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

4
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CONFIDENTIAL

1 **Abstract**

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

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

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

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

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

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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

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

3 4 **2. Methods**

5 Male mice were housed under temperature controlled ($22 \pm 2^\circ\text{C}$) and light conditions
6 (12h light: 12h dark cycle) with ad libitum access to drinking water and standard rodent
7 chow. Animal experiments were carried out in accordance with the British Home Office
8 Animals Scientific Procedures Act 1986. All compounds were purchased from Tocris
9 Biosciences unless otherwise stated.

10
11 **2.1 Islet isolation:** Mouse islets were isolated from male CD1 mice aged 8-12 weeks
12 (Charles River Laboratories, Margate, U.K.) by collagenase digestion (1mg/ml; type
13 XI; Sigma-Aldrich, Poole, U.K.) and separated on a histopaque gradient (Histopaque
14 1077; Sigma-Aldrich)[24]. Human islets were obtained from the pancreata of non-
15 diabetic heart-beating donors with appropriate ethical approval [25]. All islets were
16 incubated overnight at 37°C (5% CO_2) prior to experiments.

17
18 **2.2 qPCR:** Total RNA was extracted from approximately 200 islets using an RNAeasy
19 kit (Qiagen, Sussex, UK). Reverse transcription was performed using the High-
20 capacity cDNA Reverse Transcription kit (Applied Biosystems, U.K.). Quantitative
21 PCR amplification was performed as previously described [26]. A list of primers used
22 can be found in the supplemental data.

23
24 **2.4 Islet apoptosis *in vitro*:** Islets were cultured for 48h with PYY (1-36), PYY(3-36),
25 [$\text{Leu}^{31}\text{Pro}^{34}$]-NPY or Y190 in the absence or presence of a cytokine cocktail (IL- 1β ,
26 0.2ng/ml, TNF- α , 100ng/ml, IFN- γ , 100ng/ml) for the last 20h of culture. In further
27 studies, islets were cultured for 48h with [$\text{Leu}^{31}\text{Pro}^{34}$]-NPY with and without sodium
28 palmitate (500 μM) or streptozotocin (5mM) for the last 20h of culture. Following
29 cytokine treatment Caspase 3/7 activity was measured using the CaspaseGlo 3/7 kit
30 (Promega, Southampton, U.K.) [18]. To determine the signalling cascades islets were
31 treated with various intracellular inhibitors, pertussis toxin (1ng/ml), U73122 (5 μM),
32 Go6983 (1 μM), gallein (10 μM) or MEK inhibitor U0126 (5 μM) in the absence or
33 presence of cytokines. PKC was depleted using PMA (200nM) for 21h prior to
34 treatment with cytokines [24]. TUNEL staining was used to localise the anti-apoptotic

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

3
4 **2.5 Nuclear NFκB and Nitrite production:** Groups of 50 islets (n=4) were cultured
5 for 48h in RPMI in the absence or presence of a cytokine cocktail (IL-1β, 1ng/ml; TNF-
6 α, 5ng/ml, IFN-γ, 5ng/ml) for the last 20h of culture, with or without 1nM [Leu³¹Pro³⁴]-
7 NPY. Nitrite production in the culture media was measured using the Measure-iT High
8 Sensitivity Nitrite Assay Kit (Molecular Probes). NFκB p65 in nuclear extracts was
9 measured using an elisa (Abcam).

10
11 **2.6 In vitro islet proliferation:** Islets were cultured in RPMI (2% FBS, 1% penicillin-
12 streptomycin, 1% l-glutamine) containing 1mg/ml BrdU with the addition of 10nM
13 [Leu³¹Pro³⁴]-NPY or 20nM exendin-4 for 7 days.

14 **2.7 In vivo administration of Y190:** CD1 mice (n=8) were administered
15 intraperitoneally with Y190 (75nmol/kg; i.p.) or vehicle for 11 days (days -1-10). Low
16 dose STZ (75mg/kg) was administered on days 0-2. Glucose tolerance was
17 determined following i.p. glucose ~~2g/kg or 1g/kg during the STZ study on day 21, as~~
18 ~~previously described [27]. The anti-apoptotic effect of Y190 in a low dose~~
19 ~~streptozotocin model was also determined. Mice received daily ip. dosing of Y190 (25~~
20 ~~or 75nmol/kg/day) for ten days [1].~~ Beta-cell area was calculated following insulin
21 ~~staining, beta~~staining, ~~beta~~ cell apoptosis was quantified on day 9 by TUNEL staining.

