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1 **Islet neuropeptide Y receptors are functionally**
2 **conserved and novel targets for the preservation of**
3 **beta-cell mass.**

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Abstract

Objective: Two unmet therapeutic strategies for diabetes treatment are prevention of beta-cell death and stimulation of beta-cell replication. Our aim was to characterise the role of Neuropeptide Y receptors in the control of beta-cell mass.

Methods: We used endogenous and selective agonists of the NPY receptor system to explore its role in the prevention of beta-cell apoptosis and proliferation in islets isolated from both mouse and human donors. We further explored the intra-cellular signalling cascades involved using chemical inhibitors of key signalling pathways. As proof of principle we designed a long-acting analogue of [Leu³¹Pro³⁴]-NPY, an agonist of the islet expressed Y receptors, to validate if targeting this system could preserve beta-cell mass *in vivo*.

Results: Our data reveal NPY Y1, 4 and 5 receptor activation engages a generalised and powerful anti-apoptotic pathway which protects mouse and human islets from damage. This anti-apoptotic effects were dependent on stimulating a G α i-PLC-PKC signalling cascade, which prevented cytokine induced NF κ B signalling. NPY receptor activation functionally protected islets by restoring glucose responsiveness following chemically induced injury in both species. NPY receptor activation attenuated beta-cell apoptosis, preserved functional beta-cell mass and attenuated the hyperglycaemic phenotype in a low dose streptozotocin model of diabetes.

Conclusion: Taken together, our observations identify the islet Y receptors as promising targets for the preservation of beta-cell mass. As such targeting these receptors could help to maintain beta-cell mass in both Type 1 and 2 diabetes and may also be useful for improving islet transplantation outcomes.

Keywords: Neuropeptide Y; Beta-cell mass; apoptosis; proliferation; Islets; diabetes.

Word count 347255

1. Introduction

Maintenance and replenishment of beta-cell mass are key targets of novel therapeutic interventions for diabetes. We have previously identified the gut hormone peptide YY (PYY) and the neuropeptide Y (NPY) receptor family as having a novel role in the control of beta-cell survival [1]. PYY is a gut hormone [2] that is best known for its role in regulating appetite [3-6]. However, recent data are shining a light on its role in beta-cell function. It is a 36 amino acid peptide synthesized by enteroendocrine L-cells [7]. The enzymatic cleavage of secreted PYY1-36 by dipeptidyl peptidase IV (DPP-IV) produces PYY3-36 [8]. The ratio of circulating PYY1-36 to PYY3-36 has not been consistently determined, although PYY3-36 is often cited as the major circulating form [9]. PYY exerts its effects through the NPY family of receptors [10]. PYY1-36 binds with similar affinity to all Y receptors (Y1R, Y2R, Y4R and Y5R), while PYY3-36 is somewhat selective for the Y2R receptor [11]. As well as the gut, PYY is also present in foetal and adult pancreas, but its role in glucose homeostasis and beta-cell function is not well defined [12-14]. In the foetus it is expressed in the earliest formed islet endocrine cells [15, 16], suggesting a role in islet development. Whilst in the adult pancreas, PYY production is restricted to subpopulations of α -, δ - and pancreatic polypeptide (PP) -cells [13, 17].

A large body of evidence suggests activation of islet NPY receptors inhibits insulin secretion both *in vitro* and *in vivo*. NPY and PYY1-36 have been shown to inhibit stimulated insulin secretion from isolated islets in a number of species [18, 19]. *In vivo* intravenous administration of PYY1-36 inhibits insulin secretion [13] and PYY knockout mice are hyperinsulinaemic [20]. In contrast, PYY3-36 has no effect on insulin secretion, consistent with the absence of Y2R expression in islets [21]. In addition to the reported effects of the NPY receptors on the regulation of insulin secretion, it is becoming apparent these receptors could be targets for the preservation of beta-cell mass. Our previous data suggests PYY may provide an anti-apoptotic tone helping to maintain beta-cell mass [1]. Additionally, NPY receptor activation in other cell types protects against apoptosis [22] and more recently their activation has tentatively revealed anti-apoptotic and mitotic potentials in insulin producing cells, albeit in transformed clonal cell lines [23]. Together these data point to NPY receptor signalling being a prime candidate for further investigation as a target for the maintenance of beta-cell mass. To this end, we investigated the role of NPY receptor

activation using endogenous and selective agonists on beta-cell mass in isolated primary islets and in a whole animal model of diabetes.

