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**LH-21 and Abn-CBD improve  $\beta$ -cell function in isolated human and mouse islets through GPR55-dependent and -independent signalling**

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3 **LH-21 and Abn-CBD improve  $\beta$ -cell function in isolated human and mouse islets**  
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5 **through GPR55-dependent and -independent signalling**  
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**ABSTRACT**

**Aims:** CB1 and GPR55 are GPCRs expressed by islet  $\beta$ -cells. Pharmacological compounds have been used to investigate their function, but off-target effects of ligands have been reported. This study examined the effects of Abn-CBD (GPR55 agonist) and LH-21 (CB1 antagonist) on human and mouse islet function, and islets from GPR55<sup>-/-</sup> mice were used to determine signalling via GPR55.

**Materials and methods:** Islets isolated from human organ donors and mice were incubated in the absence or presence of Abn-CBD or LH-21 and insulin secretion, [Ca<sup>2+</sup>]<sub>i</sub>, cAMP, apoptosis,  $\beta$ -cell proliferation and CREB and AKT phosphorylation were examined by standard techniques.

**Results:** Abn-CBD potentiated glucose-stimulated insulin secretion and elevated [Ca<sup>2+</sup>]<sub>i</sub> in human islets and islets from both GPR55<sup>+/+</sup> and GPR55<sup>-/-</sup> mice. LH-21 also increased insulin secretion and [Ca<sup>2+</sup>]<sub>i</sub> in human islets and GPR55<sup>+/+</sup> mouse islets, but concentrations of LH-21 up to 0.1  $\mu$ M were ineffective in islets from GPR55<sup>-/-</sup> mice. **Neither ligand affected basal insulin secretion or islet cAMP levels.** Abn-CBD and LH-21 reduced cytokine-induced apoptosis in human islets and GPR55<sup>+/+</sup> mouse islets, and these effects were suppressed following GPR55 deletion. They also increased  $\beta$ -cell proliferation: the effects of Abn-CBD were preserved in islets from GPR55<sup>-/-</sup> mice, while those of LH-21 were abolished. Abn-CBD and LH-21 increased AKT phosphorylation in mouse and human islets.

**Conclusions:** This study demonstrated that Abn-CBD and LH-21 improve human and mouse islet  $\beta$ -cell function and viability. Use of islets from GPR55<sup>-/-</sup> mice suggests that designation of Abn-CBD and LH-21 as GPR55 agonist and CB1 antagonist, should be revised.

## 1- INTRODUCTION

Cannabinoids are chemicals produced by the cannabis plant (phytocannabinoids) and vertebrates (endocannabinoids) or manufactured commercially (synthetic cannabinoids), and they all act at cannabinoid G protein-coupled receptors (GPCRs) to regulate cell function. CB1 and CB2 are the canonical receptors for cannabinoids, whilst another GPCR, GPR55, which has low sequence homology with CB1 and CB2 [1] and lacks the typical 'cannabinoid binding pocket' [2], is also activated by some cannabinoids [3-9]. CB1 and GPR55 have in common an abundant expression in the central nervous system and metabolic tissues and a proposed role in energy balance, [10-16] that may be secondary to their regulation of insulin secretion [10, 11, 13, 14, 17]. We, and others, have demonstrated that CB1 and GPR55 activation in isolated rodent and human islets is associated with insulinotropic properties [10, 11, 13, 14, 18], although a shared consensus in the scientific community has still not been reached with respect to the role of CB1 receptors in islets [19, 20].

Some of the discrepancies between studies may arise through lack of specificity of the cannabinoid ligands used. For example, we have demonstrated that antagonists of CB1 and CB2 have the same stimulatory effects on insulin release from human islets as agonists of these receptors, suggesting that the antagonists act via CB1/CB2-independent pathways [17]. The CB1 antagonist AM251 activates GPR55 [21-23] and we have recently confirmed that GPR55 is expressed by mouse and human islet  $\beta$ -cells, where it plays a positive role in regulating  $[Ca^{2+}]_i$  and insulin secretion [14]. In addition, the anti-obesity drug rimonabant, which was developed as a CB1 antagonist, also acts as a GPR55 agonist [21-23] thus suggesting that its anti-obesity and insulin sensitising properties might be mediated, at least in part, by activation of this receptor, rather than by antagonising CB1 [24, 25].