22
23 **2.8 Immunostaining:** Islets or pancreata were fixed with 4% paraformaldehyde, 5 μm
24 sections underwent antigen retrieval with trypsin-EDTA (BrdU, insulin) or heat
25 mediated antigen retrieval (insulin, Ki67) before blocking. Sections were incubated
26 with guinea pig anti-insulin (Dako,1:200) and a mouse anti-BrdU (Sigma,1:100) ~~or a~~
27 ~~polyclonal rabbit anti-Ki67 (Abcam,1:200)~~ for 1 h at room temperature. Primary
28 antibody binding was detected using either goat anti-mouse Alexa Fluor 488 or donkey
29 anti-guinea pig Alexa Fluor 594 or a donkey anti-rabbit Alexa Fluor 488. All secondary
30 antibodies were used at 1:200 and purchased from Jackson ImmunoResearch
31 Laboratories. For beta-cell area (STZ study) an insulin biotinylated goat anti-guinea
32 pig primary antibody (Jacksons,1:200) was used and visualised with horseradish
33 peroxidase-conjugated streptavidin (Vector Laboratories) and Diaminobenzidine
34 (DAB) detection. Apoptotic cells were detected by a TUNEL assay according to

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

10
11 **2.9 Data analysis:** Data are expressed as mean \pm SEM and analysed using one or
12 two-way ANOVA as appropriate and Bonferroni's post-hoc test or t-tests where only
13 two groups were compared. A p-value <0.05 between treatments was considered
14 significant.

15 16 **3. RESULTS**

17 **3.1 Expression of NPY receptors in mouse and human islets**

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

30 31 **3.2 Islet NPYR activation protects islets from cytokine induced apoptosis**

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

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

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

8
9 **3.5 Islet NPYR activation rescues glucose responsiveness in islets following**
10 **injury.** To establish if the protective effect of NPYR agonism could maintain the insulin
11 secretory function of islets, we performed static insulin secretion studies following
12 cytokine treatment. We empirically determined a less aggressive cytokine cocktail to
13 allow us to more sensitively determine insulin secretion in these functional studies
14 (supplementary fig 3). As expected cytokine exposure, completely impaired insulin
15 secretion in response to 20mmol/l glucose (Fig. 2 C and D). This impaired secretory
16 response was completely rescued by treatment with either 1nM [Leu³¹Pro³⁴]-NPY (Fig
17 2 C and D) or 1nM Y141(Y4R agonist, supplementary fig 4) in both mouse and human
18 islets, demonstrating islet Y receptor activation functionally protects islets from injury.

19
20 **3.6 Which intracellular signalling pathways do islet NPYR's employ to protect**
21 **islets from injury?** We used chemical blockade of various intracellular signalling
22 cascades to delineate the anti-apoptotic pathways employed by select NPY receptors.
23 [Leu³¹Pro³⁴]-NPY protected islets from cytokine-induced apoptosis by coupling to Gai,
24 since pertussis toxin significantly attenuated its protective effects (Fig 2. E, F). To
25 determine if islet NPYR's engage intracellular kinases we used the non-specific
26 inhibitor staurosporine which blocked [Leu³¹Pro³⁴]-NPY anti-apoptotic effects
27 (supplementary fig 4). Subsequently, we sought to define which kinases were
28 involved. Protection against injury was blocked by the PLC inhibitor U73122 (Fig 2 G
29 and H), by PKC depletion with PMA (supplementary fig 4) and by the PKC inhibitor
30 Go6983 (Fig 2, I and J). Demonstrating the protective effect of NPYR activation signals
31 via PLC and PKC downstream of Gai engagement in both mouse and human islets.
32 Similarly, we found the protective effects of both the Y4R agonist (mouse and human
33 islets) and the Y5R agonist (human islets) could be blocked by pertussis toxin,
34 U73122, and Go6983 (Fig 3 A-I).