2. Methods

Male mice were housed under temperature controlled ($22 \pm 2^{\circ}\text{C}$) and light conditions (12h light: 12h dark cycle) with ad libitum access to drinking water and standard rodent chow. Animal experiments were carried out in accordance with the British Home Office Animals Scientific Procedures Act 1986. All compounds were purchased from Tocris Biosciences unless otherwise stated.

2.1 Islet isolation: Mouse islets were isolated from male CD1 mice aged 8-12 weeks (Charles River Laboratories, Margate, U.K.) by collagenase digestion (1mg/ml; type XI; Sigma-Aldrich, Poole, U.K.) and separated on a histopaque gradient (Histopaque 1077; Sigma-Aldrich)[24]. Human islets were obtained from the pancreata of non-diabetic heart-beating donors with appropriate ethical approval [25]. All islets were incubated overnight at 37°C (5% CO_2) prior to experiments.

2.2 qPCR: Total RNA was extracted from approximately 200 islets using an RNAeasy kit (Qiagen, Sussex, UK). Reverse transcription was performed using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, U.K.). Quantitative PCR amplification was performed as previously described [26]. A list of primers used can be found in the supplemental data.

2.4 Islet apoptosis *in vitro*: Islets were cultured for 48h with PYY (1-36), PYY(3-36), [Leu³¹Pro³⁴]-NPY or Y190 in the absence or presence of a cytokine cocktail (IL-1 β , 0.2ng/ml, TNF- α , 100ng/ml, IFN- γ , 100ng/ml) for the last 20h of culture. In further studies, islets were cultured for 48h with [Leu³¹Pro³⁴]-NPY with and without sodium palmitate (500 μM) or streptozotocin (5mM) for the last 20h of culture. Following cytokine treatment Caspase 3/7 activity was measured using the CaspaseGlo 3/7 kit (Promega, Southampton, U.K.) [18]. To determine the signalling cascades islets were treated with various intracellular inhibitors, pertussis toxin (1ng/ml), U73122 (5 μM), Go6983 (1 μM), gallein (10 μM) or MEK inhibitor U0126 (5 μM) in the absence or presence of cytokines. PKC was depleted using PMA (200nM) for 21h prior to treatment with cytokines [24]. TUNEL staining was used to localise the anti-apoptotic

effect of 1nM [Leu³¹Pro³⁴]-NPY to beta-cells. Results shown are representative of data from 3 independent studies.

2.5 Nuclear NFkB and Nitrite production: Groups of 50 islets (n=4) were cultured for 48h in RPMI in the absence or presence of a cytokine cocktail (IL-1 β , 1ng/ml; TNF- α , 5ng/ml, IFN- γ , 5ng/ml) for the last 20h of culture, with or without 1nM [Leu³¹Pro³⁴]-NPY. Nitrite production in the culture media was measured using the Measure-iT High Sensitivity Nitrite Assay Kit (Molecular Probes). NFkB p65 in nuclear extracts was measured using an elisa (Abcam).

2.6 In vitro islet proliferation: Islets were cultured in RPMI (2% FBS, 1% penicillin-streptomycin, 1% l-glutamine) containing 1mg/ml BrdU with the addition of 10nM [Leu³¹Pro³⁴]-NPY or 20nM exendin-4 for 7 days.

