Development and characterisation of a peptidergic N- and C-terminally stabilised mammalian NPY1R agonist which protects against diabetes induction

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Abstract

Background: PYY(1-36) peptides from phylogenetically ancient fish, such as sea lamprey, have previously been shown to function as specific neuropeptide Y1 receptor (NPYR1) agonists. Although, sea lamprey PYY(1-36) is N-terminally stable, we reveal in this study that the peptide is subject to endopeptidase mediated C-terminal dipeptide degradation. In an attempt to prevent this, (D-Arg^{35})-sea lamprey PYY(1-36) was developed

Methods: *In vitro* bioassays assessed enzymatic stability, insulinostatic activity as well as beta-studies examined the impact of twice daily administration of sea lamprey PYY(1-36) or (D-Arg^{35})-sea lamprey PYY(1-36) in multiple low dose STZ-induced diabetic mice.

Results: (D-Arg^{35})-sea lamprey PYY(1-36) was fully resistant to plasma enzymatic degradation. The peptide possessed similar significant NPYR1-mediated insulinostatic, as well as positive beta-cell proliferative and anti-apoptotic biological actions, as the parent peptide. Sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) delayed diabetes progression in STZ mice. Both treatment interventions induced a significant decrease in body weight, food and fluid intake as well as glucose and glucagon concentrations. In addition, glucose tolerance, plasma and pancreatic insulin were partially normalised. (D-Arg^{35})-sea lamprey PYY(1-36) was significantly more effective than sea lamprey PYY(1-36) in terms of enhancing glucose-stimulate insulin release. Both treatments improved pancreatic islet morphology, linked to augmented proliferation and decreased apoptosis of beta-cells.

Conclusion: We present (D-Arg^{35})-sea lamprey PYY(1-36) as the first-in-class N- and C-terminally stable PYY(1-36) peptide analogue.

General significance: Enzymatically stable, long-acting PYY(1-36) peptides highlight the therapeutic benefits of sustained activation of NPYR1's in diabetes.

1. Introduction

The beneficial effects of sustained modulation of pancreatic beta-cell neuropeptide Y1 receptors (NPY1R's) by the 36 amino acid gut-derived peptide hormone, Peptide Tyrosine Tyrosine (PYY), are now acknowledged [1,2]. As such, acute activation of beta-cell NPY1R's was initially shown to encourage inhibition of glucose-stimulated insulin secretion (GSIS) and produce beta-cell rest [3]. However, later studies revealed positive effects of more prolonged PYY(1-36)-induced NPY1R activation on growth, survival and overall secretory function of pancreatic beta-cells [1,2,4,5], with obvious related benefits for the treatment of diabetes [1]. However, the therapeutic efficacy of native PYY(1-36) is significantly hampered by rapid enzymatic degradation and loss of NPY1R specificity. In this regard, the ubiquitous enzyme dipeptidyl peptidase-4 (DPP-4) removes the N-terminal Tyr^{1}-Ala^{2} dipeptide from PYY(1-36) to yield PYY(3-36) [6], a degradation fragment with postulated anti-obesity actions [7]. However, this DPP-4 mediated enzymatic reaction dramatically reduces the affinity of PYY for NPY1R's [8], severely diminishing therapeutic applicability for diabetes.

To circumvent this issue, a number of PYY(1-36) peptide analogues with N-terminally stability and postulated NPYR1 specificity have recently been described [2,9]. At first, utilisation of available structure/function knowledge in the field of PYY/NPYR1 led to the generation of PYY(1-36) molecules with logically introduced amino acid substitutions or secondary modifications, namely ($P^{3}L^{31}P^{34}$)PYY(1-36) and PYY(1-36)(Lys^{12}PAL), respectively [9]. However, the biological effectiveness of these synthetic PYY(1-36) analogues was markedly reduced when compared to the parent peptide [9], dismissing antidiabetic utility. Following on from this, PYY(1-36) peptide sequences from phylogenetically ancient fish, with intrinsic DPP-4 resistance, were also investigated as potential specific NPYR1 modulators [2]. As such, *Amia calva* (bowfin), *Oncorhynchus mykiss* (trout), *Petromyzon marinus* (sea lamprey) and *Scaphirhynchus albus* (sturgeon) effectively activated rodent and human pancreatic beta-cell NPYR1's, and possessed a pharmacodynamic profile superior to native

PYY(1-36) [2]. Sea lamprey PYY(1-36) proved to be the most efficacious fish-derived compound and induced notable improvements on pancreatic islet morphology and insulin secretion following sustained treatment in multiple low dose streptozotocin (STZ)-induced diabetic mice [2].

Despite the clear antidiabetic benefits of N-terminally stabilised sea lamprey PYY(1-36), the importance of C-terminal stability for preserved bioactivity of PYY-based peptides has been documented of late [10,11]. Thus, C-terminal dipeptide cleavage of PYY(3-36), to yield PYY(3-34), occurs naturally within the circulation and annuls the well-described anorexic effects of this PYY fragment-peptide [10]. For PYY(1-36) a similar C-terminal degradation process occurs, rendering the peptide inactive at the level of the pancreatic beta-cell [11]. Notably, the C-terminal region of sea lamprey PYY(1-36) exhibits distinct amino acid sequence homology with native human PYY(1-36) [2]. Therefore, in the present study we initially demonstrated degradation of sea lamprey PYY(1-36) to (1-34) in plasma. Further studies examined the impact of substitution of L-Arg^{35} with its enantiomer D-Arg^{35} in sea lamprey PYY(1-36), to yield (D-Arg^{35})-sea lamprey PYY(1-36), on metabolic stability as well as *in vitro* pancreatic beta-cell function, growth and survival, alongside NPYR specificity. Essentially, (D-Arg^{35})-sea lamprey PYY(1-36) represents the parent sea lamprey PYY(1-36) peptide, where the L-Arg amino acid residue at position 35 is replaced with its own mirror image, known as D-Arg^{35}. In addition, the impact twice daily treatment with (D-Arg^{35})-sea lamprey PYY(1-36), or the parent peptide, on metabolic control and pancreatic architecture was investigated in multiple low dose streptozotocin (STZ)-induced diabetic mice.

2. Methods

2.1 Peptides

All peptides (Table 1) were supplied by Synpeptide Ltd (Shanghai, China) at greater than 95% purity and characterised in-house by HPLC with MALDI-TOF, as described previously [12].

2.2 Plasma degradation

Sea lamprey and (D-Arg^{35})-sea lamprey PYY(1-36) peptides (50 µg) were incubated at 37ºC on a plate-shaker in 50 mM triethanolamine/HCl (pH 7.8) with 5 µl of fasted murine plasma for 0 and 8 hours. Reactions were terminated by addition of 10 µl of 10% (v/v) trifluoroacetic acid/water. Reaction mixes were separated on RP-HPLC using a (250 x 4.6 mm) Phenomenex C-18 analytical column, with absorbance monitored at 214 nm using a Thermoquest, SpectraSystem UV2000 detector. Defined HPLC peaks were collected and identified via MALDI-TOF MS on a PerSeptive Biosystems Voyager-DE Biospectrometer (Hertfordshire, UK).

