tubes were centrifuged at 12,000 rpm for 30 s at RT after which the flow through was discarded. At this stage on-column DNase digestion was performed to remove any DNA contamination using an RNase free DNase set (Qiagen). DNase I was mixed with RDD buffer (10 µl DNase I/ 70 µl RDD) and 80 µl of the mix was added directly onto the membrane in each column and incubated for 15 min at RT. Wash buffer RW1 (350 µl) was added to each column and tubes were centrifuged at 12,000 rpm for 30 s at RT and the flow through discarded. Next RPE wash buffer (500 µl) was added to each column and centrifuged at 12,000 rpm for 30 s at RT to wash the membrane and prevent carry over of salt traces. This step was repeated however the columns were centrifuged for 2 min. The collection tube was discarded with the flow through, the columns transferred to new 2 ml collection tubes and centrifuged at 15,000 rpm for 1 min to avoid ethanol carry over. The columns were transferred again to new 1.5 ml collection tubes and 20-25 µl of RNase-free water was added directly to the membranes followed by centrifugation at

12,000 rpm for 1 min at RT. To concentrate the final RNA, the elute was pipetted directly onto the membranes of the respective columns and centrifuged again using the same 1.5 ml collection tubes.

2.7.1.1 Determination of RNA concentration and purity

The concentration of RNA was quantified on a NanoVue Plus spectrophotometer (GE Healthcare) which can detect the concentration and purity of RNA in a 2 µl sample. RNA purity was determined from two absorbance ratios, 260/230 and 260/280. The first ratio indicates phenol and salt contamination and the sample was considered pure if the ratio was <2.2. The latter (260/280) was used to determine protein contamination and the sample was considered pure at 1.9-2.2. The RNA concentration was quantified according to the absorbance at 260 nm since nucleic acids absorb at 260 nm. Following quantification the RNA samples were stored at -80 °C.

2.7.2 cDNA reverse transcription

Reverse transcription by a DNA polymerase (reverse transcriptase) allows DNA synthesis from an RNA template resulting in production of complementary DNA or cDNA. RNA was reverse transcribed using a High capacity cDNA reverse transcription kit (Applied Biosystems™, UK) as follows. A 2x reverse transcription master mix was prepared as indicated in Table 2.7.4.1 and placed on ice.

RNA was diluted as required in RNase-free water in a maximum volume of 10 µl and a 10 µl master mix added to each tube containing the RNA samples. Tubes were briefly centrifuged and loaded onto a thermal cycler (BIO-RAD T100™ Thermal cycler). Reverse transcription was performed using the

settings indicated in table 2.7.1. Following completion of reverse transcription the cDNA samples were stored at -20 °C.

Table 2.7.1 Thermal cycler settings for cDNA reverse transcription

Settings	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	Infinite hold

2.7.3 Quantitative polymerase chain reaction (qPCR)

The mRNA expression of target genes was determined by quantitative real-time PCR. Here the mRNA of a specific target is quantified by a fluorescent probe that can bind to double-stranded DNA in a non-specific (SYBR green) or sequence specific (TaqMan) manner. A house-keeping gene is also included in the assay and the mRNA expression of the genes of interest is calculated relative to the mRNA expression of the house-keeping gene. In this study gene expression was determined using a QuantiFast SYBR green PCR reagent kit (Qiagen) and a real-time PCR machine (StepOne Plus, Applied Biosystems™, UK) as follows. All reagents and tubes were kept on ice. SYBR® green master mix was made as indicated in table 2.7.4.2. Working primer stocks were prepared at 10 µM in molecular biology grade water. Master mix (18 µl) was added to 0.2 ml reaction tubes and 2 µl of cDNA or RNase-free water (no template control) were added to respective tubes which were vortexed and centrifuged briefly prior to adding 9.8 µl of each reaction mix per well in a MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems™, UK). The plate was covered with a film, centrifuged at 2000 rpm for 1 min and

loaded onto a StepOnePlus™ Real-Time PCR System (Applied Biosystems™). A 2-step cycling protocol with the parameters indicated in table 2.7.2 was used to perform the assay.

Table 2.7.2 Parameters for 2-Step qPCR

1. Pre-incubation	5 min 95 °C	denaturation
2. PCR (40 cycles)	10 s 95 °C 30 s 60 °C	annealing/extension

Data was analysed using the ΔΔCT method where genes of interest were expressed relative to house-keeping genes *Ppia* and *Hprt* (Table 2.7.3) (Pfaffl *et al.*, 2002) or using primer efficiency values as described by Amisten *et al.* (2011).

Table 2.7.3 Primers for housekeeping genes (designed in-house)

Gene	Primer sequence	PCR product (bp)	T annealing (°C)
Ppia	FW: TACAGGTCCTGGCATCTTGTC RV: CTCCATGGCTTCCACAATG	154	60
Hprt	FW: AGTCCCAGCGTCGTGATTAG RV: ACAGAGGGCCACAATGTGAT	186	60

2.7.4 Reagents

cDNA reverse transcription

A 2x master mix was prepared by adding the reagents indicated in Table 2.7.4.1 except for the transcriptase enzyme into an RNase-free tube and it was vortexed and centrifuged. The enzyme was added last and mixed with pipette only.

Table 2.7.4.1 Master mix for cDNA reverse transcription

Reagent	Volume (µl)
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
Nuclease-free H2O	4.2
MultiScribe™ Reverse Transcriptase	1.0

qPCR

To make up 1x qPCR master mix SYBR® green was mixed with RNase-free water as indicated in Table 2.7.4.2. Forward and reverse primers were mixed separately and added to the master mix.

Table 2.7.4.2 Master mix for qPCR

Reagent	Volume (µl)
2X SYBR® green	10
RNase-free water	6
Primers (10 µM)	2

2.8 Determination of protein expression

2.8.1 Preparation of clonal cell and islet lysates

2.8.1.1 Preparation of beta cell lysates

MIN6 cell were trypsinised, washed in ice cold PBS and lysed with 200-500 µl 1x radioimmunoassay precipitation (RIPA) + 1x Halt cocktail (stock 100x, ThermoFisher Scientific, UK). Tubes were kept on ice for 20 min with the cell suspension being vortexed every 5 min to ensure cell lysis. After 20 min cell lysates were centrifuged for 15 min at 12,000 rpm at 4 °C. Supernatants were stored at -80 °C.

2.8.1.2 Preparation of islet lysates

Islets of mixed sizes (120-150) were handpicked into 1.5 ml tubes and washed three times with ice cold PBS. Islet pellets were lysed by adding 40-60 µl 1x RIPA+ 1x Halt cocktail buffer and lysing for 5-10 s at 2 A until completely lysed using a cell disruptor (Microson™ ultrasonic cell disruptor XL, Misonix Inc, USA).

2.8.1.3 Preparation of tissue lysates

Stomach and hypothalamus isolated following removal of pancreas for islet isolation were kept frozen at -80 °C. To prepare lysates, 2-3 mg of each tissue was cut, quickly rinsed in ice cold PBS and transferred to 1.5 ml tubes kept on ice. 250-300 µl 1x RIPA+ 1x Halt buffer was added to each tube and the content lysed 5-10 s at 5 A utilizing a cell disruptor. Lysates were left on an orbital shaker for 2 h at 4 °C to ensure complete lysis. Lysates were centrifuged at 4 °C for 15 min at 12,000 rpm and supernatants were stored at -80 °C.

2.8.2 Protein quantification

Lysates of beta cell lines, islets and tissues were quantified for protein content using Bio-Rad DC™ protein assay (BIO-RAD, UK cat no. 500-0116). The DC™ (detergent compatible) assay is a colourimetric assay based on the Lowry protein assay that has been modified to reduce procedure time (Bio-Rad DC™ protein assay manual LIT448). The assay kit consists of a reagent A (alkaline tartrate solution), reagent B (a diluted Folin reagent) and a proprietary reagent S. Where a lysis buffer containing detergents were used, a solution to neutralise them (solution AS) was made by adding 20 µl of solution S to each 1 ml of reagent A.

A serial doubling dilution of 10 mg/ml BSA ranging from 0.0-1.4 mg/ml in dH_2O was prepared and 5 µl of each standard and sample was added in duplicates to a clear 96-well plate. 25 µl of solution AS was added to each well followed by 200 µl of reagent B. The plate was shaken using a plate shaker for 1 min at 300 rpm and incubated for 15 min at room temperature. Absorbance was read at 750 nm using a Multiscan plate reader (ThermoFisher Scientific, UK). A representative standard curve is shown in figure 2.8.1. Following determination of protein concentration in the lysates 20 µg of protein of each sample was diluted 1:5 using 5x sample buffer (Table 2.8.5.1) and stored at -20 °C.

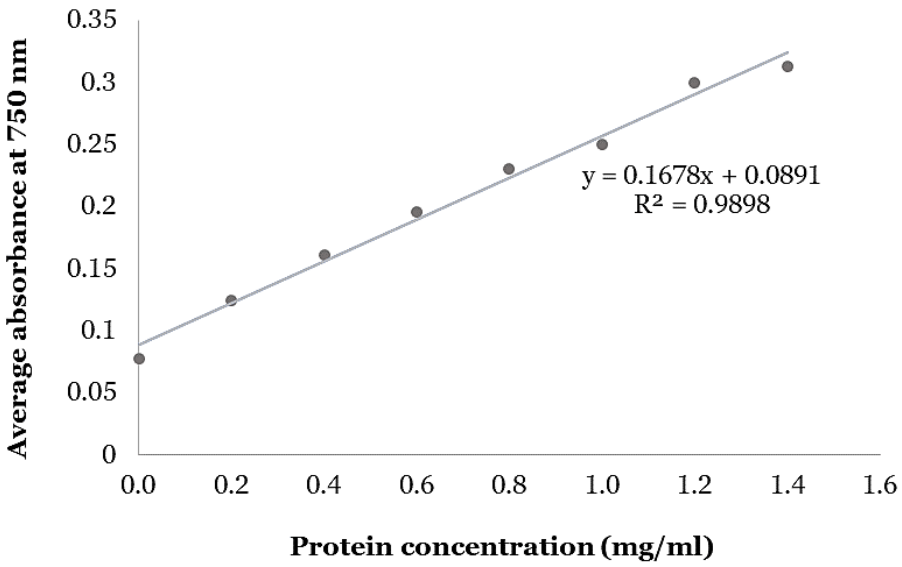

Figure 2.8.1 Example standard curve of BSA protein standards. A standard curve was plotted using average absorbance values against protein concentrations. A line of best fit was drawn, and its equation was used to calculate the protein concentrations in unknown samples.

2.8.3 Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

Poly-acrylamide gel electrophoresis is employed to separate proteins by molecular weight by exploiting the migration of charged proteins in an electric field. SDS binds to proteins and confers a negative charge and partially unfolds proteins. In addition, 2-mercaptoethanol further denature the proteins by breaking disulfide bonds. The overall negatively charged proteins then migrate from cathode to anode in an electric field where the polyacrylamide gel acts as a molecular sieve by separating the proteins by molecular weight. A molecular weight ladder is used as a reference to identify the molecular weight of protein of interest.

In-house made 12 or 15% polyacrylamide gels (resolving gels Table 2.8.5.2 and stacking gel 2.8.5.3) were used in this study. Lysates diluted in sample buffer were boiled at 100 °C for 5 min and allowed to cool on ice. Samples were briefly centrifuged, mixed and loaded into wells at 20 µg/well. 4 µl of protein ladder (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, ThermoFisher Scientific, UK) was added to designated wells. Any empty wells were filled with 6 µl of sample buffer. Gels were loaded onto a BIO-RAD Mini PROTEAN® Tetra Cell tank and run for 1.5-2 h at 120 V until proteins were completely separated according to molecular weight in 1x Tris-glycine-SDS buffer (BIO-RAD).

2.8.4 Western blot

Western blot consists of the transfer of proteins separated by SDS-PAGE on to a membrane for visualization. This process is performed in an electric field that is perpendicular to the surface of the gel. The transfer of proteins takes place from cathode to anode within a sandwich of filter papers, membrane and gel as shown in figure 2.8.2 and is known as an electrophoretic transfer that takes place in semi-dry or wet conditions (Mahmood and Yang, 2012).

Western blotting was performed using a Trans-Blot Turbo semi-dry transfer machine (BIO-RAD, UK) onto a Nitrocellulose (GE Healthcare, UK) membrane at 25 V/gel for 7 min using ice-cold transfer buffer (Table 2.8.5.4). Successful transfer was visually determined using ponceau S stain. Following a quick wash with dH_2O to remove the excess ponceau S the membrane was blocked with 5% non-fat milk for 45 min and incubated overnight with primary antibodies. Mouse anti-beta actin (Abcam cat no. ab8224) was used as loading control.

The next day, the membrane was washed for 3 x 20 min using 1x Tris Buffered Saline (TBS, Table 2.8.5.5) containing 0.001% Tween 20 (TTBS) then incubated with fluorescent secondary antibodies for 1.5 h at RT (Li-Cor IRdye® donkey anti-rabbit 680RD and donkey anti-mouse 800CW). This was followed by 3 x 15 min washes using TTBS. The membrane was imaged and the band intensity was quantified using a Licor Odyssey FC imaging system with Image Studio software version 5.2. The expression of proteins of interest were calculated relative to the loading control, beta-actin.

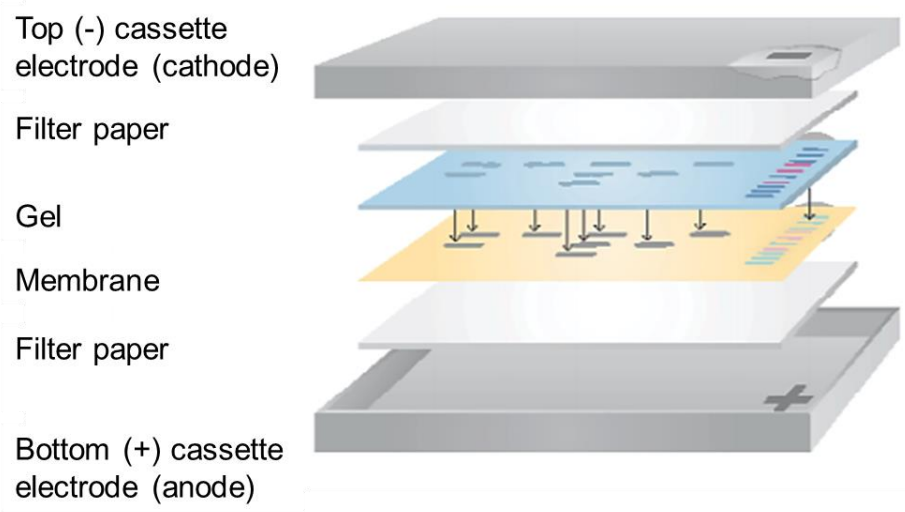

Figure 2.8.2 Western blot sandwich. The SDS-PAGE gel is placed on top of the blotting membrane in between two sets of filter paper. The proteins are transferred from cathode to anode. (Modified from BIO-RAD, no date:12).

2.8.5 Reagents

Sample buffer

To prepare sample buffer or loading buffer first a 312 mM Tris-HCl solution was prepared by dissolving 3.8 g of tris in 100 ml of distilled water and pH was adjusted to 6.8 using HCl. Then the reagents in Table 2.8.5.1 were gradually mixed in the given order to obtain a 5x solution. Aliquots of 1 ml were stored at -20 °C.

Table 2.8.5.1 Preparation of 5x sample buffer

Reagent	Amount (for 20 ml)	Final concentration
312 mM Tris-HCl, pH 6.8	13 ml	202 mM
SDS	2 g	10% w/v
glycerol	5 ml	25% v/v
2-mercaptoethanol	2 ml	10% v/v
bromophenol blue	0.003 g	0.015% w/v

Polyacrylamide gels

Resolving gels of 1.5 mm thickness were prepared using the reagents indicated in Table 2.8.5.2. First Tris, acrylamide solution, SDS and water were added to a 50 ml tube. Just prior to adding APS, TEMED was pipetted in and quickly mixed. Once APS was added the solution was quickly transferred between the casting plates using a 10 ml pipette ensuring that ~1 cm space was left at the top for the stacking gel.

During polymerisation of the resolving gel, the reagents for stacking gels were mixed with the exception of TEMED and APS that were incorporated just before adding on top of the polymerised resolving gels (Table 2.8.5.3).

Immediately a comb was inserted to make the wells. Once polymerised the gels were either used immediately or stored overnight in running buffer at 4 °C. Prior to loading the samples, the combs were removed ensuring the wells were intact.

Table 2.8.5.2 12% and 15% polyacrylamide resolving gels

Reagent	**Volume**		**Final concentration**	
	12% gel	**15% gel**	**12% gel**	**15% gel**
1 M Tris (pH 8.8)	3.75 ml	3.75 ml	0.4 M	0.4 M
30% v/v acrylamide (protogel)	3.6 ml	5 ml	12%	15%
20% w/v SDS	50 µl	50 µl	0.1%	0.1%
Distilled water	2.3 ml	0.9 ml	-	-
TEMED (*N*,*N*,*N′*,*N′*-Tetramethylethylenediamine)	12.5 µl	12.5 µl	0.1%	0.1%
10% w/v ammonium persulfate (APS)	300 µl	300 µl	0.3%	0.3%

Table 2.8.5.3 Polyacrylamide stacking gel

Reagent	**Volume**	**Final concentration**
1 M Tris (pH 6.8)	625 µl	0.1 M
30% v/v acrylamide (protogel)	900 µl (5%)	5%
20% w/v SDS	25 µl	0.1%
Distilled water	3.3 ml	-
TEMED	12.5 µl	0.25%
10% w/v APS	150 µl	0.3%

Transfer buffer

In order to perform semi-dry transfer of proteins transfer buffer was prepared by mixing Tris, glycine, methanol and topping up with distilled water up to 1L (Table 2.8.5.4). The solution was stored at 4 °C.

Table 2.8.5.4 1x transfer buffer

Reagent	Amount (for 1 L)	Final concentration
Tris	3 g	25 mM
Glycine	15 g	200 mM
Methanol	200 ml	20% v/v
dH_2O	Up to 1 L	-

Wash buffer

A 10x tris-buffered saline solution was prepared by dissolving tris and glycine in distilled water (Table 2.8.5.5). The pH of the solution was adjusted to pH 8.0 and stored at RT. For experimental use the buffer was diluted 1:10 in dH_2O and 0.001% Tween®20 was added.

Table 2.8.5.5 10x tris-buffered saline, pH 8.0

Reagent	Amount (for 1 L)	Final concentration
Tris	18.2 g	150 mM
NaCl	87 g	1.5 M
dH_2O	Up to 1 L	-

2.9. Statistical analysis

Raw data was processed and analysed using Microsoft Office 365 ProPlus Excel (version 1902). Statistical analysis was carried out using GraphPad Prism software version 8.3 and using the following statistical tests:

For two sets of data paired and/or unpaired Student's T-tests, and for three or more One-way analysis of variance (ANOVA) with post-hoc multiple comparison test Bonferroni were applied as indicated in the figure legends. Differences were considered significant at $p<0.05$.

CHAPTER 3 - The role of somatostatin and SSTR3 in MIN6 beta cell secretory function and survival

3.1 Introduction

Somatostatin (SST) is produced in the brain, retina, immune system, gastrointestinal tract and in the delta cells of pancreatic islets (reviewed by Martinez, 2013). The neuropeptide and hormone mainly acts as an inhibitor of secretion of hormones, and in particular pancreatic SST has been shown to act in a paracrine manner to inhibit insulin secretion from beta cells and glucagon secretion from alpha cells (Hauge-Evans *et al.*, 2009). Although the function of SST in islet hormone secretion is well characterised there is little evidence about its role in beta cell survival. It has been found that SST promotes apoptosis in certain cancer cell lines including lung, gastric, colorectal and brain (reviewed by Ferjoux *et al.*, 2000; Pyronnet *et al.*, 2008). However, there are other studies indicating a protective role for SST in conditions such as retinal neurodegeneration that can lead to diabetes retinopathy (Carrasco *et al.*, 2007), attenuation of liver damage when administered with prostaglandin E1 as well as suppression of inflammation in nerves (e.g. sciatica) (Jia *et al.*, 2013; reviewed by Pintér *et al.*, 2006). Findings in somatostatin knockout mice indicated that beta cell volume was reduced in one-year old mice lacking the somatostatin gene, and that the lack of *Sst* in early development resulted in increased apoptosis and decreased proliferation in pancreatic cells (Richardson *et al.*, 2015). Recent studies conducted in our lab showed that pre-treatment of MIN6 cells and mouse islets with SST resulted in protection against fatty acid-induced endoplasmic reticulum stress or cytokine-induced apoptosis (Damsteegt *et al.*, 2019). It is therefore evident that SST protects beta cells and islets from detrimental

effects of stressors typical of type 2 diabetes. However the somatostatin receptor subtypes conveying this effect are yet to be identified.

SST acts via five G-protein coupled receptors (SSTR 1-5). In rodents it has been found that SSTR2 and 5 are primarily expressed in beta cells, whereas alpha cells express mainly SSTR2 (reviewed by Strowski and Blake, 2007). The gene expression of *Sstr3* in beta cells and *Sstr2* and *3* in alpha cells only was recently detected in single-cell transcriptomic analyses utilising cells sorted from mouse islets (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). Similarly, we have found that mRNA expression level of *Sstr3* was significantly greater in MIN6 beta cells compared to that of other somatostatin receptors (Damsteegt *et al.*, 2019). A study by Pasternak *et al.* (2012) reported that SSTR3 contributed to the insulinostatic effect of SST on mouse islets using a SSTR3 selective antagonist and the SST analogue, octreotide. It was also revealed that in the presence of the antagonist a reduction in glucose excursion was observed in wild-type mice following intraperitoneal dextrose injection. This effect was not observed in global *Sstr3*$^{-/-}$ mice suggesting that SSTR3 is involved in mediating the insulinostatic effect of SST (Pasternak *et al.*, 2012). These results are discordant with the consensus that the activity of SST via SSTR 1,2 and 5 in mice and SSTR2 in humans is responsible for its inhibitory effect on insulin secretion (reviewed by Ludvigsen, 2007; Strowski *et al.*, 2003; Strowski and Blake, 2007). It was therefore necessary to ascertain whether SSTR3 is involved in mediating the insulinostatic and pro-survival effects of SST in clonal beta cells.

As described in 1.3.1.2, MK4256 is a recently identified SSTR3 competitive antagonist (He *et al.*, 2012). In oral glucose tolerance tests (OGTT) performed

in mice increasing doses of MK4256 resulted in reduced blood glucose levels. This was confirmed in *Sstr3*$^{-/-}$ mice where the reduction in blood glucose following administration of MK4256 was not significant (He *et al.*, 2012).

Based on the studies outlined above, the aims of this chapter were to investigate whether the insulinostatic and pro-survival effects of SST were conveyed via SSTR3 with the use of SSTR3 antagonist MK4256.

3.2 Aims and objectives

3.2.1 To further evaluate the role of SST in beta cell survival.

3.2.2 To confirm the expression of *Sstr3* at mRNA level and detection of SSTR3 at protein level in MIN6 beta cells and pancreatic mouse islets.

3.2.3 To determine whether SSTR3 mediates the inhibitory effect of SST on insulin secretion in MIN6 cells.

3.2.4 To investigate whether SSTR3 is implicated in the effect of SST on MIN6 beta cell survival.

3.3 Methods

The somatostatin used in this study was somatostatin-14 (Bachem, Switzerland) and this will be referred to as somatostatin or SST in the subsequent sections.

3.3.1 Static insulin secretion and radioimmunoassay

Static incubation experiments with MIN6 cells were conducted as described in section 2.4.1 and insulin release was assessed in response to 20 mM glucose, forskolin (FSK, 10 μM) and 3-isobutyl-1-methylxanthine (IBMX, 100 μM) with or without SST and the SSTR3 antagonist, MK4256 (MedChemExpress, Sweden). Samples were diluted and insulin content was measured by RIA as described in 2.4.4.1. Since MIN6 cells are a clonal beta cell line that is grown in medium containing 25 mM glucose it has been reported that their glucose responsiveness is lost at later passages 35-40 (Kayo *et al.*, 1996). Indeed it has been observed by us and others that MIN6 cell glucose responsiveness was not consistently reproducible despite using cells of passage number 35 or below. Therefore, FSK and IBMX were used to potentiate glucose-induced insulin secretion from MIN6 cells. In the presence of glucose FSK stimulates adenylate cyclase activity that in turn increases cAMP levels resulting in elevated insulin release (Wiedenkeller and Sharp, 1983). IBMX is a phosphodiesterase inhibitor that prevents breakdown of cAMP therefore causing increase in insulin secretion from beta cells (Rabinovitch *et al.*, 1978). Since SST has been shown to exert its inhibitory activity via interference with adenylate cyclase and cAMP production (Hildebrandt and Kohnken, 1990; Tentler *et al.*, 1997), FSK and IBMX were chosen as the appropriate potentiators of glucose-induced insulin secretion.

3.3.2 Beta cell apoptosis

The effect of SST on MIN6 or INS-1 cell apoptosis was tested in the presence of a range of selected stressors linked to type 2 diabetes:

Sodium palmitate (lipotoxicity)

Fatty acid (FA)-free BSA was prepared as a 10% w/v solution in 2% FBS DMEM and filter sterilised. A 50 mM solution of sodium palmitate was prepared by adding 1 ml of 50:50 v/v ethanol/H_2O to 13.9 mg of sodium palmitate and solubilised at 70 °C for 10 min. Palmitate and control solutions (50:50 v/v ethanol/H_2O) were diluted 1:10 in FA-free BSA solution and both were incubated at 37 °C for 1 h to allow binding of palmitate to BSA. At the end of the incubation both solutions were further diluted 1:10 in 2% FBS DMEM to obtain 0.5 mM palmitate and 0.5% ethanol in 0.95% BSA.

Thapsigargin (ER stress)

Thapsigargin was prepared as a 1 mM solution in DMSO and further diluted in 2% FBS DMEM to obtain a final concentration of 1 μM.

Pro-inflammatory cytokines

TNF-α (activity $1x10^7$) and IL-1β (activity $5x10^8$, Peprotech, UK) were re-constituted in glucose-free RPMI containing 2% FBS at 1000 U/ml and 100 U/ml respectively. The cytokines were used at final concentrations of 500 U/ml (TNF-α) and 50 U/ml (IL-1β).

20 mM glucose (glucotoxicity)

RPMI media containing 11 mM glucose and 2% FBS was supplemented with 9 mM glucose (45 % w/v stock solution) to prepare a 20 mM glucose solution.

20 mM glucose and 0.5 mM palmitate (glucolipotoxicity)

A 0.5 mM palmitate solution was prepared as described above for lipotoxicity using RPMI medium that was supplemented with 9 mM glucose as described for glucotoxicity.

Cell apoptosis experiments were performed as described in section 2.6. Briefly, MIN6 cells (15,000 cells/well) were pre-treated for 48 h with or without 0.1 or 1 μM SST and exposed to the cellular stressors indicated above for 20 h. INS-1 cells (5,000 cells/well) were cultured for two days and exposed to glucotoxicity, lipotoxicity or glucolipotoxicity for 20 h. Apoptosis was detected using the Promega Caspase-Glo assay as described in 2.6.1. Pertussis toxin (PTX) was prepared as a 100 μg/ml solution in dH_2O and used at 100 ng/ml final concentration.

For the assessment of the role of SSTR3 on MIN6 cell apoptosis, MIN6 cells were pre-incubated with or without 0.1 or 1 μM SST ± 1 or 5 μM MK4256 for 48 h, followed by exposure to 0.5 mM palmitate for 20h. Apoptosis was detected using the Promega Caspase-Glo assay as described in 2.6.1.

3.3.3 Gene expression analysis

MIN6 cells were harvested at 70-80% confluency following normal tissue culture maintenance, islets were harvested following maintenance in complete medium for two days and hypothalamic tissue was lysed as described in 2.7.1.1. RNA extraction, cDNA reverse transcription and qPCR were performed as described in section 2.7 with the use of primers for house-keeping genes (section 2.7.3) and *Sstr3* (Damsteegt *et al.*, 2019, Table 3.1). Gene expression was determined relative to *Ppia* and *Hprt*.

Table 3.1 *Sstr3* primers

Gene	Primer sequence (5'-3')	PCR product (bp)	T annealing (°C)
Sstr3	FW: TGGTGATCTACGTGGTCCTG RV: AGACGGCACATGAGAGATCC	163	59.9

3.3.4 Protein expression

In order to prepare the protein samples MIN6 cells were harvested at 70-80% confluency following normal tissue culture maintenance, islets were harvested following maintenance in complete medium for two days and hypothalamic tissue was prepared as described in 2.8.1.3. Protein concentration was quantified and expression of targets of interest was detected by Western blot as described in section 2.8. Rabbit polyclonal SSTR3 antibody (ThermoFisher Scientific, cat no. PA3-207) was used at 1:5000 dilution with beta actin as loading control.

3.4 Results

In this study MIN6 cells were used as a model to test whether the observed effects of SST in islets are mediated via the somatostatin receptor-3. The monotypic MIN6 cells differ from the composite structure of islets containing SST-secreting delta cells, therefore a number of experiments were conducted on MIN6 cells to test that somatostatin used in this study was active.

3.4.1 The role of SST in MIN6 cells

3.4.1.1 Insulinostatic effect of SST in MIN6 cells

In order to assess the effect of SST on insulin secretion from MIN6 cells, a static insulin secretion experiment followed by radioimmunoassay was conducted. Exposure to 20 mM glucose alone did not significantly affect insulin secretion from MIN6 cells ($p<0.05$ vs 2 mM glucose; Fig. 3.4.1), but adjunct treatment with FSK and IBMX significantly increased insulin secretion from these cells ($p<0.0001$, $n=8$). Treatment with 1 μM SST significantly reduced both 20 mM glucose-induced ($p<0.01$, $n=8$) and FSK+IBMX-induced ($p<0.001$, $n=8$) insulin secretion (Fig. 3.4.1).

3.4.1.2 Effect of SST on MIN6 beta cell survival in response to cellular stressors

Lipotoxicity

In order to confirm the protective effect of SST reported in Damsteegt *et al.* (2019) MIN6 cells pre-treated with or without SST were exposed to a number of cellular stressors typical of type 2 diabetes as described in 3.3.2. Treatment with both 0.1 and 1 μM SST resulted in significant reduction of 0.5 mM palmitate-induced apoptosis in MIN6 cells ($p<0.05$, Fig. 3.4.2).

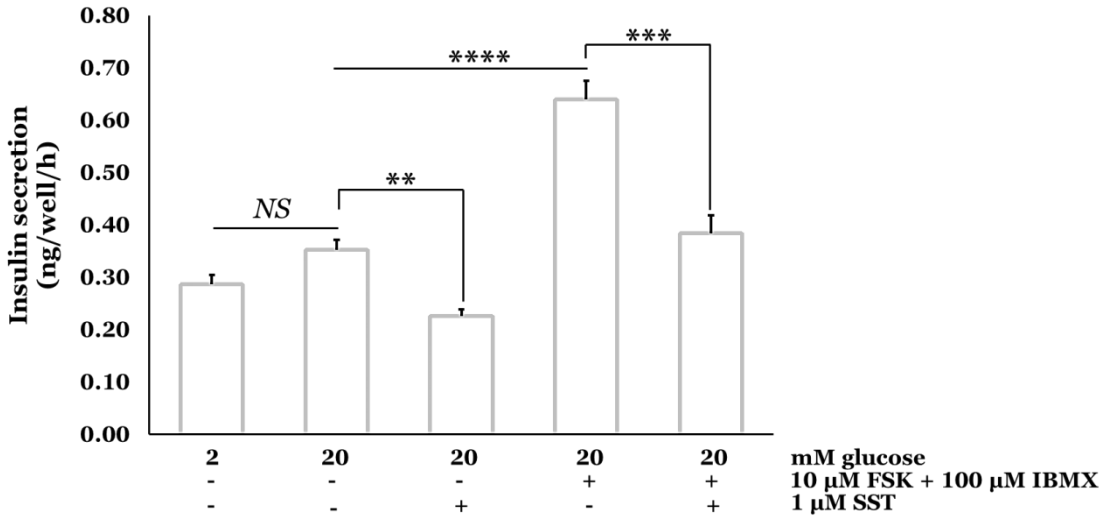

Figure 3.4.1 The effect of SST on insulin secretion from MIN6 beta cells. MIN6 cells were seeded at 20,000 cells/well and incubated for 48 h. The cells were pre-incubated with 2 mM glucose for 2 h and treated as indicated for 1 h. Supernatants were assayed for insulin concentration by an in-house radioimmunoassay. Data is presented as mean+SEM, n=8, one experiment. One-Way ANOVA with posthoc test Bonferroni **p<0.01, ***p<0.001, ***p<0.0001. NS = not significant p>0.05.

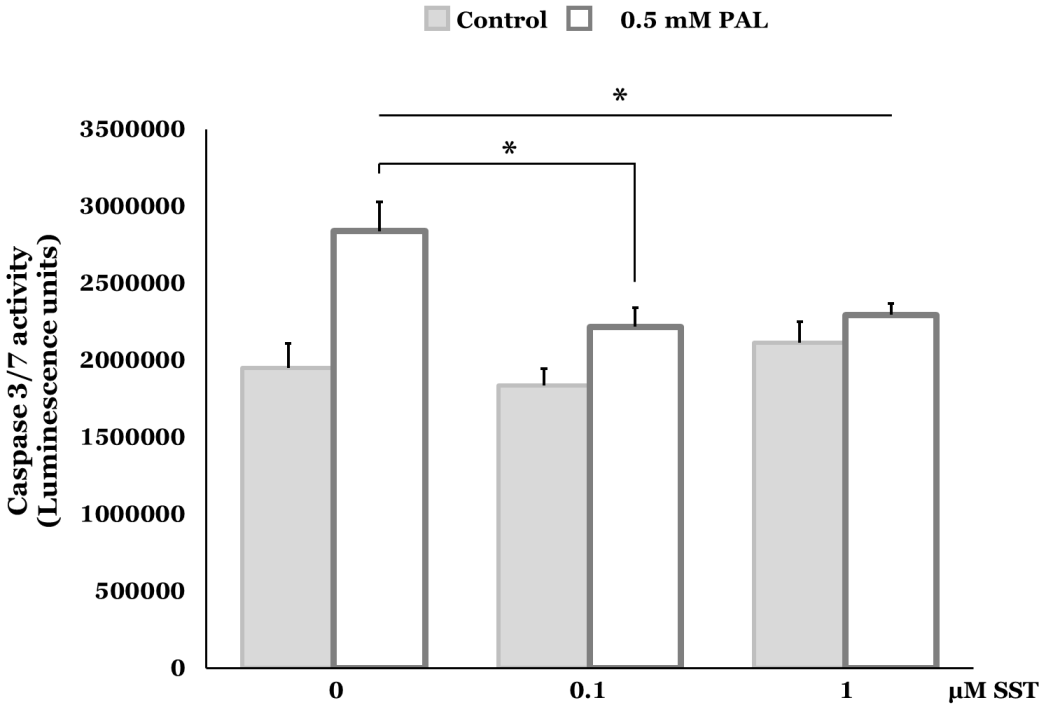

Figure 3.4.2 The effect of SST on MIN6 beta cell apoptosis induced by sodium palmitate. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48 h with either 0.1 or 1 µM SST then exposed to 0.5 mM palmitate for 20 h with or without SST. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=4-5, one independent experiment. One-Way ANOVA with posthoc test Bonferroni *$p<0.05$.

ER-stress

Incubation with the ER-stress inducer, thapsigargin, caused a significant elevation in apoptosis in MIN6 cells ($p<0.0001$), however treatment with 0.1 or 1 µM SST did not significantly reduce the increased caspase 3/7 activity ($p>0.05$ vs 1 µM Tg; Figure 3.4.3).