2.7 In vivo administration of Y190: CD1 mice (n=8) were administered intraperitoneally with Y190 (75nmol/kg; i.p.) or vehicle for 11 days (days -1-10). Low dose STZ (75mg/kg) was administered on days 0-2. Glucose tolerance was determined following i.p. glucose 2g/kg or 1g/kg during the STZ study on day 21, as previously described [27]. The anti-apoptotic effect of Y190 in a low dose streptozotocin model was also determined. Mice received daily ip. dosing of Y190 (25 or 75nmol/kg/day) for ten days [1]. Beta-cell area was calculated following insulin staining, betastaining, beta-cell apoptosis was quantified on day 9 by TUNEL staining.

2.8 Immunostaining: Islets or pancreata were fixed with 4% paraformaldehyde, 5 μ m sections underwent antigen retrieval with trypsin-EDTA (BrdU, insulin) or heat mediated antigen retrieval (insulin, Ki67) before blocking. Sections were incubated with guinea pig anti-insulin (Dako,1:200) and a mouse anti-BrdU (Sigma,1:100) or a polyclonal rabbit anti-Ki67 (Abcam,1:200) for 1 h at room temperature. Primary antibody binding was detected using either goat anti-mouse Alexa Fluor 488 or donkey anti-guinea pig Alexa Fluor 594 or a donkey anti-rabbit Alexa Fluor 488. All secondary antibodies were used at 1:200 and purchased from Jackson ImmunoResearch Laboratories. For beta-cell area (STZ study) an insulin biotinylated goat anti-guinea pig primary antibody (Jacksons,1:200) was used and visualised with horseradish peroxidase-conjugated streptavidin (Vector Laboratories) and Diaminobenzidine (DAB) detection. Apoptotic cells were detected by a TUNEL assay according to

manufacturer's instructions (TACS 2 TdT In Situ Apoptosis Detection Kit, Fluorescein AMS Biotechnology) with insulin fluorescently stained as above or using the TACS 2 TdT-DAB kit visualised with Nickel/DAB and the insulin biotinylated primary imaged with ImmPACT NovaRED peroxidase (Vector Laboratories). Image-J was used for analysis; beta-cell area was automatically calculated and adjusted for total pancreatic area, a minimum of 75 islets per treatment, > 3500 cells, were counted for measuring Ki67-BrDU positive beta-cells and TUNEL positive beta-cells in vitro. For in vivo TUNEL staining a minimum of 30 islets, >1500, cells per independent sample, n=4, were counted, –

2.9 Data analysis: Data are expressed as mean $\pm$ SEM and analysed using one or two-way ANOVA as appropriate and Bonferroni's post-hoc test or t-tests where only two groups were compared. A p-value <0.05 between treatments was considered significant.

3. RESULTS

3.1 Expression of NPY receptors in mouse and human islets

It has been reported that mouse and human islets express the Y1R and Y4R, whilst human islets additionally express the Y5R and neither express the Y2R [18, 26, 28]. Next generation sequence of specific islet cell types has somewhat confused this picture. In mouse, RNAseq of beta, alpha and delta cells suggest only the Y1R is expressed at detectable levels and it was expressed in all cell types [29, 30]. However, data from sorted human alpha and beta cells suggests all NPY receptor subtypes are present in all cell types [31]. We confirmed the expression of NPY receptors in islets from our CD1 male mice and in human islets. The Y1R receptor was the most abundantly expressed NPY receptor subtype in mouse and human islets. The Y4R was expressed at low levels in both species, while the Y5R was additionally expressed in human islets but not by mouse tissue and the Y2R receptor was not detectable in either species (supplementary fig 1).