2.3 In vitro insulin secretion

In vitro effects of PYY(1-36) peptides on insulin secretion were determined using rodent BRIN BD11 beta-cells. The characteristics of this cell line, including glucose-sensitivity and insulin secretory function, have been described in detail elsewhere [13]. Importantly, BRIN BD11 cells have been shown to abundantly express the NPYR1 [4], and have been successfully utilised for assessment of beta-cell actions of various PYY peptides [1,2,9,11]. Cells were cultured in RPMI 1640 media (Gibco Life Technologies Ltd), supplemented with 10% v/v foetal bovine serum (Gibco), 1% v/v antibiotics (0.1 mg/ml streptomycin and 100 U/ml penicillin) at 37ºC in 5% atmospheric CO_2. For experimentation, cells were seeded into 24-well plates (Falcon Ltd) at a density of 150,000 cells per well. Following overnight attachment, media was aspirated and cells were pre-incubated in 1.1 mM glucose KRB buffer for 40 minutes. Following pre-incubation, the 1.1 mM glucose solution was removed and 1 ml of

KRB test solution, containing either 5.6 or 16.7 mM glucose with either sea lamprey PYY(1-36) or (D-Arg^{35})-sea lamprey PYY(1-36) (10^{-12} – 10^{-6} M) was added. We have routinely shown that 5.6 mM glucose has insulin secretory stimulatory effects in BRIN-BD11 cells above basal output, which is further augmented at 16.7 mM glucose [11,14-16]. In a second series of experiments, insulin secretory effects of PYY(1-36) peptides were determined following incubation in the presence of alanine (10 mM) at 16.7 mM glucose. For all experiments, following a 20 min incubation period, supernatant was collected and stored at -20ºC until required for insulin concentration determination using a dextran coated charcoal insulin radioimmunoassay [17].

2.4 Beta-cell proliferation and apoptosis

To assess the effects (10^{-8} and 10^{-6} M) of sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) on beta-cell proliferation and protection against apoptosis, rodent BRIN-BD11 and human 1.1B4 beta-cells [18] were used. Functional characteristics and suitability of 1.1B4 cells as a proxy for primary human beta-cells in studies of insulin secretion, beta-cell growth and protection are provided by the original publication and subsequent papers that have been reviewed [15,19]. 1.1B4 cells used in the present studies were well differentiated as confirmed by routine measurements of cellular insulin content and responsiveness to a range of secretagogues, which were in line with these earlier studies. Cells were seeded onto sterilised, clear-glass coverslips (16 mm diameter) and placed in 12-well plates (Falcon Ltd) at a density of 40,000 cells per well and cultured for 18 h. Media control, GLP-1 (10^{-8} and 10^{-6} M) and a human cytokine cocktail mix (IL-1β (100 U/mL), IFNγ (20 U/mL), TNFα (200 U/mL), Sigma Aldrich UK) were employed as controls, as appropriate. Cells were then rinsed with PBS and fixed using 4% paraformaldehyde. After antigen retrieval with sodium citrate buffer at 95ºC for 20 min, blocking was performed using 2% BSA for 45 min. To assess the ability of PYY(1-

36) peptides to protect against cytokine-induced apoptosis, BRIN BD11 and 1.1B4 cells were seeded, washed and fixed as above, with the exception that the media was supplemented with the cytokine mix. The coverslips were then incubated at 37ºC with TUNEL reaction mix (Roche Diagnostics Ltd, UK) for 60 min, and mounted onto polysine-coated microscope slides using a 50:50 Glycerol:PBS solution and stored at 4ºC until required for analysis. For proliferation studies, the coverslips were incubated at 37ºC with rabbit anti-Ki-67 primary antibody (Abcam, ab15580), and subsequently with Alexa Fluor® 488 secondary antibody (Invitrogen, UK), and mounted onto microscope slides as above. In a separate study, beta-cell proliferative effects were examined in INS1 832/13 cells with CRISPR-Cas9 induced knockout (KO) of either the *Npy1r* or *Npyr2*, with generation and characterisation of both KO cell lines described in detail elsewhere [2]. INS1 832/13 cells were selected for KO studies because of their better transfection efficiency when compared to BRIN BD11 cells [2]. All slides were viewed using a fluorescent microscope (Olympus System Microscope, model BX51; Southend-on-Sea, UK) and photographed by DP70 camera adapter system. Proliferation/TUNEL positive frequency was determined using the cell-counter function on ImageJ Software and expressed as a percentage of total cells in proliferation experiments or as a fold-change of the cytokine cocktail control in apoptosis experiments.

2.5 Sub-chronic in vivo effects

NIH male Swiss TO mice (14 weeks of age, Envigo Ltd, UK) received twice-daily treatment with either saline vehicle, sea lamprey PYY(1-36) or ($_D$-Arg^{35})-sea lamprey PYY(1-36) (both at 25 nmol/kg bw; n=10) for the first three days of the experiment. On day 3, mice received multiple low dose streptozotocin (STZ) injection (4 h fast, 50 mg/kg bw, i.p., in sodium citrate buffer, pH 4.5) for 5 consecutive days to induce diabetes. Twice-daily treatment interventions continued during this diabetes induction phase and up until day 14. An additional group of

control mice (n=10) were maintained on normal laboratory chow throughout (10% fat, 30% protein and 60% carbohydrate, Trouw Nutrition, UK), were injected twice-daily with saline rather than STZ. Cumulative energy and fluid intake, body weight, circulating glucose and insulin concentrations were assessed at regular intervals, with plasma glucagon measured on day 14. At the end of the treatment period, glucose tolerance and glucose-induced insulin secretion in response to an i.p. glucose load (18 mmol/kg bw) was assessed. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU for animal experiments. The study was approved by Ulster University Animal Welfare Ethical Review Body (AWERB). Terminal analyses included extraction of pancreatic tissue and appropriate processing for measurement of hormone content following acid/ethanol protein extraction or islet architecture, as described previously [2]. This included co-staining for insulin (1:500; Abcam, ab6995) or glucagon (PCA2/4, 1:200; raised in-house) with either Ki-67 (1:400; Abcam ab15580) to assess proliferation rate or TUNEL reaction mixture (Roche Diagnostics Ltd, UK) to quantify cell apoptosis. As such, following incubation with primary antibodies, appropriate secondary antibodies were employed, namely Alexa Fluor® 594 goat anti-mouse IgG (1:400), Alexa Fluor® 488 goat anti-guinea pig IgG (1:400) and Alexa Fluor® 488 goat anti-rabbit IgG as appropriate. Slides were viewed under a FITC (488 nm) or TRITC filter (594 nm) using a fluorescent microscope (Olympus system microscope, model BX51) and photographed using a DP70 camera adapter system. Islet parameters were analysed using Cell^{F} image analysis software (Olympus Soft Imaging Solutions, GmbH).