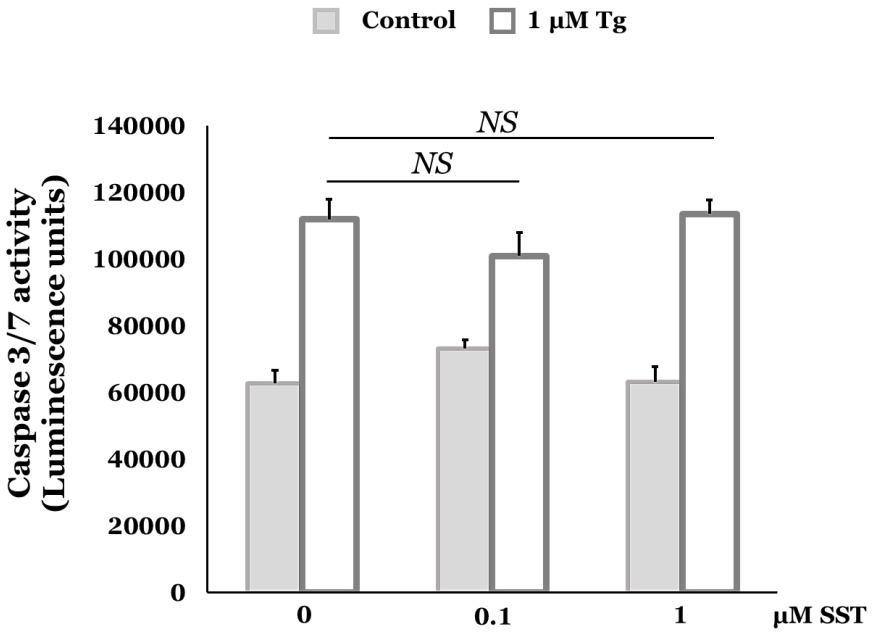

Figure 3.4.3 The effect of SST on MIN6 beta cell apoptosis induced by thapsigargin. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48h with either 0.1 or 1 µM SST then exposed to 1 µM thapsigargin for 20 h ± SST. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=5, graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni. NS = not significant $p>0.05$.

Pro-inflammatory cytokines

Exposure to a combination of cytokines (500 U/ml TNF-α and 50 U/ml IL-1β) significantly increased apoptosis in MIN6 cells ($p<0.0001$; Fig. 3.4.4). Treatment with 0.1 and 1 µM SST however could not significantly reduce apoptosis induced by cytokines ($p>0.05$).

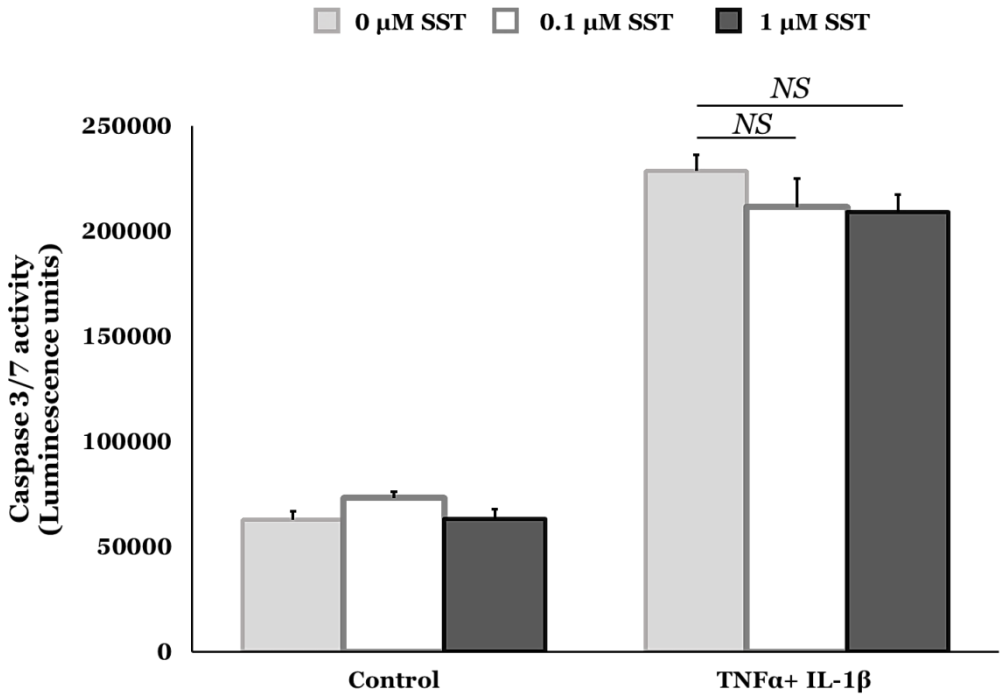

Figure 3.4.4 The effect of SST on MIN6 beta cell apoptosis induced by exposure to pro-inflammatory cytokines. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48 h with either 0.1 or 1 µM SST then exposed to a combination of cytokines (500 U/ml TNFα+ 50 U/ml IL-1β) for 20 h ± SST. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=5, graph represents results of one experiment out of two independent experiments. One-Way ANOVA with posthoc test Bonferroni. NS = not significant $p>0.05$.

Glucotoxicity and glucolipotoxicity

When testing cellular stressors typical of type 2 diabetes glucotoxicity and glucolipotoxicity should also be considered since hyperglycaemia and simultaneous presence of high circulating free fatty acid contribute to the pathogenesis of type 2 diabetes (reviewed by Hasnain *et al.*, 2016). MIN6 cells are maintained in 25 mM glucose therefore do not constitute an ideal model for studying glucotoxicity. Hence INS-1 cells maintained in 11 mM glucose were tested. In our experimental model glucotoxicity with 20 mM glucose

alone was not sufficient to cause a significant increase in apoptosis in INS-1 cells compared to 11 mM glucose ($p>0.05$, Fig. 3.4.5). Incubation with 0.5 mM palmitate and 0.5 mM palmitate + 20 mM glucose significantly increased apoptosis compared to control in MIN6 cells (both $p<0.0001$). However, addition of 20 mM glucose to 0.5 mM palmitate did not cause a significant synergistic increase in INS-1 cell apoptosis compared to 0.5 mM palmitate alone ($p>0.05$, Fig. 3.4.5). Therefore, the effect of SST on glucotoxicity or glucolipotoxicity was not tested in this research model.

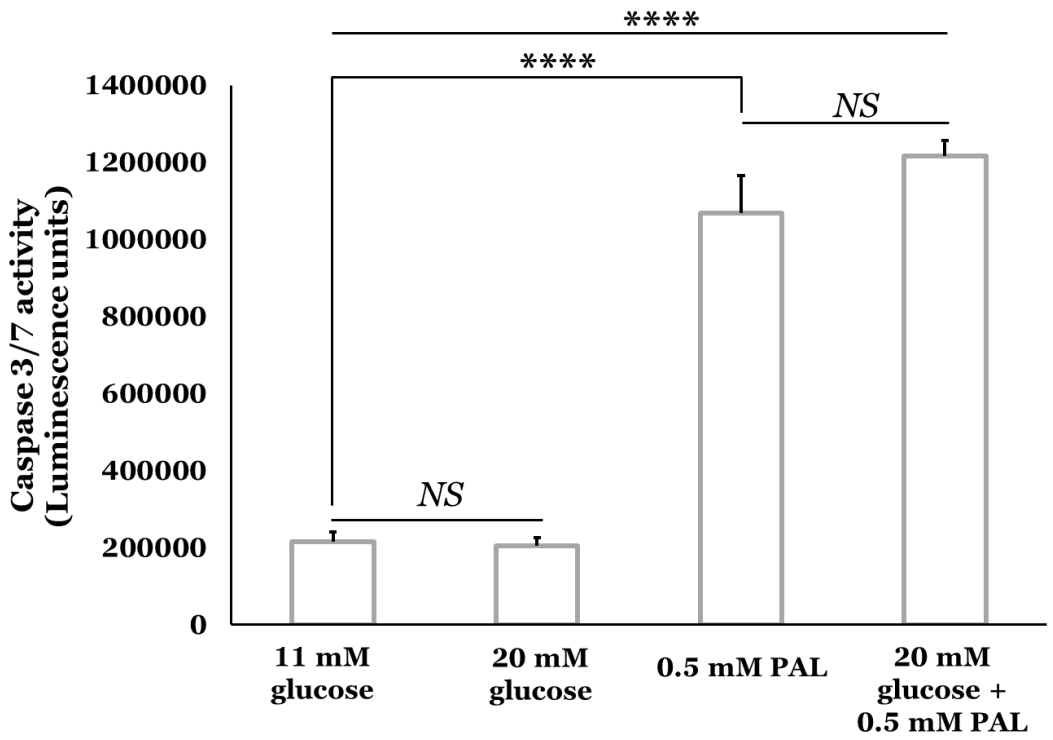

Figure 3.4.5 Comparison of the effect of glucotoxicity, lipotoxicity and glucolipotoxicity on INS-1 beta cell apoptosis. INS-1 beta cells were plated at 5,000 cells/well and incubated for 48 h then exposed to either 11 mM glucose 20 mM glucose, 0.5 mM palmitate or 20mM glucose + 0.5 mM palmitate for 20 h as indicated. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=5, graph represents results of one experiment out of four independent experiments. One-Way ANOVA with posthoc test Bonferroni. ****$p<0.0001$. NS = not significant $p>0.05$.

3.4.2 Role of somatostatin receptor -3 in beta cell function

Prior to testing whether the observed effects of SST in clonal beta cells were mediated by SST receptor-3, a number of experiments were conducted to detect the expression of SSTR3 at mRNA and protein levels in MIN6 cells and mouse islets.

3.4.2.1 mRNA expression of *Sstr3*

In order to detect mRNA expression, qPCR analysis was performed, and it revealed that *Sstr3* was expressed in MIN6 cells, mouse islets and in murine hypothalamus (Fig. 3.4.6). Mean Ct values are indicated in Table 3.4.1.

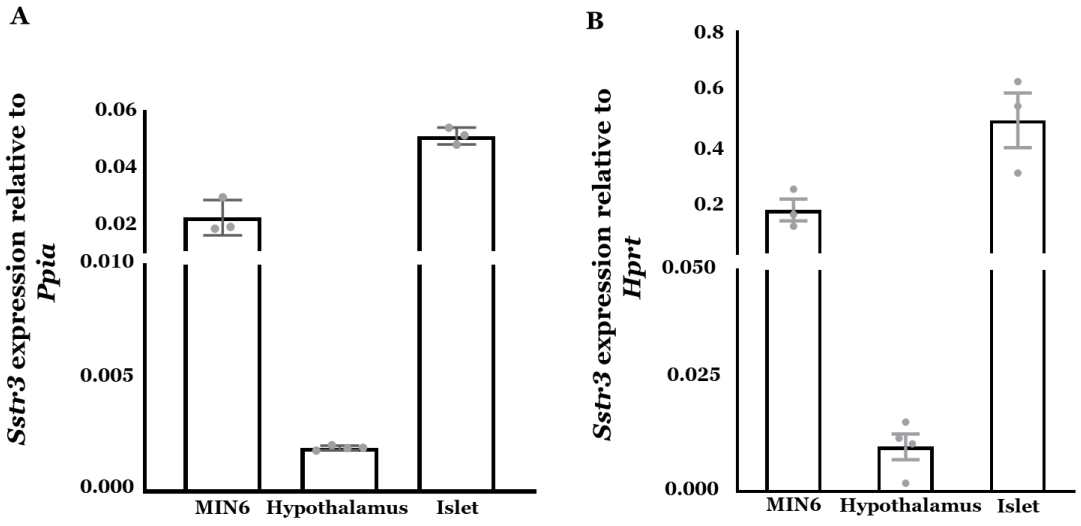

Figure 3.4.6 mRNA expression of *Sstr3*. The mRNA expression of *Sstr3* was detected in hypothalamus and isolated islets from mice and in MIN6 beta cells. Mean mRNA expression ± SEM relative to housekeeping genes *Ppia* (A) and *Hprt* (B) is shown (n=3-4, separate biological samples).

Table 3.2 Mean Ct values ± SEM of *Sstr3* and housekeeping genes (n=3)

	MIN6	Hypothalamus	Islet
Sstr3	21.73 ± 0.49	25.09 ± 0.23	21.79 ± 0.39
Ppia	16.29 ± 0.33	16.08 ± 0.23	17.51 ± 0.34
Hprt	19.46 ± 0.38	18.12 ± 0.49	20.74 ± 0.18

3.4.2.2 Protein expression of SSTR3

Western blot analysis showed that SSTR3 was expressed in clonal beta cells (MIN6 and INS-1, Fig. 3.4.7 A and B) and in the murine hypothalamus (Fig. 3.4.7 C). However, multiple unclear bands were observed for SSTR3 in primary mouse islets (Fig. 3.4.7 D). Expression levels relative to beta actin are indicated in Fig. 3.4.7 E, where expression of highest expression of SSTR3 was detected in MIN6 cells.

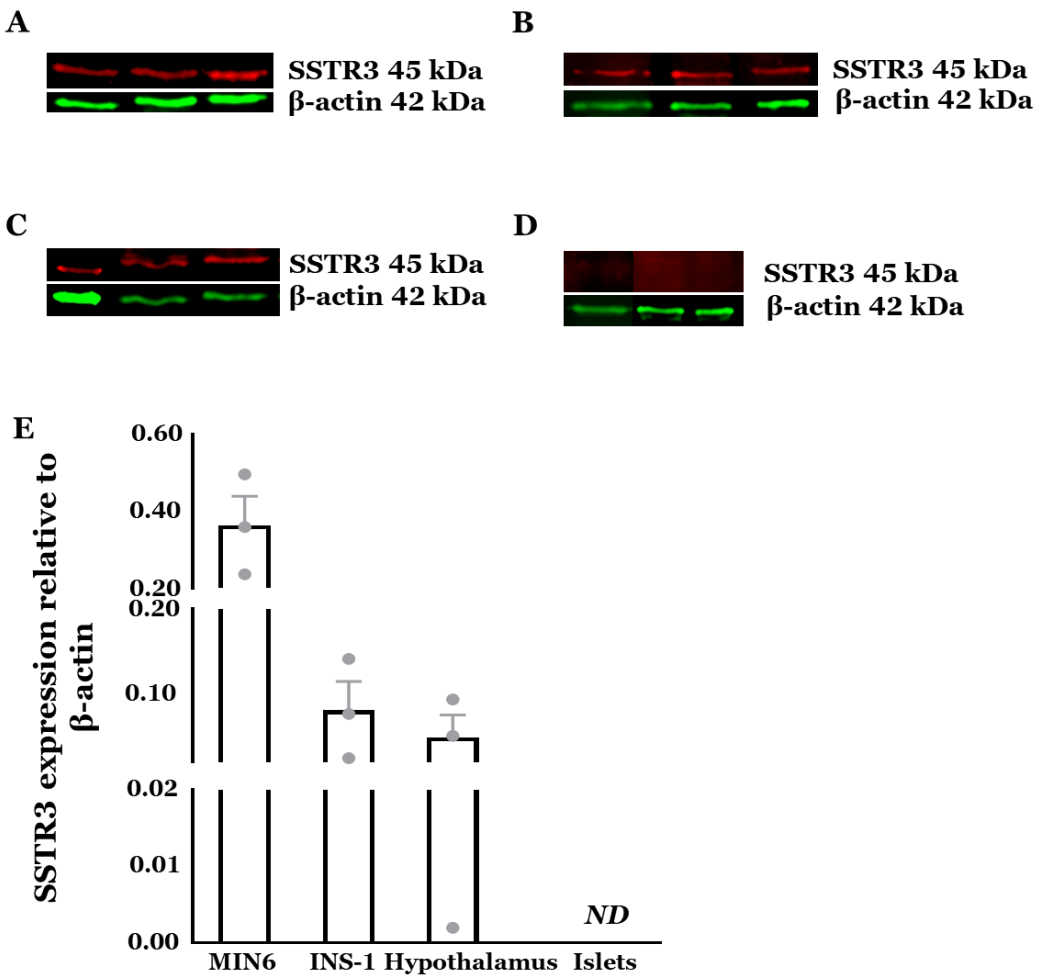

Figure 3.4.7 Protein expression of SSTR3. SSTR3 was detected in beta cells MIN6 (A), INS-1 (B) and in murine hypothalamus (C) but not in murine islets (D). Panel E shows mean + SEM relative expression levels of SSTR3 compared to loading control beta actin (n=3, separate biological samples). 20 µg protein was loaded per sample and resolved using a 12-15% polyacrylamide gel. Proteins were detected by Western blot. Primary antibodies used: SSTR3 1:5000 and beta actin as loading control 1:8000. Detected using Li-Cor fluorescent secondary antibodies and image studio software.

3.4.2.3 The role of SSTR3 in insulin secretion

In order to test whether the insulinostatic effect of SST is mediated via SSTR3 in MIN6 cells, static incubations were conducted as described in 2.4.1 with the SSTR3 antagonist, MK4256. Acute insulin secretion in the presence of FSK and IBMX was significantly reduced by exposure to 1 μM SST ($p<0.001$, Fig. 3.4.8), whereas 1 and 5 μM MK4256 alone did not significantly affect insulin release from MIN6 cells ($p>0.05$ vs 20 mM glucose+ 10 μM FSK + 100 μM IBMX). However, co-treatment of 1 μM SST with 5 μM MK4256 resulted in significant reversal of the insulinostatic effect of SST ($p<0.01$; Fig. 3.4.8). Although an increase in insulin secretion was observed with co-treatment of 1 μM SST with 1 μM MK4256 this was not statistically significant compared to the effect of 1 μM SST alone ($p>0.05$).

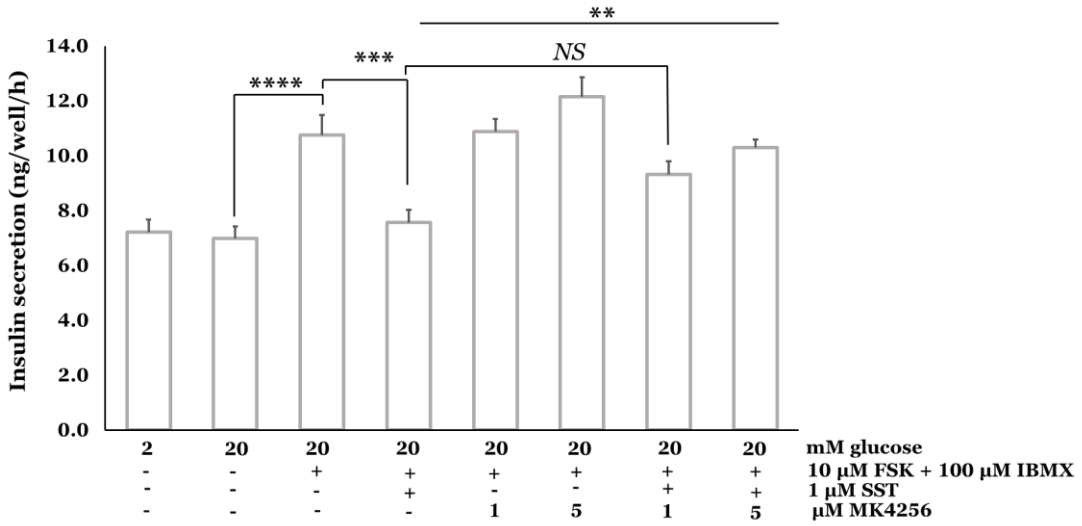

Figure 3.4.8 The effect of SST and the SSTR3 antagonist, MK4256, on insulin secretion from MIN6 beta cells. MIN6 cells were plated at 20,000 cells/well and incubated for 48 h. The cells were then pre-incubated with 2 mM glucose and treated as indicated for 1 h. Supernatants were assayed for insulin content by an in-house radioimmunoassay. N=8, Data is presented as mean+SEM, graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni **p<0.01, ***p<0.001, ***p<0.0001. NS = not significant p>0.05.

3.4.2.4 The role of SSTR3 in mediating the pro-survival effects of SST

Following confirmation that SSTR3 antagonist MK4256 could significantly reverse the insulinostatic effect of SST in MIN6 cells, it was investigated whether the observed pro-survival effect of SST could be reversed by the same compound.

Exposure of MIN6 cells to 0.5 mM palmitate significantly increased apoptosis in MIN6 cells which was significantly reduced by treatment with 1 μM SST ($p<0.05$). Addition of 1 and 5 μM MK4256 did not reverse the protective effect of SST in MIN6 cells ($p>0.05$; Fig. 3.4.9) and instead resulted in a significant reduction of 0.5 mM palmitate-induced apoptosis ($p<0.01$).

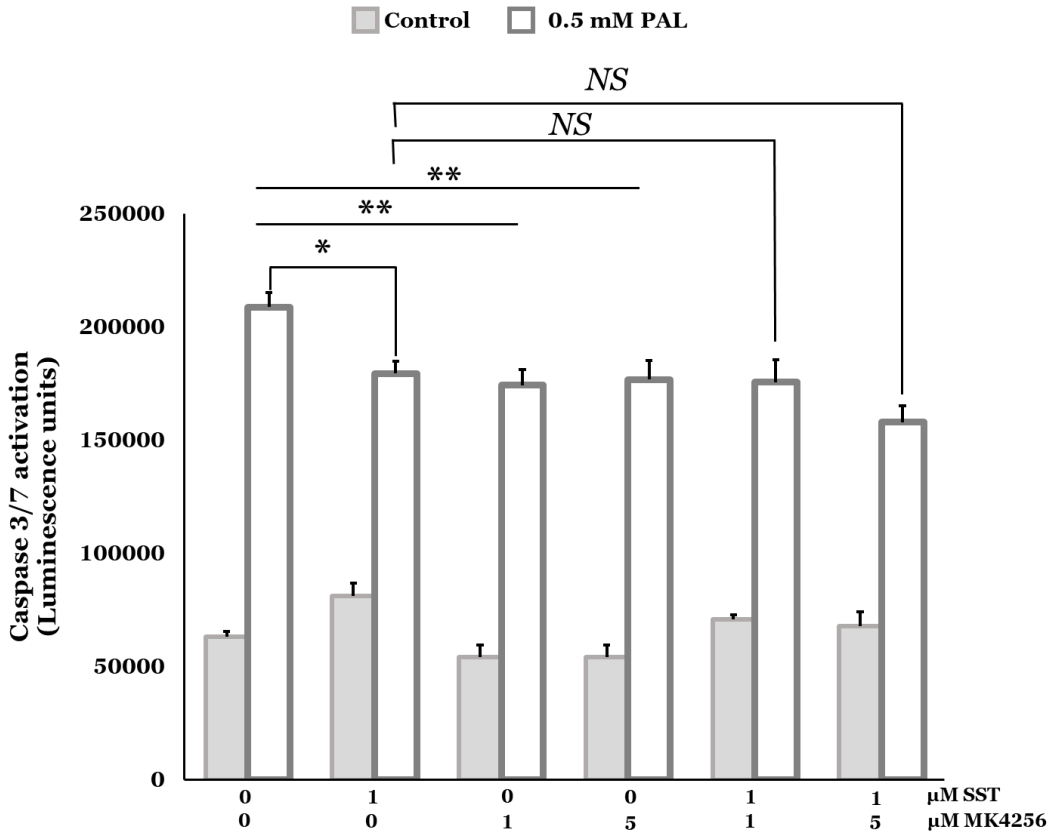

Figure 3.4.9 The effect of SST and SSTR3 antagonist MK4256 on MIN6 beta cell apoptosis. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48h with 1 µM SST ± 1 or 5 µM MK4256 then exposed to 0.5 mM palmitate for 20h with or without compounds of interest. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=5, graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni. *p<0.05, **p<0.01. NS = not significant p>0.05.

3.4.3 The anti-apoptotic effect of SST in MIN6 cells is $G_{\alpha i}$ signalling pathway dependent

Since the protective effect of SST could not be reversed by MK4256, it was tested whether treatment with 100 ng/ml pertussis toxin (PTX) could reverse this effect. PTX is a bacterial adenine diphosphate (ADP)-rybosylating toxin that acts on $G_{\alpha i}$ signalling pathway to exert its inhibitory effects. ADP-rybosylation holds the $G_{\alpha i}$ in an inactive state thereby preventing interaction with GPCRs and hence preventing inhibition of adenylate cyclase (Mangmool and Kurose, 2011). Since SSTRs signal via both $G_{\alpha i}$ dependent and independent signalling pathways (reviewed by Ferjoux *et al.*, 2000) PTX was used as an antagonist of SST. Exposure to 1 μM SST significantly reduced

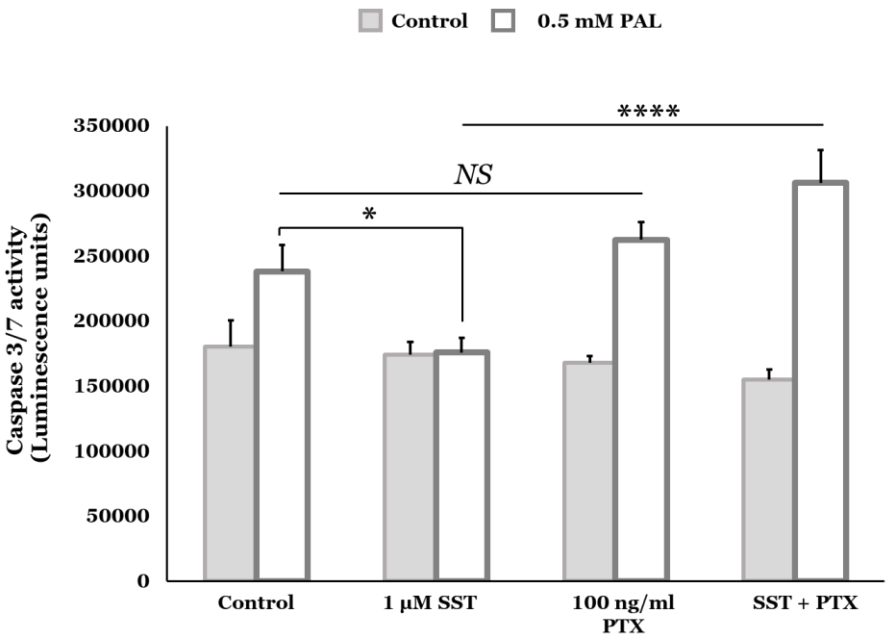

Figure 3.4.10 The effect of SST on MIN6 beta cell apoptosis. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48 h with either 1 μM SST14 or 100 ng/ml PTX then exposed to 0.5 mM palmitate for 20 h. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=6, graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni *p<0.05, ****p<0.0001. NS = not significant p>0.05.

apoptosis induced by 0.5 mM palmitate ($p<0.05$; Fig. 3.4.10) which was significantly reversed in the presence of PTX ($p<0.0001$ vs 0.5 mM palmitate +1 μM SST).

3.5 Discussion

Beta cell dysfunction and apoptosis are central in the development of type 2 diabetes. Strategies to overcome detrimental effects of cellular stressors such as lipotoxicity and inflammation are fundamental for treatment of the condition (reviewed by Hasnain *et al.*, 2016; Wajchenberg *et al.*, 2007). The experiments described in this chapter aimed to affirm the effect of somatostatin on beta cell function and the identification of the receptor mediating its effects.

3.5.1 The effects of somatostatin on MIN6 beta cell function

As detailed in section 3.4.1 treatment with somatostatin significantly inhibited glucose and FSK+IBMX-induced stimulation of insulin secretion confirming that the compound used in this study is active and fulfils its function as an insulinostatic hormone (Hauge-Evans *et al.*, 2009). Pre-treatment with SST for 48 h significantly decreased palmitate-induced apoptosis in MIN6 beta cells. These results are in corroboration with the previous work in our lab reported in Damsteegt *et al.* (2019) where the same effect was observed. Previously others have reported both pro and anti-apoptotic roles for SST. Jia *et al.* (2013) found that hepatic injury following 90% hepatectomy was alleviated by treatment with SST in combination with prostaglandin E1 (PGE1). Here the percentage survival of hepatocytes was greater compared to hepatectomy control for the compounds individually and in combination. It was also found that expression of caspase-3, CHOP as well as of pro-inflammatory cytokines TNF-α and interleukin 6 (IL6) was reduced by co-treatment of SST with PGE1 (Jia *et al.*, 2013). It was reported that SST is present in the neuroretina and retinal pigment epithelium of both diabetic and

non-diabetic patients. However, its levels were lower in the neuroretina of patients with diabetes where a higher level of apoptotic cells was observed. This elevation in apoptosis was inversely correlated with the level of SST suggesting that lower levels of SST are associated with higher level of retinal neurodegeneration (Carrasco *et al.*, 2007). Others have shown that prolonged treatment with a high dose of SST analogue octreotide conveyed protection against incidence of severe acute pancreatitis (SAP) in patients with predicted SAP and reduced the number of patients diagnosed with SAP by elevating plasma SST that in turn decreased TNF-α and IL6 levels (Wang *et al.*, 2013). Similarly, in rats fed a high fat diet causing high plasma TNF-α levels, injection of octreotide reduced elevation in plasma TNF-α (Liu *et al.*, 2012). These studies suggest that similar to our observations SST plays a pro-survival role in a number of tissues. Others however have suggested that SST and its analogues caused apoptosis in cancer tissues including breast cancer where treatment with SST induced activation of caspase-8 and increased the number of apoptotic cells in clonal breast cancer cell line Michigan Cancer Foundation-7 or MCF7 (Liu *et al.*, 2000). It has also been reported that SST inhibits proliferation in a number of cancers including lung, brain and colorectal (reviewed by Ferjoux *et al.*, 2000 and Pyronnet *et al.*, 2008), and displayed anti-angiogenic activity in the highly vascularised Kaposi sarcoma tumour induced by subcutaneous injection in nude mice (Florio *et al.*, 2003). Here treatment with SST inhibited tumour growth and the sarcomas were highly lacking in vascularisation. The effects were found to be mediated by SST receptor 3 (Florio *et al.*, 2003). Taken together these data suggest that the effects of SST on apoptosis are divergent and tissue specific as evident by the

anti-apoptotic activity observed mainly in tumour cells (reviewed by Pyronnet *et al.*, 2008).

However, the anti-apoptotic effect of SST was not observed in MIN6 cells exposed to 500 U/ml TNF-α + 50 U/ml IL-1β in contrast to our previous observations (Damsteegt *et al.*, 2019). It is possible that in the current experiments the detrimental effect of cytokines could not be overcome by the addition of SST. In addition, treatment of MIN6 cells with SST could not significantly reduce apoptosis caused by ER-stress inducer thapsigargin. This was interesting because both palmitate and thapsigargin have been shown to affect intercellular Ca^{2+} concentrations and activate unfolded protein response (UPR) (Cnop *et al.*, 2010). Our previous findings reported in Damsteegt *et al.* (2019) indicated that exposure to palmitate upregulated downstream modulators of UPR in MIN6 cells including DNA damage-inducible transcript 3 (*Ddit3*) that encodes pro-apoptotic transcription factor C/EBP-homologous protein (CHOP) that is also induced by the activity of thapsigargin (Yamaguchi and Wang, 2004). We have shown that treatment with SST significantly downregulated *Ddit3* mRNA expression in MIN6 cells incubated with both control medium and medium containing 0.5 mM palmitate (Damsteegt *et al.*, 2019). Therefore, the ineffectiveness of SST in protecting MIN6 cells against apoptosis caused by thapsigargin may be due to its highly potent pharmacological activity (García-Casas *et al.*, 2018) that could not be overcome by exposure to SST in the current study. Indeed, thapsigargin induces ER-stress by inhibiting sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps that regulate intracellular calcium levels (Thastrup *et al.*,

1990). Binding of thapsigargin to the ATPase was found to be irreversible despite further dilution of the compound (Sagara *et al.*, 1992).

Since the current study investigated the role of SST in overcoming the detrimental effects of stressors typical of type 2 diabetes it was important to include glucotoxicity and gluco-lipotoxicity as part of the study. With the use of the INS-1 cell line it was observed that treatment with 0.5 mM palmitate (lipotoxicity) and 0.5 mM palmitate + 20 mM glucose (glucolipotoxicity) but not 20 mM glucose (glucotoxicity) significantly increased apoptosis in INS-1 cells. Beta cell dysfunction as a result of glucotoxicity typically results following prolonged exposure to high glucose levels (Donath *et al.*, 2005; Kaiser *et al.*, 2003). It is therefore possibly that the 20 h incubation period used in the current study was not long enough to observe any increase in apoptosis due to elevated glucose levels. In addition, no synergistic effect was observed for glucolipotoxicity compared to lipotoxicity alone in increasing caspase 3/7 activity in INS-1 cells (Damsteegt *et al.*, 2019). As reported in El-Assaad *et al.* (2003) prolonged exposure to hyperglycaemic conditions resulted in a synergistic increase in beta cell apoptosis following exposure to 0.4 mM palmitate (El-Assaad *et al.*, 2003), again implying that prolonged exposure to high levels of glucose is necessary to observe a greater glucolipotoxic effect. In addition, it is possible that as a clonal beta cell line, INS-1 cells are more susceptible to the detrimental effects of 0.5 mM palmitate within the 20 h incubation period model used in this study compared to 20 mM glucose. Indeed, it has been previously reported that greater cellular dysfunction in hepatocytes was caused by exposure to free fatty acid oleate than 30 mM glucose within the same incubation period (Pang *et al.*, 2013)

suggesting that lipotoxicity is a faster and more potent inducer of cell apoptosis than glucotoxicity.

One of the downstream signalling pathways activated upon binding of SST to its receptors is mediated via $G_{\alpha i}$. Therefore, pertussis toxin, a known inhibitor of $G_{\alpha i}$ (Mangmool and Kurose, 2011), was used to test the hypothesis that the effects of SST on MIN6 cells were mediated by the activation of $G_{\alpha i}$. As shown in section 3.4.3 and also in Damsteegt *et al.* (2019) treatment with PTX significantly reversed the anti-apoptotic effect of SST in response to 0.5 mM palmitate in MIN6 cells, indicating that the protective effect of SST is mediated via a $G_{\alpha i}$ dependent pathway in accordance with observations made by Sharma *et al.* (1996) for octreotide. It is therefore possible that by inhibiting insulin secretion, SST potentially lessens the metabolic burden of MIN6 cells improving their response to apoptosis induced by some cellular stressors typical of type 2 diabetes. However, the binding of SST to its receptors also activate downstream signalling pathways that are independent of $G_{\alpha i}$. These include inhibition of Ca^{2+} and activation of K^{+} channels that result in the hyperpolarisation of cell membrane causing inhibition of insulin secretion (reviewed by Theodoropoulou and Stalla, 2013), further supporting the possibility that inhibition of insulin secretion in MIN6 cells may play a role in their survival. Acute exposure to free fatty acids promotes insulin secretion from beta cells (Turk *et al.*, 1993; Cen *et al.*, 2016) that can result in increased strain on MIN6 cell secretory function. Indeed, insulin secretion is a highly active process that lead to production of reactive oxygen species and oxidative stress (Fridlyand and Philipson, 2004). Furthermore, it was reported that co-treatment of PGE1with somatostatin resulted in the accumulation of anti-

apoptotic protein Bcl-2 in hepatocytes (Jia *et al.*, 2013) and over-expression of human *Bcl-2* in mouse islet beta cells was found to inhibit apoptosis induced by a protein kinase inhibitor, staurosporine (Allison *et al.*, 2000). Therefore, it is possible to suggest that SST performs its pro-survival activity in MIN6 cells via both reduction in apoptosis and upregulation of pro-survival Bcl-2.