3.2 Islet NPYR activation protects islets from cytokine induced apoptosis

Our previous data suggested that PYY signalling may provide trophic support to beta-cells and either the Y1R or Y2R were the most likely receptors through which this occurred [1]. To further explore the protective role of NPY receptors we investigated

the potential of endogenous and selective agonists to prevent apoptosis in islets exposed to a cocktail of cytokines designed to approximate the toxic cytokine milieu evident during diabetes. Subsequently, we measured caspase 3/7 activity as a surrogate marker of apoptosis. The selectivity of the ligands was determined using cAMP assays against NPY receptor subtypes (supplementary fig 2). PYY1-36 (a non-selective agonist for all Y receptors) potently and significantly inhibited cytokine-induced apoptosis reaching a maximal inhibition at 1nM. Human islets appeared more sensitive to the effect of PYY1-36 having a maximal inhibitory effect at 10pM (Fig. 1A, B). As expected, the Y2R-selective agonist PYY3-36 did not protect against cytokine-mediated apoptosis in islets from either species (supplementary fig 3). [Leu³¹Pro³⁴]-NPY which is selective for the islet expressed receptors Y1, Y4 and Y5 also potently protected mouse and human islets from cytokine-induced apoptosis (Fig 1. C, D), even at very low doses (supplementary fig 3). In human tissue the inhibition of apoptosis was again more profound, with complete blockade of apoptosis at a dose of 100pM. BVD10, a selective Y1R antagonist [32, 33], blocked the anti-apoptotic properties of [Leu³¹Pro³⁴]-NPY in mouse and human islets (Fig 1. E, F), suggesting most of the protective effect of [Leu³¹Pro³⁴]-NPY was mediated through Y1R activation consistent with its 10-fold higher potency at this receptor compared to Y4R and Y5R. To determine if the timing of NPYR activation was critical for reducing cytokine-induced apoptosis we cultured islets using our standard cytokine protocol, with a 24h [Leu³¹Pro³⁴]-NPY pre-treatment followed by 20h cytokine exposure in the presence or absence of [Leu³¹Pro³⁴]-NPY. All treatments significantly inhibited cytokine-induced apoptosis in mouse and human islets suggesting the anti-apoptotic effect of [Leu³¹Pro³⁴]-NPY was immediate and lasted at least 24 hours following removal of the ligand (supplementary fig 3). Selective agonists at both Y4R (Y141, supplementary fig 2) and Y5R (BW46) [34] also potently inhibited cytokine induced apoptosis (Fig 1 G-I). We did not test Y5R in mouse as in our hands this receptor is not expressed by mouse islets. In human islets, the anti-apoptotic effect of BW46 was blocked by addition of the selective Y5R antagonist L152 [35] demonstrating a specific receptor mediated effect (Fig 1 J). We could not conduct similar experiments for Y141 (Y4R) as there are no selective Y4R antagonists, however in mouse the effect of Y141 was not blocked by the addition of the Y1R antagonist BVD10, suggesting by extrapolation, that Y141's protective effect was driven by the Y4R and not by promiscuous activity at the Y1R (supplementary fig 3). Given activation of all islet expressed NPYRs either

individually or selectively reduced islet caspase activity, we used [Leu³¹Pro³⁴]-NPY to probe whether the NPYR mediated reductions in caspase translated into reduced beta-cell apoptosis. [Leu³¹Pro³⁴]-NPY significantly reduced the number of TUNEL positive beta-cells suggesting NPY receptor activation directly protected beta-cells from cytokine induced apoptosis (Fig 2 A and B). Together the data demonstrates that islet expressed NPYR's are functionally conserved with respect to their ability to protect islet cell types from cytokine induced injury.

3.5 Islet NPYR activation rescues glucose responsiveness in islets following injury. To establish if the protective effect of NPYR agonism could maintain the insulin secretory function of islets, we performed static insulin secretion studies following cytokine treatment. We empirically determined a less aggressive cytokine cocktail to allow us to more sensitively determine insulin secretion in these functional studies (supplementary fig 3). As expected cytokine exposure, completely impaired insulin secretion in response to 20mmol/l glucose (Fig. 2 C and D). This impaired secretory response was completely rescued by treatment with either 1nM [Leu³¹Pro³⁴]-NPY (Fig 2 C and D) or 1nM Y141(Y4R agonist, supplementary fig 4) in both mouse and human islets, demonstrating islet Y receptor activation functionally protects islets from injury.