2.6 Biochemical analyses

Blood samples were collected from the cut tip on the tail vein, of conscious mice, at times indicated in Figures. Blood glucose was measured immediately using a hand-held Ascencia

Contour blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK). For plasma insulin and glucagon, blood was collected in chilled fluoride/heparin coated micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged using a Beckman micro-centrifuge (Beckman Instruments, Galway, Ireland) for 10 minutes at 12,000 rpm. Plasma was separated and stored at -20^{o}C, until determination of plasma insulin by radioimmunoassay [17] or glucagon by a commercially available ELISA kits (EZGLU-30K, Merck Millipore).

2.7 Statistical analysis

Statistical analyses were performed using GraphPad PRISM software (Version 5.0). Values are expressed as mean ± S.E.M. Comparative analyses between groups were carried out using a One-way ANOVA with Bonferroni's post hoc test or student's unpaired t-test, as appropriate. The difference between groups was considered significant if $p < 0.05$.

3. Results

3.1 In vitro murine plasma stability

Incubation of human PYY(1-36) with murine plasma for 8 h resulted in generation of the N- and C-terminally truncated products PYY(3-36) and PYY(1-34) (Fig. 1A). In the same experimental system, the only observable degradation product of sea lamprey PYY(1-36) was sea lamprey PYY(1-34) (Fig. 1B). However, (D-Arg^{35})-sea lamprey PYY(1-36) was completely resistant to enzymatic degradation over the 8 h period with no evident degradation peaks (Fig. 1C).

3.2 Effects of PYY(1-36) peptides on insulin secretion from BRIN BD11 beta-cells

All PYY peptides, namely human PYY(1-36), sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36), significantly ($p < 0.05$ to $p < 0.001$) inhibited insulin secretion from BRIN BD11 cells at both 5.6 and 16.7 mM glucose concentrations (Fig. 2A,B). Notably, there was

an approximate 30% increase in insulin secretory output at 16.7 mM glucose, when compared to 5.6 mM glucose (Fig. 2A,B). At 5.6 mM glucose, sea lamprey PYY(1-36) was more effective ($p < 0.05$ to $p < 0.001$) that human PYY(1-36) at concentrations of 10^{-8} M and above (Fig. 2A). In addition, all PYY(1-36) peptides (10^{-7} and 10^{-6} M) inhibited ($p < 0.01$ to $p < 0.001$) alanine-induced elevations of insulin release (Fig. 2C). However, only human PYY(1-36) and sea lamprey PYY(1-36) exerted inhibitory ($p < 0.05$ to $p < 0.001$) effects at concentrations below 10^{-7} M (Fig. 2C).

3.3 Effects of PYY(1-36) peptides on beta-cell proliferation and protection against cytokine-induced apoptosis

All PYY(1-36) peptides (10^{-8} and 10^{-6} M) significantly ($p < 0.05$ to $p < 0.001$) augmented BRIN BD11 and 1.1B4 beta-cell proliferation, to a similar, or greater, extent as observed with the positive control GLP-1 (Fig. 3A,B). Notably, (D-Arg^{35})-sea lamprey PYY(1-36) was significantly ($p < 0.05$) more effective than native PYY(1-36) at 10^{-6} M in 1.1B4 cells (Fig. 3B). In order to determine receptor interactions involved in these beta-cell proliferative actions, *Npy1r* and *Npyr2* KO INS1 832/13 cells were employed (Fig. 3C). As expected, all peptides (at either 10^{-6} or 10^{-8} M) effectively ($p < 0.05$ to $p < 0.01$) promoted beta-cell proliferation in INS1 832/13 wild-type cells (Fig. 3C). However, all PYY(1-36) peptides were ineffective in terms of stimulating proliferation in *Npy1r* KO INS1 832/13 cells, but did induce ($p < 0.05$ to $p < 0.01$) beta-cell growth in *Npy2r* KO cells (Fig. 3C). All test peptides, including GLP-1 positive control, imparted similar significant ($p < 0.05$ to $p < 0.001$) protective effects against cytokine-induced apoptosis in both BRIN BD11 (Fig. 3D) and 1.1 B4 (Fig. 3E) beta-cells.

3.4 Sub-chronic effects of PYY(1-36) peptides on various metabolic parameters as well as pancreatic hormone content in STZ-induced diabetic mice

Initiation of STZ injection on day 3 resulted in progressive ($p < 0.05$ to $p < 0.01$) weight loss during the observation period in saline treated mice, with this weight reducing effect annulled by twice daily sea lamprey PYY(1-36) or (D-Arg^{35})-sea lamprey PYY(1-36) injection (Fig. 4A). Accumulated food and fluid intake were elevated ($p < 0.05$ to $p < 0.01$) in STZ-diabetic mice on days 12 and 14 (Fig. 4B,C). Sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) treatment significantly ($p < 0.05$ to $p < 0.01$) suppressed STZ-induced effects on food and fluid intake (Fig. 4B,C), with food intake in these mice being similar to lean controls (Fig. 4B). Circulating blood glucose was substantially elevated ($p < 0.001$) in STZ-diabetic mice from day 8 onwards when compared to lean controls (Fig. 4D). This rise in blood glucose was significantly ($p < 0.05$ to $p < 0.01$) reduced by both PYY(1-36) treatment regimens, and indeed non-fasting glucose levels in these mice were not significantly different from lean controls on day 14 (Fig. 4D). Corresponding insulin concentrations were dramatically decreased ($p < 0.001$) in saline treated STZ-diabetic mice (Fig. 4E). Both sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) slowed the progression of decreases in insulin concentrations (Fig. 4E). In terms of plasma glucagon concentrations, STZ-induced diabetes significantly ($p < 0.001$) elevated this parameter on day 14, with both sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) fully reversing the effect (Fig. 4F). Indeed, twice-daily treatment with sea lamprey PYY(1-36) significantly ($p < 0.05$) reduced circulating glucagon when compared to STZ-diabetic controls (Fig. 4F).