3.5.2 The role of SSTR3 in mediating the effects of SST in MIN6 cells

As mentioned before SST acts via five GPCRs (SSTR1-5). Many studies have reported their expression in islets and beta cells (e.g. Ludvigsen *et al.*, 2004; Strowski *et al.*, 2000). However, in most studies SSTR3 expression was not prominent and based on pharmacological studies and *in vivo* studies with SST receptor knockout mice, other SST receptor subtypes have been thought to be functionally important in the beta cell (Kailey *et al.*, 2012; reviewed by Strowski and Blake, 2007). We have detected SST receptor-3 mRNA to be expressed at the highest level in MIN6 beta cells compared to the other SSTR subtypes (Damsteegt *et al.*, 2019). These results were in corroboration with recent single-cell transcriptomic studies where only *Sstr3* expression was detected in primary beta cells (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). The results described in 3.4.2.1 also showed that *Sstr3* was present in clonal beta cell lines and mouse islets. Next it was explored whether SSTR3 was also expressed at protein level in MIN6 cells and in mouse islets in the current study. In addition, due to contrasting evidence about functional role of the SST receptor subtypes it was tested whether SST inhibited insulin secretion and promoted cell survival via its activation of SSTR3 in MIN6 cells.

As described in 3.4.2.2 it was found that SSTR3 is expressed in both MIN6 and INS-1 clonal beta cell lines and in the mouse hypothalamus. Despite detecting

Sstr3 mRNA in mouse islets its protein expression could not be identified by Western blot suggesting that perhaps in the islet as a composite tissue the expression of SSTR3 may not be as high, consistent with previous literature describing SSTR1, 2 and 5 as SST receptors playing the most prominent roles in the mouse islets (Kailey *et al.*, 2012; Ludvigsen *et al.*, 2004). Nonetheless it appears that in clonal beta cell lines the high expression of SSTR3 (Damsteegt *et al.*, 2019) may still have a functional role as also suggested by single-cell transcriptomic analyses for isolated primary beta cells (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). In the current study, the role of SSTR3 was further explored in functional studies investigating the effect of SST on insulin secretion in MIN6 cells.

In corroboration with Pasternak *et al.* (2012) where a precursor of MK4256 was used together with octreotide, it has been confirmed here that the insulinostatic effect of SST was reversed by SSTR3 antagonist MK4256 in MIN6 beta cells, however at the higher concentration of 5 μM. Therefore, it is not possible to disregard that the inhibitory effect of SST on insulin secretion in MIN6 cells has been mediated via SST receptors other than SSTR3. Indeed, it was reported that MK4256 displayed antagonistic activity of SSTR3 at IC50 of 0.95 nM and showed 83% inhibition of human functional cAMP at 2 μM. It also exhibited 42% inhibitory activity at 20 μM for SSTR4 and 34% at 5 μM for SSTR5 (He *et al.*, 2012). These findings can be further evaluated by using small interfering RNA (siRNA) to knockdown SSTR3 in MIN6 beta cells and to test whether the insulinostatic effect of SST is similar to the reports of Pasternak *et al.*, (2012), where a global *Sstr3* knockout mouse model was used. Despite MK4256 antagonising the inhibitory activity of SST on insulin

secretion in MIN6 cells, my findings did not satisfy the hypothesis that the pro-survival effect of SST is mediated via SSTR3. This might be because SST-mediated activation of other SSTR subtypes may be involved in conveying its pro-survival effect in beta cells. However, even at 5 μM the antagonist MK4256 did not significantly reverse the protective effect of SST. Instead a significant reduction in apoptosis was observed similar to that of SST alone. This could be due to agonistic effect of the compound at high concentration because according to He *et al.* (2012) at 5 μM, the compound showed 27% agonistic activity on SSTR4.

As detailed in 3.5.1 previous studies have associated SST and its receptors with both an anti-proliferative role for SST in cancer (reviewed by Ferjoux *et al.*, 2000; reviewed by Pyronnet *et al.*, 2008) and a pro-survival role in retinal neurodegeneration as well as neuronal inflammation (Hernández *et al.*, 2013; reviewed by Pintér *et al.*, 2006). The majority of these studies indicated SSTR2, 4 or 5 as the receptor(s) mediating the reported effects irrespective of the outcome, with only a few exploring the role of SSTR3. War *et al.* (2011) investigated the role of the C-terminal domain of SSTR3 in the anti-proliferative action of SST in human kidney cells (HEK) transfected with wild type (wt) SSTR3 and a mutant SSTR3 with a deleted C-terminus and found that the anti-proliferative effect of SST was mediated via wt-SSTR3 (War *et al.*, 2011). Previously, Sharma *et al.* (1996) had shown that the pro-apoptotic signalling of octreotide was mediated exclusively via SSTR3 following induction of p53 and Bax in Chinese Hamster Ovary (CHO) cells expressing SSTR1-5. Since SSTRs are GPCRs that activate a number of downstream signalling proteins, it is possible that their activation is dependent on the type

of tissue hence its needs, resulting in tissue-specific variations in SST receptor expression and function.

3.5.3 Summary

The results reported in this thesis indicate that clonal beta cell apoptosis in response to lipotoxicity was reduced by treatment with SST and that SSTR3 is expressed at protein level in MIN6 beta cells and facilitates inhibition of insulin secretion by SST. However, the findings do not indicate that the anti-apoptotic effect of SST is also mediated via SSTR3, although the findings confirm that it is Gi dependent. In conclusion, somatostatin protects beta cells against lipotoxicity and cytokine-induced (Damsteegt *et al.*, 2019) apoptosis. Although SSTR3 may partly contribute to the mediation of the insulinostatic effect of SST in MIN6 beta cells it is unlikely that SST conveys its anti-apoptotic effect via this receptor in MIN6 cells.

CHAPTER 4 - The effect of ghrelin on MIN6 cell and murine islet function

4.1 Introduction

Ghrelin is an orexigenic hormone that is mainly produced in the stomach, but also expressed in the upper intestines, brain, heart and pancreas (Gnanapavan, 2002; Sato *et al.*, 2011; Wierup *et al.*, 2002; Weirup *et al.*, 2014). The two main forms of ghrelin are acyl-ghrelin and des-acyl-ghrelin. The acylation of des-acyl ghrelin is catalysed by the enzyme ghrelin-*O*-acyltransferase (GOAT). This modification is essential for binding to its receptor (reviewed by Castañeda *et al.*, 2010; reviewed by Sivertsen *et al.*, 2013). In this chapter the word ghrelin will refer to acyl-ghrelin unless otherwise specified.

As described in section 1.3.2 ghrelin plays a role in the regulation of glucose homeostasis by interfering with glucose-induced insulin secretion in both rodents and humans (Reimer *et al.*, 2003; Tong *et al.*, 2010). Therefore, in the pancreas, ghrelin has been mainly characterised for its role in regulating insulin secretion from beta cells (reviewed by Dezaki, 2013; reviewed by Granata *et al.*, 2010) with some studies reporting that ghrelin inhibits insulin secretion from clonal beta cells and rodent islets (Dezaki *et al.*, 2004; Esler *et al.*, 2007; Wierup *et al.*, 2004). Others, however, have found that insulin secretion was increased or remained unchanged in the presence of ghrelin in clonal beta cells, rat and human islets (Adeghate and Ponery, 2002; Date *et al.*, 2002; Diaz-Ganete *et al*, 2015; Granata *et al.*, 2007; Salehi *et al.*, 2004).

As stated before, most studies have focused primarily on the role of ghrelin in the regulation of insulin secretion and there is less information with regard to the potential impact of the peptide on beta cell and islet survival. With the use of a subclone of the beta cell line INS-1 (INS-1E) it was found that ghrelin

promoted clonal beta cell survival in response to a combination of cytokines typical of type 1 diabetes (Diaz-Ganete *et al.*, 2015). Granata *et al.* (2007) have reported that serum starved clonal beta cell lines hamster islet transformed-tioguanine resistant clone-15 (HIT-T15) and INS-1E showed elevated proliferation with increasing concentrations of both acyl and desacyl-ghrelin. In a research model of type 1 diabetes induced by TNF-α and interferon gamma (IFN-γ), apoptosis was partly prevented by treatment with ghrelin in HIT-T15 cells (Granata *et al.*, 2007). In the same study, human islet apoptosis induced by serum starvation and/or exposure to a combination of TNF-α, INF-γ and IL-1β was inhibited by exposure to ghrelin (Granata *et al.*, 2007). However, the pro-survival function of ghrelin seems to be tissue specific and dependent on experimental conditions. In certain cancers such as aldosteronomas and in two human adrenocortical carcinoma cell lines treatment with ghrelin increased basal apoptosis whereas in non-cancerous tissues and cells such as human umbilical vein endothelial cells (HUVEC), rat osteoblasts and human normal adrenocortical cells ghrelin had no effect on basal apoptosis (Belloni *et al.*, 2004). Taken together these studies imply that the investigations into the role of ghrelin in beta cell and islet survival is limited and its inhibitory role on insulin secretion remains controversial. Furthermore, only a very small number of studies have assessed the role of ghrelin on beta cell survival in response to cellular stress-inducing conditions typical of type 2 diabetes (Wang *et al.*, 2010). These questions therefore remain to be further investigated.

It was reported in chapter 3 of this thesis and in Damsteegt *et al.* (2019) that somatostatin (SST) promotes beta cell and islet survival under conditions of

cellular stress typical of type 2 diabetes. Both somatostatin and ghrelin act on the same tissues such as brain and gut (reviewed by Martinez, 2013; reviewed by Pradhan *et al.*, 2013), suggesting a potential functional interaction between the two hormones. Exogenous ghrelin was found to stimulate circulating SST levels in humans (Arosio *et al.*, 2003) and increased total (both acyl and des-acyl) ghrelin levels were detected in SST knockout mice (Luque and Kineman, 2007). Previous work in our laboratory has found that *GHSR1a* mRNA was upregulated in islets isolated from somatostatin-deficient mice (Hauge-Evans *et al.*, unpublished) further suggesting the interaction between the two. Similarly, SST was shown to suppress plasma ghrelin levels and the SST mimetic octreotide was found to inhibit ghrelin secretion in a pre-perfused rat stomach (Shimada *et al.*, 2003). Furthermore, centrally administered ghrelin reportedly enhanced growth hormone release by blocking the effect of SST in rats (Wargner *et al.*, 2009). These studies suggest that the interaction between somatostatin and ghrelin is tissue-specific.

Recently, it was shown that ghrelin can influence SST secretion in the pancreas as proposed in a study by DiGruccio *et al.* (2016) and confirmed by Adriaenssens *et al.* (2016), where ghrelin was suggested to act on islet delta cells and promote glucose-induced SST secretion, thus mediating an inhibitory effect of ghrelin on insulin secretion indirectly via release of SST from delta cells. We therefore hypothesized that the reported effect of ghrelin on beta cell and islet survival may similarly be mediated indirectly by SST. However, this is in contrast to studies described earlier proposing that ghrelin promoted clonal beta cell survival, which indicates a direct, delta-cell independent effect of ghrelin on the beta cells (Diaz-Ganete *et al.*, 2015; Granata *et al.*, 2007).

Hence, establishing the effect of ghrelin on beta cell and islet function constitutes an initial step in the process leading to the investigation of its potential interaction with SST in the pancreatic islets.

4.2 Aims and objectives

4.2.1 To investigate the effect of ghrelin on palmitate-induced apoptosis in MIN6 cells.

4.2.2 To assess the effect of ghrelin on glucose and forskolin-induced insulin secretion from MIN6 cells.

4.2.3 To investigate the effect of ghrelin on mouse islet apoptosis induced by pro-inflammatory cytokines typical of type 2 diabetes.

4.2.5 To analyse the role of ghrelin in glucose-induced insulin secretion from mouse islets.

4.3 Methods

4.3.1 Assessment of clonal beta cell viability and apoptosis

Cell viability

MIN6 cell viability was tested as described in section 2.5. Briefly, MIN6 cells were seeded at 25,000 cells/well and pre-incubated for 48 h in complete DMEM with or without 0-500 nM ghrelin (Tocris, UK) with replacement of treatment following 24 h of incubation. After the 48 h pre-incubation period the cells were exposed to 0.5 mM palmitate for 20 h and MTT assay was performed as described in section 2.5 to determine cell viability.

Apoptosis

Assessment of MIN6 cell apoptosis was performed by measurement of caspase 3/7 activity as described in 2.6.1. Briefly, 15,000 cells/well were seeded in white-walled 96 well plates and pre-treated as indicated above for cell viability assay. Following exposure to 0.5 mM palmitate or 500 U/ml TNF-α and 50 U/ml IL-1β, Caspase-Glo assay was performed as described in 2.6.1.

4.3.2 Assessment of apoptosis in mouse islets

Islets were pre-incubated in complete RPMI for 48 h as indicated above for MIN6 cells with or without 100 nM ghrelin. Next the islets were exposed to 1000 U/ml TNF-α and 50 U/ml IL-1β ± 100 nM ghrelin and immediately handpicked into a white-walled 96 well plate (5 islets/well) in 40 µl of incubation media. Following 20 h incubation Caspase-Glo assay was performed as described in 2.6.2.

4.3.3 Static incubation experiments

Insulin secretion from clonal beta cells

Insulin secretion from MIN6 cells in response to 2 mM glucose, 20 mM glucose, 10 μM forskolin (FSK) + 100 μM 3-isobutyl-1-methylxanthine (IBMX) ± 100 nM ghrelin was determined as described in 2.4.1.

Insulin secretion from mouse islets

Islet insulin secretion was assessed in response to treatment with 2 mM glucose and 20 mM glucose with or without 100 nM ghrelin as described in 2.4.2.

All samples were diluted to concentrations detectable within the range of the standard curve and insulin contents were measured by radioimmunoassay as described in section 2.4.4.1.

4.3.4 H&E staining

Female mice used in this study were tested for oestrus cycle stage as described in 2.3. Briefly, vaginal smears were fixed, hydrated and stained with haematoxylin and eosin. The stained smears were dehydrated and mounted with a glass coverslip. The images of slides were taken with a bright field microscope and oestrus cycle stage determined as described in 2.3.

4.4 Results

4.4.1 The role of ghrelin in MIN6 cell secretory function and apoptosis

Previous studies investigating the functional effects of ghrelin in clonal beta cells and islets have reported the peptide to be effective at 10-100 nM (Diaz-Ganete *et al.*, 2015; Granata *et al.*, 2007; Wierup *et* al., 2004). For experimental purposes a concentration response test was conducted to identify the concentration of ghrelin to be used in the current research model. MIN6 cells were pre-treated for 48 h with 0-500 nM ghrelin with the medium being replaced after 24 h since the acylated form of the compound is unstable and the acyl group can be cleaved by esterases in the medium (reviewed by Delporte, 2013). Cell viability was significantly reduced in the presence of palmitate ($p<0.0001$), but incubation with ghrelin did not significantly prevent cell death at all concentrations tested ($p>0.05$) (Fig. 4.4.1). Further testing utilising the Promega CellTitre-Glo assay that detects cell viability by measurement of ATP content confirmed the above findings (data not shown).

In accordance with the literature, 10 and 100 nM ghrelin were therefore chosen for further testing since these concentrations have been shown to be effective in other experimental in vitro settings.

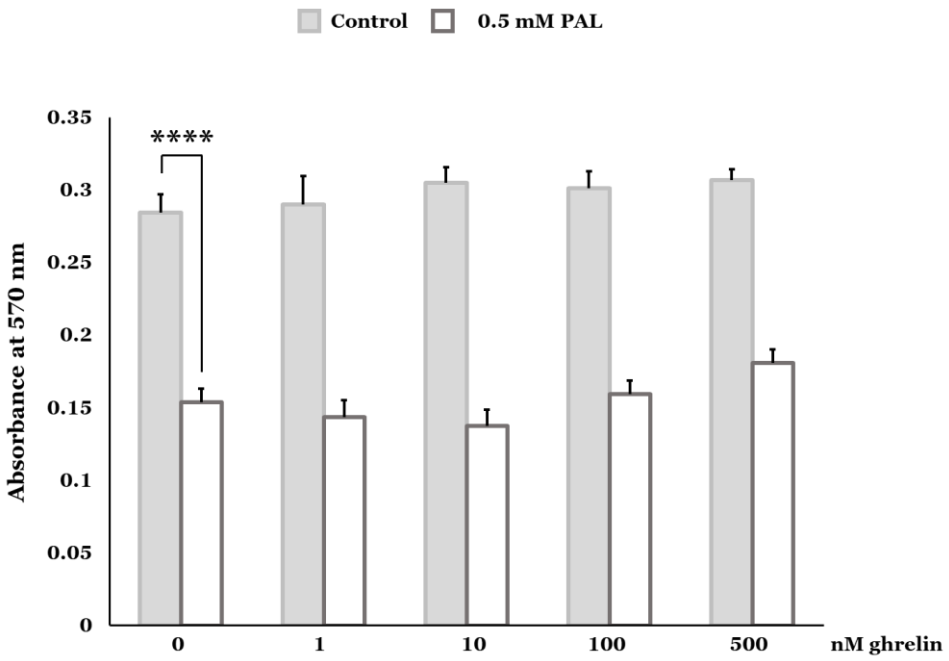

Figure 4.4.1 The effect of ghrelin on MIN6 cell viability. MIN6 beta cells were plated at 25,000 cells/well and pre-treated for 48 h with or without a range of concentrations of ghrelin as indicated and exposed to 0.5 mM palmitate for 20 h ± ghrelin. Cell viability was detected by measurement of MTT incorporation. Data is presented as mean+SEM, n=6, graph represents results of one experiment out of two independent experiments. One-Way ANOVA with posthoc test Bonferroni ****$p<0.0001$.

The cell viability assays used were based on the measurement of cellular metabolic activity and provided only an indication of how many cells were viable rather than the specific modulators of cell survival such as proliferation and reduction in apoptosis. Therefore, measurement of caspase 3/7 activity was employed to detect cell apoptosis in order to elucidate whether ghrelin affects specific underlying regulators of cell survival. Similar to cell viability experiments, apoptosis induced by 0.5 mM palmitate ($p<0.05$; Fig. 4.4.2A) or 500 U/ml TNF-α and 50 U/ml IL-1β ($p<0.0001$) was not significantly prevented following treatment of the cells with ghrelin ($p>0.05$) (Fig. 4.4.2B).

Since ghrelin did not affect MIN6 beta cell apoptosis, the functional responsiveness of MIN6 cells to ghrelin in general was investigated by assessment of insulin secretion. Since MIN6 cells are grown in medium containing 25 mM glucose and do not consistently respond to stimulation with 20 mM glucose, FSK and IBMX were used to potentiate glucose-induced insulin secretion from MIN6 cells. Incubation with 100 nM ghrelin did not significantly reduce insulin secretion stimulated by FSK and IBMX ($p>0.05$) nor did it affect insulin release at 20 mM glucose alone ($p>0.05$, Fig. 4.4.3).

These results implied that ghrelin may not directly modulate beta cell function and survival. This is consistent with recent single-cell transcriptomic studies (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016), where ghrelin's effect on insulin secretion from pancreatic beta cells was found to be mediated via release of SST from pancreatic delta cells. MIN6 cells are a monotypic, monolayer beta cell line therefore useful to study direct effects of any compounds on beta cells. However, to study interaction between different cell types, the islet research model is required. Hence mouse islets that are a composite organ containing both beta and delta cells were used in the subsequent experiments. For this purpose, islets from male CD-1 mice were used in initial experiments.

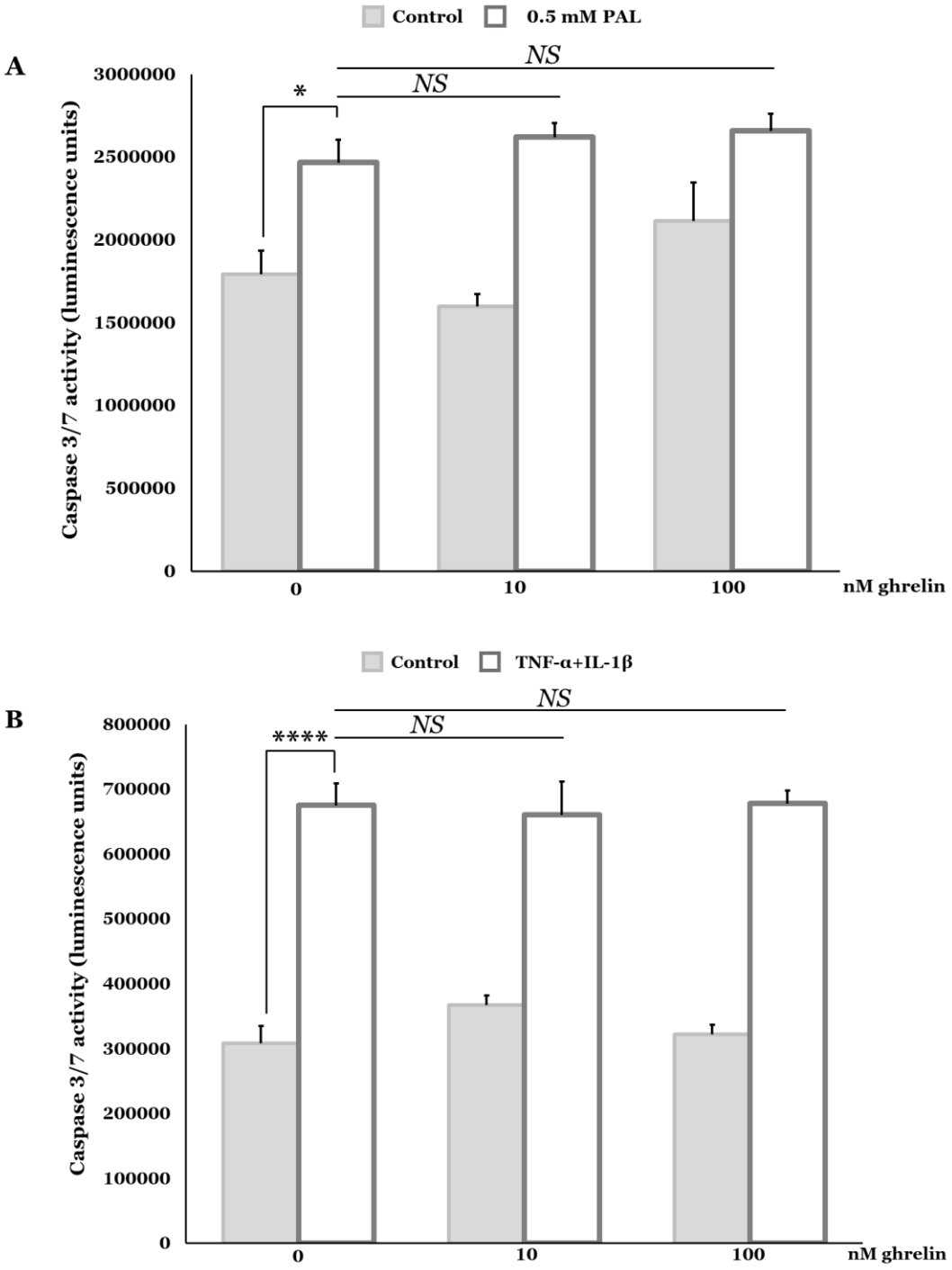

Figure 4.4.2 The effect of ghrelin on MIN6 cell apoptosis. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48 h with or without 10 or 100 nM ghrelin and exposed to 0.5 mM palmitate ± ghrelin (A) or 500 U/ml TNF-α+ 50 U/ml IL-1β ± ghrelin (B) for 20 h. Apoptosis was detected by measurement of caspase 3/7 activity. Data is presented as mean+SEM, n=5. Graph A represents results of one experiment out of three independent experiments and graph B of one experiment. One-Way ANOVA with posthoc test Bonferroni *$p<0.05$ and ****$p<0.0001$. NS = not significant $p>0.05$.

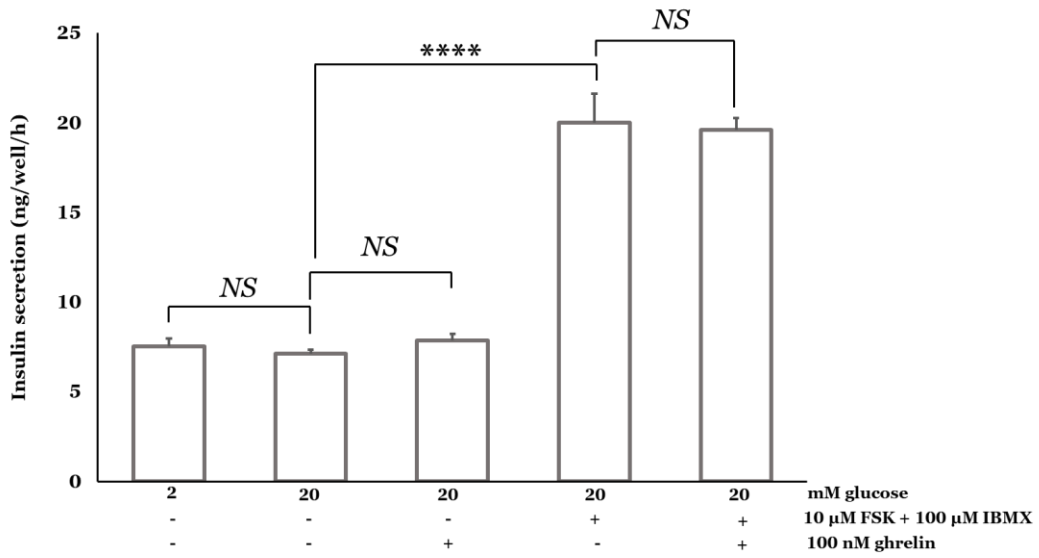

Figure 4.4.3 The effect of ghrelin on MIN6 insulin secretion. MIN6 cells were plated at 20,000 cells/well and incubated for 48 h. The cells were then pre-incubated with 2 mM glucose for 2 h and subsequently treated as indicated for 1 h. Supernatants were assayed for insulin content by radioimmunoassay. Data is presented as mean+SEM, n=6-8 and graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni ****$p<0.0001$. NS = not significant, $p>0.05$.

4.4.2 The role of ghrelin in islet survival and function

First a concentration response study with 10 and 100 nM ghrelin was conducted, followed by a time course experiment, where the impact of treatment duration was assessed. A combination of pro-inflammatory cytokines (1000 U/ml TNF-α and 50 U/ml IL-1β) was used as a cellular stress inducing agent to ensure that an adequate apoptotic signal was obtained, as the detrimental effect of palmitate on survival in intact islets has been inconsistent in previous studies in our lab (Hauge-Evans *et al.*, unpublished).

Exposure to ghrelin for 24 h did not alter cytokine-induced apoptosis at 10 or 100 nM of the peptide. Based on the literature (Diaz-Ganete *et al.*, 2015; Granata *et al.*, 2007) and MIN6 studies 48 h pre-treatment with 24 h replenishment and 10 and 100 nM concentrations were chosen for the subsequent experiments.

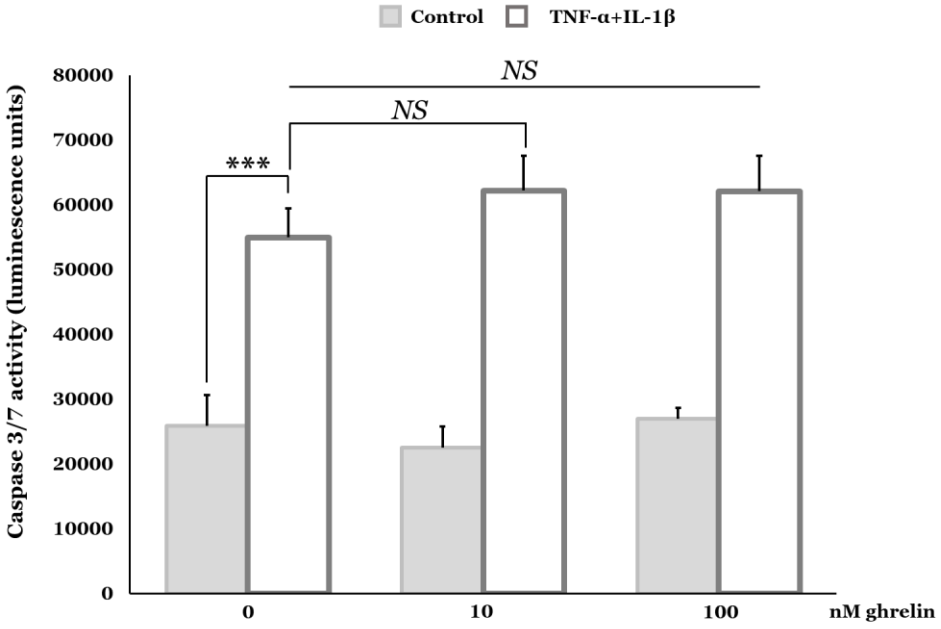

Figure 4.4.4 The effect of ghrelin on apoptosis in islets from male mice. Isolated mouse islets were pre-treated for 48 h with 10 or 100 nM ghrelin and exposed to 1000 U/ml TNF-α + 50 U/ml IL-1β for 20 h ± ghrelin. Cell apoptosis was detected by measurement of caspase 3/7 activity. Data is presented as mean+SEM, n=6. Graph represents results of one experiment out of 3-4 independent experiments. One-Way ANOVA with posthoc test Bonferroni ***$p<0.001$. NS = not significant $p>0.05$.

Islets from male mice pre-treated with or without ghrelin and exposed to cytokines as described above were further assessed for apoptosis. As shown in figure 4.4.4 islet apoptosis was significantly increased in the presence of

cytokines ($p<0.001$), however this was not significantly reversed by exposure to 10 or 100 nM ghrelin ($p>0.05$).

These results implied that either ghrelin does not affect islet apoptosis or that the peptide used in the study was inactive. Therefore, it was tested whether ghrelin modified glucose-induced insulin secretion in mouse islets. Incubation with 20 mM glucose for 1 h significantly increased islet insulin secretory response compared to basal levels ($p<0.001$), however this was not affected by treatment with 100 nM ghrelin ($p>0.05$) (Fig. 4.4.5).

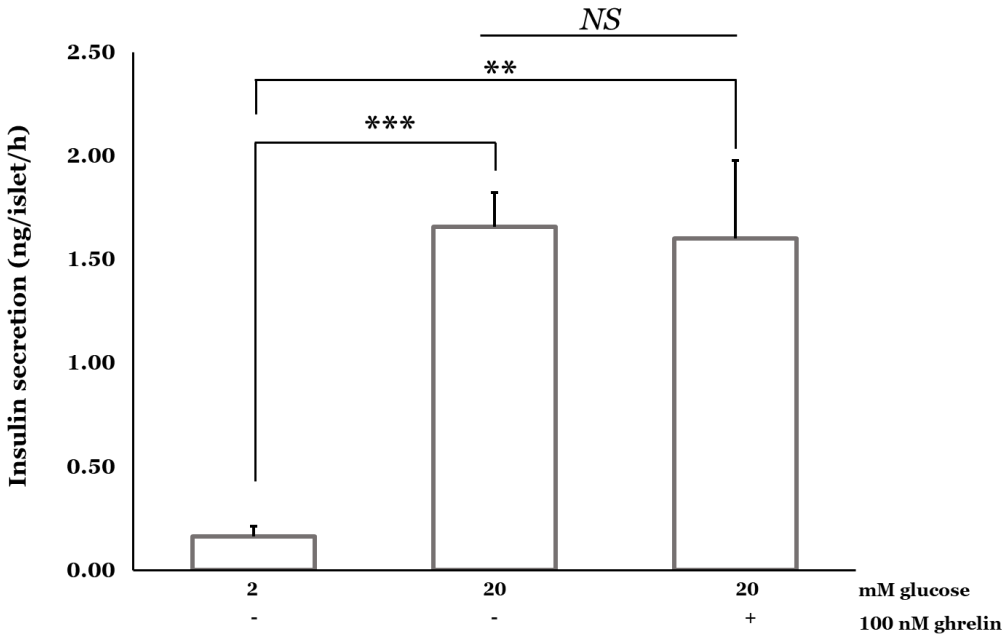

Figure 4.4.5 The effect of ghrelin on insulin secretion from islets from male mice. Isolated islets were pre-treated with 2mM glucose for 1 h followed by treatments as indicated for 1 h. Secreted insulin was detected by radioimmunoassay. Data is presented as mean+SEM, n=5-6, and graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni **p<0.01, ***p<0.001. NS = not significant p>0.05.

Further to the results described above, a literature search was conducted to check for any reports of sex-dependent effects of ghrelin as these experiments were carried out with islets from male mice only. This revealed that ghrelin displays sexual dimorphism with regards to circulating levels and orexigenic effects in both rodents and humans (Choi *et al.*, 2017; Clegg *et al.*, 2007). It was therefore hypothesized that ghrelin's effect in the pancreatic islets may depend on the biological sex of the animal from whom the islets were derived. Therefore, the role of ghrelin on survival and insulin secretory function was assessed in islets from age-matched female CD-1 mice.

Prior to the assessment of the functional effects of ghrelin, female mice were assessed for their oestrus cycle stage at time of islet isolation as described in section 2.3. H&E staining revealed that approximately 60% of animals were at metestrus stage, 31% at oestrus and the remaining at pro-oestrus stage (Fig. 4.4.6). This pattern was observed in mice used for all but two experiments where the distribution was 50:50 metestrus and oestrus stages.

In order to test whether treatment with ghrelin could reverse apoptosis induced by cytokines in islets from female mice, isolated islets were pre-treated for 48 h with or without ghrelin and exposed to cytokines for 20 h as indicated for islets from male mice. As shown in figure 4.4.7, incubation with 10 and 100 nM ghrelin significantly reduced cytokine-induced apoptosis in islets from female mice ($p<0.0001$).

Next, it was tested whether ghrelin modified glucose-induced insulin secretion in islets from female mice. Incubation with 20 mM glucose significantly increased islet insulin secretory response compared to basal level ($p<0.001$)

however this was not affected by treatment with 100 nM ghrelin ($p>0.05$, Fig. 4.4.8) similar to observations in islets from male mice and MIN6 cells.

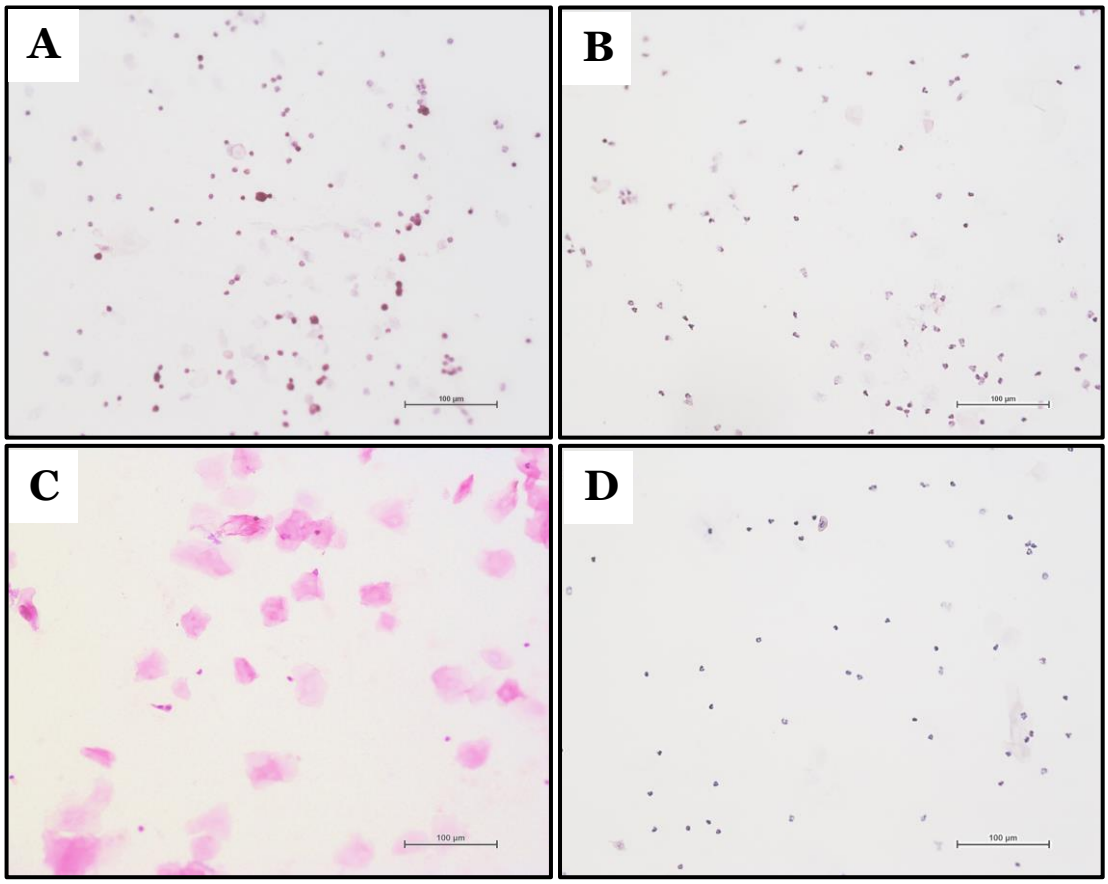

Figure 4.4.6 Determination of oestrus cycle stage of female mice. Vaginal smears were fixed in 3.7% formaldehyde and stained with H&E. Oestrus cycle stage was determined according to cell morphology. Images were taken with X20 objective and scale bar = 100 µm. Four representative images out of 48 are shown. 31% of female mice used were at oestrus stage (C) characterised by cornified squamous epithelial cells and high plasma oestrogen levels during the day. About 60% of female mice were at metestrus stage (A, B and D) characterised by leukocytes (dots) and a few nucleated or cornified squamous epithelial cells where plasma oestrogen levels are lowest during the mouse oestrus cycle.