LH-21 (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-hexyl-1H-1,2,4-triazole; supplementary Figure 1) was initially identified through screening of 1,2,4-triazole compounds for cannabinoid receptor activity, and it was found to antagonize the effects of the CB1 agonist WIN 55,212-2 [26].

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3 Subsequently, other authors identified LH-21 as a neutral antagonist [24, 27] or weak inverse agonist  
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5 of CB1 [25] with limited brain penetration, and, similar to rimonabant, it is reported to have  
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7 anorexigenic effects in animal models of obesity [27, 28]. Treatment of rats with LH-21 for 10 days  
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9 resulted in a significant up-regulation of GPR55 expression in visceral adipose tissue, suggesting  
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11 involvement of peripheral GPR55-related regulatory mechanisms in its effects [27]. We have recently  
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13 demonstrated that LH-21 improves glucose metabolism and reduces anxiety in obese pre-diabetic  
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15 mice, this latter effect being prevented by a GPR55 antagonist [29]. Due to the promising  
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17 therapeutic effects of the phytocannabinoid cannabidiol (CBD), synthetic CBD derivatives such as  
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19 abnormal cannabidiol (Abn-CBD; trans-4-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-  
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21 1,3-benzenediol, supplementary Figure 1) have been synthesized [30]. Abn-CBD has vasodilator  
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23 effects that were blocked by the CB1 antagonist SR141716A, but were maintained in CB1/CB2  
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25 double knockout mice, which led to the proposal that Abn-CBD acted as an agonist at a novel  
26  
27 cannabinoid receptor [31]. Abn-CBD is now considered to be a potent and selective GPR55 agonist  
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29 [11]. It potentiates glucose-dependent insulin secretion from mouse islets and the BRIN-BD11 cell  
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31 line *in vitro* [11, 13], and improves glucose tolerance *in vivo* that is associated with increased insulin  
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33 sensitivity and  $\beta$ -cell proliferation [11, 13]. The importance of GPR55 in promoting proliferation is  
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35 supported by studies with the GPR55 agonist O-1602 in various cell types [14, 15, 21, 32-34], which  
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37 demonstrated activation of pathways known to regulate  $\beta$ -cell mass [35, 36].  
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44 In the current study we have investigated the effects of LH-21 and Abn-CBD on the secretory  
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46 function, apoptosis and proliferation of islets isolated from human non-diabetic donors and C57BL/6  
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48 mice. The availability of islets from GPR55<sup>-/-</sup> mice has allowed us to define whether the effects of  
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50 these compounds on insulin secretion, [Ca<sup>2+</sup>]<sub>i</sub>, cAMP,  $\beta$ -cell proliferation and apoptosis are mediated  
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52 via GPR55-dependent or -independent signalling.  
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## 2- MATERIALS AND METHODS

### 2.1 Materials

Culture media and supplements, collagenase type XI, histopaque-1077, Fura-2 AM, tolbutamide, carbachol, adenosine triphosphate (ATP) 5-bromo-2'-deoxyuridine (BrdU), mouse monoclonal anti-BrdU antibody, Tween-20, ethylenediaminetetraacetic acid (EDTA), 3-isobutyl-1-methylxanthine (IBMX) and bovine serum albumin (BSA): Sigma-Aldrich (Dorset, UK). LH-21 and Abn-CBD: Cayman Chemical (Cambridge, UK). Caspase-Glo 3/7, HRP-conjugated secondary antibody and ECL western blotting reagents: Promega (Hampshire, UK). Recombinant TNF $\alpha$ , IFN $\gamma$  and IL-1 $\beta$ : PeproTech EC (London, UK). Guinea pig anti-insulin primary antibody: Dako (Cambridge, UK). AlexaFluor<sup>®</sup> 488 and AlexaFluor<sup>®</sup> 594-conjugated secondary antibodies: Jackson ImmunoResearch Laboratories (Suffolk, UK). Bicinchoninic acid (BCA) protein assay, 4-12% polyacrylamide gels, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), Hank's balanced salt solution (HBSS), NuPAGE<sup>®</sup> sample and transfer buffers: Thermo Fisher Scientific (Paisley, UK). Anti phospho- AKT, anti phospho-CREB, anti-AKT and anti-CREB primary antibodies: Cell Signaling (Hitchin, UK). Rainbow molecular weight markers and PVDF membrane: Millipore (Watford, UK). Cyclic AMP HiRange assay: Cisbio (Codolet, France).