1 **3.7 How does islet NPYR activation prevent apoptosis in islets?**

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

18 **3.8 Islet NPYR activation induces beta cell proliferation.**

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

27 **3.9 Islet NPYR activation protects islets from injury in vivo.**

28 If the islet NPYR's are to be considered novel targets for the maintenance of beta-cell
29 mass, it is critical to demonstrate efficacy *in vivo*. To selectively target the NPYR's
30 expressed by the islet and not the Y2R which has been previously demonstrated to
31 increase the release of GLP-1, we lipidated [Leu³¹Pro³⁴]-NPY to produce Y190.
32 Lipidation is a simple and well known strategy to extend biological activity, usually
33 providing 8-18h of half-life extension. This modification did not alter [Leu³¹Pro³⁴]-NPY
34 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.
11

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 (BW446). 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 G α -PLC-PKC pathway which significantly attenuated cytokine
27 inhibition of the anti-apoptotic Bcl-2 gene and inhibited cytokine activation of NF κ B
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-

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

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

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

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

6
7 **Author Contributions:** ZF, AT and PFP gathered the data and contributed to the
8 manuscript writing, GAB gathered data, wrote the manuscript and managed the
9 project, AK contributed to the manuscript writing, SP contributed to the manuscript and
10 management of the studies, GH provided human islets.

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

20 21 **Figure Legends**

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

31
32 **Figure 2. NPY receptor activation inhibits cytokine induced beta-cell apoptosis
33 and restores GSIS via a Giα-PLC-PKC pathway.** (A) Percentage Number of-TUNEL
34 positive beta-cells per insulin-stained area following cytokine exposure with or without
35 [Leu³¹Pro³⁴]-NPY (1nM). (B) Representative images of TUNEL staining in each
36 condition, TUNEL (green) insulin (red). (C and D) Islets were treated with [Leu³¹Pro³⁴]-
37 NPY for 48h in the absence or presence of a cytokine cocktail for the last 20h.
38 Following treatment, groups of islets underwent a static secretion assays. *p<0.05,

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

9
10 **Figure 3. The anti-apoptotic effects of Y4R and Y5R are also mediated via a Giα-
11 PLC-PKC pathway.** Islets were treated for 48h with the 1nM Y141 (Y4R agonist) in
12 the absence presence of 100ng/ml pertussis toxin (A and B), 10µM U73122 (C and D)
13 or 1µM Go6983 (E and F). Islets were treated with BWX46 a Y5R agonist in the
14 presence of pertussis toxin (G), U73122 (H) or Go6983 (I). (J) Islets were treated with
15 Y141 for 48h in the absence or presence of a cytokine cocktail for the last 20h.
16 Following treatment, groups of islets underwent a static insulin secretion assays.
17 **p<0.01, ***p<0.001 vs. control, Δp<0.05, ΔΔp<0.01, ΔΔΔp<0.001 vs. cytokines,
18 ++p<0.01, +++p<0.001 vs. treatment, one-way ANOVA and Bonferroni's multiple
19 comparisons test. Data are presented as mean ± SEM, n=6 biological replicates per
20 treatment group.

21
22
23 **Figure 4. NPY receptor activation inhibits cytokine induced gene transcription
24 and increases proliferation of beta-cells.** Mouse islets were treated for 48h with
25 [Leu³¹Pro³⁴]-NPY in the absence or presence of cytokines for the last 20h of treatment
26 Islet expression of Bcl-2 (A) iNOS (B) and CHOP(C) were measured by qPCR. Nitrite
27 production was measured using the greiss assay (D) and nuclear NFκB p65 measured
28 using an elisa (E). (F) Mouse islets were cultured for 7 days with 10nM [Leu³¹Pro³⁴]-
29 NPY and 20nM exendin-4 in the presence of 1mg/ml BrdU, β-cell proliferation is
30 expressed as BrdU positive cells per total β-cells. (H) representative images of staining
31 BrdU (green) insulin (red). Data are presented as mean ± SEM, . *p<0.05, **p<0.01 vs.
32 control, Δp<0.05, ΔΔp<0.01, ΔΔΔp<0.001 vs. cytokines one-way ANOVA and
33 Bonferroni's multiple comparisons test, n=6 observations-biological replicates per
34 group. (I) Graphical representation of NPY receptor mechanisms of action. PTX,
35 pertussis toxin, U7312 PLC inhibitor, G06983 PKC inhibitor, iNOS inducible nitric oxide
36 synthase, NO nitric oxide, CHOP C/EBP homologous protein, Bcl-2 B-cell lymphoma
37 2.