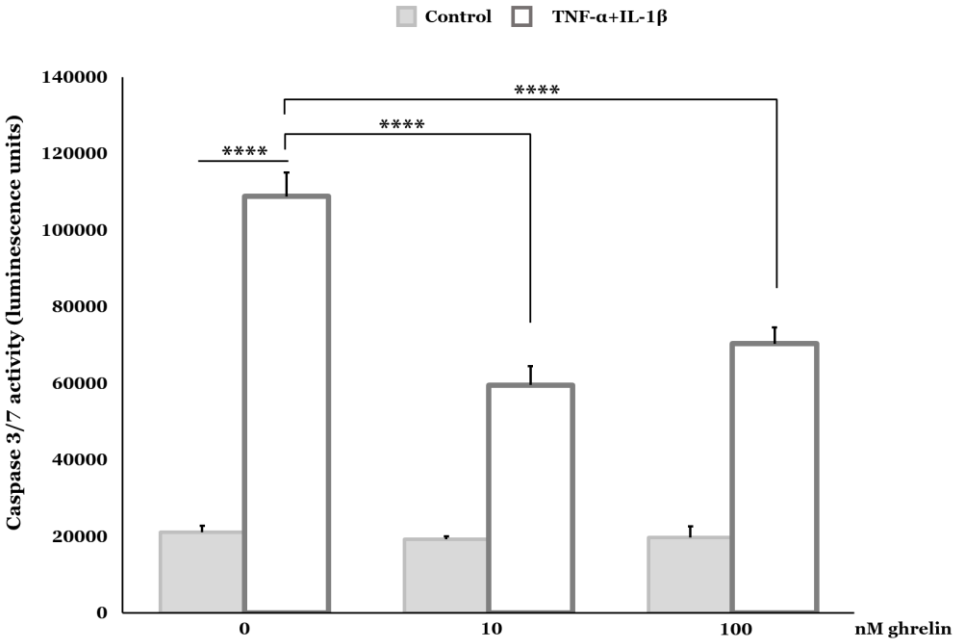

Figure 4.4.7 The effect of ghrelin on apoptosis in islets from female mice. Isolated mouse islets were pre-treated for 48 h with 10 or 100 nM ghrelin and exposed to 1000 U/ml TNF-α + 50 U/ml IL-1β for 20 h ± ghrelin. Cell apoptosis was detected by measurement of caspase 3/7 activity. Data is presented as mean+SEM, n=6, and graph represents results of one experiment out of 2-3 independent experiments. One-Way ANOVA with posthoc test Bonferroni ****$p<0.0001$.

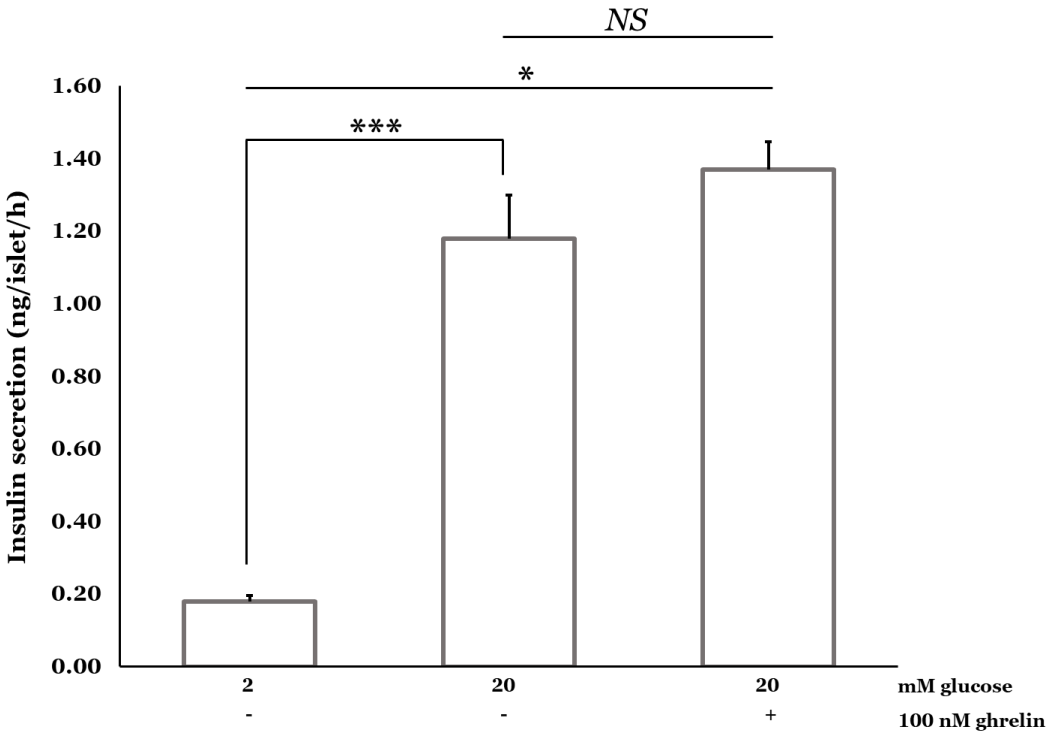

Figure 4.4.8 The effect of ghrelin on insulin secretion from islets from female mice. Isolated islets were pre-treated with 2 mM glucose for 1 h followed by treatments as indicated for 1 h. Secreted insulin was detected by radioimmunoassay. Data is presented as mean+SEM, n=5 and graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni *p<0.05, ***p<0.001.

4.5 Discussion

Ghrelin, an orexigenic hormone, has been shown to inhibit insulin secretion in beta cells and to promote beta cell and islet survival (Dezaki *et al.*, 2004; Diaz-Ganete *et al.*, 2015; Granata *et al.*, 2007). However, these actions remain contested due to reports that ghrelin either did not affect or it increased insulin secretion in rodent islets (Adeghate and Ponery, 2002; Salehi *et al.*, 2004) and promoted apoptosis in other tissues (Belloni *et al.*, 2004). In addition, the evidence supporting a pro-survival role for ghrelin in response to cellular stressors typical of type 2 diabetes remains limited. Furthermore, some have found that ghrelin interacts with somatostatin in a number of tissues such as brain and gastro-intestinal tract (Arosio *et al.*, 2003; Shimada *et al.*, 2003). Most recently it was reported that the insulinostatic action of ghrelin is due to an indirect and stimulatory action of ghrelin on pancreatic delta cells leading to the release of SST, which acts on the beta cell to inhibit insulin secretion (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). It was therefore hypothesized that the effect of ghrelin on beta cell and islet survival may also be mediated by the action of SST. However, this is discordant with studies showing a pro-survival action of ghrelin in clonal beta cell lines exposed to stressors typical of type 1 diabetes (Diaz-Ganete *et al.*, 2015; Granata *et al.*, 2007). Therefore, the experiments conducted in this chapter aimed to evaluate whether treatment with ghrelin could modify MIN6 cell and mouse islet survival and insulin secretory responses.

4.5.1 The role of ghrelin in MIN6 cell function

First of all it was necessary to establish whether the potential actions of ghrelin on pancreatic beta cell survival and insulin secretion are direct or indirect. For

this the MIN6 cell line was used as a model. Since MIN6 are a monotypic beta cell line it was employed to investigate the direct effects of ghrelin without any paracrine regulation by islet hormones released from other islet cell types.

Treatment with ghrelin did not significantly affect MIN6 cell apoptosis induced by 0.5 mM palmitate or a combination of cytokines as indicated in 4.4.1 in contrast to results reported by some (Diaz-Ganete *et al.*, 2015; Granata *et al.*, 2007; Zhang *et al.*, 2007). It is unlikely that this was due to 0.5 mM palmitate and the concentration of cytokines used being too potent. For example, Wang *et al.* (2010) used 0.4 mM palmitate at similar conditions for a duration of 24 h with replacement of treatment every 8 h suggesting that the apoptosis induced by palmitate could in part be prevented by treatment with ghrelin in the same cell line. It is important to highlight that there are only a limited number of studies investigating the role of ghrelin on beta cell apoptosis, and any discrepancies in results may be due to different experimental models. Despite Wang *et al.* (2015) reporting an effect in MIN6 cells, having conducted over three sets of repeats it is possible to confidently state that ghrelin did not significantly affect beta cell apoptosis induced by palmitate or a combination of cytokines in the current study as discussed next. When comparing the treatment with cytokines, although Diaz-Ganete *et al.* (2015) used a combination of three cytokines (100 ng/ml IFN-γ, 50 ng/ml TNF-α and 0.05 ng/ml IL-1β), the concentrations of those used in the current study did not differ much (50 ng/ml TNF-α and 0.1 ng/ml IL-1β) further suggesting that incubation with ghrelin could overcome the cytokine-induced apoptosis in clonal beta cells despite a more powerful combination of three cytokines than the two used in the current study. Importantly exposure to

ghrelin did not affect basal apoptosis levels in MIN6 cells in accordance with the observations by Diaz-Ganete *et al.* (2015) but not by Wang *et al.* (2010) where ghrelin also reduced basal apoptosis. It is therefore possible that the differences in experimental set up or absence of direct effect of ghrelin on MIN6 cells may have contributed to the discrepancy in results compared to the other studies.

Following lack of effect being observed for ghrelin on MIN6 cell apoptosis it was tested whether it could affect insulin secretion in the clonal beta cells in order to ascertain the general responsiveness of MIN6 cells to exogenous ghrelin. Incubation with ghrelin did not modify insulin secretion from MIN6 cells both in the presence of 20 mM glucose and 20 mM glucose with FSK and IBMX. These results are also discordant with a number of studies where acyl-ghrelin significantly reduced glucose-induced and basal insulin secretion in rodents (Dezaki *et al.*, 2004) or in clonal beta cells (Wierup *et al.*, 2004). However our results conform with other reports where treatment with ghrelin did not alter insulin secretory function in rodent islets (Salehi *et al.*, 2004).

These results suggest that either the ghrelin peptide used in the current study was not active or that MIN6 cells do not respond to treatment with exogenous ghrelin. The latter may either be due to the presence of endogenous ghrelin or the absence of ghrelin receptors on MIN6 cells. It has been reported that beta cells displayed both ghrelin-like and ghrelin receptor-like immunoreactivity (Kageyama *et al.*, 2005) and that *ghrelin* was detected at mRNA level in HIT-T15 clonal beta cell line (Granata *et al.*, 2007). The most plausible explanation, however, supports the findings in single-cell transcriptomic analyses, where the ghrelin receptor was only detected in the islet delta cells and it was revealed

that glucose-induced release of somatostatin was potentiated by the action of ghrelin on the islet delta cell (Adriaenssens *et al.*, 2016; DiGruccio *et al.* 2016). Therefore, both studies suggested that the insulinostatic effect of ghrelin was due to the direct action of somatostatin and not ghrelin on beta cells. In accordance with these reports the findings of this chapter indicate that ghrelin may not directly influence clonal beta cell survival and insulin secretion. Hence mouse islets were used in the subsequent experiments.

4.5.2 The role of ghrelin in mouse islet function

Experiments conducted using islets from male mice as described in 4.4.2 found that ghrelin did not significantly alter cytokine-induced apoptosis in islets in accordance with observations in MIN6 cells. However this result was divergent from the findings by Granata *et al.* (2007) where treatment with ghrelin reduced cytokine-induced apoptosis in human islets. Similar to the results in MIN6 cells ghrelin had no effect on basal apoptosis in mouse islets.

It is well documented that both ghrelin and its receptor are present within the islets of Langerhans (Kageyama *et al.*, 2005; Volante *et al.*, 2002). However, to my knowledge there is no report investigating involvement of the ghrelin receptor in mediating the action of ghrelin on islet survival, but instead many have characterised this for its effects on insulin secretion. Therefore, the role of ghrelin on islet insulin secretion was assessed and no significant outcome was observed. These results conform with the reports that exogenous ghrelin does not affect glucose-induced insulin secretion in islets from rodents (Adeghate and Ponery, 2002; Salehi *et al.*, 2004). Taken together these results reveal that the role of ghrelin in the regulation of islet function may be more complex than initially presumed.

The orexigenic effects and plasma levels of ghrelin have been reported to display sexually dimorphic characteristics in rodents and humans (Choi *et al.*, 2017; Clegg *et al.*, 2007; Yamada *et al.*, 2015). Based on the hypothesis that the action of ghrelin may be influenced by the sex of the mice from whom the islets were derived, it was tested whether ghrelin modified cytokine-induced apoptosis in islets from female mice. As reported in 4.4.2, treatment with ghrelin significantly reduced cytokine-induced apoptosis in islets from female mice suggesting a sex-dependent role for ghrelin in islet survival. This result confirmed that the peptide used in the current study was active and exposure to ghrelin only altered cytokine-induced but not basal apoptosis in accordance with observations in islets from male mice and MIN6 cells. Subsequent experiments investigating the effect of ghrelin on insulin secretion from islets from female mice found, however, no significant changes in glucose-induced insulin secretion in the presence of the peptide. It is important to highlight that the islets demonstrated good glucose-responsiveness therefore the lack of effect was not due to a substandard quality of islets. In addition, it is possible that research models used and differences in experimental set up may have contributed to observed effects. For example Dezaki *et al.* (2006) measured dynamic insulin secretion in a perfused isolated pancreas model where the effects of ghrelin on insulin secretion at different time points could be identified. In the current study a static insulin secretion model was used where overall insulin secretion at 1 h was detected. Furthermore by using the intact pancreas it is possible to better mimic physiological environment and input from non-endocrine tissues in this experimental setting cannot be discounted. As described in 1.3.2 a number of studies suggested that ghrelin regulates glucose homeostasis by altering islet responses to glucose (Broglio *et al.*, 2003;

Tong *et al.*, 2010). The results of the current study however suggest that the effect of ghrelin on glucose metabolism does not appear to be mediated via its effects on islet secretory responses as also suggested by Salehi *et al.* (2004). Others have reported that ghrelin also acts on liver and promotes hepatic gluconeogenesis (Barazzoni *et al.*, 2007), suggesting that *in vivo* effects of ghrelin on glucose metabolism may be regulated by inputs from a number of organs.

A study by Clegg *et al.*, (2007) explored how ovariectomising and treatment with oestradiol affected ghrelin's orexigenic action in rats. Here with the use of wild-type male, female and ovariectomised (OVX) female rats it was revealed that males and OVX females were more susceptible to orexigenic effects of ghrelin. Indeed, OVX female rats had increased ghrelin levels, food intake and weight gain compared to normal females. The ablation of the ghrelin receptor negated these effects (Clegg *et al.*, 2007). In another investigation by Yamada *et al.* (2015), the effects of two weeks of social isolation resulting in physiological stress were studied in mice and their observations focused on changes in body weight, feeding behaviours and plasma ghrelin levels. Young male mice showed increased plasma ghrelin levels compared to females following one week of isolation. This was also the case for older male mice although to a lesser extent. Furthermore, it was reported that male patients affected by functional dyspepsia, a condition affecting the upper gastrointestinal tract, had lower plasma ghrelin levels compared to female patients (Choi *et al.*, 2017). In another study involving male and female twins it was found that females had higher plasma ghrelin compared to males and that this level was higher in younger participants

(Makovey *et al.*, 2007). Overall these findings indicate that there are differences between males and females with regards to plasma ghrelin levels and responsiveness.

It is possible to argue that the presence of oestrogen in female individuals may have contributed to the divergent effects of ghrelin observed in mouse islets. It is well known that oestrogens protect against apoptosis; for example it was found that in a mouse model where oxidative stress was induced by streptozotocin (STZ), wild-type female mice showed normal islet architecture and beta cell number whereas male mice were more vulnerable to STZ treatment and had reduced beta cell mass and insulin content. In particular, male mice showed increased beta cell apoptosis and treatment with oestradiol suppressed this effect (Le May *et al.*, 2006). In another study it was found that the expression of the oestrogen receptor alpha (ERα) naturally protected beta cells from oxidative stress in mice (Killic *et al.*, 2014). Furthermore, in some mouse models such as those fed a high-fat diet, male mice were more prone to hypothalamic inflammation resulting in impaired glucose tolerance (Morselli *et al.*, 2014). In spontaneously diabetic mice, glycaemia was worse in males compared to females with aging (Diaz *et al.*, 2019). In both studies either the presence of oestrogen (Diaz *et al.*, 2019) or ERα-dependent pathways (Morselli *et al.*, 2014) were identified to be responsible for the differential outcomes. Sex-specific differences can arise not only due to the action of oestrogens but also androgens and it has been reported that the androgen receptor (AR) is expressed on islet beta cells (Mauvais-Jarvis, 2016). The studies investigating the role of androgens on beta cells have primarily focused on insulin secretory function. In males testosterone enhanced GSIS, whereas

in females excessive androgens resulted in hypersecretion of insulin and oxidative injury (Navarro *et al.*, 2018; Xu *et al.*, 2019). Indeed, female mice treated with dihydrotestosterone developed hyperinsulinaemia, insulin resistance and hyperglycaemia indicating beta cell failure, but this effect was not observed in beta cell-specific AR knockout mice (Navarro *et al.*, 2018). Similarly, hyperinsulinaemia was detected in a female rat model of polycystic ovary syndrome which was found to be caused by elevated *Ins1* transcription (Mishra *et al.*, 2018). Hence, it appears that only oestrogen conveys a protective effect on beta cell function in islets from both male and female mice. Therefore, it is possible that the protective effect of ghrelin in islets from female mice may be due to the presence of oestrogen rather than androgens, although isolated islets were used in the current investigation. Hence, at the time of the experiments islets from female mice were no longer exposed to elevated levels of E2 due to the ex-vivo experimentation. To my knowledge there are no reports on how long sex-related differences may last following islet isolation and as also discussed in section 7.2 it is possible that the observed sex-specific differences may be due to inherent differences between islets from male and female mice.

A study examining variations in plasma ghrelin levels in pre and post-menopausal women in response to oestradiol reported that there was no significant difference in ghrelin levels from start to end of treatment. However, post-menopausal women had significantly lower plasma ghrelin levels compared to pre-menopausal women (Dafopoulos *et al.*, 2010). This result conflicts with the observations by Clegg *et al.* (2004) described earlier where ovariectomised females had greater plasma ghrelin levels despite circulating

oestrogen being lower in both conditions. A study assessing whether the stages of oestrus cycle influenced ghrelin levels in rats found no significant changes in plasma and stomach ghrelin levels between cycle stages. However, similar to the findings by Dafopoulos *et al.* (2010) rats over the age of 37 weeks showed significantly less plasma ghrelin levels (Johnson *et al.*, 2016). Similarly, the female mice used in the current study presented different stages of the oestrus cycle and the studies by Dafopoulos *et al.* (2010) and Johnson *et al.* (2016) confirm the findings of H&E staining that the effect of ghrelin on islet survival is independent of the oestrus cycle stage of the animals used. Taken together these studies suggest that ovariectomy and menopause characterised by decreased circulating oestrogen affect plasma ghrelin levels, which, however, do not seem to be influenced by fluctuations in oestrogen levels during oestrus cycle.

Since ghrelin displays sexual dimorphism in its levels and action in some tissues it implies that the expression of ghrelin receptor may also depend on the sex of the animal. A study conducted in yellow catfish found *Ghsr1a* mRNA expression to be elevated in stomach, gut and hypothalamus of males whereas it was less expressed in pituitary, heart and muscle compared to females (Zhang *et al.*, 2016). Hence further elucidation of the expression of GHSR1a would aid to confirm whether the sex-specific effects of ghrelin depend on the expression of its receptor and this will be further investigated in chapter 5 of this thesis. As discussed previously, single-cell transcriptomic analyses suggest that *Ghsr1a* expression is cell-type specific within the islets, with exclusive expression on the islet delta cells (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). It is therefore possible that in islets from female mice

the pro-survival action of ghrelin may depend on altered expression of the ghrelin receptor thereby influencing somatostatin secretion and subsequent activation of SSTR-dependent intracellular pathways.

4.5.3 Summary

The results described in this chapter indicate that exogenous ghrelin does not play a role in apoptosis and insulin secretory function of MIN6 beta cells confirming the hypothesis that the effect of ghrelin on beta cell function may be indirect via its action on the delta cell and stimulation of secretion of somatostatin. However, experiments conducted in islets have revealed a sex-specific role for ghrelin where a reduction in apoptosis by ghrelin was only detected in islets from female mice. This function may depend on differences in the expression of ghrelin receptor, endogenous ghrelin, prolonged exposure to oestrogen or crosstalk with somatostatin. Some of these potential mediators of ghrelin action are further characterised in chapter 5 and 6 of this thesis.

CHAPTER 5 - The role of GHSR1a in mediating the functional effects of ghrelin in MIN6 cells and islets

5.1 Introduction

Ghrelin is known to act via the G-protein-coupled receptor (GPCR) GHSR1a and the acylation of the peptide has been found to be necessary for binding to this receptor (Kojima *et al.*, 1999; Davenport *et al.*, 2005). GHSR1a has been reported to be expressed almost ubiquitously in the body including in brain, stomach, pancreas, blood vessels, reproductive organs (testis and ovaries), intestines and heart (Gnanapavan *et al.*, 2002; Katugampola *et al.*, 2001; Uenberg *et al.*, 2009). However, within the islet, the specific location of its expression remains disputed. GHSR1a-like staining was observed by Kageyama *et al.* (2005) in both beta and alpha cells in rat pancreatic islets and in pancreatic tumours by Volante *et al.* (2002). Chuang *et al.* (2011) have found GHSR1a to be expressed only in alpha cells, suggesting that the effect on islet function is due to ghrelin's action on alpha-cells. But in 2016, single-cell transcriptomic analyses identified the mRNA expression of *Ghsr* exclusively in another murine islet cell type, the pancreatic delta cell (DiGruccio *et al.*, 2016); a finding that was confirmed by Adriaenssens *et al.* (2016). In both studies ghrelin's effect on beta cells was found to be mediated via release of somatostatin from delta cells. A number of studies have shown that ghrelin modulates insulin secretion in beta cells using a clone of the INS-1 cell line (Wierup *et al.*, 2004) and beta cells isolated from rat islets (Date *et al.*, 2002; Dezaki *et al.*, 2004), indicating a direct action either via GHSR1a expressed at very low levels (Kageyama *et al.*, 2005) or alternatively via another ghrelin-binding receptor being expressed on beta cells (Granata *et al.,* 2007). These studies highlight the controversy regarding the expression pattern of GHSR1a in the pancreatic islets that warrants further investigation.

In the process of elucidating the mechanisms conveying the findings reported in chapter 4, identification of the role of GHSR1a in islets therefore constitutes a fundamental step.

As described in 1.3.2, a number of GHSR1a antagonists have been developed to date. In the present study the synthetic GHSR1a antagonist YIL781 and the endogenous antagonist LEAP2 were used to identify the role of GHSR1a in mediating the functional effects of ghrelin in clonal beta cells and islets reported in the previous chapter. Furthermore, based on the findings in chapter 4, the experiments of this chapter have also assessed whether a potential differential expression of GHSR1a in the islets from male and female mice may have contributed to the observed sex-specific response to cytokine-induced apoptosis following exposure to ghrelin.

5.2 Aims and objectives

5.2.1 To explore whether the effect of ghrelin on islet apoptosis is mediated by GHSR1a in islets from female mice.

5.2.2 To investigate additional roles of GHSR1a in apoptosis in islets from male mice and in MIN6 cells.

5.2.3 To determine mRNA expression of *Ghsr1a* and protein expression of GHSR1a in islets and clonal beta cells.

5.2.4 To assess the role of GHSR1a on glucose-induced insulin secretion in islets.

5.3 Methods

5.3.1 Clonal beta cell and islet apoptosis

MIN6 cells

Assessment of MIN6 cell apoptosis was performed by measurement of caspase 3/7 activity as described in 2.6.1. Briefly, 15,000 cell/well were seeded in white-walled 96 well plates. Cells were pre-treated with or without 100 nM ghrelin ± GHSR1a antagonist (YIL781-Tocris, UK and LEAP2 – Peptides International, USA) in DMEM for 48 h with refreshment of treatment after 24 h. Following exposure to 0.5 mM palmitate for 20 h with or without ghrelin ± GHSR1a antagonist Caspase-Glo assay was performed as described in 2.6.1.

Islets

Islets were pre-treated in complete RPMI for 48 h as indicated above for MIN6 cells with or without 100 nM ghrelin ± GHSR1a antagonist. Islets were then exposed to 1000 U/ml TNF-α and 50 U/ml IL-1β ± 100 nM ghrelin ± GHSR1a antagonist and immediately handpicked into a white-walled 96 well plate (5 islets/well) in 40 µl of supplemented media. Following 20 h incubation, Caspase-Glo assay was carried out as described in 2.6.2.

5.3.2 Gene expression analysis

RNA extraction, cDNA reverse transcription and qPCR were performed as described in section 2.7 with the use of primers for house-keeping genes (section 2.7.3); and Ghrelin receptor (*Ghsr1a*) made in-house (Table 5.1). Gene expression was determined relative to the house-keeping genes *Ppia* and *Hprt*.

Table 5.1 Details of *Ghsr1a* primers

Gene	Primer sequence (5'-3')	PCR product (bp)	T annealing (°C)
Ghsr1a	FW: GCACCACCACCAACCTCTAC RV: GCAGCTCTCGCTGACAAACT	155	60

5.3.3 Static incubation experiments for assessment of insulin secretion from mouse islets

Islet insulin secretion was assessed as described in 2.4.2 in response to treatment with 2 mM glucose and 20 mM glucose with or without 100 nM AG ± 5 μM YIL781 or 100 nM LEAP2. All samples were diluted to concentrations detectable within the range of the standard curve and the concentration of insulin was measured by radioimmunoassay as described in section 2.4.4.1.

5.3.4 Detection of protein

Cell and tissue lysates were prepared using 1x RIPA buffer containing 1x protease inhibitor cocktail as described in 2.8.1. The lysates of leukaemia cell lines were kindly provided by Dr Yoana Arroyo. Following SDS-PAGE, proteins were transferred on to a nitrocellulose membrane by Western blot as described in 2.8.4. Rabbit polyclonal antibody to GHSR1a was purchased from Life Technologies (ThermoFisher Scientific, UK, cat no. 720278) and used at a final concentration of 1 μg/ml. GHSR1a expression was expressed relative to loading control beta-actin.

5.4 Results

5.4.1 The effect of GHSR1a antagonists on islet and MIN6 apoptosis following co-treatment with ghrelin.

Following the discovery in chapter 4 of ghrelin conveying a protective effect on pro-inflammatory cytokine-induced apoptosis only in islets from female mice, two GHSR1a antagonists were used to determine whether this effect of ghrelin could be reversed to identify whether ghrelin acts via the known receptor GHSR1a.

YIL781

First the competitive antagonist YIL781 was used. Treatment with 100 nM ghrelin significantly reduced cytokine-induced apoptosis in islets from female mice ($p<0.0001$; Fig. 5.4.1A), however exposure to 5 μM YIL781 did not significantly reverse this effect ($p>0.05$). Instead, incubation with 5 μM YIL781 alone resulted in a significantly reduction ($p<0.0001$) in islet apoptosis compared to cytokine-treated control.

The finding led to the investigation whether YIL781 alone had a similar effect in islets from male mice and MIN6 cells to test the potential non-specific nature of the compound, and/or whether the observed effect was dependent on the presence of non-beta cells in islets. As reported in chapter 4, incubation with exogenous ghrelin did not significantly influence apoptosis induced by cytokines in islets from male mice ($p>0.05$; Figure 5.4.1B) and palmitate-induced apoptosis in MIN6 cells ($p>0.05$; Figure 5.4.2). However, similar to the results observed for islets from female mice, treatment with 5 μM YIL781 alone significantly reduced ($p<0.01$) apoptosis in MIN6 cells (Fig. 5.4.2).

Although a similar trend was observed in islets from male mice treated with 5 μM YIL781, it was not significantly different compared to the cytokines only control (p>0.05) (Fig. 5.4.1B).

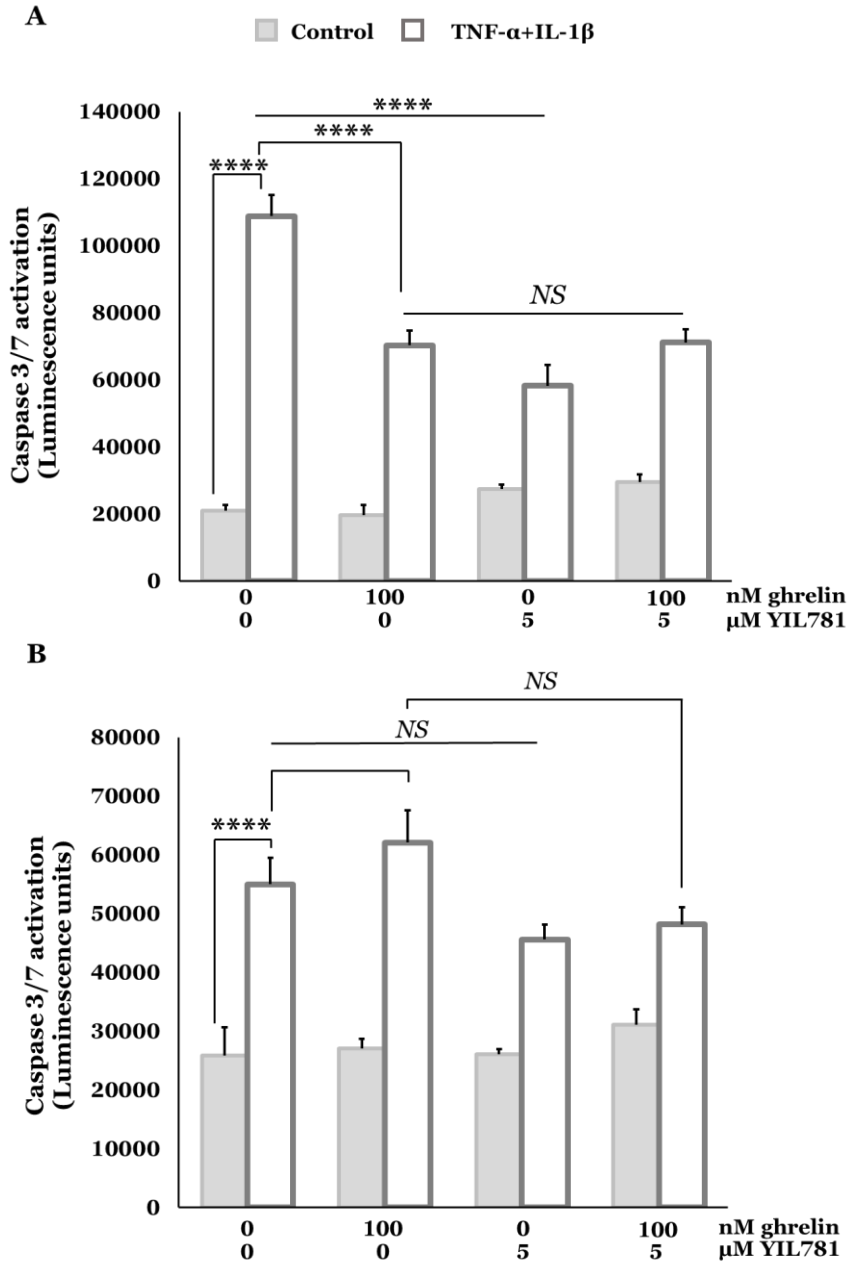

Figure 5.4.1 The effect of ghrelin and YIL781 on apoptosis in islets from female (A) and male (B) mice. Isolated islets were pre-treated for 48h with or without 100 nM ghrelin ± 5 μM YIL781 and exposed to 1000 U/ml TNF-α+50 U/ml IL-1β for 20 h ±100 nM ghrelin ± 5 μM YIL781. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=6. Each graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni ****p<0.0001. NS = not significant p>0.05.

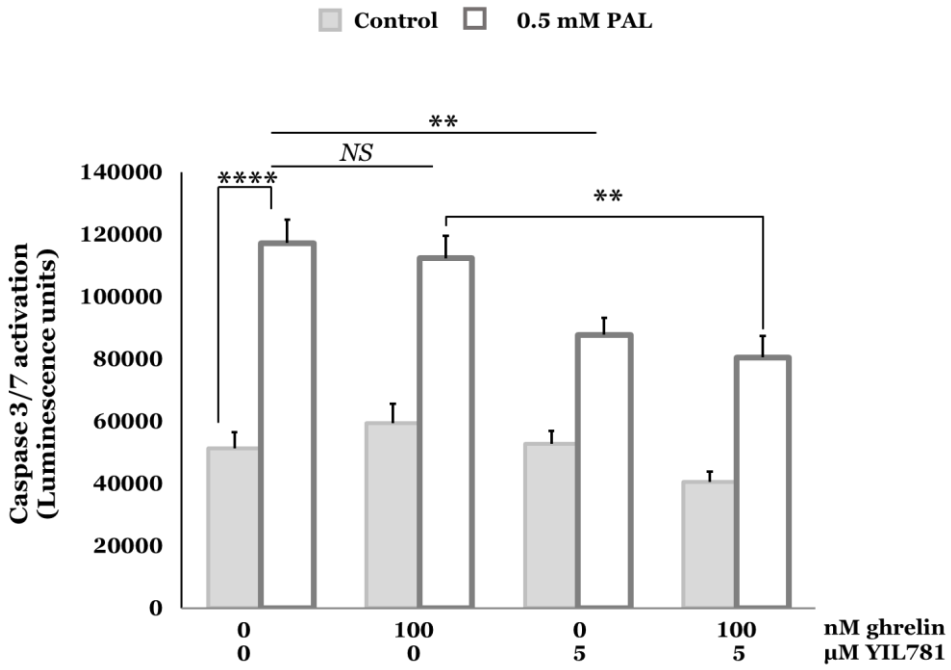

Figure 5.4.2 The effect of ghrelin and YIL781 on MIN6 beta cell apoptosis. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48 h with or without 100 nM ghrelin ± 5 µM YIL781 and exposed to 0.5 mM palmitate for 20 h ± 100 nM ghrelin ± 5 µM YIL781. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=5. Graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni **p<0.01 and ****p<0.0001. NS = not significant p>0.05.