### 2.2 Experimental animals

A colony of C57BL/6J GPR55 homozygous knockout mice (GPR55<sup>-/-</sup>) [14] was maintained at King's College London, with food and water supplied *ad libitum*. Age-matched wild-type (GPR55<sup>+/+</sup>) male C57BL/6 mice were purchased from Envigo (Bicester, UK) and maintained in the same conditions as the GPR55<sup>-/-</sup> mice prior to islet isolation.

### 2.3 Isolation of mouse and human islets

Islets were isolated from 8-12 week old male GPR55<sup>-/-</sup> C57BL/6 mice and age-matched GPR55<sup>+/+</sup> mice by collagenase digestion of the exocrine pancreas [37], giving a yield of approximately 200



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3 **islets per mouse**. All animal procedures were approved by the King's College London Ethics  
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5 Committee and carried out in accordance with the UK Home Office Animals (Scientific Procedures)  
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7 Act 1986. Mouse islets were incubated at 37°C in RPMI-1640 (supplemented with 10% FBS, 2 mM  
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9 glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin) for up to 48 h before use. **Human islets**  
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11 **were isolated from 18 non-diabetic, heart beating pancreas donors (age: 45±2; BMI: 28.5±1.4 kg/m<sup>2</sup>,**  
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13 **6 male, 12 female)** at the King's College Hospital Islet Transplantation Unit with appropriate ethical  
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15 approval [38]. Human islets were maintained at 37°C in CMRL-1066 (supplemented with 2% human  
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17 albumin, 4 mM glutamine, 2 mM HEPES, pH 7.2-7.4, and 10 mM nicotinamide) for up to 48 h before  
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19 use [39].  
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#### 24 **2.4 Insulin secretion**

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26 Assessment of dynamic insulin secretion from groups of 40 mouse or 50 human islets was performed  
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28 using a temperature-controlled perfusion apparatus [39]. Islets were perfused with a physiological  
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30 salt solution [40] supplemented with 2 mM glucose or 20 mM glucose in the absence or presence of  
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32 compounds of interest (vs. vehicle). Perifusate fractions were collected every 2 min and insulin  
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34 contents were determined by radioimmunoassay [41].  
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#### 39 **2.5 Single cell calcium microfluorimetry**

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41 **As islets are clusters of 1-2,000 endocrine cells, for single cell microfluorimetry experiments whole**  
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43 **islets were dissociated by short-term incubation with 0.02% EDTA solution, as previously described**  
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45 **[42].** Groups of approximately 100,000 dispersed mouse and human islet cells were seeded onto  
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47 glass coverslips, maintained in culture overnight then loaded with 5 μM Fura-2 AM for 30 min. Cells  
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49 on coverslips were perfused (37°C, 1 ml/min) with a physiological salt solution [40] containing 2 mM  
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51 glucose in the absence or presence of test agents. Real-time changes in  $[Ca^{2+}]_i$  were determined by  
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53 illuminating cells alternately at 340 nm and 380 nm, with the emitted light being filtered at 510 nm  
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55 and data were recorded with a CCD camera every 3 seconds.  
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## 2.6 Cyclic AMP

Groups of 4 mouse islets or 5 human islets were transferred to white-walled 96 well plates in HBSS medium supplemented with 10 mM HEPES, 0.2% BSA and 2 mM IBMX, and incubated for 1 h at room temperature in the absence or presence of test agents. Islet cAMP levels were quantified according to the manufacturer's protocol, with measurement of the fluorescence emission intensity ratio at 665/620 nm using a Pherastar FS microplate reader (BMG LABTECH Ltd., Bucks, UK).