During glucose tolerance tests at the end of the treatment period, plasma glucose levels were significantly ($p < 0.01$ to $p < 0.01$) elevated in STZ-diabetic mice when compared to lean controls (Fig. 4G). Sea lamprey PYY(1-36) treatment reduced glucose levels at 15, 30, 60 and 90 min post-injection (Fig. 4G), with (D-Arg^{35})-sea lamprey PYY(1-36) inducing a significant ($p < 0.05$) reduction at 30 mins post-injection (Fig. 4G). Related AUC analysis confirmed an elevated ($p < 0.01$) glycaemic excursion in saline treated STZ-diabetic mice, which was not

apparent in either of the PYY(1-36) peptide treated groups (Fig. 4I). As expected, the corresponding glucose-induced insulin secretory response in STZ-diabetic control mice animals was significantly ($p < 0.05$ to $p < 0.001$) reduced in terms of individual and overall values when compared to lean control mice (Fig. 4H,J). Both sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) also had a reduced ($p < 0.05$ to $p < 0.001$) plasma insulin secretory response when compared to lean mice, but overall AUC values were significantly ($p < 0.01$) greater in (D-Arg^{35})-sea lamprey PYY(1-36) mice when compared to diabetic controls (Fig. 4H,J). Assessment of both pancreatic insulin and glucagon content on day 14 revealed dramatically ($p < 0.001$) decreased insulin and increased glucagon concentrations in STZ-diabetic mice (Fig. 4K,L). Both PYY(1-36) treatments almost fully reversed these detrimental effects (Fig. 4K,L), however sea lamprey PYY(1-36) treated mice still presented with slightly ($p < 0.05$) decreased pancreatic insulin concentrations compared to lean controls (Fig. 4K).

*3.5 Sub-chronic effects of PYY(1-36) peptides on pancreatic morphology in STZ-induced diabetic mic***e**

Histological analysis of pancreatic tissue highlighted the negative morphological consequences STZ injection, which appeared to be countered by twice-daily sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) treatment (Fig. 5A-D). Indeed, quantification of pancreatic islet area revealed substantially ($p < 0.001$) smaller islets in STZ-diabetic mice, which were significantly ($p < 0.001$) enlarged in all PYY(1-36) peptide treated mice (Fig. 5E). In keeping with this, percentage beta-cell area was significantly ($p < 0.001$) lower in all STZ mice, but increased ($p < 0.001$ and $p < 0.01$; respectively) by sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) treatment (Fig. 5F). In addition, percentage α-cell area was elevated ($p < 0.001$) in all STZ mice, but reduced by both treatment interventions (Fig. 5G). However, it should also be noted that these islet, beta- and alpha-cell parameters were still significantly

different from lean controls in all PYY(1-36) peptide treated mice (Fig. 5E-G). Despite this, alpha:beta cell ratios were similar in both sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) treated STZ mice as compared to lean controls, and significantly ($p < 0.001$) reduced when compared to STZ-diabetic controls (Fig. 5H). Furthermore, islets of saline treated STZ-diabetic mice had significantly ($p < 0.001$) more centrally-located α-cells, a phenomenon which was reversed by both PYY(1-36) peptide treatments (Fig. 5I). In addition, both sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) returned islet numbers to lean control levels (Fig. 5J). To uncover the mechanisms behind islet morphological changes, alpha- and beta-cell proliferation and apoptosis frequency was examined (Fig. 6). STZ-diabetic mice exhibited significantly ($p < 0.001$) decreased beta-cell proliferation and increased beta-cell apoptosis (Fig. 6A,C). Both treatment interventions significantly ($p < 0.001$) increased beta-cell proliferation and returned beta-cell apoptosis rates to lean control levels (Fig. 6A,C). There was no change in alpha-cell proliferation in any of the groups of mice (Fig. 6B), but alpha-cell apoptosis was significantly ($p < 0.001$) elevated in STZ controls (Fig. 6D). Effects on alpha-cell apoptosis were fully or partially reversed by (D-Arg^{35})-sea lamprey PYY(1-36) and sea lamprey PYY(1-36), respectively (Fig. 6D).

4. Discussion

Recent studies with sea lamprey PYY(1-36) confirm that this peptide, derived from a phylogenetically-ancient fish, represents an N-terminally stabilised mammalian NPYR1 activator, with notable beneficial beta-cell effects following chronic administration in STZ-treated mice with established insulin deficiency and diabetes [2]. However, in the present study we reveal, for the first time, that sea lamprey PYY(1-36) is susceptible to C-terminal dipeptide degradation. This is of particular importance given that C-terminal truncation of PYY(1-36) dramatically impairs NPYR1 activation profile of the peptide [11].

To circumvent C-terminal degradation of sea lamprey PYY(1-36), (D-Arg^{35})-sea lamprey PYY(1-36) was generated and characterised. In this regard, it is suggested that the Arg^{35} residue is the target for the, as yet unidentified, C-terminally directed endopeptidase that degrades PYY peptides [10,20]. Thus, although the angiotensin-converting-enzyme (ACE) inhibitor, captopril, blocks C-terminal dipeptide cleavage of PYY peptides [11], there is no direct evidence for ACE-mediated truncation of PYY(1-36) [21]. Moreover, co-incubation of PYY(1-36) with captopril in plasma still leads to generation of the C-terminally truncated product PYY(1-35), which also lacks ability to modulate NPYR1 function [11]. Nevertheless, substitution of naturally occurring L- for D-isomers in specific amino acid residues targeted by enzymes within peptide hormones has been demonstrated as a relatively straightforward strategy to induce enzyme resistance, with minimal detrimental impact on receptor binding and overall bioactivity [22-25]. In full agreement, (D-Arg^{35})-sea lamprey PYY(1-36) was completely stable against plasma enzyme mediated degradation and possessed a comparable *in vitro* biological action profile as the parent peptide. As such, (D-Arg^{35})-sea lamprey PYY(1-36) had clear insulinostatic actions in BRIN BD11 cells, and also promoted beta-cell growth and protection against apoptosis [2], which were dependent on NPYR1 activation.

Based on these observations, the ability of (D-Arg^{35})-sea lamprey PYY(1-36), or the parent peptide, to protect against STZ-induced insulin deficiency was examined. As observed previously with sustained NPYR1 activation [2], twice-daily treatment of mice with either of the PYY(1-36) peptides, partially or fully, protected from the characteristic weight loss, increased fluid and food intake, as well as elevated blood glucose levels, evoked by repeated low-dose STZ administration. In keeping with this, pancreatic and plasma glucagon levels were noticeably decreased by both treatments, this being despite knowledge that PYY peptides do not directly modulate glucagon secretion [26], indicating indirect effects on alpha-cell function. Interestingly, pancreatic insulin content was markedly elevated by both interventions, and this

translated to protection from a decline in circulating insulin. In addition, there was a significant improvement of beta-cell secretory function in (D-Arg^{35})-sea lamprey PYY(1-36) treated mice, as evidenced following exogenous glucose injection at the end of the treatment period. In agreement, augmented NPYR1 activation has been linked to the well-established beta-cell secretory benefits following Roux-en Y gastric bypass (RYGB) surgery in humans [5]. Moreover, circulating levels of PYY(1-36) are diminished in type 2 diabetic patients, who are recognised to present with impaired beta-cell secretory function [27]. In addition, metabolic improvements induced by (D-Arg^{35})-sea lamprey PYY(1-36) can also be linked, in part, to *in vitro* observations highlighting clear NPY1R-mediated insulinostatic actions. As such, the antidiabetic benefits of beta-cell rest has previously been demonstrated, including reduced beta-cell oxidative stress [28], restoration of cellular insulin stores [29] and a reduction of islet fibrosis [30].