These results implied that YIL781 may display non-specific effects. As the number of commercially available GHSR1a antagonists is limited the discovery of LEAP2 was of great interest for the current project. LEAP2 was then used to test if the effect of ghrelin in islets from female mice could be reversed. In addition, LEAP2 was also utilised to assess whether the observed change in apoptosis following YIL781 treatment was specific to YIL781, or was

a general effect of GHSR1a antagonists, including the endogenous antagonist LEAP2.

LEAP2

Based on the results reported in Ge *et al.* (2018), where 100 nM LEAP2 exerted the greatest inhibition of ghrelin-mediated activation of GHSR1a, LEAP2 was used at 100 nM in the current study. Treatment with 100 nM ghrelin significantly reduced cytokine-induced apoptosis in islets from female mice

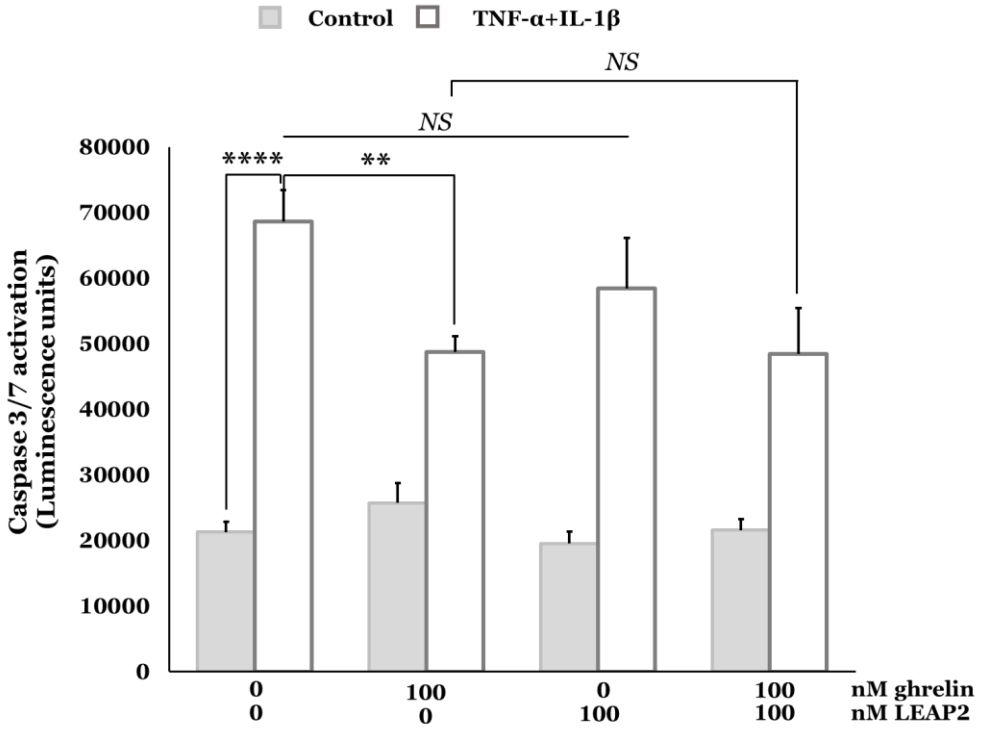

Figure 5.4.3 The effect of ghrelin and LEAP2 on apoptosis in islets from female mice. Isolated islets were pre-treated for 48 h with or without 100 nM ghrelin ± 100 nM LEAP2 and exposed to 1000 U/ml TNF-α+50 U/ml IL-1β for 20 h ± 100 nM ghrelin ± 100 nM LEAP2. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=5-6. Graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni **p<0.01 and ****p<0.0001. NS = not significant p>0.05.

($p<0.0001$; Fig. 5.4.3), however incubation with 100 nM LEAP2 did not significantly reverse this ($p>0.05$).

Next, the role of LEAP2 was tested in islets from male mice and MIN6 cells. Similar to previous experiments, 100 nM ghrelin did not significantly affect apoptosis induced by cellular stressors in these groups ($p>0.05$). In addition, incubation with 100 nM LEAP2 alone did not significantly affect palmitate-induced apoptosis in both groups ($p>0.05$) (Fig. 5.4.4 and 5.4.5).

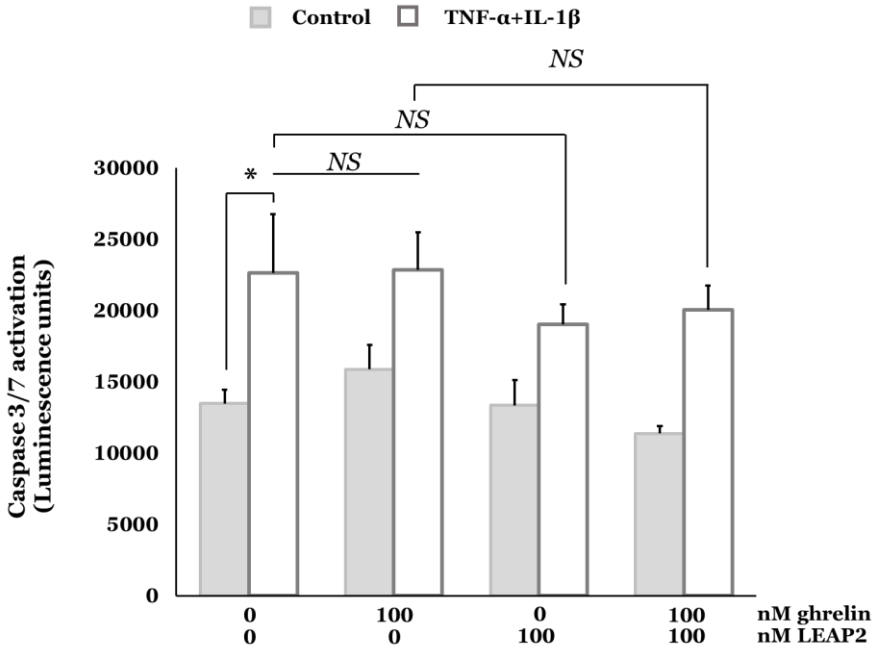

Figure 5.4.4 The effect of ghrelin and LEAP2 on apoptosis in islets from male mice. Isolated islets were pre-treated for 48 h with or without 100 nM ghrelin ± 100 nM LEAP2 and exposed to 1000 U/ml TNF-α+50 U/ml IL-1β for 20 h ±100 nM ghrelin ± 100 nM LEAP2. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=5-6. Graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni **$p<0.01$. NS = not significant $p>0.05$.

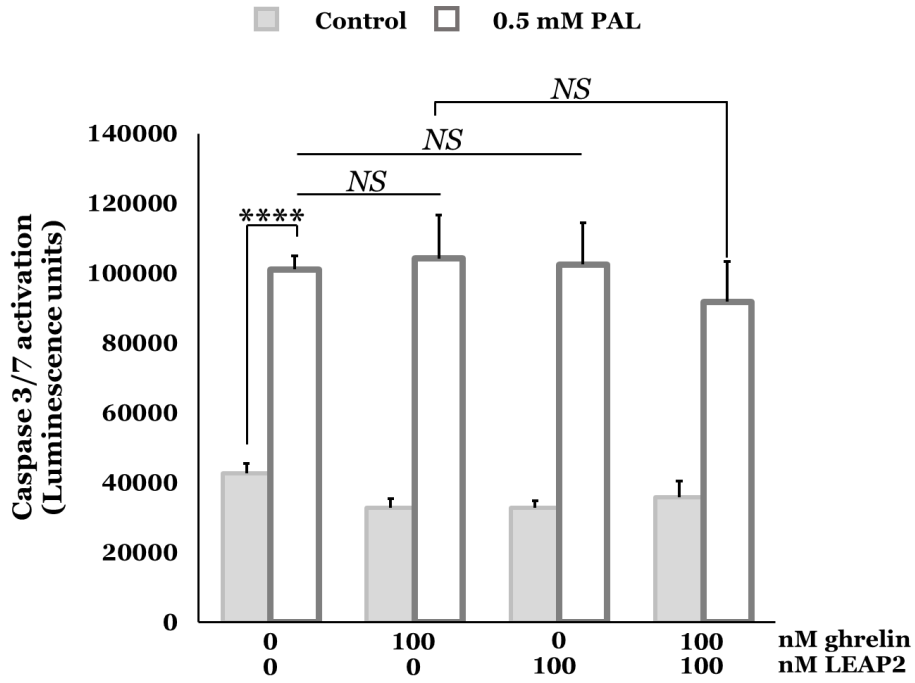

Figure 5.4.5 The effect of ghrelin and LEAP2 on MIN6 beta cell apoptosis. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48 h with or without 100 nM ghrelin ± 100 nM LEAP2 and exposed to 0.5 mM palmitate for 20 h ± 100 nM ghrelin ± 100 nM LEAP2. Cell apoptosis was detected by measuring caspase 3/7 activity. Data is presented as mean+SEM, n=5. Graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni ****$p<0.0001$. NS = not significant $p>0.05$.

5.4.2 mRNA expression and protein expression of GHSR1a in islets and clonal beta cells.

Following the observation that GHSR1a antagonists could not reverse the protective effect of ghrelin observed in islets from female mice, it was explored whether *Ghsr1a* and GHSR1a are present in the groups tested at mRNA and protein levels respectively.

mRNA expression analysis

First, mRNA expression of *Ghsr* was assessed in control tissues, hypothalamus and fundus of male and female CD-1 mice. *Ghsr1a* was found to be expressed in the hypothalamic tissue in both male and female mice and the level of expression was not significantly different between the two groups ($p>0.05$; Fig. 5.4.6) (Table 5.2). However, *Ghsr1a* was not detected in the fundus of male and female mice and the expression of *Ghsr1a* was only traceable in MIN6 cells (Table 5.2). Next, the mRNA expression of *Ghsr1a* in islets from male and female mice was explored and it was also tested whether its expression was modified by 48 h exposure to 100 nM ghrelin, similar to the experimental conditions used when assessing apoptosis. The *Ghsr1a* expression was determined relative to house-keeping genes and fold change was calculated relative to male day 0 control. The results showed that *Ghsr1a* was expressed at a significantly higher level in islets from female mice compared to those from males when compared to *Ppia* ($p<0.05$, Fig. 5.4.7A), but not *Hprt* ($p>0.05$, Fig. 5.4.7B) in control islets at day 3 of culture. *Ghsr1a* mRNA expression remained unchanged in the presence of 100 nM ghrelin relative to both house-keeping genes compared to control islets ($p>0.05$) (Fig. 5.4.7 and Table 5.3).

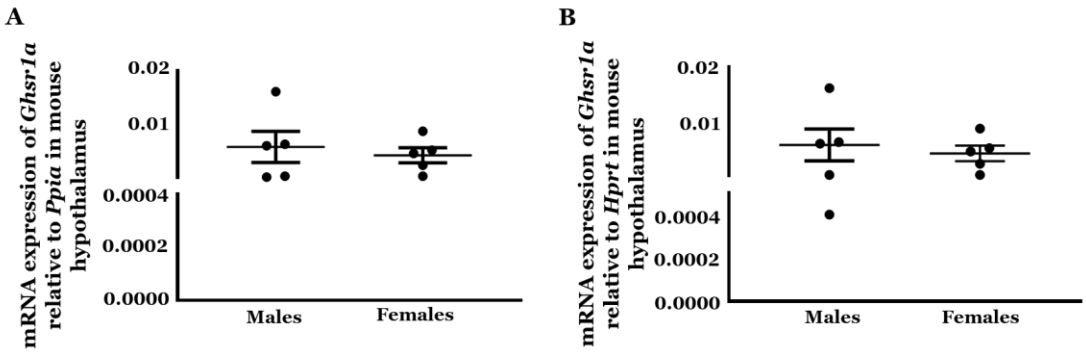

Figure 5.4.6 Expression of *Ghsr1a* mRNA in the murine hypothalamus. The data represent mean mRNA expression ± SEM of *Ghsr1a* relative to the housekeeping genes *Ppia* (A) and *Hprt* (B). Data is presented as mean±SEM, n=5.

Table 5.2 Mean Ct values ± SEM of *Ghsr1a* and housekeeping genes in hypothalamic and fundus tissues (n=5)

	Hypothalamus males	Hypothalamus females	Fundus males	Fundus females	MIN6
Ghsr1a	24.6±1.2	24.4±0.7	36.2±0.1	ND	29.0±1.4
Ppia	16.1±0.2	16.2±0.2	18.3±0.6	18.3±0.6	16.2±0.3
Hprt	19.3±0.3	19.6±0.2	21.8±0.7	22.2±0.7	19.7±0.3

*ND= not detected

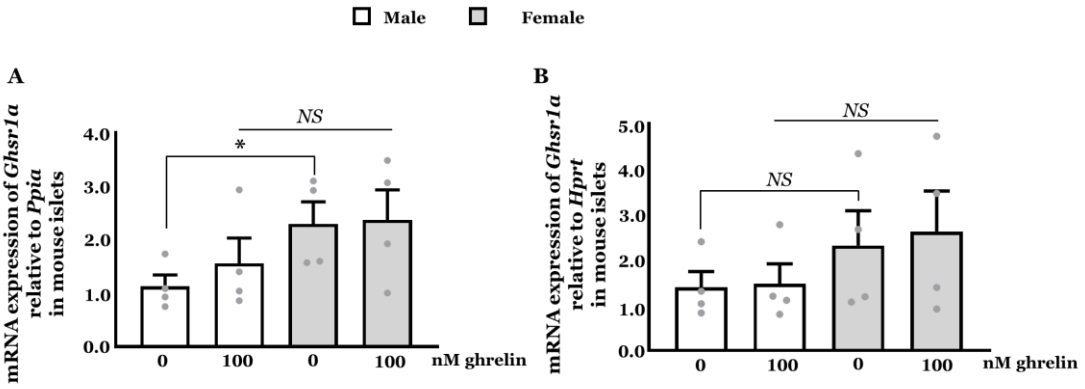

Figure 5.4.7 Expression of *Ghsr1a* mRNA in islets from male and female mice. Isolated islets were treated for 48 h as indicated and the mRNA expression of *Ghsr1a* was detected by qPCR. mRNA expression relative to housekeeping genes *Ppia* (A) and *Hprt* (B) and male day 0 control is shown. Data is presented as mean+SEM, n=4. Paired student's T-test *p=0.02. NS = not significant p>0.05.

Table 5.3 Mean Ct values ± SEM of *Ghsr1a* and housekeeping genes in islets (n=4)

	Islets males (Day 0 control)	Islets males (Day 3 control)	Islets males (+ 100 nM ghrelin)	Islets females (Day 0 control)	Islets females (Day 3 control)	Islets females (+ 100 nM ghrelin)
Ghsr1a	27.4±0.8	26.0±0.9	26.7±1.1	26.6±0.8	26.1±1.1	26.1±1.1
Ppia	17.8±0.2	17.4±0.2	17.6±0.2	18.0±0.4	17.6±0.3	17.6±0.3
Hprt	20.7±0.1	20.6±0.1	20.4±0.0	21.1±0.3	20.3±0.2	20.5±0.2

Protein expression studies

To evaluate more specifically whether the protein expression of GHSR1a was different between islets from male and female mice, Western blot analysis was performed. Prior to this a pilot study was conducted to assess the expression of GHSR1a in control tissues: hypothalamus and fundus of CD-1 mice. GHSR1a was expressed in hypothalamic tissue of male and female mice at a similar level ($p>0.05$) (Fig. 5.4.8 A, B and E), however it was not consistently detectable in the fundus of both groups (Fig. 5.4.8 C&D). Next it was tested whether GHSR1a was detectable in clonal beta cell lines MIN6 and INS-1. Both cell lines were found to express GHSR1a and its expression was not significantly different between the two cell lines ($p>0.05$) (Fig. 5.4.9 A-C). As shown in Fig. 5.4.7A, gene expression of *Ghsr1a* was higher in islets from female mice under control conditions, therefore the presence of GHSR1a was tested in untreated islets maintained in complete medium. GHSR1a was found to be present in both groups tested. Although in islets from female mice there was a trend towards a higher level of expression of the protein, this was not significantly different from the level in islets from male mice ($p>0.05$) (Fig. 5.4.10 A-C). Since GHSR1a is almost omnipresent and not many studies have shown the detectability of GHSR1a at protein level in islets using Western blot due to limited availability of suitable antibodies, leukaemic cell lines THP-1 (human monocytes), REH (lymphoblasts) and Kasumi-1 (myeloblasts) were used as negative controls. GHSR1a was not detected in these cells, however all three cell lines expressed beta-actin indicating that the observed expression levels of GHSR1a are not due to non-specific binding (Fig. 5.4.11).

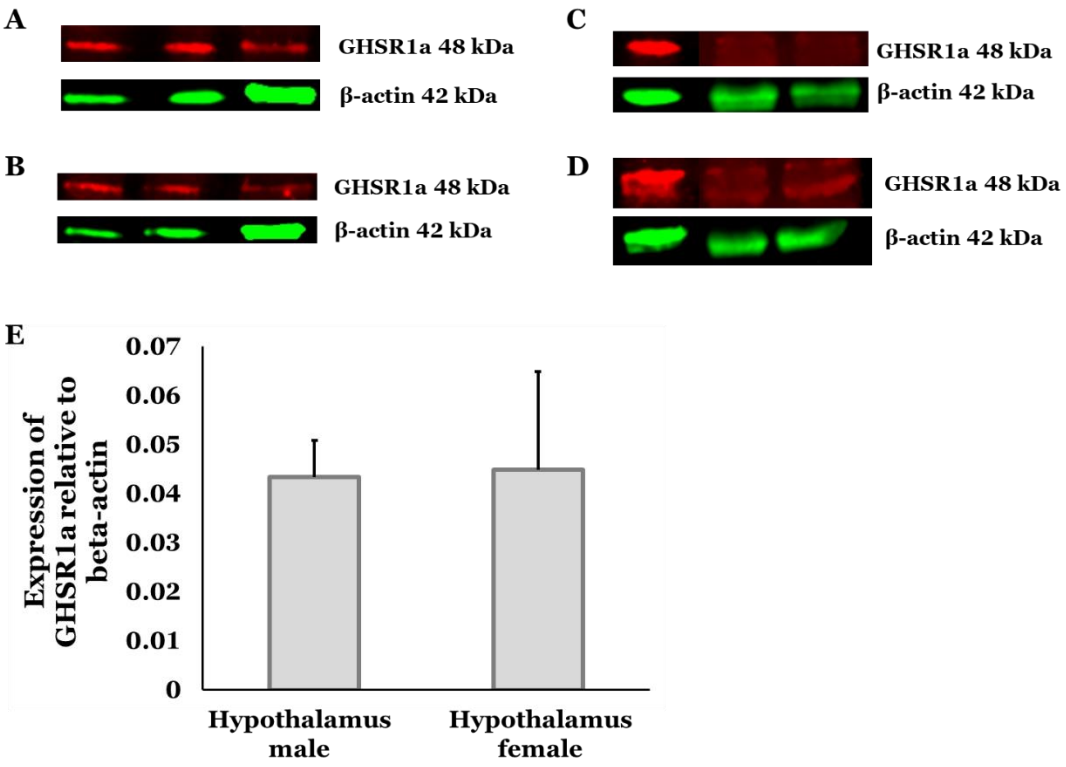

Figure 5.4.8 Protein expression of GHSR1a in murine hypothalamus and fundus of male and female mice. GHSR1a expression was detected in hypothalamus of male (A) and female (B) mice but was not consistently detectable in the fundus of both groups (C and D). Each lane corresponds to one biological sample. Panel E shows mean relative expression levels + SEM of GHSR in hypothalamus compared to loading control beta actin (n=3). 20 µg protein was loaded per sample and resolved using a 12% polyacrylamide gel. Proteins were detected by Western blot. Primary antibodies used: GHSR1a (1 µg/ml) and beta actin as loading control (1:8000). Secondary antibodies: Li-Cor IRdye® donkey anti-rabbit 680RD and donkey anti-mouse 800CW. Membranes were imaged, and band intensities were quantified using Li-Cor Image Studio software.

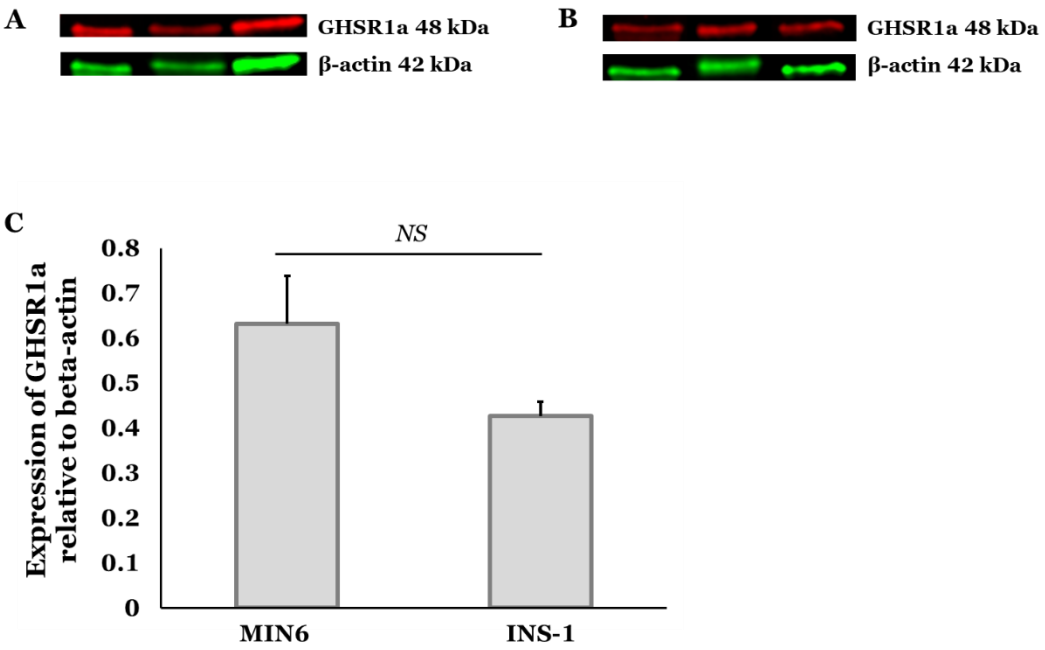

Figure 5.4.9 Protein expression of GHSR1a in two clonal beta cell lines. GHSR1a expression was detected in MIN6 (A) and INS-1 (B) cells. Each lane corresponds to one biological sample. Panel C shows mean relative expression levels + SEM of GHSR compared to loading control beta actin (n=3). 20 µg protein was loaded per sample and resolved using a 12% polyacrylamide gel. Proteins were detected using Western blot. Primary antibodies used: GHSR1a (1 µg/ml) and beta actin as loading control 1:8000. Secondary antibodies: Li-Cor IRdye® donkey anti-rabbit 680RD and donkey anti-mouse 800CW. Membranes were imaged, and band intensities were quantified using Li-Cor Image Studio software. Unpaired Student's T-test NS = not significant $p>0.05$.

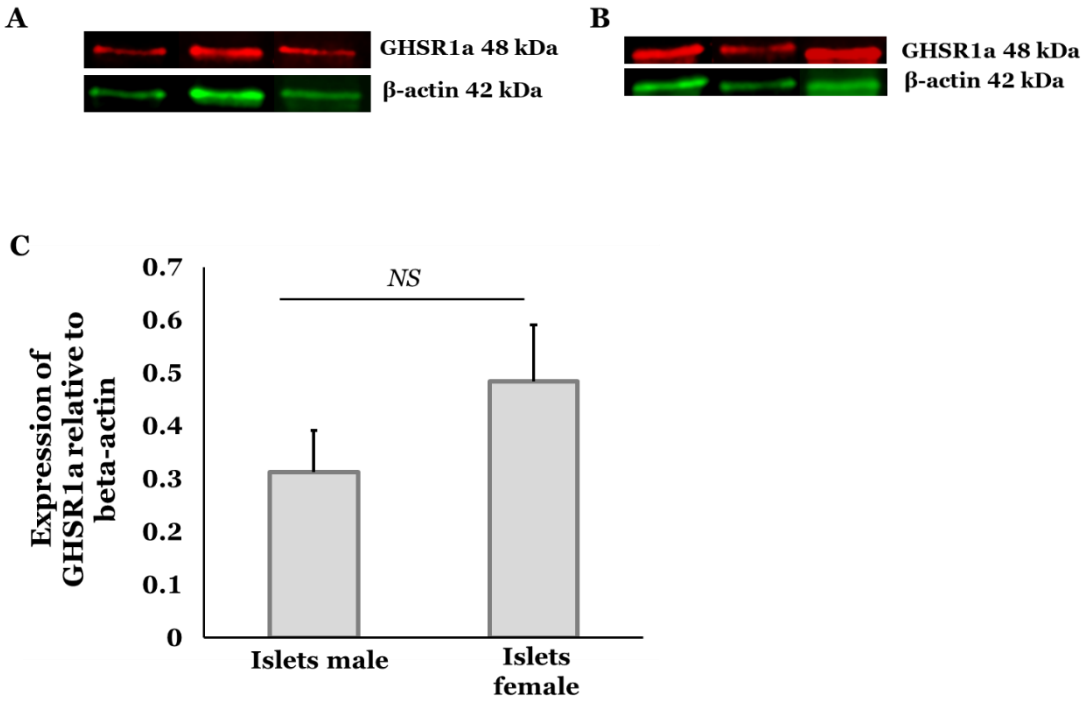

Figure 5.4.10 Protein expression of GHSR1a in islets from male (A) and female mice (B). GHSR1a expression was detected in islets from both male and female CD-1 mice. Each lane corresponds to one biological sample. Panel C shows mean relative expression levels + SEM of GHSR compared to loading control beta actin (n=3). 20 µg protein was loaded per sample and resolved using a 12% polyacrylamide gel. Proteins were detected using Western blot. Primary antibodies used: GHSR1a (1 µg/ml) and beta actin as loading control 1:8000. Secondary antibodies: Li-Cor IRdye® donkey anti-rabbit 680RD and donkey anti-mouse 800CW. Membranes were imaged, and band intensities were quantified using Li-Cor Image Studio software. Unpaired Student's T-test NS = not significant $p>0.05$.

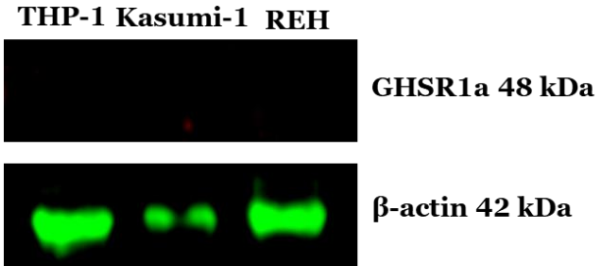

Figure 5.4.11 Protein expression of GHSR1a in three leukaemia cell lines. GHSR1a expression was not detected in any of the leukaemia cell lines tested as negative control, n=1. 20 μg protein was loaded per sample and resolved using a 12% polyacrylamide gel. Proteins were detected using Western blot. Primary antibodies used: GHSR1a (1 μg/ml) and beta actin as loading control 1:8000. Secondary antibodies: Li-Cor IRdye® donkey anti-rabbit 680RD and donkey anti-mouse 800CW. Membranes were imaged using Li-Cor Image Studio software.

5.4.3 The role of GHSR1a on glucose-induced insulin secretion in islets.

As reported above, the *Ghsr1a* was detected at both mRNA and protein levels in MIN6 cells and islets. Since the two GHSR1a antagonists used in this study have been characterised for their effects on insulin secretion (YIL781) and antagonising orexigenic effects of ghrelin (YIL781 and LEAP2), their effect on glucose-induced insulin secretion was assessed in islets from male and female mice to evaluate 1) whether ghrelin-independent effects of antagonists are seen with regards to insulin secretion and therefore whether potential action of endogenous ghrelin can be blocked by the antagonists; 2) whether the effects of antagonists vary between males and females with regards to secretory function.

As shown in chapter 4, ghrelin did not significantly affect glucose-induced insulin secretion in both groups ($p>0.05$) (Fig. 5.4.12 and 5.4.13). Treatment with 5 μM YIL781 alone or in combination with ghrelin also did not significantly affect glucose-induced insulin secretion in islets ($p>0.05$) (Fig. 5.4.12 A and B). Treatment with 100 nM LEAP2 however resulted in significantly greater glucose-induced insulin secretion in islets from male mice ($p<0.01$) (Fig. 5.4.13 B), but not females ($p>0.05$) (Fig. 5.4.13 A), and this effect was significantly reversed in the presence of 100 nM ghrelin in 2/3 experiments ($p<0.05$; Fig. 5.4.13 B). In the third experiment the reversal did not reach statistical significance ($p=0.06$)

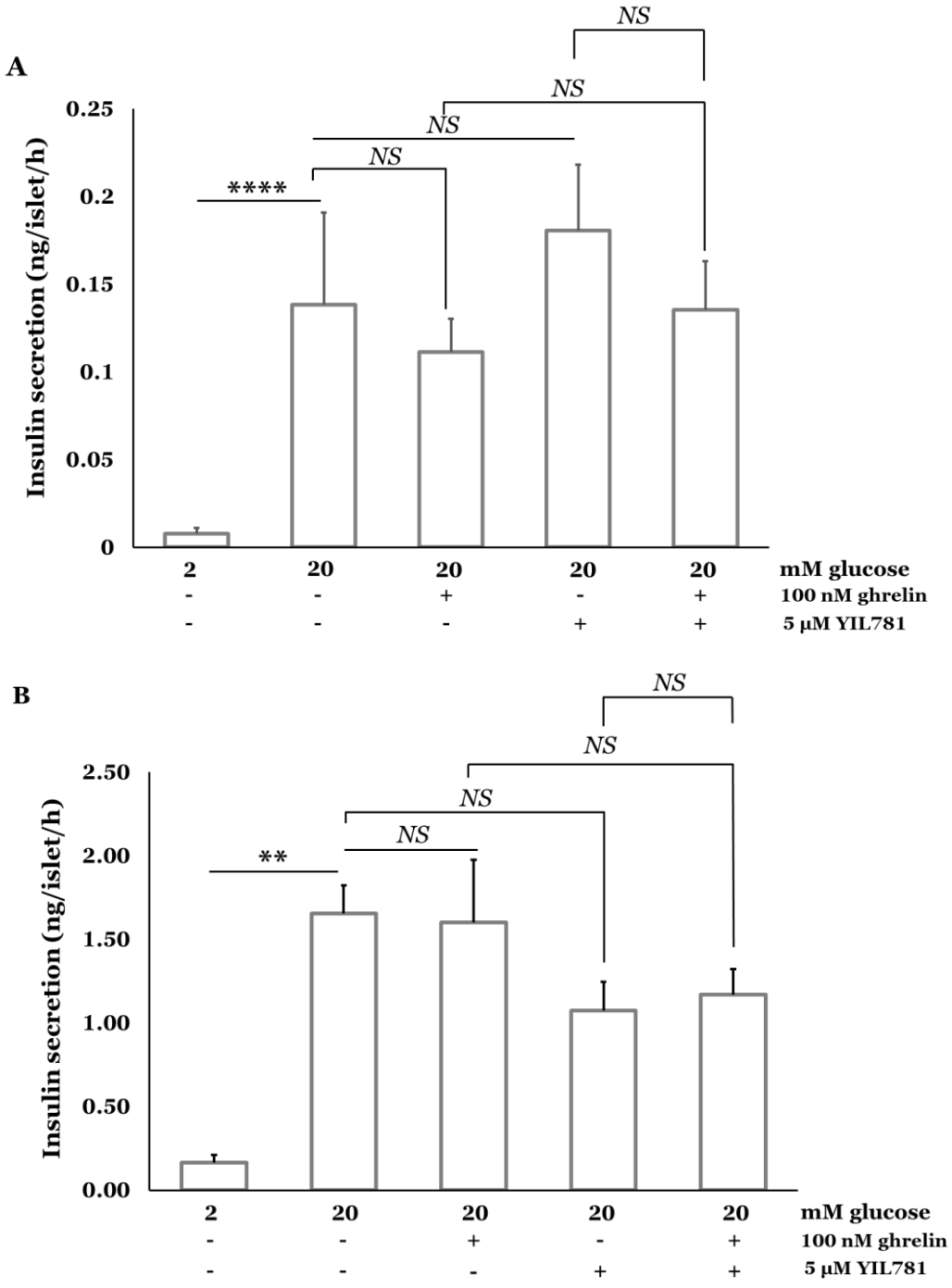

Figure 5.4.12 The effect of ghrelin and GHSR1a antagonist YIL781 on insulin secretion in islets from female (A) and male (B) mice. Isolated islets were pre-treated with 2 mM glucose for 1 h then treated as indicated for 1 h. Secreted insulin was detected by an in-house radioimmunoassay. Data is presented as mean+SEM, n=4-6. Each graph represents results of one experiment out of three independent experiments. One-Way ANOVA with posthoc test Bonferroni **p<0.01, ****p<0.0001. NS = not significant p>0.05.

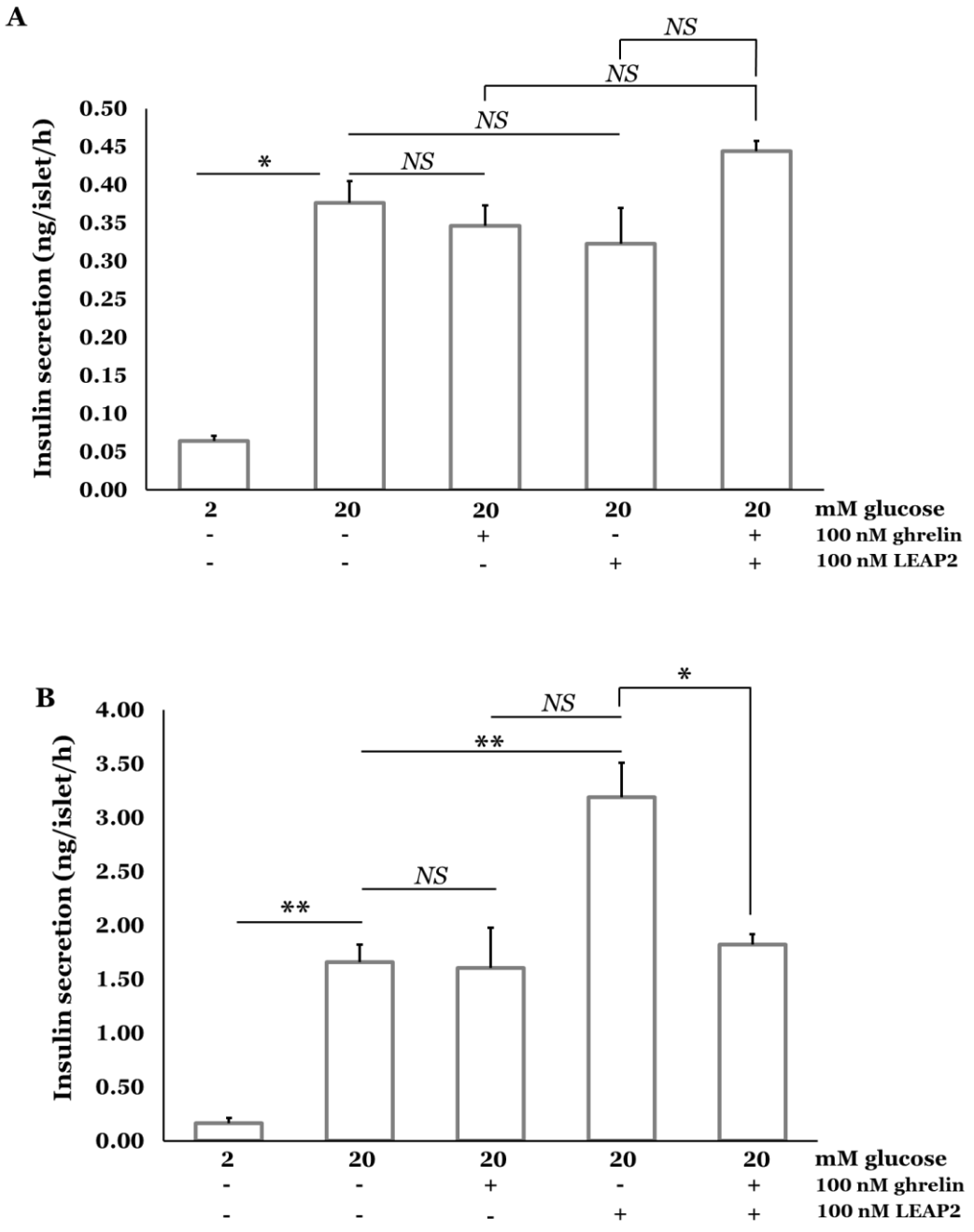

Figure 5.4.13 The effect of ghrelin and GHSR1a antagonist LEAP2 on insulin secretion in islets from female (A) and male (B) mice. Isolated islets were pre-treated with 2 mM glucose for 1h then treated as indicated for 1 h. Secreted insulin was detected by an in-house radioimmunoassay. Data is presented as mean+SEM, n=6. Graph A represents results of one out of two independent experiments and graph B of one out of three experiments. One-Way ANOVA with posthoc test Bonferroni *$p<0.05$, **$p<0.01$. NS = not significant $p>0.05$.