3.6 Which intracellular signalling pathways do islet NPYR's employ to protect islets from injury? We used chemical blockade of various intracellular signalling cascades to delineate the anti-apoptotic pathways employed by select NPY receptors. [Leu³¹Pro³⁴]-NPY protected islets from cytokine-induced apoptosis by coupling to G_{ai}, since pertussis toxin significantly attenuated its protective effects (Fig 2. E, F). To determine if islet NPYR's engage intracellular kinases we used the non-specific inhibitor staurosporine which blocked [Leu³¹Pro³⁴]-NPY anti-apoptotic effects (supplementary fig 4). Subsequently, we sought to define which kinases were involved. Protection against injury was blocked by the PLC inhibitor U73122 (Fig 2 G and H), by PKC depletion with PMA (supplementary fig 4) and by the PKC inhibitor Go6983 (Fig 2, I and J). Demonstrating the protective effect of NPYR activation signals via PLC and PKC downstream of G_{ai} engagement in both mouse and human islets. Similarly, we found the protective effects of both the Y4R agonist (mouse and human islets) and the Y5R agonist (human islets) could be blocked by pertussis toxin, U73122, and Go6983 (Fig 3 A-I).

3.7 How does islet NPYR activation prevent apoptosis in islets?

To further elucidate the mechanisms of the anti-apoptotic effect we measured expression of genes involved in the regulation of apoptosis. As expected, the anti-apoptotic gene Bcl-2 was downregulated in response to cytokines and [Leu³¹Pro³⁴]-NPY significantly attenuated this inhibition. In beta-cells cytokines increase the nuclear translocation of NFkB p65 initiating pro-apoptotic gene transcription. For example, NFkB increases the expression of inducible nitric oxide synthase (iNOS) increasing the production of nitric oxide inducing beta-cell death [36] in part by driving endoplasmic reticulum and oxidative stress and the subsequent production of the pro-apoptotic C/EBP homologous protein CHOP [37]. [Leu³¹Pro³⁴]-NPY attenuated cytokine induced translocation of NFkB, the induction of iNOS expression, the production of nitrite (a marker of nitric oxide) and the induction of CHOP expression (Fig 4. A-E). Taken together these data suggest that NPYR's engage a conserved Gi-PLC-PKC pathway to protect islets from injury and this pathway promotes islet survival by decreasing cytokine induced upregulation of pro-apoptotic signals and increasing mitochondrial pro-survival signalling (Fig 4, I).

3.8 Islet NPYR activation induces beta cell proliferation.

Strategies designed to maintain beta-cell mass are likely to be more successful if they target more than one mechanism. For example, protecting beta-cells from injury whilst also increasing proliferation. We assessed the role of YR activation on beta-cell proliferation in mouse islets. Exposure of islets to [Leu³¹Pro³⁴]-NPY *in vitro* for 7 days significantly increased BrdU positive cells per islet and the percentage of BrdU positive beta-cells (Fig. 4. F, G and H). This suggest NPYR activation could help maintain beta-cell mass by inhibiting beta-cell apoptosis and increasing proliferation.

3.9 Islet NPYR activation protects islets from injury in vivo.

If the islet NPYR's are to be considered novel targets for the maintenance of beta-cell mass, it is critical to demonstrate efficacy *in vivo*. To selectively target the NPYR's expressed by the islet and not the Y2R which has been previously demonstrated to increase the release of GLP-1, we lipidated [Leu³¹Pro³⁴]-NPY to produce Y190. Lipidation is a simple and well known strategy to extend biological activity, usually providing 8-18h of half-life extension. This modification did not alter [Leu³¹Pro³⁴]-NPY selectivity or potency (supplementary fig 2). In agreement with our *in vitro* data, Y190