Consistent with pancreatic islet benefits following (D-Arg^{35})-sea lamprey PYY(1-36) treatment, beta-cell proliferation was enhanced and apoptosis decreased in these mice, fully consistent with *in vitro* observations, albeit sea lamprey PYY(1-36) evoked essentially similar positive effects. Thus, the previously documented pancreatic morphological benefits of sustained administration of sea lamprey PYY(1-36) in established STZ-diabetic mice [2], were largely recapitulated during development and establishment of diabetes in the current study by both PYY(1-36) peptide treatments. Related mechanisms are most likely connected to NPYR1 downstream signalling and increased cellular PKC epsilon activity [31], which couples via mitochondrial pathways to inhibit cell apoptosis [32]. Furthermore, PYY has been shown to protect beta-cells against cytokine-induced apoptosis via inhibition of nuclear factor κB (NF-κB) [33]. Notable pancreatic benefits in the current study included increased islet numbers and beta-cell area, reduced alpha-cell area and glucagon positively stained central islet cells, as well as improved alpha:beta cell ratios. Notably, increased centralised pancreatic alpha-cells

represents a unique indicator of islet disturbance in rodents [34]. Such observations also correlate closely with knowledge that selective knockout of PYY expressing cells diminishes beta-cell area and moderates secretory function of these cells [35]. Furthermore, apoptotic rate of pancreatic alpha-cells was decreased by both sea lamprey PYY(1-36) treatment regimens, yet proliferation remained unchanged, with this effect being much more pronounced in (D-Arg^{35})-sea lamprey PYY(1-36) treated mice. This observation, coupled with the related impact on alpha-cell area and pancreatic glucagon content, could imply positive effects of sea lamprey PYY(1-36) peptides on pancreatic islet cell plasticity and lineage reprograming [36], but confirmation of this requires further detailed study.

Although, as noted above, some marginal benefits of (D-Arg^{35})-sea lamprey PYY(1-36) over the parent peptide were evident, it is somewhat surprising that C-terminal enzymatic stability did not result in more discernible improvements in STZ-diabetic mice, especially since *in vitro* bioactive profiles of the peptides are relatively similar. It is recognised that the C-terminus of the NPY family of peptides is fundamentally important for receptor binding [37]. In this respect, while Arg^{35} has been noted to be integral for NPYR2 activation, less importance is associated with NPYR1 binding [37], supporting our use of the Arg^{35} enantiomer to evoke enzyme resistance in sea lamprey PYY(1-36). However, earlier studies do suggest that the guanidine moiety of Arg^{35} may be involved in NPYR1 binding to some extent [38]. Thus, although exchanging the L- for the D-isomer of Arg^{35} in sea lamprey PYY(1-36) affords full C-terminal stability, there is a small possibility that this could impede efficient NPYR1 binding and downstream signalling, and further study would be required to confirm this. In addition, any impact of the D-Arg^{35} change on the characteristic 'PP-fold' conformation of PYY peptides, responsible for peptide recognition by the NPYR1 [39], would also need to be investigated. However, lack of clear distinction between the antidiabetic efficacy of the two sea lamprey PYY(1-36) treatment options could also simply be a consequence of the dose

chosen, and severity of the diabetes induced, by repeated low-dose STZ administration, and the need to activate complementary signalling pathways to adequately curtail the development of overt diabetes. Examination of the pharmacokinetic profile of both PYY(1-36) peptides would also be useful in this regard, but beyond the scope of the current study.

In conclusion, the present study has generated (D-Arg^{35})-sea lamprey PYY(1-36) as a novel NPY1R agonist with dual N- and C-terminal enzymatic stability. Benefits of (D-Arg^{35})-sea lamprey PYY(1-36) to prevent progression of STZ-induced insulin deficiency and diabetes were broadly similar to the parent peptide, sea lamprey PYY(1-36). Previous studies have highlighted the difficulty of generating bioactive C-terminally modified PYY peptides [40], due to involvement of the C-terminus of the peptide in receptor binding and activation [10,11]. However, renal clearance of (D-Arg^{35})-sea lamprey PYY(1-36) is unlikely to be altered by the C-terminal modification, and could be an important factor in terms of overall pharmacological efficacy. Thus, secondary modifications to extend the circulating half-life of (D-Arg^{35})-sea lamprey PYY(1-36), such as fatty acid derivation or PEGylation, as successfully employed for other related gut-derived hormones [41-44], may be required for realisation of the full antidiabetic potential of this treatment approach and future translational benefits.

Conflict of interest

PRF and NI are named on patents filed by the University of Ulster for exploitation of peptide therapeutics.

Acknowledgments

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Table 1. Amino acid sequences, expected and calculated masses as well as purity and plasma stability for PYY(1-36) peptides

Peptide	Amino acid sequence	Expected mass (Da)	Calculated mass (Da)	Peptide purity	% Plasma degradation at 8 h
Human PYY(1-36)	Y-P-I-K-P-E-A-P-G-E-D-A-S-P-E-E-L-N-R-Y-Y-A-S-L-R-H-Y-L-N-L-V-T-R-Q-R-Y- NH_2	4309.8	4309.7	96.8	83.8
Sea Lamprey PYY(1-36)	M-**P**-P-**K-P**-D-N-**P**-S-P-**D-A-S-P-E-E-L-**S-K-**Y**-M-L-A-V-**R-N-Y**-I-**N-L**-I-**T-R-Q-R-Y**- NH_2	4285.9	4284.2	98.5	47.4
(D-Arg^{35})-Sea Lamprey PYY(1-36)	M-**P**-P-**K-P**-D-N-**P**-S-P-**D-A-S-P-E-E-L-**S-K-**Y**-M-L-A-V-**R-N-Y**-I-**N-L**-I-**T-R-Q-(DR)-Y**- NH_2	4285.9	4283.5	97.8	0

Amino acids in **bold** text are conserved in human PYY(1-36). Peptide masses were calculated using MALDI-TOF MS on a PerSeptive Biosystems Voyager-DE Biospectrometer. Peptide purity was assessed by HPLC and analysis of AUC data. For plasma degradation, peptides (50 µg) were incubated at 37°C in 50 mM triethanolamine/HCl with murine plasma (5 µL) for 0 and 8 hours. Defined HPLC peaks were collected and identified via MALDI-TOF MS on a PerSeptive Biosystems Voyager-DE Biospectrometer.

Figure legends

Fig. 1. HPLC profiles obtained following incubation of (A) PYY(1-36), (B) sea lamprey PYY(1-36) and (C) (D-Arg^{35})-sea lamprey PYY(1-36) with non-fasted murine plasma. Peptides (50 µg; n=3) were incubated at 37ºC with 5 µl plasma in 50 mM triethanolamine-HCL. Reactions were stopped using 10% (v/v) trifluoroacetic acid/water and reaction mixes separated by HPLC. Peptide or peptide fragment masses were determined by MALDI-TOF.