5.6 Discussion

Based on the findings reported in chapter 4, where treatment with ghrelin significantly reduced cytokine-induced apoptosis only in islets from female mice as well as lack of effect of ghrelin in MIN6 cells and islets from male mice, the role of GHSR1a in mediating the functional effects of ghrelin in these tissues was tested. Particular interest was given to the role of GHSR1a in the context of sexual dimorphism in islets from male and female mice.

A number of studies have reported that ghrelin acts via the known ghrelin receptor GHSR1a. Kojima *et al.* (1999) identified ghrelin as the target of the orphaned GHSR1a. Dezaki *et al.* (2004) have reported that simultaneous injection of ghrelin and glucose in oral glucose tolerance tests in mice resulted in reduced insulin response and increased glucose responses compared to a control without ghrelin. Also, the use of two GHSR1a antagonists reversed the effects of ghrelin (Dezaki *et al.*, 2004). In addition, in dispersed rat islets reduction in glucose-induced insulin secretion by treatment with ghrelin was reversed by the addition of the GHSR1a antagonist YIL781 (Esler *et al.*, 2007). Furthermore, in mice administered with ghrelin, stimulation of growth hormone release was significantly inhibited by the GHSR1a antagonist LEAP2 (Ge *et al.*, 2018). In the same study LEAP2 also significantly reversed the ghrelin-induced increase in food intake in mice. However, some studies have suggested that the function of ghrelin may be conveyed via another unknown receptor in beta cells (Granata *et al.*, 2007) and in other tissues (Muccioli *et al.*, 2007). Therefore, in this study it was assessed whether the protective effect of ghrelin observed in islets from female mice was conveyed via the known

receptor GHSR1a with the use of two GHSR1a antagonists, YIL781 and LEAP2.

5.5.1 The role of GHSR1a in MIN6 cell and mouse islet apoptosis

As described in section 5.4.1, both GHSR1a antagonists used in this study did not significantly reverse the observed protective effect of ghrelin on cytokine-induced apoptosis in islets from female mice. Instead, cytokine-induced apoptosis was significantly reduced in the presence of YIL781 alone in islets from female mice and in MIN6 cells. However, in all groups treatment with LEAP2 alone or in combination with ghrelin did not significantly influence apoptosis induced by palmitate or cytokines.

In the study by Ge *et al.* (2018) where LEAP2 was reported to be a non-competitive inhibitor of GHSR1a, cells expressing GHSR1a were pre-treated with LEAP2 for 30 min prior to addition of ghrelin. Whereas in the current study, both compounds were added simultaneously. More recently others have reported that LEAP2 could acts as a competitive or non-competitive antagonist (Wang *et al.*, 2019) or as an inverse agonist (M'Kadmi *et al.*, 2018) depending on the experimental set up. Wang *et al.* (2019) have found that in ligand-receptor binding studies addition of LEAP2 before ghrelin resulted in a non-competitive effect of the antagonist whereas simultaneous addition of the two compounds resulted in a competitive-type antagonism. It was reported that these effects were potentially dependent on the slow dissociation of LEAP2 from GHSR1a (Wang *et al.*, 2019) suggesting that subsequent addition of ghrelin may be unable to displace the antagonist.

Furthermore, a recent study identified YIL781 as a partial agonist that only activated the G protein subunits G_q and G_{12} of GHSR1a, therefore resulted in

the stimulation of phospholipase C (PLC) (Mende *et al.*, 2018). PLC activates PKC that is a regulator of cell survival (Cantley, 2013). Indeed it has been previously reported that activation of PLC subunits is fundamental for PKC-dependent cell survival during oxidative stress (Bai *et al.*, 2002b) and heat-induced stress (Bai *et al.*, 2002a). It is therefore possible that in the current study the reduction in apoptosis following exposure to YIL781 observed in all three groups, although not statistically significant in islets from male mice, may have been due to the biased activation of GHSR1a by YIL781.

Taken together the results of section 5.4.1 and the above discussed studies suggested that the GHSR1a antagonists are not specific in testing ghrelin's effects on apoptosis, that ghrelin does not act via GHSR1a to exert its action on survival, that it acts via an unknown receptor and/or that GHSR1a is not present in the tissues tested.

5.5.2 The expression of GHSR1a in MIN6 cells and islets

qPCR results confirmed that the *Ghsr1a* is present at mRNA level in islets as has been reported by others (Date *et al.*, 2002), and to a lower extent in the clonal beta cell line MIN6. However, in a study by Granata *et al.* (2007) *Ghsr1a* expression was not detected in the beta cell line HIT-T15 as was also reported in single-cell transcriptomic analysis using primary beta cells (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). Furthermore, in the current study it was also established that GHSR1a is present at protein level in both islets and the clonal beta cell lines tested. Previously, it was only demonstrated with the use of immunohistochemical staining and anti-sera that GHSR-like staining was detected in primary beta cells and alpha cells (Kageyama *et al.*, 2005) and in alpha cells only (Date *et al.*, 2002). Despite GHSR1a being expressed in clonal

beta cell lines, no effect of ghrelin was observed on MIN6 cell apoptosis or insulin secretion as reported in chapter 4. It is possible that endogenous ghrelin may be present in these cells as suggested by Granata *et al.* (2007) in HIT-T15 cells and in primary beta cells as reported by Kageyama *et al.* (2005), and potentially counteract the effect of exogenous ghrelin. Alternatively, it is possible that ghrelin does not act via the known receptor GHSR1a in MIN6 cells. In addition, the expression level of GHSR1a was not significantly different between islets from male and female mice. At functional level however the pro-survival effect of ghrelin could not be reversed by GHSR1a antagonists suggesting ghrelin may exert its effects via an alternative receptor or that biological sex influences GHSR1a activity in a manner that is independent of its level of expression in the tissue as further explored in chapter 6 of this thesis.

5.5.3 The role of GHSR1a in mouse islet insulin secretory responses

Treatment with YIL781 alone or in combination with ghrelin had no effect on glucose-induced insulin secretion in islets from both male and female mice. However, based on the earlier described suggestion by Mende *et al.* (2018) that binding of YIL781 to GHSR1a activated PLC-dependent signalling, an increase in insulin secretion should have been observed. This is because activation of PKC promotes Ca^{2+} influx and exocytosis of insulin granules from beta cells (reviewed by Nesher *et al.*, 2002). The lack of effect of YIL781 on insulin secretion in the current study implies either that endogenous ghrelin may have influenced its effect or that the antagonist is ineffective in the current experimental set up.

In contrast, treatment with the novel antagonist LEAP2 significantly increased glucose-induced insulin secretion sex-dependently in islets from male mice only. It is possible that in this group LEAP2 may have antagonised the action of endogenous ghrelin present in islets from male mice as observed in rat islets by Dezaki *et al.* (2004). Alternatively, LEAP2 may have antagonised the constitutive activity of the receptor. Indeed GHSR1a belongs to a small group of GPCRs that present constitutive activity (Holst *et al.*, 2007) and GHSR1a has been shown to present about 50% intrinsic constitutive activity (Damian *et al.*, 2011; Veldhuis and Bowers, 2010).

A recent study by M'Kadmi *et al.* (2018) has found that LEAP2 displayed the characteristics of an inverse agonist and specifically caused reduction in the activation of G_q and G_{13} subunits of GHSR1a. Since LEAP2 antagonises GHSR1a, which recently has been found to be expressed exclusively on the islet delta cell, the reduction in G_q activation may imply a reduction in SST secretion. Consequently, this may have abolished the inhibition of SST on insulin secretion with concomitant increase in release resulting in the observed elevation in insulin secretion in the presence of LEAP2 in the current study. It is, however, unclear why the same effect of LEAP2 was not observed in islets from female mice. Since the measurable effect of LEAP2 in islets from male mice suggests that ghrelin may exert its action via GHSR1a as previously reported (Kojima *et al.*, 1999), it is possible that sex-related differences in islet function (as reviewed by Gannon *et al.*, 2018) may have contributed to divergent outcomes. Furthermore the insulinotropic effect of LEAP2 was significantly reduced in the presence of ghrelin in islets from male mice suggesting a competition between the two compounds. Therefore, sex-related

differences in the effect of LEAP2 on glucose-induced insulin secretion in islets further implies a role for endogenous ghrelin. Further experiments were conducted to assess whether potential differences in endogenous ghrelin between islets of male and female mice may have played a role in the observed differential effects of ghrelin and GHSR1a antagonists and the findings are reported in the next chapter of this thesis.

5.5.5 Summary

The results presented and discussed in this chapter aimed to elucidate the role of GHSR1a in mediating the functional effects of ghrelin in pancreatic islets and clonal beta cells. All experiments were conducted using islets from both male and female mice to identify the existence of any sex-related differences and are summarised in the Table 5.4. GHSR1a is present in mouse islets and MIN6 cells, and its expression level was comparable in islets from both sexes. GHSR1a antagonists however could not reverse the pro-survival effect of ghrelin in islets from female mice. Studies conducted in MIN6 cells indicated that a direct effect of ghrelin on beta cells was not evident despite GHSR1a being expressed in these cells. This may imply that endogenous ghrelin present within these cells may have influenced the activity of exogenous ghrelin. Indeed in islets from male mice the insulinotropic effect of GHSR1a antagonist LEAP2 indicated that the antagonist may have reversed the effect of endogenous ghrelin. Taken together these results indicate that the effect of ghrelin on islet function and survival are governed by sex related differences that may be both dependent and independent of GHSR1a.

Table 5.4 Sex-specific effects of GHSR1a-dependent factors tested in the current study

Effect or feature	Sex-specific differences
The effect of YIL781 on islet apoptosis	Reduced apoptosis in islets from female mice but no effect in islets from male mice.
The effect of LEAP2 on islet apoptosis	No overall difference in apoptosis in both groups
Ghsr1a mRNA expression	Increased level of expression in islets from female mice when expressed relative to *Ppia*, but not relative to *Hprt*. Treatment with ghrelin did not affect *Ghsr1a* expression in both groups.
GHSR1a expression	No overall difference in expression between both groups
The effect of YIL781 on islet insulin secretory responses	No difference in glucose-induced insulin secretion in both groups
The effect of LEAP2 on islet insulin secretory responses	Increased insulin secretion in islets from male mice that was reversed by exposure to ghrelin. No effect on islets from female mice.

CHAPTER 6 - GHSR1a-independent factors mediating the functional effects of ghrelin in MIN6 cells and islets

6.1 Introduction

6.1.1 Expression of ghrelin in the islet

The 28 amino acid peptide ghrelin is expressed in the pancreatic islets of Langerhans (Volante *et al.*, 2002). However its localisation within specific islet cell types remains controversial. In 2002 Wierup *et al.* identified a novel cell type, pancreatic epsilon cells, as the ghrelin producing cells in the islets. Others have reported that ghrelin-like immunoreactivity was detected in beta cells in rat islets (Kageyama *et al.*, 2005), and strong ghrelin immunoreactivity was observed in beta cells in human islets (Volante *et al.*, 2002). However, Date *et al.* (2002) have found ghrelin to be expressed in alpha cells of human and rat pancreatic islets as confirmed by Dezaki *et al.* (2004) using rat pancreatic islets. The number of ghrelin expressing cells has been found to be higher in the human foetal pancreatic islets compared to those in the adults, suggesting that islet-derived ghrelin may play a greater role during pancreatic development (Wierup *et al.*, 2002). It is possible that the reported differentiation of epsilon cells, predominantly into alpha and PP cells in mature islets may have contributed to the reduced number of epsilon cells in adult pancreatic islets (Arnes *et al.*, 2012). These studies suggest that ghrelin is expressed endogenously in a number of pancreatic islet cell types and that endogenous pancreatic ghrelin therefore may play a role in regulating islet function. Indeed, a study by Dezaki *et al.* (2004) revealed that in the presence of two GHSR1a antagonists, insulin secretion was significantly higher at 5.6 mM glucose in isolated rat islets. The same effect was observed in the presence of anti-serum against ghrelin (Dezaki *et al.*, 2004) suggesting that endogenous ghrelin negatively affects insulin secretion. The results reported in chapter 4

of this thesis have shown that islets from female mice respond differently to treatment with ghrelin compared to the ones from males. It is necessary to ascertain whether this sex difference in sensitivity to ghrelin is linked to a differential islet-specific production or release of the hormone between the two groups.

6.1.2 Interaction between ghrelin, oestradiol and their receptors

As discussed in chapter 4, ghrelin displays sexual dimorphism in its orexigenic activity and plasma levels in rodents and humans. However, whole-body observations cannot always be reproducible *in vitro* where cell models are used as introduced in section 1.4. Nonetheless our findings indicate that ghrelin significantly reduced cytokine-induced apoptosis only in islets from female mice suggesting that the action of ghrelin may be modulated directly or indirectly by oestrogen, thus potentially rendering islets from female mice more sensitive to a protective effect of ghrelin.

A number of studies point towards a potential mechanistic interaction between oestradiol (E2) and ghrelin (Sakata *et al*., 2006). In the stomachs of both male and female rats gastric oestrogen was found to increase production and expression of ghrelin (Sakata *et al*., 2006), whereas administration of E2 suppressed orexigenic effects of ghrelin observed in male and OVX female rats (Clegg *et al*., 2007). There are contrasting reports on the effect of E2 on circulating ghrelin levels in humans with one study reporting that E2 replacement therapy in postmenopausal women resulted in increased levels of plasma ghrelin (Kellokoski *et al*., 2005), however another study by Dafopoulus *et al*. (2010) reported that the treatment with E2 in both pre- and postmenopausal women had no effect on plasma ghrelin levels. Taken together

these studies suggest that E2 modulates the expression and/or action of ghrelin in a tissue or species-specific manner. It has not, however, been explored whether this interaction exists in pancreatic islets and what implications this might have on beta cell function.

Oestrogen acts via three receptors: oestrogen receptor alpha (ERα or ESR1), beta (ERβ or ESR2) and a G-protein coupled receptor (GPER) (Geisler *et al.*, 2002; Nadal *et al.*, 2000). In the hypothalamus of OVX mice both ghrelin receptor (GHSR1a) and ERs have been reported to be co-expressed and treatment with E2 resulted in significantly greater increase in expression of *Ghsr1a* mRNA in arcuate nucleus (Frazao *et al.*, 2014).

The results presented in chapter 5 however suggested that overall there was no significant difference in GHSR1a expression in islets of male and female mice. This implies that chronic exposure to E2 may not have resulted in a measurable change in GHSR1a expression in islets from female mice. It is however possible that differences in ER expression may have indirect effects on the action of ghrelin. Based on the above factors it was investigated whether there are underlying sex-specific differences in mRNA expression of ERs and if treatment with ghrelin modified their expression patterns. Secondly, it was assessed whether the presence of E2 could in the first instance modify the effect of ghrelin on clonal beta cell apoptosis.

6.1.3 Interaction between somatostatin and ghrelin

As reported in the previous chapters, several studies have suggested that somatostatin and ghrelin interact in the regulation of their plasma levels (Arosio *et al.*, 2003; Luque and Kineman, 2007), actions on growth hormone

release (Wagner *et al.*, 2009) and insulin secretion (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016).

The lack of effect of ghrelin on MIN6 beta cell function, despite GHSR1a being expressed in these cells, confirmed this possibility. However, the pro-survival effect of ghrelin being limited to islets from female mice implied that ghrelin's functional effects on beta cells may not only be indirect via the action of SST released by delta cells, but that it is also sex specific. Nevertheless, the expression of GHSR1a was not found to be different between islets from male and female mice. This suggests that should the effect of ghrelin be dependent on the action of SST, then sex-specific differences in islet SST receptor expression or SST levels may mediate its actions. Therefore, as part of this chapter it was explored whether *Sstr3* mRNA expression displayed sex-specific differences in mouse islets and whether it was modified by treatment with ghrelin. Next it was tested whether antagonising SSTR3 could modify the functional effects of ghrelin on insulin secretory responses in the first instance.

6.2 Aims and objectives

6.2.1 To assess mRNA expression and endogenous ghrelin content in mouse islets and MIN6 cells.

6.2.2 To investigate the interaction between E2 and ghrelin in beta cells and islets with particular focus on:

- The effect of ghrelin on sex-specific expression of oestrogen receptors in islets.
- The effect of oestradiol on the functional role of ghrelin in MIN6 cell apoptosis.

6.2.3 To determine sex-specific expression of SSTR3, and whether the functional effects of ghrelin in murine islets are mediated by this receptor.

6.3 Methods

6.3.1 Gene expression analysis

RNA extraction, cDNA reverse transcription and qPCR were performed as described in section 2.7 with the use of primers for house-keeping genes (section 2.7.3), *Ghrelin* (kindly provided by Dr Simone Nascimento, Table 6.1), oestrogen receptor-1 (*Esr1*) and oestrogen receptor-2 (*Esr2*) purchased from Sigma (KiqStart primers), and *Sstr3* (Table 3.1). Gene expression was determined relative to the house-keeping genes *Ppia* and *Hprt*

Table 6.1 *Ghrelin* primers

Gene	Primer sequence (5'-3')	PCR product (bp)	T annealing (°C)
Ghrelin	FW: GCTGTCTTCAGGCACCATCT RV: TTCTCTGCTGGGCTTTCTGG	113	60

6.3.2 Static incubation experiments for assessment of ghrelin secretion

Islet ghrelin secretion was assessed as described in 2.4.2 in response to treatment with 11 mM glucose and 2 mM glucose with or without 10 μM noradrenaline (NA) +100 μM ascorbic acid. Samples were diluted 1:4 for detection of total ghrelin and used neat for acyl-ghrelin, and their concentrations were measured by radioimmunoassay as described in section 2.4.4.2.

6.3.3 Detection of endogenous ghrelin content in MIN6 cells and tissue

Cell pellets and tissue samples were prepared as described in 2.4.3 and incubated in 0.5 M acetic acid for 15 min at 100 °C. Following cooling down, the samples were stored at -80 °C and assayed as described in 2.4.4.2.

6.3.4 Clonal beta cell apoptosis

Prior to experiments MIN6 cells grown in T75 flasks were washed once with PBS and incubated for three days in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS, 6 mM L-glutamine and penicillin (100 U/ml)/ streptomycin (0.1 mg/ml). FBS contains other hormones including oestrogens (Cao *et al.*, 2009). Therefore, to investigate the effect of exogenously added oestradiol (E2) it is necessary to use a serum that does not contain any additional amounts of the hormone. Charcoal stripping uses activated charcoal that removes lipophilic substances such as viruses and hormones by binding to them (De Faria *et al.*, 2016). In addition, phenol red has a similar structure to oestrogens, therefore can bind oestrogen receptors (De Faria *et al.*, 2016). This can be prevented by using a phenol red-free medium. Hence in the current study phenol red-free medium supplemented with charcoal-stripped FBS was used. Furthermore, to ensure that any effect of oestrogens present in normal culture medium is neutralised, the cells were pre-incubated for three days with the modified medium as reported by others (Weinstein-Oppenheimer *et al.*, 2002).

Assessment of MIN6 cell apoptosis was performed by measurement of caspase 3/7 activity as described in 2.6.1. Briefly, 15,000 cell/well were seeded in white-walled 96 well plates. Cells were pre-treated with or without 10 or 100

nM ghrelin ± 100 nM E2 in DMEM for 48 h with refreshment of treatment after 24 h. Following exposure to 0.5 mM palmitate for 20 h with or without 10 or 100 nM ghrelin ± 100 nM E2, Caspase-Glo assay was performed as described in 2.6.1.

6.3.5 Static incubation experiments for assessment of insulin secretion from mouse islets

Islet insulin secretion was assessed as described in 2.4.2 in response to treatment with 2 mM glucose and 20 mM glucose with or without 100 nM ghrelin or 1 μM SST ± 1 μM MK4256. All samples were diluted to concentrations detectable within the range of the standard curve and the concentration of insulin was measured by radioimmunoassay as described in section 2.4.4.1.

6.4 Results

6.4.1 Determination of ghrelin content in islets and MIN6 cells

Based on the reports that ghrelin in addition to epsilon cells is present in other islet cells including beta and alpha cells, and endogenous ghrelin influenced insulin secretion in rat islets (Dezaki *et al.*, 2004), it was hypothesised that endogenous ghrelin may play a role in the differential effect of exogenous ghrelin in islets from male and female mice as well as MIN6 cells. mRNA expression of *Ghrelin* was detected by qPCR. An in-house radioimmunoassay to detect both acylated and total ghrelin in the tissues of interest was set up by Dr M. Patterson.

6.4.1.1 mRNA expression studies

First the mRNA expression levels of *Ghrelin* were detected in MIN6 cells as well as in fundus, hypothalamus and islets from male and female mice. *Ghrelin* mRNA was traceable in MIN6 cells (Table 6.2). Its expression was highest in the fundus of both male and female mice (Fig. 6.4.1; Table 6.2). The mRNA levels of *Ghrelin* however did not significantly differ between fundus, hypothalamus (Fig. 6.4.1) and islets (Fig. 6.4.2; Table 6.3) from both sexes ($p>0.05$).

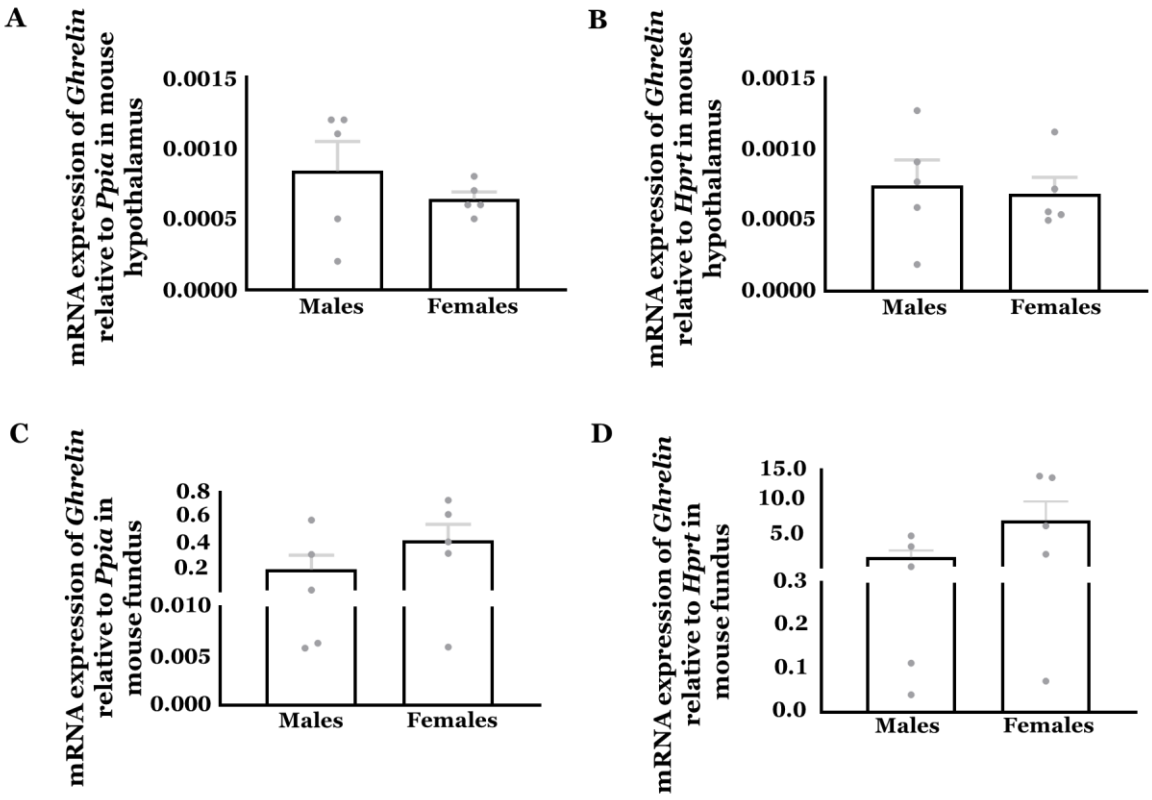

Figure 6.4.1 Expression of Ghrelin mRNA in the murine hypothalamus and fundus. The data represent mean mRNA expression of *Ghrelin* relative to the housekeeping genes *Ppia* (A, C) and *Hprt* (B, D) in hypothalamus (A and B) and fundus (C and D) of male and female CD-1 mice. Data is presented as mean+SEM, n=5.

Table 6.2 Mean Ct values ± SEM of *Ghrelin* and housekeeping genes in mouse hypothalamic tissue and fundus (n=5)

	Fundus males	Fundus females	Hypothalamus males	Hypothalamus females	MIN6
Ghrelin	22.9±1.4	20.6±1.7	26.6±0.7	26.8±0.2	29.5±0.6
Ppia	18.3±0.6	18.3±0.6	16.1±0.2	16.2±0.2	16.2±0.3
Hprt	21.8±0.7	22.2±0.7	19.3±0.3	19.6±0.2	19.7±0.3

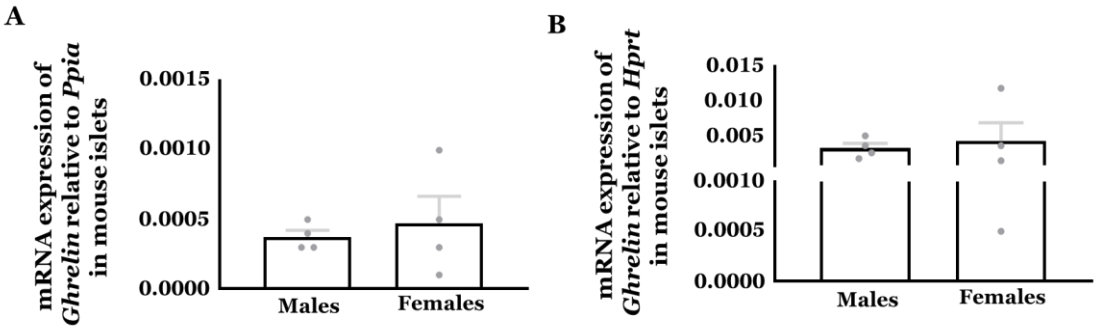

Figure 6.4.2 Expression of Ghrelin mRNA in islets from male and female mice. The data represent mRNA expression of *Ghrelin* relative to the housekeeping genes *Ppia* (A) and *Hprt* (B) in islets of male and female CD-1 mice. Data is presented as mean+SEM, n=4.

Table 6.3 Mean Ct values ± SEM of *Ghrelin* and housekeeping genes in mouse islets (n=4)

	Islets males	Islets females
Ghrelin	28.8±0.3	29.0±0.9
Ppia	17.4±0.2	17.6±0.3
Hprt	20.6±0.1	20.3±0.2

6.4.1.2 Detection of secreted and endogenous ghrelin

Static incubation experiments where islets were treated with 11 mM, 2 mM glucose and 2 mM glucose + 10 µM NA + 100 µM ascorbic acid for 1 h were first conducted and supernatants were analysed by radioimmunoassay. It was not possible to detect basal (11 mM glucose) or stimulated (2 mM glucose ± NA) release of either acyl-ghrelin or total ghrelin in the islet supernatants. These results were confirmed by enzyme-linked immunosorbent assay (ELISA, assessed by Dr M. Patterson, data not shown). In contrast, both total

and acyl-ghrelin were detected in stomach samples, which were used as positive controls (data not shown).

Next, it was tested whether the islet content of ghrelin differed between male and female mice. In islets from female mice a significantly higher level of total ghrelin was detected compared to islets from male mice ($p<0.01$; Fig. 6.4.3A). The concentration of acyl-ghrelin did not differ significantly between the two groups (Fig. 6.4.3B). Since *Ghrelin* mRNA was detected in MIN6 cells it was tested whether ghrelin was detectable in these cells using RIA. Both total and acyl-ghrelin were detected in MIN6 cells confirming findings of qPCR experiments (acyl-ghrelin: 130±12 pmol/4x10^6 cells and total ghrelin: 107±22 pmol/4x10^6 cells).

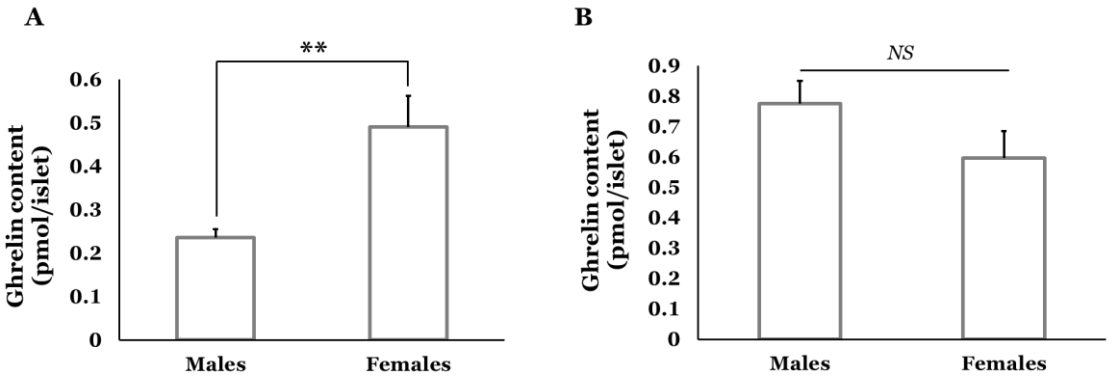

Figure 6.4.3 Endogenous total ghrelin (A) and acyl-ghrelin (B) content in mouse islets. Following the ghrelin secretion experiment, islets were pelleted, washed and lysed in 0.5 M acetic acid. Ghrelin content was assayed by an in-house radioimmunoassay. Data is presented as mean+SEM, n=3, one experiment. Unpaired Student's T-test **$p<0.01$. NS = not significant $p>0.05$.

6.4.2 The sex-specific expression of islet ERs, and the effect of interaction between E2 and ghrelin on clonal beta cell survival

6.4.2.1 Sex-specific expression of Esr1 and Esr2 in the mouse islet

The expression of *Esr1* and *Esr2* was detected by qPCR. Hypothalamic expression was tested as positive control and it was found that the expression of both receptor subtypes in the hypothalamus was not significantly different between the two groups (p>0.05; Fig. 6.4.4; Table 6.4).

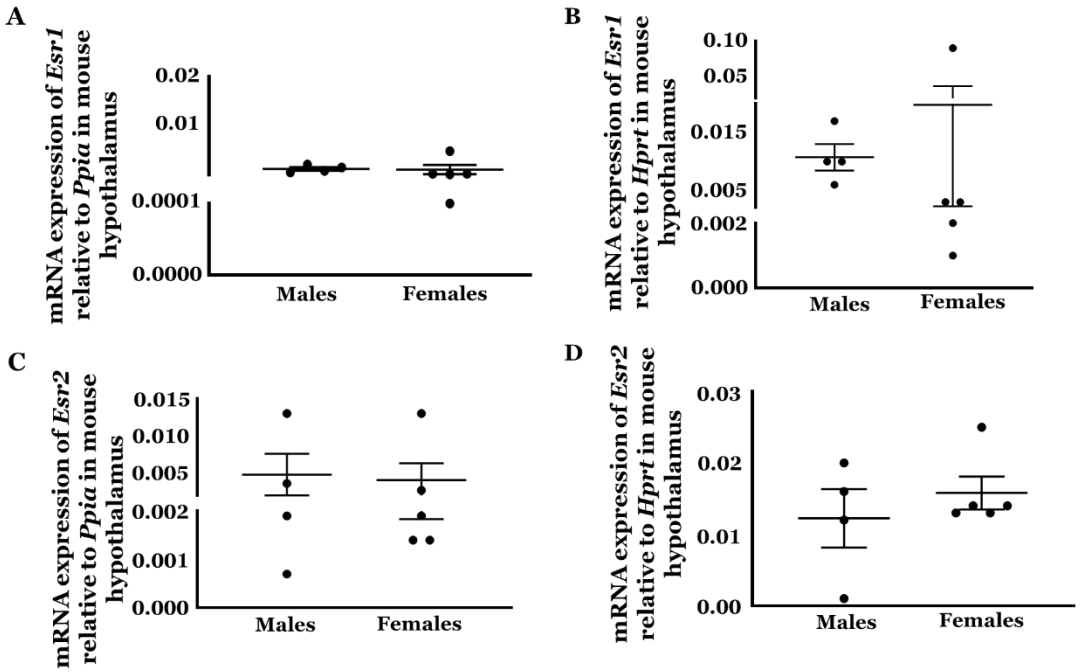

Figure 6.4.4 mRNA expression of oestrogen receptors in hypothalamus of male and female mice. The data represent mRNA expression of *Esr1* (A and B) and *Esr2* (C and D) relative to the housekeeping genes *Ppia* (A, C) and *Hprt* (B, D) in hypothalamus of male and female CD-1 mice. Data is presented as mean+SEM, n=4-5.

Table 6.4 Mean Ct values ± SEM of *Esr1* and *Esr2* and housekeeping genes in mouse hypothalamic tissue (n=4-5)

	Hypothalamus males	Hypothalamus females
Esr1	25.7±0.5	27.4±0.9
Esr2	26.2±1.3	25.3±0.3
Ppia	15.9±0.1	16.2±0.2
Hprt	19.1±0.2	19.6±0.2

Next, the expression of *Esr1* and *Esr2* was assessed in islets from male and female mice in the presence and absence of 100 nM ghrelin. Only *Esr1* and not *Esr2* was detectable in islets from both groups and its expression was not different between male and female mice ($p>0.05$). *Esr1* mRNA expression was not significantly influenced by 48 h exposure to 100 nM ghrelin ($p>0.05$) (Fig. 6.4.5) (Table 6.5).