1 significantly inhibited cytokine and streptozotocin (STZ) induced apoptosis in mouse
2 islets (Fig. 5A and B). In a model of diabetes induced by multiple low doses of STZ,
3 Y190 (75nmol/kg) protected mice against hyperglycaemia (Fig 5, C). Y190 treated
4 mice exhibited improved glucose tolerance (Fig 5, D) and significantly greater beta-
5 cell area than STZ/vehicle treated mice (Fig 5, E and F). We further examined the
6 islets histologically and found beta-cell apoptosis was significantly reduced in treated
7 mice compared to controls (Fig 5, G and H). These data demonstrate that NPY
8 receptor activation preserves functional beta-cell mass *in vivo* in a model of diabetes
9 by reducing STZ induced beta-cell apoptosis. Taken together our data validate the
10 islet expressed NPY receptors (Y1, 4 and 5) as targets for preserving beta-cell mass.

12 **4.1 Discussion**

13 Peptide YY is best known for its appetite regulating properties. However, recent data
14 has shifted focus toward PYY and the NPY receptor system's potential role in islet cell
15 function and survival [1]. We used cytokine exposure as a model of islet injury and
16 found that PYY1-36 powerfully protected mouse and human islets from apoptosis, but
17 PYY3-36 did not. We chose to explore the anti-apoptotic effect of the NPY receptors
18 further using [Leu³¹Pro³⁴]-NPY, as it is selective for the islet expressed receptors Y1,
19 4 and 5, and compounds selective for the Y4R (Y141) and the Y5R (BW46). All three
20 agonists protected islets from injury to a similar extent to PYY1-36. NPYR activation
21 likely attenuated beta-cell apoptosis since [Leu³¹Pro³⁴]-NPY reduced the number of
22 TUNEL positive beta-cells in response to cytokine exposure. The reduction in
23 apoptosis had a direct positive effect on islet secretory function, both [Leu³¹Pro³⁴]-NPY
24 and Y141 restored glucose stimulated insulin secretion in mouse and human islets
25 exposed to cytokines. The anti-apoptotic effect of islet NPY receptor activation was
26 initiated by activating a Giα-PLC-PKC pathway which significantly attenuated cytokine
27 inhibition of the anti-apoptotic Bcl-2 gene and inhibited cytokine activation of NFκB
28 pro-apoptotic signalling. Whether other cytokine induced pro-apoptotic pathways are
29 inhibited by NPY receptor activation will require further investigation. As will the
30 precise mechanism linking NPY receptor signalling with inhibition of apoptosis.

31 PYY1-36 is a non-selective receptor agonist which circulates at concentrations which
32 may activate islet NPY receptors. It is also produced and released locally in the islet
33 [38, 39]. NPY, another non-selective agonist [18] which is released into the islet via
34 nerve endings [40], has also been suggested to be produced and released by beta-

cells [41] and circulates in similar concentrations to PYY. Furthermore, Pancreatic polypeptide, a selective Y4R agonist (100 fold over Y5R), is also produced locally in the islet [42-44]. Since NPY receptors appear pharmacologically conserved, future experiments designed to tease apart the relative importance of each ligand, whether a locally produced or circulating source plays a role, and the collective or individual contribution of each receptor, will help to clarify the physiological role of this system in the protection of the islet from injury.

Since we used whole islets we could not determine whether the protective effect of NPYR activation was a function of activating receptors expressed directly on beta-cells or whether receptors expressed by other islet cell types such as alpha and delta cells play an important role. This will require some thought as antibodies against NPYR's are notoriously poor, whilst quantitative PCR and cell sorting coupled with next generation sequencing have so far provided conflicting results. What has emerged is the consistent finding that Y1R is the most abundantly expressed receptor whether using whole islets or sorted islet cell types [30, 31]. The inconsistency of results exploring the expression of the other receptors maybe due to very low levels of expression. However, Y2R is generally absent whilst the Y5R is found only in human samples and the Y4R is either absent or expressed at low concentrations in both mouse and human [18, 26, 28]. It may therefore be more useful to determine if a receptor is functionally present. In our hands Y1R, Y4R and Y5R are functionally present and in the case of Y1R and Y5R the effects can be blocked with selective antagonists. These functional readouts would be further strengthened by using islets from mice with targeted deletion of each receptor.