Fig. 2. Effects of sea lamprey PYY(1-36) peptides on insulin release from rodent BRIN-BD11 beta-cells. Incubations were performed at (A) 5.6, (B) 16.7 mM glucose or (C) 16.7 mM glucose supplemented with alanine (10 mM) and the effects of PYY(1-36) peptides (10^{-6} – 10^{-12} M) on insulin secretion determined. Values are mean ± SEM (n=8). *p < 0.05, **p < 0.01, ***p < 0.001 compared to (A,B) respective glucose control or (C) 16.7 mM glucose plus 10 mM alanine. $^{\Delta}$p < 0.05, $^{\Delta\Delta}$p < 0.01, $^{\Delta\Delta\Delta}$p < 0.001 compared to (A) human PYY(1-36) or (C) 16.7 mM glucose.

Fig. 3. Effects of sea lamprey PYY(1-36) peptides on (A-C) beta-cell proliferation and (D,E) protection against cytokine-induced apoptosis. (A) BRIN-BD11, (B) 1.1B4 and (C) INS1 832/13, as well as *Npy1r* or *Npy2r* KO INS1 832/13, beta-cells were cultured (16 h) with GLP-1 or PYY(1-36) peptides (10^{-8} and 10^{-6} M) and proliferation assessed by Ki-67 staining. (D) BRIN-BD11 and (E) 1.1 B4 beta-cells were cultured (16 h) with GLP-1 or PYY(1-36) peptides (10^{-8} and 10^{-6} M) in the presence of a cytokine cocktail and apoptosis detected using the TUNEL assay. All values are mean ± SEM (n=3). (A-C) *p < 0.05, **p < 0.01, ***p < 0.001 compared to respective media control. (B) $^{\Delta\Delta}$p < 0.01 compared to PYY(1-36) (C) $^{\Delta}$p < 0.05, $^{\Delta\Delta\Delta}$p < 0.001 compared to INS1 832/13 wild-type cells at each condition. (D,E) *p < 0.05, **p

< 0.01, ***p < 0.001 compared to cytokine-cocktail. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ compared to RPMI media control.

Fig. 4. Effects of sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) on various metabolic parameters as well as pancreatic insulin and glucagon content in STZ-induced diabetic mice. (A-E) Parameters were measured at regular intervals for 3 days before and 14 days during treatment with twice-daily injection of sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) (both peptides at 25 nmol/kg bw). As indicated in panels A, D and E, STZ administration was initiated on day 3. (F-J) Parameters were measured on day 21. (F) Plasma glucagon was measured using a commercially available ELISA kit. (G,I) Blood glucose and (H,J) plasma insulin were measured prior to and after i.p. administration of glucose alone (18 mmol/kg) at t = 0 min. (K,L) Pancreatic insulin and glucagon content was measured by RIA or ELISA, respectively, following acid-ethanol extraction. All values are expressed as mean ± SEM for 10 mice. *p < 0.05, **p < 0.01, ***p < 0.001 compared with STZ diabetic control mice. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ and $^{\Delta\Delta\Delta}p < 0.001$ compared with lean control mice.

Fig. 5. Effects of sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) on pancreatic morphology in STZ-induced diabetic mice. All parameters were measured at after 14 days treatment with twice-daily injection of sea lamprey PYY(1-36) or (D-Arg^{35})-sea lamprey PYY(1-36) (both at 25 nmol/kg bw). (A-D) Representative images of islets showing insulin (red) and glucagon (green) immunoreactivity from each treatment group. (E) Total islet area, percentage (F) beta- and (G) alpha-cell area, (H) alpha/beta cell ratio, (I) percentage centrally located alpha-cells as well as (J) numbers of islets/mm^2. All values are expressed as mean ± SEM for 10 mice, with 100-150 islets being analysed per group. *p < 0.05, **p < 0.01,

***p < 0.001 compared with STZ diabetic control mice. $^{\Delta}$p < 0.05, $^{\Delta\Delta}$p < 0.01 and $^{\Delta\Delta\Delta}$p < 0.001 compared with lean control mice.

Fig. 6. Effects of sea lamprey PYY(1-36) and (D-Arg^{35})-sea lamprey PYY(1-36) on β- and α-cell survival in STZ-induced diabetic mice. All parameters were measured at after 14 days treatment with twice-daily injection of sea lamprey PYY(1-36) or (D-Arg^{35})-sea lamprey PYY(1-36) (both at 25 nmol/kg bw). Beta- and alpha-cell proliferation (A,B) and apoptosis (C,D) were assessed by Ki-67 and TUNEL immunoreactive staining, respectively. (E-H) Representative images of insulin and glucagon positive cells, co-stained with Ki-67 or TUNEL, for each treatment group are provided. Values are expressed as a fold-change of the lean control and are mean ± SEM for 10 mice, with 100-150 islets being analysed per group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with STZ diabetic control mice. $^{\Delta}$p < 0.05, $^{\Delta\Delta}$p < 0.01 and $^{\Delta\Delta\Delta}$p < 0.001 compared with lean control mice.

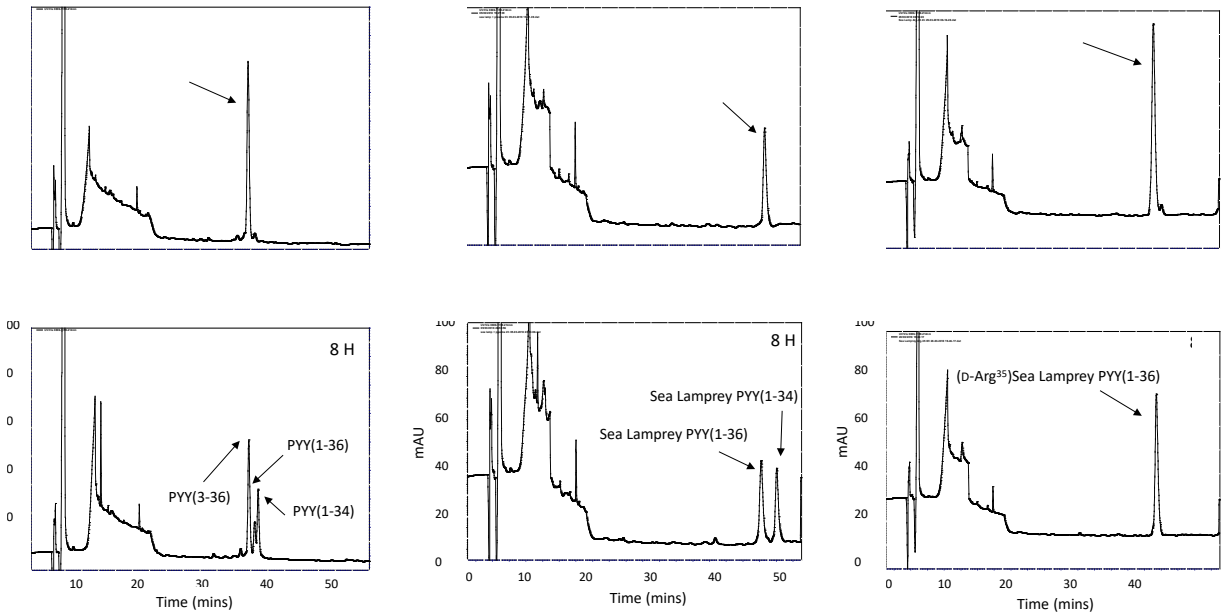

8 H
PYY(1-36)
PYY(3-36)
PYY(1-34)
Time (mins)
8 H
Sea Lamprey PYY(1-34)
Sea Lamprey PYY(1-36)
mAU
Time (mins)
(D-Arg35)Sea Lamprey PYY(1-36)
mAU
Time (mins)