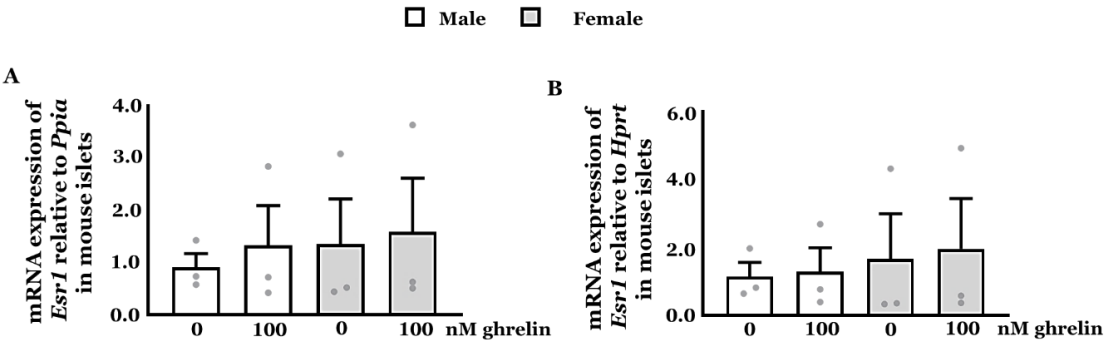

Figure 6.4.5 mRNA expression of *Esr1* in islets from male and female mice. Isolated islets were treated for 48 h as indicated and the mRNA expression of *Esr1* and *Esr2* was detected by qPCR. *Esr1* mRNA expression relative to housekeeping genes *Ppia* (A) and *Hprt* (B) and male day 0 control is shown. Data is presented as mean+SEM, n=3. *Esr2* was not detected in islets.

Table 6.5 Mean Ct values ± SEM of *Esr1* and *Esr2* and housekeeping genes in mouse islets (n=3)

	Islets males (untreated Day 0)	Islets males (untreated Day 3)	Islets males (+100 nM ghrelin Day 3)	Islets females (untreated Day 0)	Islets females (untreated Day 3)	Islets females (+100 nM ghrelin Day 3)
Esr1	30.2±1.0	30.1±0.6	30.0±0.5	29.4±0.5	30.0±0.3	29.8±0.4
Esr2	33.1±0.2	32.8±0.6	32.5±0.6	33.9±0.2	33.5±0.4	32.7±0.7
Ppia	17.9±0.3	17.6±0.3	17.6±0.3	18.0±0.5	17.6±0.4	17.6±0.4
Hprt	20.7±0.3	20.6±0.2	20.4±0.1	21.1±0.4	20.2±0.1	20.3±0.4

6.4.2.2 Effect of E2 and ghrelin on MIN6 cell apoptosis

In order to test whether treatment with oestrogen could modify the MIN6 cell survival responses to treatment with ghrelin, preliminary cell apoptosis experiments were conducted as described in 6.3.4. Treatment with 10 or 100 nM ghrelin or 100 nM E2 alone did not significantly affect palmitate-induced apoptosis in MIN6 cells ($p>0.05$, Fig. 6.4.6). Prolonged exposure of MIN6 cells to 100 nM E2 did not modify the lack of effect of ghrelin on MIN6 cell survival ($p>0.05$, Fig. 6.4.6).

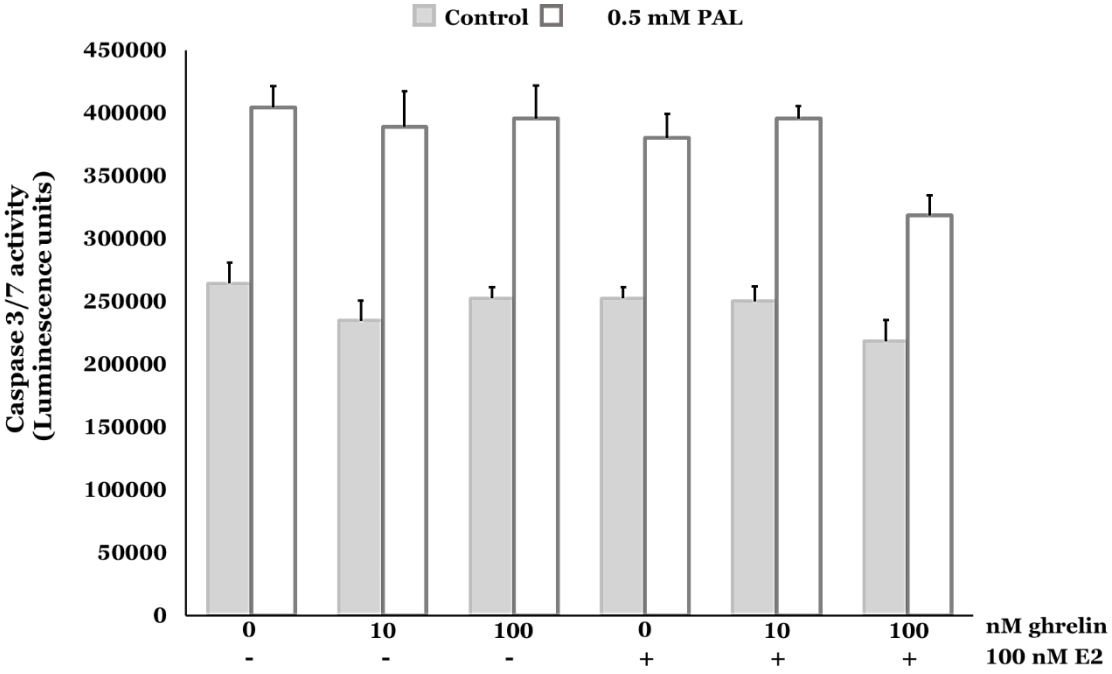

Figure 6.4.6 The effect of ghrelin and E2 on MIN6 cell apoptosis. MIN6 beta cells were plated at 15,000 cells/well and pre-treated for 48 h with or without 10 or 100 nM ghrelin ± 100 nM E2, and exposed to 0.5 mM palmitate ± ghrelin ± 100 nM E2 for 20 h. Apoptosis was detected by measurement of caspase 3/7 activity. Data is presented as mean+SEM, n=5. Graph represents results of one experiment out of two independent experiments.

Taken together these results suggest that the expression of *Esr1* in islets is neither influenced by biological sex in mice nor is it modified by treatment

with ghrelin in MIN6 cells. Furthermore, prolonged incubation with E2 alone does not affect beta cell apoptosis induced by palmitate, and E2 does not directly modify the effect of ghrelin on clonal beta cell apoptosis under these conditions.

6.4.3 SSTR3 as mediator of functional effects of ghrelin in murine islets

6.4.3.1 Sex-specific expression of Sstr3 in mouse islets

The mRNA expression of *SStr3* in islets from male and female mice was detected by qPCR at day 0 and following incubation with or without ghrelin for 48 h. Prior to the experiments using islets, mRNA expression of *Sstr3* was detected in the hypothalamus of male and female mice as a positive control, where SSTR3 was reported to be moderately distributed (Kumar, 2007; Kumar, 2012). *Sstr3* expression in the hypothalamic tissue was not significantly different between males and females ($p>0.05$; Fig. 6.4.7; Table 6.6). Likewise, *Sstr3* mRNA expression was similar in islets from male and female mice after 48 h in culture, and no significant change was observed following treatment with 100 nM ghrelin ($p>0.05$; Fig. 6.4.8 and Table 6.7).

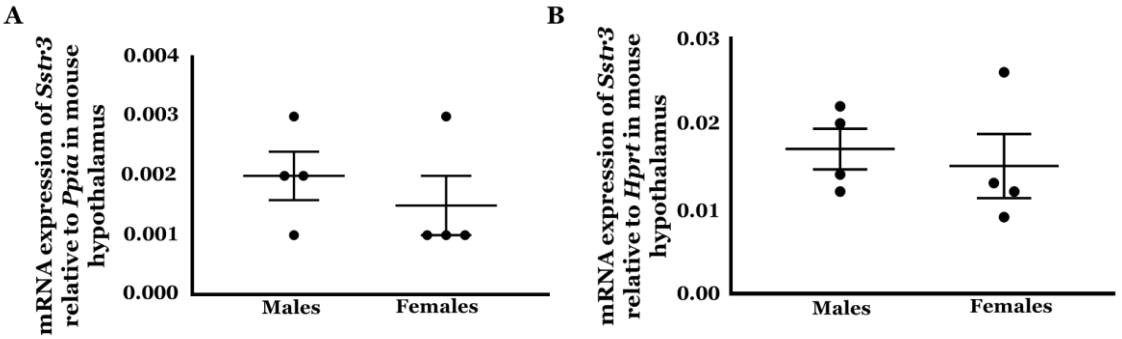

Figure 6.4.7 mRNA expression of *Sstr3* in hypothalamus of male and female mice. The data represent mRNA expression of *Sstr3* relative to the housekeeping genes *Ppia* (A) and *Hprt* (B) in hypothalamus of male and female CD-1 mice. Data is presented as mean ±SEM, n=4.

Table 6.6 Mean Ct values ± SEM of *Sstr3* and housekeeping genes in mouse hypothalamus (n=4)

	Hypothalamus males	Hypothalamus females
Sstr3	25.1±0.4	25.5±0.4
Ppia	16.1±0.2	16.2±0.2
Hprt	19.2±0.4	19.4±0.1

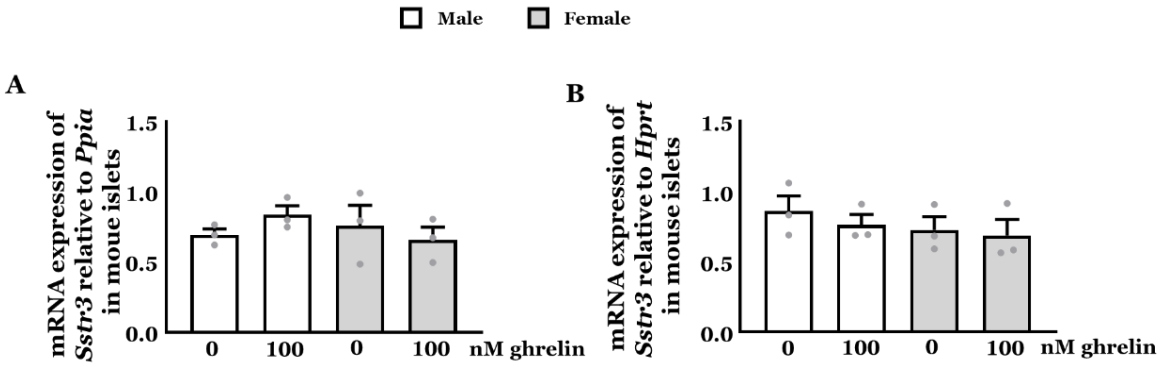

Figure 6.4.8 mRNA expression of Sstr3 in islets from male and female mice. Isolated islets were treated for 48 h as indicated and the mRNA expression of *Sstr3* was detected by qPCR. *Sstr3* mRNA expression relative to housekeeping genes *Ppia* (A) and *Hprt* (B) and male Day 0 control is shown. Data is presented as mean+SEM, n=3.

Table 6.7 Mean Ct values of ± SEM *Sstr3* and housekeeping genes in mouse islets (n=3)

	Islets males (control Day 0)	Islets males (control Day 3)	Islets males (+100 nM ghrelin Day 3)	Islets females (control Day 0)	Islets females (control Day 3)	Islets females (+100 nM ghrelin Day 3)
Sstr3	21.8±0.4	21.9±0.5	21.8±0.4	22.1±0.5	21.9±0.3	22.2±0.4
Ppia	17.9±0.3	17.5±0.3	17.7±0.2	18.1±0.5	17.6±0.4	17.7±0.4
Hprt	20.8±0.0	20.7±0.1	20.4±0.0	21.3±0.2	20.4±0.2	20.7±0.2

6.4.3.2 The effect of ghrelin and SSTR3 antagonist MK4256 on islet secretory responses

Treatment with 1 μM MK4256 on its own significantly increased glucose-induced insulin secretion in islets from male ($p<0.05$ vs 20 mM; Fig. 6.4.9B; $p<0.001$ vs 20 mM Fig. 6.4.11B), but not female mice ($p>0.05$ vs 20 mM; Fig. 6.4.9A). As also reported in chapter 4, incubation with ghrelin did not significantly reduce glucose-induced insulin secretion in both groups ($p>0.05$). This effect was not significantly altered in the presence of 1 μM MK4256 in islets from male mice in 2/3 experiments ($p>0.05$; Fig. 6.4.9B), but a significant increase in insulin secretion was observed in islets from female mice co-treated with 100 nM ghrelin + 1 μM MK4256 ($p<0.05$; Fig. 6.4.9A). However, the effect of antagonist alone was not significantly altered in the presence of ghrelin in both groups ($p>0.05$). Since MK4256 was developed against SST, it was also tested whether the action of SST on glucose-induced insulin secretion from islets could be modified in the presence of MK4256. As previously reported (Hauge-Evans *et al.*, 2009; Hauge-Evans *et al.*, 2015) addition of 1 μM SST did not significantly affect insulin secretion from isolated islets. However, co-treatment of SST with 1 μM MK4256 caused a significant increase in glucose-induced insulin secretion in islets from female mice compared to SST alone ($p<0.01$; Fig. 6.4.10). Similarly, in 2/3 experiments conducted utilising islets from male mice, incubation with 1 μM MK4256 + 1 μM SST significantly increased glucose induced-insulin secretion compared to SST alone ($p<0.01$; Fig. 6.4.11B). However, overall the effect of antagonist alone was not significantly altered in the presence of SST in islets from both sexes ($p>0.05$).

Taken together these results suggest that islets from male and female mice express *Sstr3* mRNA at a similar level, and that treatment with ghrelin does not alter its expression at transcriptional level. However, results from insulin-secretion experiments imply that SSTR3 is involved at least in part in mediating an insulinostatic effect of SST as also observed with MIN6 cells in chapter 3.

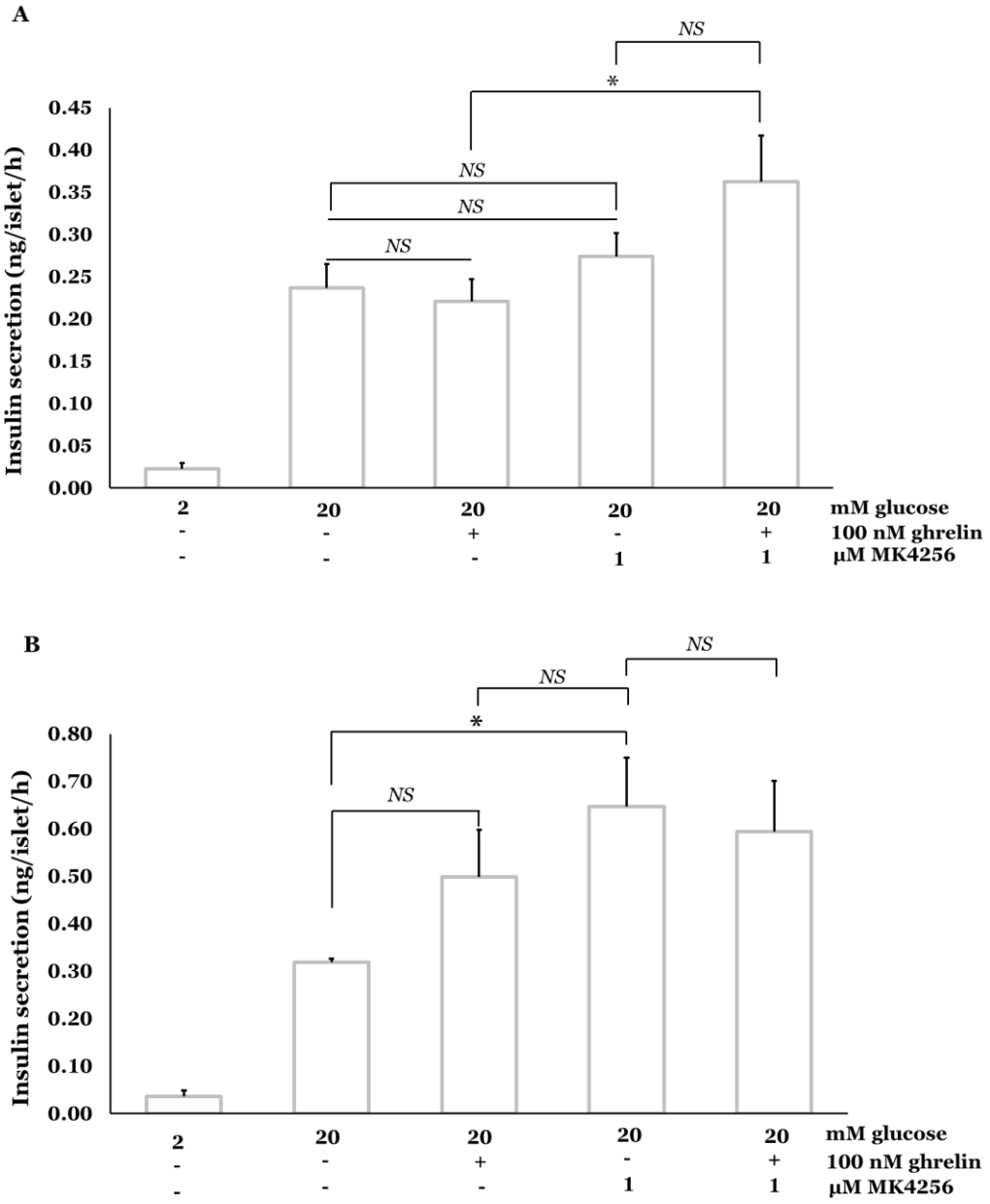

Figure 6.4.9 The effect of ghrelin and SSTR3 antagonist MK4256 on insulin secretion in islets from female (A) and male (B) mice. Isolated islets were pre-treated with 2 mM glucose for 1 h then treated as indicated for 1 h. Secreted insulin was detected by an in-house radioimmunoassay. Data is presented as mean+SEM, n=3-6. Graph A represents results of one out of two independent experiments and graph B of one out of three experiments. One-Way ANOVA with Bonferroni posthoc test *$p<0.05$. NS = not significant $p>0.05$.

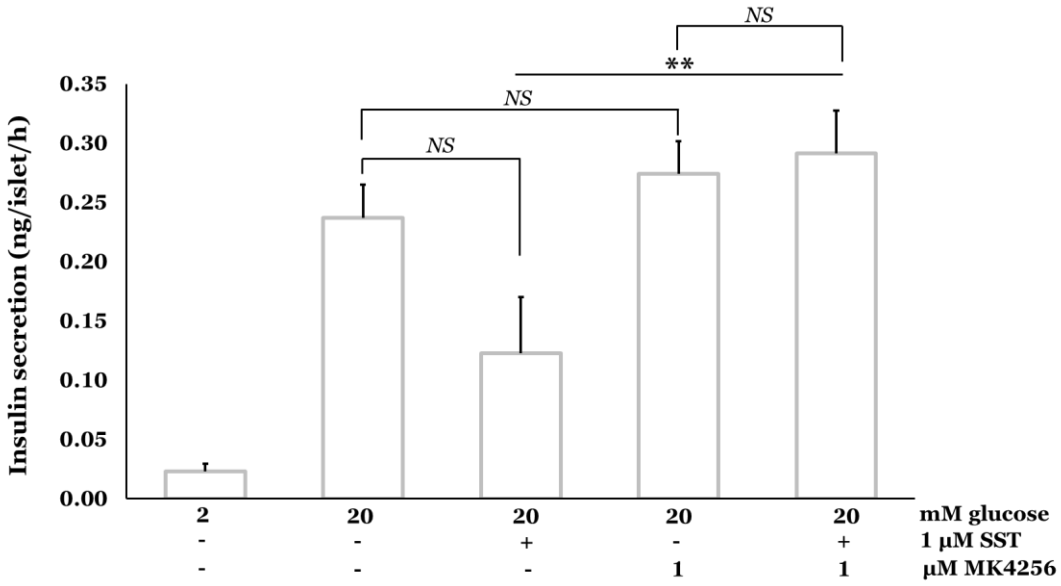

Figure 6.4.10 The effect of SST and SSTR3 antagonist MK4256 on insulin secretion in islets from female mice. Isolated islets were pre-treated with 2 mM glucose for 1 h then treated as indicated for 1 h. Secreted insulin was detected by an in-house radioimmunoassay. Data is presented as mean+SEM, n=4-6. Graph represents results of one experiment out of two independent experiments. One-Way ANOVA with posthoc test Bonferroni **p<0.01. NS = not significant p>0.05.

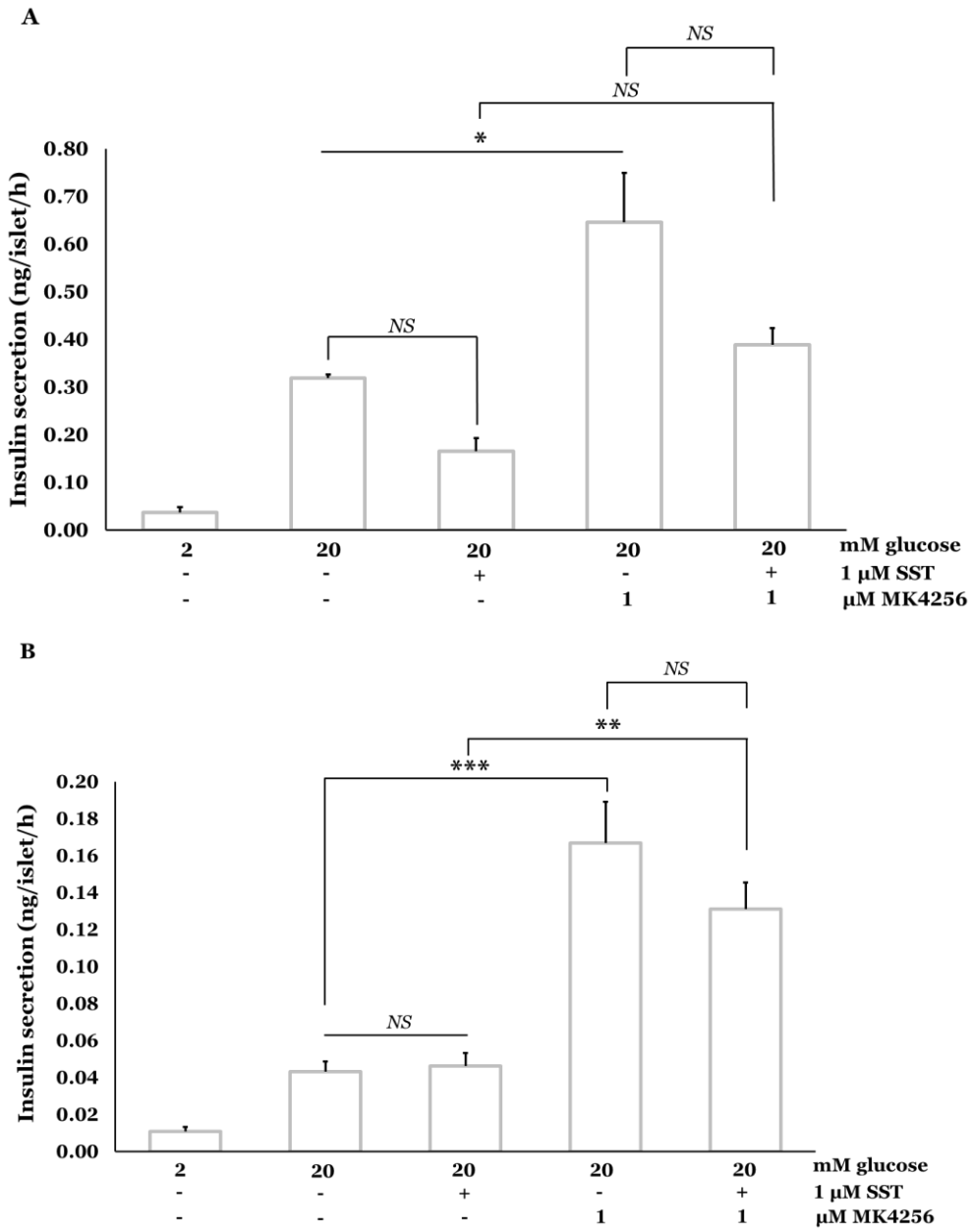

Figure 6.4.11 The effect of SST and SSTR3 antagonist MK4256 on insulin secretion in islets from male mice (A and B). Isolated islets were pre-treated with 2 mM glucose for 1 h then treated as indicated for 1 h. Secreted insulin was detected by an in-house radioimmunoassay. Data is presented as mean+SEM, n=3-6. Graph A represents results of one out of two independent experiments and graph B of one out of three experiments. One-Way ANOVA with posthoc test Bonferroni **p<0.05, **p<0.01, ***p<0.001. NS = not significant p>0.05.

6.5 Discussion

Following on from the findings in chapter 5 where it was established that sex-specific expression of GHSR1a did not appear to mediate the effect of ghrelin on islet apoptosis, other potential factors influencing ghrelin's functional effects on beta cells and islets were explored. These included the presence of endogenous ghrelin, interaction between ghrelin, oestradiol and oestrogen receptors, and sex-specific modulation of SST receptor 3 and its role in mediating the functional effects of ghrelin in mouse islets.

6.5.1 Detection of endogenous ghrelin in MIN6 cells and mouse islets

Molecular biology analysis showed that *Ghrelin* mRNA was present in all tissues tested with the level being traceable in MIN6 cells. *Ghrelin* was reported to be present also in the clonal beta cell line HIT-T15 (Granata *et al.*, 2007). The expression of *Ghrelin* did not differ between male and female mice in all tissues tested. Since when studying functional effects of a protein it is important to detect its expression at protein level, an in-house radioimmunoassay was set up.

As described in 6.4.1.2 secreted ghrelin could not be detected using this assay or a commercially available ELISA. Very few have reported the detectability of secreted ghrelin from the pancreas, instead most studies reported the presence of endogenous ghrelin content in the pancreas (Date *et al.*, 2002) and in isolated rat islets (Dezaki *et al.*, 2004). Similarly, in the current study endogenous ghrelin was detected in islets and MIN6 cells. Acyl-ghrelin levels did not differ between islets from male and female mice, whereas surprisingly total ghrelin was significantly higher in islets from female mice. This result was

unexpected, because it was hypothesised that a higher level of endogenous ghrelin would be present in islets from male mice where exogenous ghrelin had no significant effect, but the GHSR1a antagonist LEAP2 did, as reported in 5.4.3. However, it is important to highlight that the ghrelin radioimmunoassay data is preliminary and further experiments are required to ascertain whether a true difference exists in endogenous ghrelin content between the islets from male and female mice.

The detectability of ghrelin at protein level in the islets, albeit at a lower level than in circulation (Erdmann *et al.*, 2004), implies that pancreatic ghrelin may play a role locally within the islet. Indeed it was previously reported that glucose-induced insulin secretion was enhanced by pharmacologically blocking GHSR1a activity, and by neutralising endogenous ghrelin using anti-ghrelin antiserum in perfused rat pancreata (Dezaki *et al.*, 2006). However, as described in 6.1.1, in foetal islets the number of ghrelin producing cells was found to be higher compared to those in the adult islets, suggesting that the islet-derived ghrelin may play a greater role during pancreatic development (Wierup *et al.*, 2002). In contrast, others have reported that normal pancreatic development progressed in *Ghrelin*$^{-/-}$ mice, implying that ghrelin was not required for this process (Hill *et al.*, 2009). However, it has been reported that the presence of ghrelin is necessary for the embryonic development of the whole animal (Luque *et al.*, 2014). A recent publication showed that glucose-induced insulin secretion did not differ in islets from both global ghrelin null and *Ghrelin*$^{+/+}$ male and female mice (Gray *et al.*, 2019). In both genotypes insulin secretion was equally increased in response to other stimuli such as

glucagon-like peptide 1 (GLP-1) and alanine (Gray *et al.*, 2019), suggesting that endogenous ghrelin may not play a role in glucose homeostasis.

Nevertheless, as reported in the previous chapter, we observed an insulinotropic effect of LEAP2 in islets from male mice, which suggests the presence of some underlying activity of endogenous ghrelin. However, it cannot be dismissed that the observed effect of LEAP2 may have been related to the inhibition of the constitutive activity of the GHSR1a as mentioned in 5.5.3.

6.5.2 The sex-specific expression of islet ERs, and the effect of interaction between E2 and ghrelin on clonal beta cell survival

As reported in chapter 4, a sex-specific difference in response to cytokine-induced apoptosis was observed in islets from female mice. It was therefore hypothesized that prolonged exposure to oestrogen may have resulted in the sex-specific outcome. The subsequent experiments described in chapter 5 and in section 6.4.1 showed that chronic exposure to E2 in females did not have a measurable impact on GHSR1a expression and islet ghrelin content. However, it is possible that differences in islet ER expression levels between the two sexes may have indirectly affected the action of ghrelin.

It has been shown that ER receptor expression differs in male and female rats in a number of tissues such as kidney and gonads (Hutson *et al.*, 2019), and that the presence of E2 regulates islet cell secretory function and survival in a sex-dependent manner (as reviewed by Gannon *et al.*, 2018). However, to my knowledge there have been no reports of sex differences in the expression of ERs in islets. Furthermore, a potential modulation of ERs by ghrelin itself cannot be overlooked. Hence the mRNA expression of ERs was tested in islets

from male and female mice, including whether islet ER expression was modified by the presence of ghrelin. As illustrated in 6.4.2.1, *Esr1* mRNA expression did not differ between islets from male and female mice and it remained unchanged following treatment with ghrelin. In contrast to studies by Alonso-Magdalena *et al.* (2008) and Nadal *et al.* (2000) it was not possible to detect *Esr2* mRNA in the pancreatic islets, however it was detected in the hypothalamic tissues.

Previously, E2 has been shown to promote beta cell survival (Kooptiwut *et al.*, 2018; Le May *et al.*, 2006), and a number of studies have reported the influence of E2 on ghrelin levels (Choi *et al.*, 2017; Clegg *et al.*, 2007). Therefore, preliminary studies were performed to test whether incubation with E2 could modify the effect of ghrelin on MIN6 cell apoptosis. Unlike the findings in the literature, treatment with E2 did not significantly affect palmitate-induced apoptosis in MIN6 cells. Furthermore, co-treatment with E2 did not alter the lack of effect of ghrelin on palmitate-induced apoptosis in MIN6 cells, implying a lack of interaction between the two hormones in regulating clonal beta cell apoptosis. Alternatively, longer incubation with E2 may be needed. However, the overall incubation time of the current study with E2 of three days is in accordance with the duration of treatment used by others (Kooptiwut *et al.*, 2018; Le May *et al.*, 2006).

Therefore, these results suggest that ghrelin does not modify the gene expression of *Esr1* in islets; and the preliminary results on MIN6 cell apoptosis suggest that E2 does not modify the functional effects of ghrelin in clonal beta cells. However, further experiments are required to ascertain the functional effect of interaction between ghrelin, E2 and ERs. One approach would be to

use islets from OVX female mice where any influence of oestradiol would have been abolished, to assess the effects of ghrelin on cytokine-induced apoptosis and *Esr1* expression. Another method would be to administer ER antagonists such as ESR1-selective antagonist methyl-piperidino-pyrazole (MPP) *in vivo* to female mice and to test whether action of ghrelin is modified in isolated islets. Alternatively, the antagonist could be added to islets from female mice *in vitro* to assess whether any effect of ghrelin is blocked. It is also possible to expose islets isolated from male mice to E2 for a prolonged period prior to incubation with ghrelin. However, since islets can be kept in culture for a limited time only, this will need to be considered during experimental set-up. These experiments were beyond the scope of the current project therefore are considered part of future work.

6.5.3 SSTR3 as mediator of functional effects of ghrelin in murine islets

Numerous studies have reported that somatostatin and ghrelin interact in a number of tissues including in the regulation of pancreatic insulin release as described throughout this thesis. In chapter 3 it was elucidated that the insulinostatic effect of SST is mediated in part via SSTR3, which recent reports have indicated to be the only SST receptor subtype expressed in beta cells (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). However, it was not possible to clearly detect SSTR3 expression in islets at protein level by Western blot as reported in 3.4.2.2. The findings of investigations into the effect of ghrelin on MIN6 cells and mouse islet function have suggested that ghrelin may act in an indirect and sex-dependent manner to modulate beta cell function. However, it was established that this was independent of sex-specific

differences in GHSR1a or *Esr1* expression. In addition, a significant effect of ghrelin was observed on cytokine-induced apoptosis in islets from female mice only. However, if the effect of ghrelin were to be indirect via its action on the delta cell then it would be expected that the same effect would have been detected in islets from male mice, since GHSR1a expression was not different between the two groups. Thus, it was hypothesized that there may be sex-specific differences in the expression of SSTR3 that could be modulated by the presence of ghrelin. In islets from both male and female mice the levels of *Sstr3* expression were not different and they remained unchanged following treatment with ghrelin, further confirming the lack of interaction between ghrelin and SSTR3 at a transcriptional level in the current experimental setting. This however does not discount that sex-dependent differences in expression of SSTR3 may exist at protein level. However as mentioned above it was not possible to clearly detect its expression by Western blot. Therefore, further experiments using immunohistochemical staining of islet tissues may not only facilitate in identifying sex-specific differences in SSTR3 expression, but also its localisation within specific islet cell types. Although others have shown the detection of SSTR3 expression by immunohistochemistry in islets, no sex-specific comparison of SSTR3 expression in islets from male and female mice has been reported (Ludvigsen *et al.*, 2004; Somvanshi *et al.*, 2018).

The above results therefore indicate that *Sstr3* expression is neither sex-dependent nor modified by ghrelin. In addition, the findings in 3.5.2 suggested that SSTR3 appeared to be partially involved in mediating insulin secretion but not apoptosis in MIN6 beta cells. Furthermore, a sex-dependent difference in insulin-secretory responses was observed for male mice following

incubation with GHSR1a antagonist LEAP2 in 5.4.3. Based on these findings, and the fact that MK4256 has previously been reported to alter blood glucose levels during OGTT (He *et al.*, 2012), it was tested whether antagonism of SSTR3 would modify potential effects of ghrelin on islet insulin secretory responses.

Results described in 6.4.3.2 indicated that treatment with 1 μM MK4256 alone increased secretion in islets from male but not from female mice. However, co-treatment with ghrelin and 1 μM MK4256 did not modify the lack of effect of ghrelin on islet insulin secretion in islets from males, but significantly increased insulin secretion in islets from females. When co-incubated with SST, MK4256 significantly increased glucose-induced insulin secretion in islets from both groups. However, overall the increase in glucose-induced insulin secretion observed when MK4256 was co-incubated with SST or ghrelin (islets from female mice only), was not different compared to the effect seen with the antagonist alone. This implies that the insulinotropic effect was specific to the antagonist rather than counteraction of exogenous ghrelin or SST. Specifically it can be proposed that MK4256 acts via blocking endogenous SST. Indeed addition of ghrelin or SST alone did not significantly modify insulin secretory response in islets from both groups. It has previously been found that addition of exogenous SST did not alter or further decrease glucose-induced insulin secretion in islets from male mice, and it was suggested that a lack of effect was potentially due to a tonic inhibitory action of endogenous SST produced by islet delta cells (Hauge-Evans *et al.*, 2009; Hauge-Evans *et al.*, 2015). These results suggest that SSTR3 mediates insulin secretory

responses in mouse islets in a sex-dependent manner as also observed for LEAP2 in 5.4.3.

Despite ghrelin having no significant effect on glucose-induced insulin secretion in islets, it is possible that it may modulate SST release from delta cells, which may be responsible for the increase in insulin secretion observed in islets from female mice co-treated with SST + MK4256. Therefore, the effect of ghrelin on islet SST release could be investigated. Furthermore SST secretion in the presence of the GHSR1a antagonist LEAP2 could also be tested to determine whether the insulinotropic action of LEAP2 was dependent on reduction of somatostatin secretion from islet delta cells.

Nevertheless, the indirect activity of ghrelin may also depend on sexually dimorphic action or level of somatostatin itself. For example, negation of somatostatin by SST anti-serum significantly increased plasma growth hormone levels in male rats, but decreased in female rats suggesting a sex difference in hypothalamic somatostatin secretion (Painson and Tannenbaum, 1991). In another study female mice lacking *Sst* had higher expression of growth hormone and *Ghsr1a* in the pituitary compared to control animals, but this difference was not observed in males (Luque and Kineman, 2007). Furthermore, SST has been found to influence growth hormone-releasing hormone (GHRH) electrical activity in a sexually dimorphic manner by causing faster, more frequent and varied action potential firing in male mice compared to females (Osterstock *et al*., 2016). Hence exploring whether islet somatostatin levels are sexually dimorphic is of interest in studying the indirect activity of ghrelin, however these experiments were beyond the scope of the current study.