38
39 **Figure 5. NPY receptor activation protects islets from injury *in vivo*.** Mouse islets
40 were cultured with Y190 for 48h in the presence of a cytokine cocktail (A) or STZ (B)
41 for the last 20h of incubation before caspase activity was measured. (B) Mice (n=8)
42 were treated with Y190 (25 or 75nmol/kg/day), or vehicle for 11days. low dose
43 streptozotocin (75mg/kg) was given on days 1-3. Plasma glucose (C), day 21 glucose
44 tolerance (D), representative insulin staining (E), beta cell area (F). Percentage

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

8 References

- 9 [1] Sam AH, Gunner DJ, King A, *et al*. Selective ablation of peptide YY cells in adult mice
10 reveals their role in beta cell survival. *Gastroenterology*. 2012; **143**: 459-468
11 [2] Tatemoto K, Mutt V. Isolation of two novel candidate hormones using a chemical
12 method for finding naturally occurring polypeptides. *Nature*. 1980; **285**: 417-418
13 [3] Batterham RL, Cohen MA, Ellis SM, *et al*. Inhibition of food intake in obese subjects
14 by peptide YY3-36. *N Engl J Med*. 2003; **349**: 941-948
15 [4] Challis BG, Pinnock SB, Coll AP, Carter RN, Dickson SL, O'Rahilly S. Acute effects of
16 PYY3-36 on food intake and hypothalamic neuropeptide expression in the mouse.
17 *Biochem Biophys Res Commun*. 2003; **311**: 915-919
18 [5] Chelikani PK, Haver AC, Reidelberger RD. Intravenous infusion of peptide YY(3-36)
19 potently inhibits food intake in rats. *Endocrinology*. 2005; **146**: 879-888
20 [6] Vrang N, Madsen AN, Tang-Christensen M, Hansen G, Larsen PJ. PYY(3-36) reduces
21 food intake and body weight and improves insulin sensitivity in rodent models of diet-induced
22 obesity. *Am J Physiol Regul Integr Comp Physiol*. 2006; **291**: R367-375
23 [7] Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR. Human
24 distribution and release of a putative new gut hormone, peptide YY. *Gastroenterology*. 1985;
25 **89**: 1070-1077
26 [8] Medeiros MD, Turner AJ. Processing and metabolism of peptide-YY: pivotal roles of
27 dipeptidylpeptidase-IV, aminopeptidase-P, and endopeptidase-24.11. *Endocrinology*. 1994;
28 **134**: 2088-2094
29 [9] Persaud SJ, Bewick GA. Peptide YY: more than just an appetite regulator.
30 *Diabetologia*. 2014; **57**: 1762-1769
31 [10] Larhammar D. Structural diversity of receptors for neuropeptide Y, peptide YY and
32 pancreatic polypeptide. *Regul Pept*. 1996; **65**: 165-174
33 [11] Grandt D, Teyssen S, Schimiczek M, *et al*. Novel generation of hormone receptor
34 specificity by amino terminal processing of peptide YY. *Biochem Biophys Res Commun*. 1992;
35 **186**: 1299-1306
36 [12] Ali-Rachedi A, Varndell IM, Adrian TE, *et al*. Peptide YY (PYY) immunoreactivity is co-
37 stored with glucagon-related immunoreactants in endocrine cells of the gut and pancreas.
38 *Histochemistry*. 1984; **80**: 487-491
39 [13] Bottcher G, Ahren B, Lundquist I, Sundler F. Peptide YY: intrapancreatic localization
40 and effects on insulin and glucagon secretion in the mouse. *Pancreas*. 1989; **4**: 282-288
41 [14] Bottcher G, Sjoberg J, Ekman R, Hakanson R, Sundler F. Peptide YY in the
42 mammalian pancreas: immunocytochemical localization and immunochemical
43 characterization. *Regul Pept*. 1993; **43**: 115-130
44 [15] el-Salhy M, Grimelius L, Emson PC, Falkmer S. Polypeptide YY- and neuropeptide Y-
45 immunoreactive cells and nerves in the endocrine and exocrine pancreas of some vertebrates:
46 an onto- and phylogenetic study. *Histochem J*. 1987; **19**: 111-117
47 [16] Upchurch BH, Aponte GW, Leiter AB. Expression of peptide YY in all four islet cell
48 types in the developing mouse pancreas suggests a common peptide YY-producing
49 progenitor. *Development*. 1994; **120**: 245-252
50 [17] Jackerott M, Oster A, Larsson LI. PYY in developing murine islet cells: comparisons to
51 development of islet hormones, NPY, and BrdU incorporation. *J Histochem Cytochem*. 1996;
52 **44**: 809-817