There are clearly many questions to be answered with regard the physiological role of the PYY/NPY system in the control of beta-cell mass, but our *in vitro* data does suggest that targeting these receptors pharmacologically could preserve beta-cell mass in diabetes. Our lipidated [Leu³¹Pro³⁴]-NPY analogue protected mouse islets against cytokine and STZ induced apoptosis. In the whole animal, it attenuated STZ induced diabetes by maintaining a greater functional beta-cell mass caused by an inhibition of beta-cell apoptosis. Although, some of the reduced beta-cell area might be accounted for by hyperglycaemic degranulation of the beta-cell. This is the first proof of concept data validating the islet NPYR's as targets for the preservation of beta-cell mass. There are however, several caveats regarding the targeting of these receptors for the treatment of diabetes. For example, central activation of Y1R and Y5R are known to

be orexigenic [45, 46] but perhaps more importantly Y1R causes vasoconstriction [47], both undesirable side effects when treating metabolic diseases. Therefore, any strategy targeting NPYR's will likely require islet specificity or delivery in combination with compounds which alleviate the inherent drawbacks. Despite these caveats NPYR activation displays a remarkable capacity to protect human islets from injury.

Author Contributions: ZF, AT and PFP gathered the data and contributed to the manuscript writing, GAB gathered data, wrote the manuscript and managed the project, AK contributed to the manuscript writing, SP contributed to the manuscript and management of the studies, GH provided human islets.

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Duality of interest: The authors declare that there is no duality of interest.

Figure Legends

Figure 1. NPY receptor activation protects islets from apoptosis. Anti-apoptotic properties of PYY(1-36) (A and B), [Leu³¹Pro³⁴]-NPY (C and D), [Leu³¹Pro³⁴]-NPY plus BVD10 (Y1R antagonist, E and F), Y141 (Y4R agonist, G and H), BWX46 (Y5R agonist, I) and in the presence of a Y5R antagonist L152 (J), in mouse and/or human islets. Islets were incubated with treatment for 48h in the presence of a cytokine cocktail for the last 20h. Apoptosis was assessed by Caspase 3/7 activity. ***p<0.001 vs. control, ^Δp<0.05, ^{ΔΔ}p<0.01, ^{ΔΔΔ}p<0.001 vs. cytokines, **p<0.01 vs. treatment, one-way ANOVA and Bonferroni's multiple comparisons test. Data are presented as mean ± SEM, n=6 ~~observations~~ biological replicates per treatment group.

Figure 2. NPY receptor activation inhibits cytokine induced beta-cell apoptosis and restores GSIS via a Giα-PLC-PKC pathway. (A) ~~Percentage Number of~~ TUNEL positive beta-cells ~~per insulin-stained area~~ following cytokine exposure with or without [Leu³¹Pro³⁴]-NPY (1nM). (B) Representative images of TUNEL staining in each condition, TUNEL (green) insulin (red). (C and D) Islets were treated with [Leu³¹Pro³⁴]-NPY for 48h in the absence or presence of a cytokine cocktail for the last 20h. Following treatment, groups of islets underwent a static secretion assays. *p<0.05,

p<0.01, *p<0.001 vs. 2mmol/l glucose, one-way ANOVA and Bonferroni's multiple comparisons test. Data are presented as mean $\pm$ SEM, n=8 observations per treatment group. (E-J) Islets were treated for 48h with the 1nM [Leu³¹Pro³⁴]-NPY in the absence presence of 100ng/ml pertussis toxin (E and F), 10 μ M U73122 (G and H) or 1 μ M Go6983 (I and J). **p<0.01, ***p<0.001 vs. control, Δ p<0.05, $\Delta\Delta$ p<0.01, $\Delta\Delta\Delta$ p<0.001 vs. cytokines, **p<0.01, ***p<0.001 vs. [Leu³¹Pro³⁴]-NPY, one-way ANOVA and Bonferroni's multiple comparisons test. Data are presented as mean $\pm$ SEM, n=6 observations-biological replicates per treatment group.