6.5.4 Summary

The results presented and discussed in this chapter aimed to elucidate the role of GHSR1a-independent factors in mediating the effects of ghrelin in beta cells and islets, with particular focus on sex-specific differences that have been summarised in table 6.8. Ghrelin was detected both at mRNA level and protein level in MIN6 beta cells and mouse islets suggesting the presence of endogenous ghrelin, which may have influenced the effectiveness of exogenous ghrelin. However, no overall sex-specific difference in endogenous ghrelin was found in islets from male and female mice. Due to a sex-specific difference in the action of ghrelin on islet survival it was tested whether the expression of ERs or the presence of E2 may have influenced this outcome. Expression of *Esr1* was similar in islets from both sexes, and its expression was not modified at transcriptional level by treatment with ghrelin. Preliminary findings in MIN6 cells showed that E2 did not modify the effect of ghrelin on palmitate-induced apoptosis in MIN6 cells in our experimental settings, therefore implying that the two hormones do not interact in mediating clonal beta cell survival. Finally, investigations into whether sex-dependent differences in expression of *Sstr3* were responsible for divergent functional outcomes in islets from male and female mice were conducted. *Sstr3* mRNA expression was neither different in islets from male and female mice nor was its expression modified by ghrelin. Assessment of the role of SSTR3 in insulin secretion in islets from male and female mice revealed that SSTR3 is involved to some extent in mediating mouse islet insulin secretory responses in a sex-dependent manner. However, it is unlikely that any effect of ghrelin on insulin secretion is mediated by this receptor. Nevertheless, it cannot be disregarded that ghrelin may affect insulin secretion in a sex-specific

manner to modulate beta cell and islet function highlighting the need for further investigations.

Table 6.8 Sex-specific effects of GHSR1a-independent factors tested in the current study

Effect or feature	Sex-specific effects
Expression of *Ghrelin* mRNA in mouse islets	Not different between males and females
Endogenous acyl-ghrelin content in islets	Not different between both groups
Expression of *Esr1* mRNA in mouse islets	Not different between both groups and not modified by ghrelin
Expression of *Esr2* mRNA in mouse islets	Not detected in both groups
Expression of *Sstr3* mRNA in mouse islets	Not different between both groups and not modified by ghrelin
Treatment of islets with 1 μM MK4256	Increased glucose-induced insulin secretion in islets from male mice only compared to 20 mM glucose
Co-treatment of islets with ghrelin and 1 μM MK4256	Increased glucose-induced insulin secretion in islets from female mice only compared to ghrelin alone, but not compared to antagonist alone.
Co-treatment of islets with SST and 1 μM MK4256	Increased glucose-induced insulin secretion in islets from both groups compared to SST alone, but not compared to antagonist alone.

CHAPTER 7 - General discussion

The dysfunction and loss of beta cells contribute to the pathogenesis of diabetes (reviewed by Hasnain *et al.*, 2016), however the mechanism of beta cell loss is not very well understood in type 2 diabetes. One possible explanation is cellular stress caused by numerous environmental stressors such as glucotoxicity, lipotoxicity or gluco-lipotoxicity as well as increased levels of circulating cytokines resulting in an inflammatory response. These factors cause beta cell oxidative stress and ER stress that in turn can result in beta cell dysfunction leading to beta cell death (reviewed by Hasnain *et al.*, 2016). In the early stages of type 2 diabetes beta cell dysfunction plays a major role which can be considered the main driver of this condition (reviewed by Hasnain *et al.*, 2016). This may potentially be reversed and identifying target candidates that can modify the progression to beta cell apoptosis is therefore of outmost relevance (reviewed by Hasnain *et al.*, 2016, reviewed by Wajchenberg, 2007).

Somatostatin and ghrelin are two hormones that are expressed both systemically and in the pancreatic islets of Langerhans (Gnanapavan *et al.*, 2002; reviewed by Martinez, 2013). Both hormones have been associated with the regulation of islet function and glucose homeostasis by influencing insulin and glucagon secretion (Cheng-Xue *et al.*, 2013; Dezaki *et al.*, 2006; Hauge-Evans *et al.*, 2009). In addition, both SST and ghrelin have been found to promote beta cell and islet survival in response to cellular stressors typical of type 2 and type 1 diabetes, respectively (Damsteegt *et al.*, 2019; Granata *et al.*, 2007). However, the evidence supporting a pro-survival role for ghrelin in response to cellular stressors typical of type 2 diabetes remains limited.

Most recently it was reported that the insulinostatic action of ghrelin is due to an indirect and stimulatory action of ghrelin on pancreatic delta cells leading to the release of SST, which acts on the beta cell to inhibit insulin secretion (Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016). It was therefore hypothesized that the effect of ghrelin on beta cell and islet survival may also potentially be mediated via the action of SST.

The focus of the current study was therefore to explore the roles of SST and ghrelin in beta cell and/or islet function with particular focus on apoptosis induced by cellular stressors typical of type 2 diabetes, and in acute insulin secretory responses. In addition, potential underlying molecular mechanisms that mediate their actions in beta cells and islets were explored.

7.1 The roles of SST/SSTR3 and ghrelin/GHSR1a in clonal beta cell function

The findings described in chapter 3 indicate that treatment with SST reduces clonal beta cell apoptosis induced by lipotoxicity in accordance with Damsteegt *et al.* (2019). The evaluation whether SSTR3 is involved in this process found, however, that it is unlikely for the anti-apoptotic effect of SST in MIN6 cells to be mediated by this receptor. Nevertheless, it was found that SSTR3 may partly contribute to the insulinostatic effect of SST in these cells. It is important to highlight that MIN6 cell line is a transformed cell line with limitations. Indeed, a greater responsiveness to the SSTR3 antagonist MK4256 was observed in islets from male and female mice compared to MIN6 cells, despite the inability to clearly detect the receptor in islets by Western blot. In this study the effect of SST on islet apoptosis was not assessed, however

our previous work in islet cells from male mice showed that apoptosis induced by lipotoxicity was reduced by treatment with SST (Damsteegt *et al.*, 2019).

It is important to highlight that beta cell regenerative capacity is limited and is lost with age in both rodents and humans (Perl *et al.*, 2010; Rankin and Kushner, 2009). In addition, beta cell dysfunction and loss are strongly associated, and not only do these factors contribute to the onset of diabetes, but also progress with the disease (reviewed by Saisho *et al.*, 2015). Impaired beta cell function is associated with impaired glycaemic control (Ritzel *et al.*, 2006) and treatment failure (reviewed by Saisho *et al.*, 2015). Therefore, it has been suggested that preservation and recovery of dysfunctional beta cells constitute a valuable approach for the treatment of type 2 diabetes. Hence, strategies to reduce beta cell workload and improve function are suggested to be effective. Indeed, use of insulin secretagogues in type 2 diabetes therapy is limited especially due to elevated beta cell workload, with the exception of incretins, which in rodents have been found to elicit beneficial effects on beta cell mass (reviewed by Saisho *et al.*, 2015; reviewed by van Raalte and Verchere, 2017). A recent study reported that the use of an adipokine, adipsin, was shown to improve hyperglycaemia and increase tissue area of islets transplanted in the anterior chamber of the eyes of *db/db* mice (Gómez-Bannoy *et al.*, 2019), suggesting that improvement in beta cell mass aids in improved responsiveness to hyperglycaemia.

The observation in the current study that the insulinostatic hormone SST protected clonal beta cells from lipotoxicity-induced apoptosis highlights the potential beneficial effect of beta cell rest. A study conducted by Laedtke *et al.* (2000) found that overnight infusion of SST resulted in more regular insulin

secretion patterns in patients with type 2 diabetes where it is deregulated, and better insulin secretory capacity, further highlighting the importance of reducing beta cell workload. Furthermore, in type 2 diabetes patients SST release is impaired, potentially contributing to beta cell dysfunction (Strowski and Blake, 2007). Therefore, adjunct therapy of SST with insulin or novel SGLT2 antagonists may be of benefit to provide beta cell recovery. However, since SST is present physiologically and may influence growth it is important to consider development of islet-specific targeted therapy. Nevertheless, the findings in this thesis are preliminary, therefore *in vivo* studies in rodents as a next step would aid in further understanding the usefulness of this approach in the prevention of beta cell dysfunction and loss.

The results illustrated in chapter 4 showed that exogenous ghrelin did not play a role in apoptosis and insulin secretory function in MIN6 beta cells despite GHSR1a being present in these cells. Ghrelin was detected both at mRNA level and protein level in MIN6 beta cells suggesting the presence of endogenous ghrelin, which may have influenced the effectiveness of exogenous ghrelin. However, the function of endogenous ghrelin is disputed. Alternatively, this result confirms the hypothesis that the effect of ghrelin on beta cell function may be mediated indirectly via its action on the delta cell with stimulation of somatostatin secretion as suggested by Adriaenssens *et al*. and DiGruccio *et al*. in 2016, although this effect was not investigated with regards to cell survival. However, since we have shown that SST protects islet cells from stressors typical of type 2 diabetes (Damsteegt *et al*., 2019), it is, therefore, likely that any potential effect of ghrelin on islet apoptosis may be mediated by SST.

Work conducted in islets aimed to assess this, however it was first necessary to explore if ghrelin itself had any effect on rodent islet apoptosis.

7.2 Sex-dependent role of ghrelin in islet function

Experiments conducted in islets revealed a sex-specific role for ghrelin where a reduction in apoptosis by ghrelin was only detected in islets from female mice. As indicated in chapters 5 and 6, ghrelin and GHSR1a are present in mouse islets and their expression levels were comparable between islets from both sexes, suggesting that the differences in expression level of the receptor and/or influence from endogenous ghrelin were not responsible for the observed effects on islet apoptosis. GHSR1a antagonists, however, could not reverse the pro-survival effect of ghrelin in islets from female mice. Here the use of animal models with islet-specific knockout of *Ghrelin* or *Ghsr1a* may be helpful in further understanding the molecular mechanisms of actions of ghrelin.

Another interesting observation in this study was the sex-related differences in response to glucose-induced insulin secretion following treatment with GHSR1a and SSTR3 receptor antagonists, LEAP-2 and MK4256, respectively. In islets from male mice the endogenous GHSR1a antagonist LEAP2 increased insulin secretion as shown in section 5.4.3, indicating that the antagonist may have reversed the effect of endogenous ghrelin or alternatively modulated the constitutive activity of the receptor. The assessment of constitutive activity of a GPCR can be detected by measurement of cAMP levels in cells. The high constitutive activity is proportional to the level of cAMP that remains increased in the presence of a phosphodiesterase inhibitor (Prasad, 2010). However, in islets and any cell type expressing a variety of GPCRs the accurate

detection of constitutive activity of one specific receptor is hindered due to interference from the activity of other GPCRs (Damien *et al.*, 2012). Hence most studies investigating the constitutive activity of GHSR1a have relied on either overexpression of GHSR1a in a cell line that does not naturally express it (Holst *et al.*, 2003) or using lipid discs expressing the purified receptor (Damien *et al.*, 2012). However, these methods cannot provide information on sex-specific differences in the constitutive activity of GHSR1a. Nevertheless, inverse agonists have been shown to reduce the constitutive activity of a receptor (Khilnani and Khilnani, 2011) and, therefore, may potentially be useful in testing receptor constitutive activity. Hence the results on sex-specific differences in insulin secretion observed for LEAP2, recently identified as an inverse agonist (M'Kadmi *et al.*, 2018), may be indicative of inverse agonism of constitutive activity of GHSR1a. The increase in insulin secretion in islets from male mice following treatment with LEAP2 perhaps reflect the higher constitutive activity of GHSR1a in these islets compared to the ones from female mice. However, at the moment this remains speculative, and is hindered by current methods available being non-ideal for the detection of sex-specific differences in GHSR1a constitutive activity in islets.

Despite the levels of *Sstr3* mRNA expression being comparable in islets from both sexes, treatment with MK4256 modified glucose-induced insulin secretion in a sex-specific manner. It is possible to suggest that perhaps sex-specific differences in SST release may have contributed to the divergent outcomes. This may be a result of both ghrelin-dependent and independent mechanisms, since the levels and roles of SST itself have been reported to be sex-dependent in other tissues (Painson and Tannenbaum, 1991; Van Vugt *et*

al., 2008) as discussed in 6.5.3. In the present study, although exogenous ghrelin itself had no effect on insulin secretion, it may have influenced insulin release in a sex-dependent manner resulting in observed sex-specific differences in insulin secretion following incubation with MK4256. Further experiments are, however, needed to confirm this.

In general, the effects of ghrelin on glucose homeostasis are mainly negatively associated due to increase in blood glucose observed when ghrelin is administered *in vivo*, due to inhibition of insulin secretion (Broglio *et al.*, 2001; Tong *et al.*, 2010). However, others have suggested that the hyperglycaemia *in vivo* could also be related to increased hepatic gluconeogenesis by ghrelin and increased glucagon secretion (reviewed by Alamri *et al.*, 2016). Taken together these results suggest that the effect of ghrelin on islet function and survival are governed by sex related differences that may be modulated by complex systemic mechanisms.

The discovery of sex-specific differences in islets constitute an important finding in the study of diabetes where sex-dependent differences are commonly reported (Gannon *et al.*, 2018; Tramunt *et al.*, 2019), and most studies relating ghrelin to islet function have investigated islets from male rodents and sex-unspecified human islets. Epidemiological studies indicate that the prevalence of type 2 diabetes is reported to be higher in males between the ages 20-79 with greater incidence with age, and the biological reasons for the higher incidence in men are still being explored (IDF, 2019; Nordström *et al.*, 2016). Men have impaired fasting blood glucose levels reportedly due to inherent differences in systemic glucose metabolism (reviewed by Kautzky-Willer *et al.*, 2016). Although in both sexes increase in insulin secretion is

observed, males had a greater rate of insulin secretion with increasing body mass index (reviewed by Kautzky-Willer *et al.*, 2016). Nordström *et al.* (2016) suggested that the higher content of visceral fat in males was responsible for the greater incidence of the condition in males. It has been reported that female patients display better glycaemic control due to higher beta cell function and glucose responsiveness (reviewed by Gannon *et al.*, 2018). For example, in a study comparing healthy male and female participants of comparable BMIs, females had lower blood glucose during OGTT compared to males, and higher disposition and insulinogenic indices suggesting a better beta cell function in female participants (Kautzky-Willer *et al.*, 2012). The sex differences in risk and predisposition to type 2 diabetes were suggested to be associated with the greater presence of oestrogen in females, epigenetic modifications, glucagon secretion and embryonic beta cell development (reviewed by Gannon *et al.*, 2018; reviewed by Tramunt *et al.*, 2019). For example, as also discussed in chapter 4, oestrogen has been reported to protect beta cells and islets from apoptosis both *in vivo* and *in vitro* (Kooptiwut *et al.*, 2018; Le May *et al.*, 2006), and it has been suggested that *Esr1* may in part mediate this effect (Le May *et al.*, 2006).

The present results show that differences in *Esr1* expression levels did not mediate the protective effect of ghrelin on apoptosis in islets from female mice *in vitro*, however this does not discount the fact that oestrogen itself may modulate the pro-survival effect of ghrelin in islets from female mice. Preliminary data in MIN6 cell line showed that treatment with E2 did not modify the effect of ghrelin on apoptosis in these cells. However, since ghrelin had no effect on MIN6 cell apoptosis to begin with, exploring the role of E2 in

islets would be ideal as suggested in chapter 6, where a range of potential future experiments were suggested. A study comparing sex-differences in glucose responses found that male participants displayed more impaired insulin secretion compared to females. The mean age of female participants was 60 and therefore thought to be post-menopausal. Interestingly, in this study it was suggested that the beneficial effects of prolonged exposure to E2 were extended beyond menopause, however no information was available on the use of HRT or indication of level of beta cell mass/survival due to logical limitations (Russo *et al.*, 2014).

Although others have reported sex-specific differences in ghrelin levels and its orexigenic properties, this has not been reported in pancreatic islets. Investigations into the role of ghrelin on islet apoptosis was only reported in one study that utilised sex-unspecified human islets and in the conditions typical of type 1 diabetes. Therefore, the findings of the current study highlight that ghrelin displays sex-specific differences in modulating islet responses to apoptosis induced by pro-inflammatory cytokines.

Based on the findings of this thesis, the assessment of mechanisms underlying the sex-specific effect of ghrelin in mouse islets indicate that the differences in the levels of expression of GHSR1a, endogenous ghrelin, *Esr1* and *Sstr3* do not mediate the observed effect. It is therefore possible that inherent differences in islets between males and females may exist and influence islet function. So far, reports suggest that maternal obesity and nutrition can influence embryonic islet development in a sex-dependent manner (reviewed by Gannon *et al.*, 2018; reviewed by Tramunt *et al.*, 2019). For example, female offspring of obese female mice displayed reduced blood glucose and

increase insulin levels following glucose challenge. These offspring had greater mitochondrial density and respiration compared to controls from non-obese mothers (Nicholas *et al*., 2019). In the same study male offspring had impaired mitochondrial function and unchanged insulin secretion compared to those from non-obese mothers, suggesting that female progeny from obese mothers are more adapted to confront high nutrient levels after birth (Nicholas *et al*., 2019). However, in the present study wild-type CD-1® mice specifically bred for laboratory testing were used. It is believed that the mice are born from healthy females, although no information is available on the status of maternal characteristics of these mice.

Alternatively, as mentioned before, ghrelin may modulate SST secretion in a sex-specific manner or via an as-yet-unknown mechanism that is specific to the islets from female mice to protect islets from female mice from apoptosis induced by pro-inflammatory cytokines. This suggests the potential usefulness of ghrelin in personalised medicine.

7.3 Potential use of ghrelin in precision medicine to prevent beta cell apoptosis in females

Precision medicine, also known as personalised medicine, refers to the use of strategies to manage or treat a condition based on an individual's or a group's needs and/or genetic predisposition (Fitipaldi *et al*., 2018). The heterogeneity in diabetes is precisely the prerequisite for considering personalised approaches in its management and treatment, since both rodents and humans display divergence in the pathogenesis of this condition (reviewed by Bowman *et al*., 2018; Keller *et al*., 2019). In a recent study, non-diabetic male mice fed a high fat/high sucrose diet were more insulin resistant than female mice,

therefore were found to secrete more insulin than females to compensate for hyperglycaemia (Keller *et al.*, 2019), further highlighting the sex-dimorphic responses to hyperglycaemia and importance of personalised approaches for its regulation.

So far, some progress has been made in employing precision medicine for monogenic types of diabetes such as neonatal diabetes and monogenic diabetes of the young (MODY) with clear advantages in disease management (Mohan and Radha, 2019). However, precision medicine extends beyond genetics since heterogeneity of diabetes is also influenced by environmental factors such as lifestyle and availability of infrastructure (Dendup *et al.*, 2018; Groop, 2016), but approaches addressing these factors are still preliminary (Merino and Florez, 2019).

Furthermore, the differences in diabetes risk and islet function between male and female individuals also suggest that biological sex-based therapies may be advantageous in the development of treatment strategies for diabetes mellitus (Mauvais-Jarvis, 2017).

The sex-specific role of ghrelin in the prevention of beta cell apoptosis observed in the present study, therefore, constitutes an interesting therapeutic approach for women affected by prediabetes or type 2 diabetes. This is important since the majority of academic and preclinical studies in the pharmaceutical industry are performed in male rodents, which may lead to potential therapeutics that would be effective in women being overlooked. However, it is important to highlight that the results in the current study are limited by the use of *ex vivo* islets, and it has been reported that studies in rodents do not always translate into humans (reviewed by Bowman *et al.*,

2018). Nevertheless, the novel sex-specific role of ghrelin in islet apoptosis identified in the current study constitutes a promising approach for a personalised treatment approach in women, and therefore warrants further investigation. However, prior to its use in human studies, it is necessary to test whether *in vivo* ghrelin administration improves beta cell function in islets from female mice. For this mouse models of beta cell dysfunction could be used (King, 2012). Since prolonged exposure to ghrelin induces adiposity (Tschöp *et al.*, 2000), which in turn has been linked to beta cell dysfunction (reviewed by Hasnain *et al.*, 2016), a non-obese model of beta cell dysfunction such as AKITA mouse may be utilised (King, 2012). AKITA mice have a dysfunctional beta cell mass due to ER stress (Chen *et al.*, 2011), therefore could be employed to test whether administration of ghrelin improves the disease phenotype in these animals. An ideal mode of treatment would be islet specific targeted therapy with ghrelin to prevent other effects of ghrelin that may be unwanted in patients with type 2 diabetes. For example, islet-specific nanoparticles (Ghosh *et al.*, 2011), a model developed for treating islet autoimmunity in type 1 diabetes, may be a useful approach.

Concluding remarks

This study aimed to investigate the roles of SST and ghrelin in modulating beta cell and/or islet function in response to cellular stressors typical of type 2 diabetes. It was found that SST promotes beta cell survival against lipotoxicity and cytokines (Damsteegt *et al.*, 2019). This constitutes an interesting

mechanism to be targeted in the prevention of beta cell dysfunction and loss, that are characteristic of type 2 diabetes pathogenesis.

Investigations into the role of ghrelin suggested that it does not directly modulate clonal beta cell function, however the interaction between ghrelin and SST in modulating islet function remains unclear. Nevertheless, any potential effects of ghrelin do not appear to be mediated via SSTR3.

Experiments conducted utilising islets uncovered a sex-specific role for ghrelin in islet apoptosis induced by proinflammatory cytokines. This potentially has implications for diabetes as a heterogenous disease that also displays sexual dimorphism in its manifestation. The novel role of ghrelin in islets from female mice may therefore be useful in precision medicine approaches to prevent beta cell loss in women. However, these results remain preliminary for human use and warrants further investigations.

APPENDIX - Publication and conference abstracts

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Diabetes UK, 2018

Characterising the role of ghrelin and the growth hormone secretagogue receptor type 1a (GHSR1a) antagonist, YIL781, in the regulation of beta cell survival.

Hewawasam NV[1], Patterson M[1], Reeves S[1] and Hauge-Evans AC[1]

Aims: Ghrelin reportedly acts via GHSR1a to influence beta cell function. However, the receptor expression in beta cells remains controversial. The aims of this study were to evaluate the effect of the acyl-ghrelin (AG) on beta cell survival and to determine the expression of ghrelin and GHSR1a in MIN6 beta cells and islets.

Methods: Islet and MIN6 cells were pre-treated with AG or the GHSR1a antagonist, YIL781 (5μM), for 48h prior to 20h exposure to palmitate (0.5mM) or TNF-α (1000U/ml) and IL-1β (50U/ml). Apoptosis was assessed by measurement of caspase 3/7 activity. mRNA expression was determined using qPCR.

Results: Ten or 100nM AG did not affect palmitate-induced caspase activity in MIN6 cells (20h, n=6, $P>0.2$ vs. control) nor cytokine-induced apoptosis in islets (20h, n=6; $P>0.1$ vs. control). However, YIL781±AG significantly reduced apoptosis in palmitate-treated MIN6 cells compared to control (n=6; $p<0.05$ YIL781 only; $p<0.01$ YIL781+10nM AG; $p<0.01$ YIL781+100nM AG) and in cytokine-treated islets compared to 100nM AG only (n=6; $p<0.05$, YIL781+100nM AG). GHSR1a mRNA was expressed in islets and traceable in MIN6 cells. Ghrelin mRNA was detected in both tissues. GHSR1a and ghrelin mRNA expression was down-regulated in islets following 48h treatment with 100nM AG (GHSR1a:53% reduction compared to controls, ghrelin: 95%), whereas GHSR1a (61% compared to control) but not ghrelin mRNA expression (108%) was down-regulated by 5μM YIL781.

Conclusion: Although GHSR1a is expressed in the islet, exogenous AG does not affect beta cell survival. The effect of the GHSR1a antagonist suggests a potential novel role for YIL781 in apoptosis.

A Novel Role for Somatostatin in the Survival of Mouse Pancreatic Beta Cells.

Damsteegt EL[1], Hassan Z[2], Hewawasam NV[1], Sarnsamak K[1], Jones PM[2] and Hauge-Evans AC.[1]

Background/Aims: Cross-talk between different pancreatic islet cell types regulates islet function and somatostatin (SST) released from pancreatic delta cells inhibits insulin secretion from pancreatic beta cells. In other tissues SST exhibits both protective and pro-apoptotic properties in a tissue-specific manner, but little is known about the impact of the peptide on beta cell survival. Here we investigate the specific role of SST in the regulation of beta cell survival in response to physiologically relevant inducers of cellular stress including palmitate, cytokines and glucose.

Methods: Pancreatic MIN6 beta cells and primary mouse islet cells were pre-treated with SST with or without the $G_{i/o}$ signalling inhibitor, pertussis toxin, and exposed to different cellular stress factors. Apoptosis and proliferation were assessed by measurement of caspase 3/7 activity, TUNEL and BrdU incorporation, respectively, and expression of target genes was measured by qPCR.

Results: SST partly alleviated upregulation of cellular stress markers (*Hspa1a* and *Ddit3*) and beta cell apoptosis in response to factors such as lipotoxicity (palmitate), pro-inflammatory cytokines (IL1β and TNFα) and low glucose levels. This effect was mediated via a $G_{i/o}$ protein-dependent pathway, but did not modify transcriptional upregulation of the specific NFκB-dependent genes, *Nos2* and *Ccl2,* nor was it associated with transcriptional changes in SST receptor expression.

Conclusion: Our results suggest an underlying protective effect of SST which modulates the beta cell response to ER stress and apoptosis induced by a range of cellular stressors associated with type 2 diabetes.

Diabetes UK, 2019

Exploring gender differences in islet survival in response to ghrelin treatment.

Hewawasam NV[1], Austin AL[2], King A[2], Patterson M[1], Reeves S[1] and Hauge-Evans AC[1]

Aims: Ghrelin is expressed in a number of tissues including stomach and islets. It inhibits insulin secretion and promotes islet survival, however, most studies relating ghrelin to islet function have investigated islets from male rodents. The aim of this study was to explore whether treatment with acyl-ghrelin (AG) affects gene expression and apoptosis in islets from male and female mice.

Methods: Oestrus cycle stage was determined by haematoxylin and eosin (H&E) staining of vaginal smears. Islets from male and female CD-1 mice were incubated with 10 or 100nmol/l AG for 48h and exposed to TNF-α (1000U/ml) and IL-1β (50U/ml) for 20h. Gene expression was determined by qPCR and apoptosis by measurement of caspase 3/7 activity.

Results: H&E staining showed that all female mice except one were synchronised at metestrus stage (n=10). *Ghrelin* mRNA expression was 2-fold higher in stomach tissue from females compared to males, but was equal in islets from both groups. Ghrelin receptor (*Ghsr*) expression was 2-fold greater in islets from female mice. Following incubation with 100nmol/l AG, *Ghsr* expression remained unchanged, however *Ghrelin* expression was downregulated by 90% in islets from female mice only. Treatment with 10 and 100nmol/l AG reduced cytokine-induced apoptosis compared to control in islets from female (30%±0.1 (10nmol/l), $p<0.01$ and 29%±0.0 (100nmol/l) $p<0.01$, n=3) but not male mice (0.5%±0.1 (10nmol/l) $p>0.9$ and 6%±0.1 (100nmol/l), $p> 0.8$, n=4).

Conclusion: Our results suggest a differential effect of ghrelin on islet survival in male and female mice which may involve regulation of endogenous islet ghrelin.

EASD, 2019

Gender differences in the effect of ghrelin on pancreatic islet survival

Hewawasam NV[1], Austin AL[2], King A[2], Patterson M[1], Reeves S[1] and Hauge-Evans AC[1]

Background and aims: Ghrelin is expressed in a number of tissues including stomach, hypothalamus and islets of Langerhans. It inhibits insulin secretion and promotes islet survival. In addition, ghrelin displays gender dimorphism with regards to plasma levels in humans and rodents, and orexigenic responses in rodents. However, most studies relating ghrelin to islet function have investigated islets from male rodents and gender unspecified human islets. The aim of this study was to explore whether there are any gender specific differences in mRNA expression of *Ghrelin*, ghrelin receptor (*Ghsr*) and oestrogen receptor 1 (*Esr1*) in murine islets, and whether treatment with acyl-ghrelin (AG) affects their expression levels as well as apoptosis in islets from both groups. It was also tested whether any effect observed is conveyed via the Growth Hormone Secretagougue Receptor type 1a (GHSR1a).

Materials and methods: Oestrus cycle stage was determined by haematoxylin and eosin (H&E) staining of vaginal smears. Islets from male and female CD-1 mice were incubated with 10 or 100 nmol/l AG +/- 5 μmol/l GHSR1a antagonist YIL781 for 48h and exposed to TNF-α (1000 U/ml) and IL-1β (50 U/ml) for 20h. Gene expression was determined by quantitative PCR and apoptosis by measurement of caspase 3/7 activity.

Results: H&E staining showed that the majority (67%) of female mice used were synchronised at metestrus stage with the remaining at oestrus stage (33%), (n=24). Expression of *Ghrelin* was not significantly different in stomach ($p>0.5$, n=3) and hypothalamus of male and female mice. Ghrelin receptor (*Ghsr*) expression was 2-fold greater in islets from female mice ($p<0.05$), however *Ghrelin* ($p>0.2$, n=3) and oestrogen receptor 1 (n=2) mRNA expression was not significantly different between the two groups. Following incubation with 100 nmol/l AG, mRNA expression of *Ghrelin* ($p>0.9$, n=3), *Ghsr* ($p>0.09$, n=3) and *Esr1* (n=2) remained unchanged in both groups. Treatment with 10 and 100 nmol/l AG reduced cytokine-induced apoptosis compared to control in islets from female (45%±4 (10 nmol/l), $p<0.0001$ and 35%±4 (100 nmol/l) $p<0.001$, n=6, experiment representative of four repeats), but not male mice (113%±8 (10 nmol/l) $p>0.6$ and 113%±10 (100 nmol/l), $p> 0.6$, n=6, experiment representative of three repeats). Treatment with 5 μmol/l YIL781 did not reverse the observed

protective effect of ghrelin in islets from female mice. However, it resulted in significant reduction in apoptosis (47%±6 (5 μmol/l YIL781), $p<0.0001$ and 35%±.4 (5 μmol/l YIL781 + 100 nmol/l AG), $p<0.001$, n=6, experiment representative of three repeats).

Conclusion: Our results suggest a differential effect of ghrelin on islet survival in male and female mice which may involve regulation of an endogenous islet ghrelin receptor, and suggest a potential agonistic effect of GHSR1a antagonist YIL781.

Future Physiology, 2019

Gender differences in the effect of ghrelin on pancreatic islet survival.

Hewawasam NV[1], Austin AL[2], King A[2], Patterson M[1], Reeves S[1] and Hauge-Evans AC[1]

Background and aims: Ghrelin is expressed in a number of tissues including stomach, hypothalamus and islets of Langerhans. It inhibits insulin secretion and promotes islet survival. In addition, ghrelin displays sexual dimorphism with regards to plasma levels in humans and rodents, and orexigenic responses in rodents. However, most studies relating ghrelin to islet function have investigated islets from male rodents and sex unspecified human islets. The aim of this study was to explore whether there are any sex-specific differences in mRNA expression of *Ghrelin*, ghrelin receptor (*Ghsr*) and oestrogen receptor 1 (*Esr1*) in murine islets, and whether treatment with acyl-ghrelin (AG) affects their expression levels as well as apoptosis in islets from both groups. It was also tested whether any effect observed is conveyed via the Growth Hormone Secretagougue Receptor type 1a (GHSR1a).

Materials and methods: Oestrus cycle stage was determined by haematoxylin and eosin (H&E) staining of vaginal smears. Islets from male and female CD-1 mice were incubated with 10 or 100 nmol/l AG +/- 5 μmol/l GHSR1a antagonists YIL781 or +/-100 nM Liver expressed antimicrobial peptide-2 for 48h and exposed to TNF-α (1000 U/ml) and IL-1β (50 U/ml) for 20h.
Apoptosis was determined by measurement of caspase 3/7 activity, gene expression by quantitative PCR and protein expression by Western blot.

Results: H&E staining showed that the majority (60%) of female mice used were synchronised at metestrus stage with the remaining at oestrus stage (31%), (n=48). Treatment with 10 and 100 nmol/l AG reduced cytokine-induced apoptosis compared to control in islets from female (45±4% (10 nmol/l), $p<0.0001$ and 35±4% (100 nmol/l) $p<0.001$, n=6, One-Way ANOVA), but not male mice (113±8% (10 nmol/l) $p>0.6$ and 113±10% (100 nmol/l), $p>0.6$, n=6). Expression of *Ghrelin* was not significantly different in stomach and hypothalamus of male and female mice (both $p>0.05$, n=5, Student's T-test). *Ghsr* mRNA expression was 2-fold greater in islets from female mice ($p<0.05$, n=4, T-test), however *Ghrelin* and *Esr1* ($p>0.5$,n=3-4) mRNA expression levels were not significantly different between the two groups. Following incubation with 100 nmol/l

AG, mRNA expression of *Ghrelin*, *Ghsr* and *Esr1* ($p>0.1$, n=3-4, T-test) remained unchanged in both groups. GHSR expression was not significantly different in the islets from male and female mice at protein level ($p=0.09$, n=3, T-test). Treatment with either GHSR antagonists did not reverse the observed protective effect of ghrelin in islets from female mice. However treatment with YIL781 resulted in significant reduction in apoptosis (47±6% (5 µmol/l YIL781), $p<0.0001$ and 35±4% (5 µmol/l YIL781+100 nmol/l AG), $p<0.001$, n=6, One-Way ANOVA).

Conclusion: Our results suggest a differential effect of ghrelin on islet survival in male and female mice which may be independent of endogenous islet ghrelin receptor.

Diabetes UK, 2020

Pancreatic islets display sexual dimorphism in insulin secretion in response to liver-expressed antimicrobial peptide-2

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Introduction: Prevalence of type 2 diabetes, beta cell function as well as dysfunction are influenced by sex. We have found that treatment with exogenous ghrelin significantly reduced cytokine-induced apoptosis in islets from female, but not male mice. Reportedly, ghrelin acts via the G-protein coupled receptor growth hormone secretagogue receptor type 1A (GHSR1a). Liver-expressed antimicrobial peptide-2, LEAP-2, has recently been characterised as a GHSR1a antagonist. The aim of this study was to investigate whether the action of ghrelin on islet function is modified by LEAP-2 and whether the response to ghrelin and LEAP-2 is subject to sex-related differences.

Methods: Apoptosis was detected by measurement of caspase 3/7 activity. Static insulin secretion experiments were conducted and insulin content was measured by radioimmunoassay.

Results: Treatment with 100nM LEAP-2 did not significantly affect cytokine-induced apoptosis in islets from both male and female mice and it did not significantly reverse the protective effect of ghrelin observed in islets from females ($p=0.27$, $n=6$). In our study we did not observe an insulinostatic effect of ghrelin in islets from both groups ($p=0.99$ males and $p=0.76$ females, $n=5$). However, in islets from male mice treatment with 100nM LEAP-2 resulted in significantly higher glucose-induced insulin secretion compared to 20mM glucose ($p=0.001$, $n=5$-6) but not in female ($p=0.49$, $n=5$) and this was significantly reduced in the presence of ghrelin ($p=0.0016$, $n=5$-6).

Conclusion: Treatment with LEAP-2 did not significantly reverse the protective effect of ghrelin in islets from female mice. However, it significantly increased glucose-induced insulin secretion in islets from male, but not from female mice, suggesting a sexually dimorphic effect of LEAP-2 in islet function.

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