- 1 [18] Morgan DG, Kulkarni RN, Hurley JD, *et al.* Inhibition of glucose stimulated insulin
2 secretion by neuropeptide Y is mediated via the Y1 receptor and inhibition of adenylyl cyclase
3 in RIN 5AH rat insulinoma cells. *Diabetologia*. 1998; **41**: 1482-1491
- 4 [19] Greeley GH, Jr., Lluís F, Gomez G, Ishizuka J, Holland B, Thompson JC. Peptide YY
5 antagonizes beta-adrenergic-stimulated release of insulin in dogs. *Am J Physiol*. 1988; **254**:
6 E513-517
- 7 [20] Boey D, Lin S, Karl T, *et al.* Peptide YY ablation in mice leads to the development of
8 hyperinsulinaemia and obesity. *Diabetologia*. 2006; **49**: 1360-1370
- 9 [21] Chandarana K, Gelegen C, Irvine EE, *et al.* Peripheral activation of the Y2-receptor
10 promotes secretion of GLP-1 and improves glucose tolerance. *Molecular metabolism*. 2013;
11 **2**: 142-152
- 12 [22] Thiriet N, Deng X, Solinas M, *et al.* Neuropeptide Y protects against
13 methamphetamine-induced neuronal apoptosis in the mouse striatum. *J Neurosci*. 2005; **25**:
14 5273-5279
- 15 [23] Khan D, Vasu S, Moffett RC, Irwin N, Flatt PR. Islet distribution of Peptide YY and its
16 regulatory role in primary mouse islets and immortalised rodent and human beta-cell function
17 and survival. *Mol Cell Endocrinol*. 2016; **436**: 102-113
- 18 [24] Hauge-Evans AC, Reers C, Kerby A, *et al.* Effect of hyperglycaemia on muscarinic M3
19 receptor expression and secretory sensitivity to cholinergic receptor activation in islets.
20 *Diabetes Obes Metab*. 2014; **16**: 947-956
- 21 [25] Huang GC, Zhao M, Jones P, *et al.* The development of new density gradient media
22 for purifying human islets and islet-quality assessments. *Transplantation*. 2004; **77**: 143-145
- 23 [26] Amisten S, Salehi A, Rorsman P, Jones PM, Persaud SJ. An atlas and functional
24 analysis of G-protein coupled receptors in human islets of Langerhans. *Pharmacology &*
25 *therapeutics*. 2013; **139**: 359-391
- 26 [27] Gardiner JV, Campbell D, Patterson M, *et al.* The hyperphagic effect of ghrelin is
27 inhibited in mice by a diet high in fat. *Gastroenterology*. 2010; **138**: 2468-2476, 2476 e2461
- 28 [28] Burcelin R, Brunner H, Seydoux J, Thorensa B, Pedrazzini T. Increased insulin
29 concentrations and glucose storage in neuropeptide Y Y1 receptor-deficient mice. *Peptides*.
30 2001; **22**: 421-427
- 31 [29] Habib AM, Richards P, Cairns LS, *et al.* Overlap of endocrine hormone expression in
32 the mouse intestine revealed by transcriptional profiling and flow cytometry. *Endocrinology*.
33 2012; **153**: 3054-3065
- 34 [30] Adriaenssens A, Lam BY, Billing L, *et al.* A Transcriptome-Led Exploration of Molecular
35 Mechanisms Regulating Somatostatin-Producing D-Cells in the Gastric Epithelium.
36 *Endocrinology*. 2015; **156**: 3924-3936
- 37 [31] Dorrell C, Schug J, Lin CF, *et al.* Transcriptomes of the major human pancreatic cell
38 types. *Diabetologia*. 2011; **54**: 2832-2844
- 39 [32] Jois SD, Nagarajarao LM, Prabhakaran M, Balasubramaniam A. Modeling of
40 neuropeptide receptors Y1, Y4, Y5, and docking studies with neuropeptide antagonist. *J*
41 *Biomol Struct Dyn*. 2006; **23**: 497-508
- 42 [33] Jois SD, Nagarajarao LM, Prabhakaran M, Balasubramaniam A. Modeling of
43 Neuropeptide Receptors Y1, Y4, Y5, and Docking Studies with Neuropeptide Antagonist
44 Analogues: Implications for Selectivity. *J Biomol Struct Dyn*. 2004; **22**: 497-508
- 45 [34] Balasubramaniam A, Sherif S, Zhai W, Chance WT. Bis(31/31')[[Cys(31),
46 Nva(34)]NPY(27-36)-NH(2)]: a neuropeptide Y (NPY) Y(5) receptor selective agonist with a
47 latent stimulatory effect on food intake in rats. *Peptides*. 2002; **23**: 1485-1490
- 48 [35] Kanatani A, Ishihara A, Iwaasa H, *et al.* L-152,804: orally active and selective
49 neuropeptide Y Y5 receptor antagonist. *Biochem Biophys Res Commun*. 2000; **272**: 169-173
- 50 [36] Darville MI, Eizirik DL. Regulation by cytokines of the inducible nitric oxide synthase
51 promoter in insulin-producing cells. *Diabetologia*. 1998; **41**: 1101-1108
- 52 [37] Allagnat F, Fukaya M, Nogueira TC, *et al.* C/EBP homologous protein contributes to
53 cytokine-induced pro-inflammatory responses and apoptosis in beta-cells. *Cell Death Differ*.
54 2012; **19**: 1836-1846