Figure 2

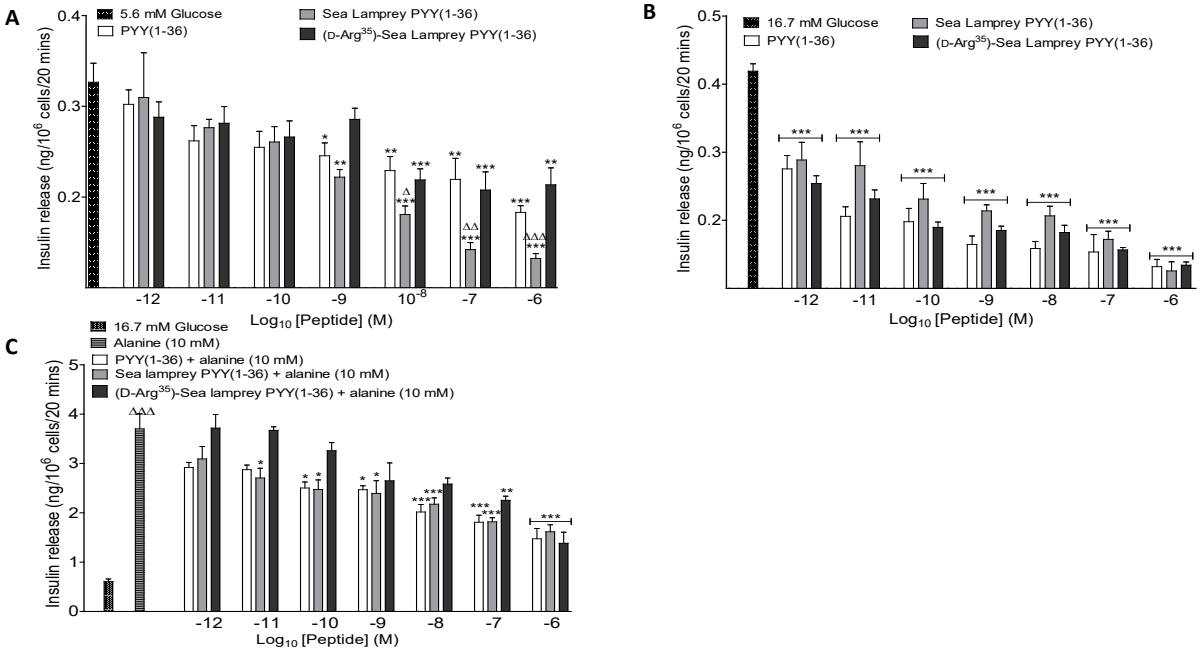
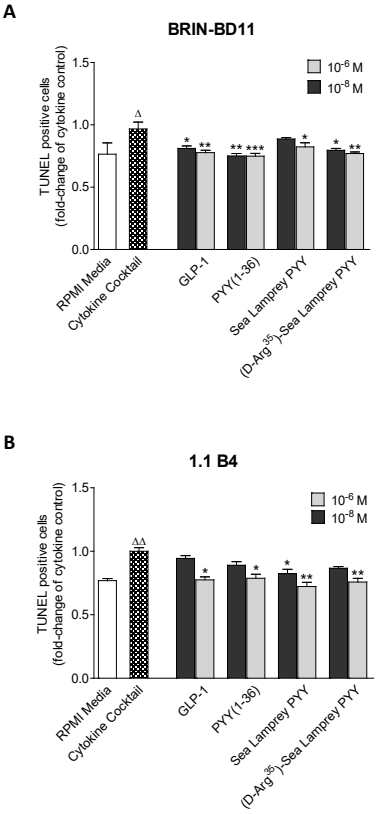
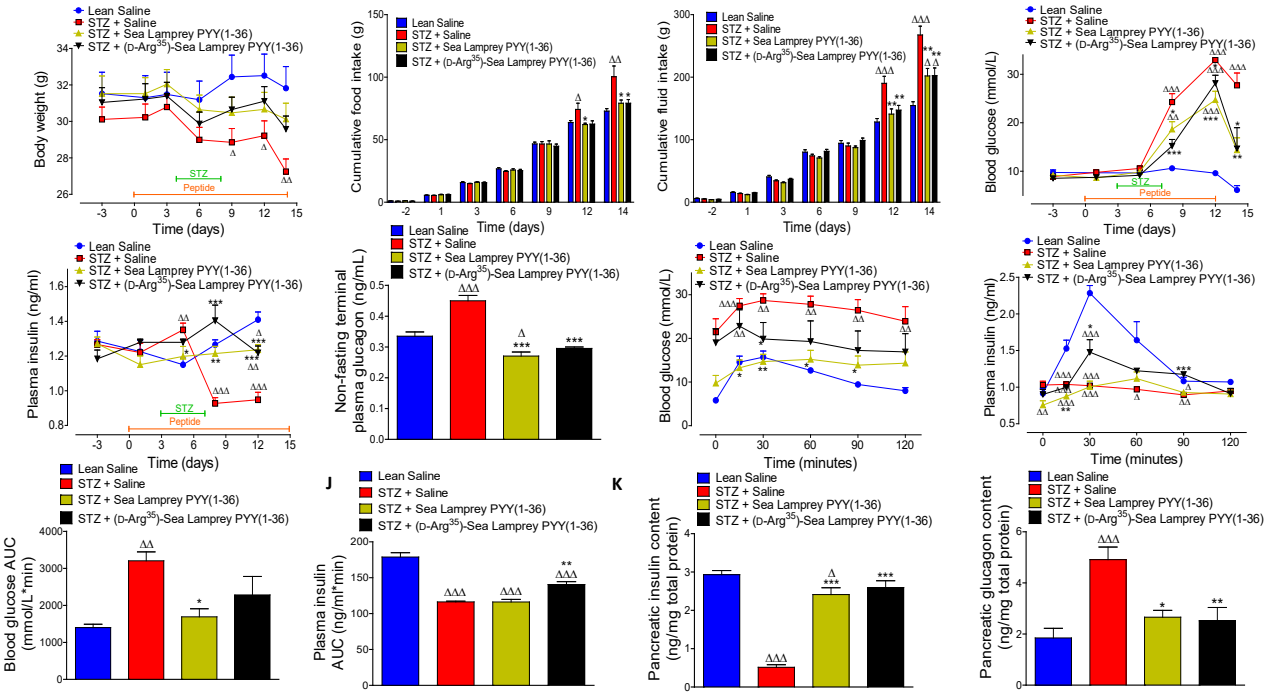