## 2.7 Caspase 3/7 activities

Mouse and human islets were maintained in culture for 24 h in the absence or presence of 0.1  $\mu$ M LH-21 or 10  $\mu$ M Abn-CBD. Groups of 3 mouse or 5 human islets were then incubated for a further 20 h in RPMI-1640 or CMRL with 2% FBS (mouse) or 0.2% albumin (human) supplemented with a cytokine cocktail (0.025 U/ $\mu$ l IL-1 $\beta$ , 1 U/ $\mu$ l TNF $\alpha$  and 1 U/ $\mu$ l IFN $\gamma$ ), and islet cell apoptosis was determined using the Caspase 3/7 Glo<sup>®</sup> assay [39].

## 2.8 Islet $\beta$ -cell proliferation

Islets isolated from GPR55<sup>+/+</sup> and GPR55<sup>-/-</sup> mice were incubated for 5 days at 37°C (95% air/5% CO<sub>2</sub>) in RPMI-1640 supplemented with 0.1  $\mu$ M LH-21, 1  $\mu$ M Abn-CBD or vehicle (0.007% DMSO) and 1 mg/ml BrdU. Media and supplements were refreshed every 48 h. Islets were then pelleted at 135 g, fixed with 4% paraformaldehyde and embedded in paraffin. 5  $\mu$ m thick sections were dewaxed then antigens were retrieved using citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0). Sections were incubated overnight at 4°C with primary anti-insulin (guinea pig) and anti-BrdU (mouse) antibodies at 1:200 and 1:100 dilutions, respectively, then incubated with anti-guinea pig AlexaFluor<sup>®</sup> 594 and anti-mouse AlexaFluor<sup>®</sup> 488 antibodies (1:50 dilution) for 1 h at room

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3 temperature. Images were visualised using a Nikon Eclipse TE2000-U microscope and analysed in a  
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5 non-blinded manner using Image J software (<https://imagej.net/>) [43].  
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## 8 9 **2.9 Western blotting**

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11 Groups of 200 mouse or human islets were incubated for 30 min in a buffer containing 25 mM  
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13 glucose in the absence or presence of Abn-CBD or LH-21. Islets were then lysed in the presence of  
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15 phosphatase and protease inhibitors and protein contents were quantified by the BCA method.  
16  
17 Forty micrograms of lysates were fractionated on 4-12% polyacrylamide gels (1 h, 200 V), transferred  
18  
19 onto 0.2 µm PVDF membranes, blocked with 5% BSA in Tris-buffered saline, 0.1% Tween-20 (TBST)  
20  
21 for 1 h at room temperature and then probed overnight at 4°C with rabbit anti-phospho CREB, anti-  
22  
23 total CREB, anti-phospho-AKT and anti-total-AKT antibodies, all diluted 1:1000 in 5% BSA in TBST.  
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25 The membranes were then incubated with anti-rabbit HRP-conjugated secondary antibody (1:10000)  
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27 for 1 h at room temperature and exposed to X-ray film after addition of ECL substrate [44].  
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## 32 33 **2.10 Statistical Analyses**

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35 Differences between selected pairs of data were analysed by unpaired Student's *t-test* while  
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37 differences between several groups were analysed by one-way ANOVA followed by Tukey's,  
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39 Dunnett's Multiple Comparison or Newman-Keuls Multiple Comparison post tests.  
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## 43 44 **3- RESULTS**

### 45 46 **3.1 Effects of Abn-CBD and LH-21 on glucose-induced insulin secretion**

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48 As shown in Figure 1A, exposure of human islets to increasing concentrations of Abn-CBD (0.1-10  
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50 µM) resulted in concentration-dependent potentiation of glucose-induced insulin secretion. The  
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52 stimulatory effects were rapid in onset, and readily reversible upon removal of Abn-CBD. LH-21 (0.1-  
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54 1 µM) also reversibly potentiated insulin secretion from human islets (Figure 1B), with a similar  
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3 stimulatory profile to that observed for Abn-CBD. Exposure of human islets to Abn-CBD or LH-21 was  
4 without effect on basal insulin secretion at 2 mM glucose (Figures 1C, 1D).