- 1 [38] Mulder H, Myrsen-Axcrona U, Gebre-Medhin S, Ekblad E, Sundler F. Expression of
2 non-classical islet hormone-like peptides during the embryonic development of the pancreas.
3 *MicroscResTech*. 1998; **43**: 313-321
- 4 [39] Chandarana K. CJ, Harrow P.W., Herrera P.L., Withers D.J., Batterham R.L. .
5 Investigating the role of intra-islet PYY. Abstracts of the 3rd International Congress on
6 Prediabetes and the Metabolic Syndrome. 2009:
- 7 [40] Ahren B. Autonomic regulation of islet hormone secretion--implications for health and
8 disease. *Diabetologia*. 2000; **43**: 393-410
- 9 [41] Whim MD. Pancreatic beta cells synthesize neuropeptide Y and can rapidly release
10 peptide co-transmitters. *PLoS One*. 2011; **6**: e19478
- 11 [42] Larsson LI, Sundler F, Hakanson R. Immunohistochemical localization of human
12 pancreatic polypeptide (HPP) to a population of islet cells. *Cell Tissue Res*. 1975; **156**: 167-
13 171
- 14 [43] Larsen PJ, Kristensen P. The neuropeptide Y (Y4) receptor is highly expressed in
15 neurones of the rat dorsal vagal complex. *Brain Res Mol Brain Res*. 1997; **48**: 1-6
- 16 [44] Cabrele C, Wieland HA, Langer M, Stidsen CE, Beck-Sickinger AG. Y-receptor affinity
17 modulation by the design of pancreatic polypeptide/neuropeptide Y chimera led to Y(5)-
18 receptor ligands with picomolar affinity. *Peptides*. 2001; **22**: 365-378
- 19 [45] Duhault J, Boulanger M, Chamorro S, *et al*. Food intake regulation in rodents: Y5 or
20 Y1 NPY receptors or both? *CanJPhysiol Pharmacol*. 2000; **78**: 173-185
- 21 [46] Lecklin A, Lundell I, Salmela S, Mannisto PT, Beck-Sickinger AG, Larhammar D.
22 Agonists for neuropeptide Y receptors Y1 and Y5 stimulate different phases of feeding in
23 guinea pigs. *Br J Pharmacol*. 2003; **139**: 1433-1440
- 24 [47] Pedrazzini T, Seydoux J, Kunstner P, *et al*. Cardiovascular response, feeding behavior
25 and locomotor activity in mice lacking the NPY Y1 receptor. *NatMed*. 1998; **4**: 722-726

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