A
5.6 mM Glucose
PYY(1-36)
Sea Lamprey PYY(1-36)
(D-Arg35)-Sea Lamprey PYY(1-36)
Insulin release (ng/10^6 cells/20 mins)
Log10 [Peptide] (M)
B
16.7 mM Glucose
PYY(1-36)
Sea Lamprey PYY(1-36)
(D-Arg35)-Sea Lamprey PYY(1-36)
Insulin release (ng/10^6 cells/20 mins)
Log10 [Peptide] (M)
C
16.7 mM Glucose
Alanine (10 mM)
PYY(1-36) + alanine (10 mM)
Sea lamprey PYY(1-36) + alanine (10 mM)
(D-Arg35)-Sea lamprey PYY(1-36) + alanine (10 mM)
Insulin release (ng/10^6 cells/20 mins)
Log10 [Peptide] (M)

Figure 3


A
BRIN-BD11
TUNEL positive cells
(fold-change of cytokine control)
1.5
1.0
0.5
0.0
10-6 M
10-8 M
Δ
*
**
***
RPMI Media
Cytokine Cocktail
GLP-1
PYY(1-36)
Sea Lamprey PYY
(D-Arg35)-Sea Lamprey PYY
B
1.1 B4
TUNEL positive cells
(fold-change of cytokine control)
1.5
1.0
0.5
0.0
10-6 M
10-8 M
ΔΔ
*
**
RPMI Media
Cytokine Cocktail
GLP-1
PYY(1-36)
Sea Lamprey PYY
(D-Arg35)-Sea Lamprey PYY


Lean Saline
STZ + Saline
STZ + Sea Lamprey PYY(1-36)
STZ + (D-Arg35)-Sea Lamprey PYY(1-36)
Body weight (g)
Time (days)
STZ
Peptide
Cumulative food intake (g)
Cumulative fluid intake (g)
Blood glucose (mmol/L)
Plasma insulin (ng/ml)
Non-fasting terminal plasma glucagon (ng/mL)
Blood glucose (mmol/L)
Time (minutes)
Plasma insulin (ng/ml)
J
K
Blood glucose AUC (mmol/L*min)
Plasma insulin AUC (ng/ml*min)
Pancreatic insulin content (ng/mg total protein)
Pancreatic glucagon content (ng/mg total protein)

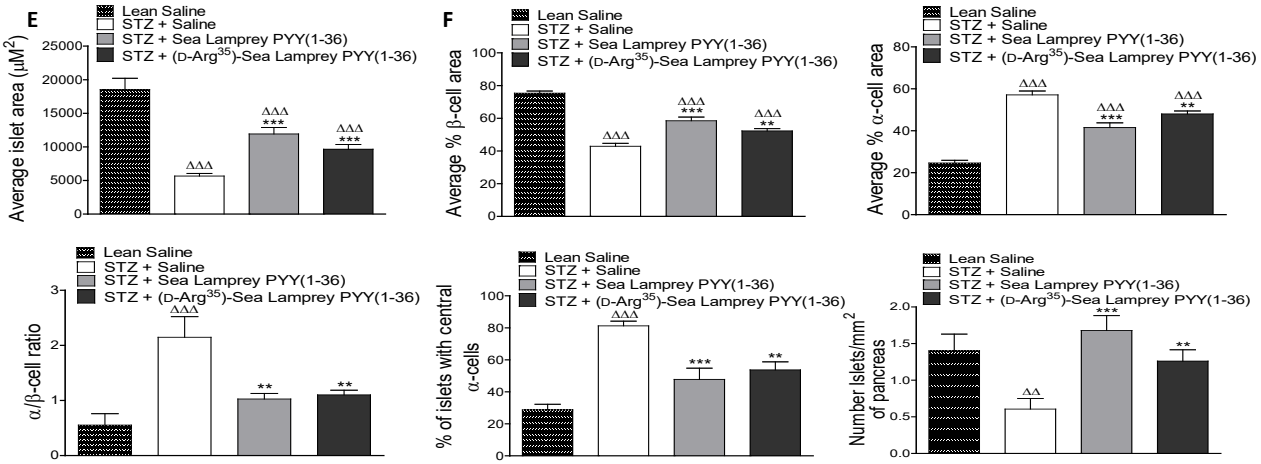
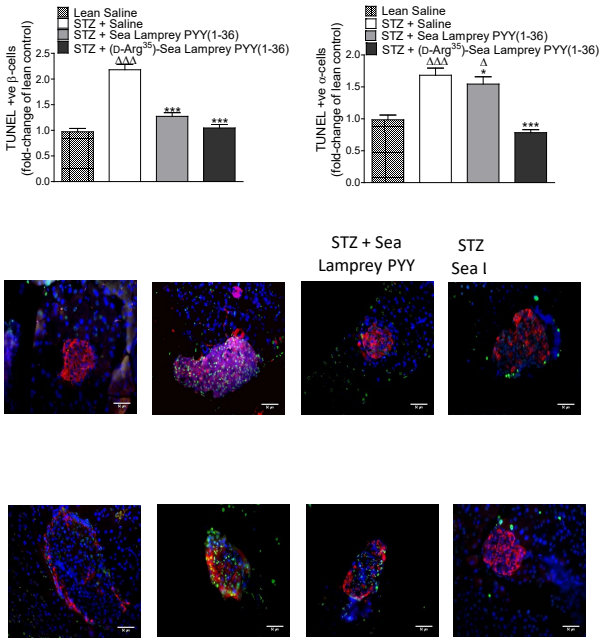

E
F
Lean Saline
STZ + Saline
STZ + Sea Lamprey PYY(1-36)
STZ + (D-Arg35)-Sea Lamprey PYY(1-36)
Average islet area (μM2)
Average % β-cell area
Average % α-cell area
α/β-cell ratio
% of islets with central α-cells
Number Islets/mm2 of pancreas

Figure 6


Lean Saline
STZ + Saline
STZ + Sea Lamprey PYY(1-36)
STZ + (D-Arg35)-Sea Lamprey PYY(1-36)
TUNEL +ve β-cells (fold-change of lean control)
TUNEL +ve α-cells (fold-change of lean control)
ΔΔΔ
***
Λ
STZ + Sea Lamprey PYY
STZ Sea l