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6 Abn-CBD (Figures 1E, 1F) and LH-21 (Figures 1H-1J) enhanced glucose-induced insulin secretion from  
7 GPR55<sup>+/+</sup> mouse islets, as they did from human islets. Stimulatory effects of 1 μM (Figure 1E) and 10  
8 μM Abn-CBD (Figure 1F) were also observed in islets isolated from GPR55<sup>-/-</sup> mice, and the  
9 potentiating effect of 10 μM Abn-CBD was actually accentuated following GPR55 deletion. In  
10 contrast, potentiation of insulin secretion by low concentrations of LH-21 was completely dependent  
11 on GPR55, with islets from GPR55<sup>-/-</sup> mice showing no response at all to 1nM and 0.1 μM of this  
12 compound (Figure 1H, 1I). Interestingly, however, it was found that deletion of GPR55 did not  
13 abrogate the stimulation of insulin secretion by 1 μM LH-21, and GPR55<sup>-/-</sup> islets secreted more  
14 insulin than their wildtype counterparts in response to this higher concentration (Figure 1J). Abn-  
15 CBD and LH-21 did not significantly stimulate basal insulin secretion from GPR55<sup>+/+</sup> or GPR55<sup>-/-</sup>  
16 mouse islets (Figure 1G, 1K).

### 3.2 Effects of LH-21 and Abn-CBD on islet [Ca<sup>2+</sup>]<sub>i</sub> and cAMP levels

17  
18 The requirement for GPR55 to mediate the stimulatory effects of 1 nM and 0.1 μM LH-21 on insulin  
19 secretion was unexpected, given that LH-21 is considered to be an antagonist or inverse agonist at  
20 CB1. We therefore explored the effects of LH-21 and Abn-CBD on changes in [Ca<sup>2+</sup>]<sub>i</sub>, since it is known  
21 that GPR55 couples to G<sub>q</sub> and Gα<sub>13</sub> pathways [14-16] that regulate Ca<sup>2+</sup> levels.

22  
23 Dispersed islet cells from both GPR55<sup>+/+</sup> (Figures 2A, 2C) and GPR55<sup>-/-</sup> mice (Figures 2B, 2D)  
24 responded to an increase in glucose concentration from 2 mM to 20 mM with a rapid and sustained  
25 elevation in [Ca<sup>2+</sup>]<sub>i</sub>. Exposure of GPR55<sup>+/+</sup> islet cells to 0.1 μM LH-21 led to a small increase in [Ca<sup>2+</sup>]<sub>i</sub>  
26 (Figure 2A) and this response was lost following deletion of GPR55 (Figure 2B), in agreement with  
27 the insulin secretion data (Figure 1I), demonstrating that low concentrations of LH-21 act via GPR55.  
28 Also consistent with the secretion data, 1 μM Abn-CBD promoted increases in [Ca<sup>2+</sup>]<sub>i</sub> in dispersed  
29 islets from both GPR55<sup>+/+</sup> and GPR55<sup>-/-</sup> mice (Figures 2C, 2D), indicating that Abn-CBD is able to  
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3 stimulate elevations in  $[Ca^{2+}]_i$  and insulin secretion independently of GPR55. In all tested batches of  
4 islets the ATP-sensitive channel blocker, tolbutamide, stimulated reversible increases in  $[Ca^{2+}]_i$ ,  
5 demonstrating that the ability to control the membrane potential was preserved after exposure to  
6 LH-21 and Abn-CBD. Dispersed human islets also responded to both Abn-CBD and LH-21 with  
7 increases in  $[Ca^{2+}]_i$ . In particular, 0.1  $\mu$ M Abn-CBD reversibly increased  $[Ca^{2+}]_i$ , while 10  $\mu$ M Abn-CBD  
8 induced a sustained elevation in  $[Ca^{2+}]_i$  (Figure 2E); 0.1  $\mu$ M LH-21 induced a rapid, reversible  
9 increase, in  $[Ca^{2+}]_i$  and the response to 1  $\mu$ M LH-21 was sustained after the drug had been removed  
10 from the perfusing buffer (Figure 2F).