Figure 3. The anti-apoptotic effects of Y4R and Y5R are also mediated via a Gi α -PLC-PKC pathway. Islets were treated for 48h with the 1nM Y141 (Y4R agonist) in the absence presence of 100ng/ml pertussis toxin (A and B), 10 μ M U73122 (C and D) or 1 μ M Go6983 (E and F). Islets were treated with BWX46 a Y5R agonist in the presence of pertussis toxin (G), U73122 (H) or Go6983 (I). (J) Islets were treated with Y141 for 48h in the absence or presence of a cytokine cocktail for the last 20h. Following treatment, groups of islets underwent a static insulin secretion assays. **p<0.01, ***p<0.001 vs. control, Δ p<0.05, $\Delta\Delta$ p<0.01, $\Delta\Delta\Delta$ p<0.001 vs. cytokines, **p<0.01, ***p<0.001 vs. treatment, one-way ANOVA and Bonferroni's multiple comparisons test. Data are presented as mean $\pm$ SEM, n=6 biological replicates per treatment group.

Figure 4. NPY receptor activation inhibits cytokine induced gene transcription and increases proliferation of beta-cells. Mouse islets were treated for 48h with [Leu³¹Pro³⁴]-NPY in the absence or presence of cytokines for the last 20h of treatment. Islet expression of Bcl-2 (A) iNOS (B) and CHOP(C) were measured by qPCR. Nitrite production was measured using the greiss assay (D) and nuclear NFkB p65 measured using an elisa (E). (F) Mouse islets were cultured for 7 days with 10nM [Leu³¹Pro³⁴]-NPY and 20nM exendin-4 in the presence of 1mg/ml BrdU, β -cell proliferation is expressed as BrdU positive cells per total β -cells. (H) representative images of staining BrdU (green) insulin (red). Data are presented as mean $\pm$ SEM, . *p<0.05, **p<0.01 vs. control, Δ p<0.05, $\Delta\Delta$ p<0.01, $\Delta\Delta\Delta$ p<0.001 vs. cytokines one-way ANOVA and Bonferroni's multiple comparisons test, n=6 observations-biological replicates per group. (I) Graphical representation of NPY receptor mechanisms of action. PTX, pertussis toxin, U7312 PLC inhibitor, G06983 PKC inhibitor, iNOS inducible nitric oxide synthase, NO nitric oxide, CHOP C/EBP homologous protein, Bcl-2 B-cell lymphoma 2.

Figure 5. NPY receptor activation protects islets from injury *in vivo*. Mouse islets were cultured with Y190 for 48h in the presence of a cytokine cocktail (A) or STZ (B) for the last 20h of incubation before caspase activity was measured. (B) Mice (n=8) were treated with Y190 (25 or 75nmol/kg/day), or vehicle for 11days. low dose streptozotocin (75mg/kg) was given on days 1-3. Plasma glucose (C), day 21 glucose tolerance (D), representative insulin staining (E), beta cell area (F). Percentage

TUNEL positive beta-cells-on day 9 of the STZ protocol (G). Representative images of TUNEL (blue/black) and insulin (red) co-localisation (H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. 2mmol/l glucose. *** $p < 0.001$ vs. control, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ vs. cytokines, one-way ANOVA and Bonferroni's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, $\Delta p < 0.01$ vs. STZ group, two-way repeated measures ANOVA and Bonferroni's multiple comparisons test. Data are presented as mean $\pm$ SEM, (n=8).

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