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CB1 receptors are coupled to  $G_i$  to reduce cAMP generation [45], so we determined whether Abn-CBD or LH-21 had any effects on islet cAMP levels. These experiments indicated that neither ligand affected basal cAMP in islets isolated from GPR55<sup>+/+</sup> (Figure 2G) or GPR55<sup>-/-</sup> (Figure 2H) mice, whereas the GLP-1 agonist exendin-4 produced the expected elevation in cAMP. In addition, Abn-CBD and LH-21 did not inhibit forskolin-stimulated cAMP accumulation, but this was significantly decreased by the  $\alpha_2$  adrenergic agonist clonidine, as expected (Figure 2I, 2J). Similar observations were made in human islets, where Abn-CBD and LH-21 neither stimulated (Figure 2K) nor inhibited (Figure 2L) cAMP generation.

### 3.3 Abn-CBD and LH-21 protect islets against cytokine-induced apoptosis via GPR55

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GPR55 has been implicated in protecting islets from programmed cell death [15, 46], thus in the current study caspase 3/7 activities were measured in islets from GPR55<sup>+/+</sup> and GPR55<sup>-/-</sup> mice. These experiments indicated that Abn-CBD and LH-21 did not affect basal levels of apoptosis in islets from GPR55<sup>-/-</sup> mice but they significantly reduced apoptosis induced by a cytokine cocktail (Figure 3A). However, although the cannabinoid ligands exerted protective effects, they did not fully block cytokine-induced apoptosis, which was significantly elevated above basal even in the presence of Abn-CBD or LH-21. Cytokines also elevated caspase 3/7 activities in islets from GPR55<sup>-/-</sup> mice and while there were small reductions in the presence of Abn-CBD or LH-21 these were not statistically

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3 significant (Figure 3B). Interestingly, as shown in Figure 3C, Abn-CBD and LH-21 totally reverted  
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5 cytokine-induced apoptosis in human islets.  
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### 9 **3.4 LH-21 and Abn-CBD stimulate $\beta$ -cell proliferation**

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11 Double immunofluorescence staining of islets from GPR55<sup>+/+</sup> and GPR55<sup>-/-</sup> mice for insulin and BrdU  
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13 expression indicated that 5 days' exposure to 1  $\mu$ M Abn-CBD or 0.1  $\mu$ M LH-21 increased BrdU  
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15 incorporation into proliferating  $\beta$ -cells of wildtype mice (Figures 4A, 4B). These stimulatory effects of  
16  
17 LH-21 were abolished following GPR55 deletion, and while Abn-CBD-induced  $\beta$ -cell proliferation was  
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19 reduced in GPR55<sup>-/-</sup> islets it was still able to significantly stimulate BrdU incorporation into  $\beta$ -cells  
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21 (Figures 4A, 4B). Further quantification of immunofluorescence images indicated that the  
22  
23 stimulatory effects of Abn-CBD and LH-21 on BrdU incorporation in GPR55<sup>+/+</sup> islets were associated  
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25 with significantly increased numbers of  $\beta$ -cells per islet (Figure 4C) and increased islet area (Figure  
26  
27 4D), and the effects of LH-21 were dependent on GPR55 since it failed to trigger the same responses  
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29 in the GPR55<sup>-/-</sup> islets. These effects of LH-21 and Abn-CBD to promote  $\beta$ -cell proliferation and  
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31 increase islet size were independent of any effect on individual  $\beta$ -cell area (Figure 4E).  
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### 37 **3.5 Effects of Abn-CBD and LH-21 on CREB and AKT phosphorylation in mouse and human islets**

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39 Exposure of mouse islets for 30 min to 10  $\mu$ M Abn-CBD or 0.1  $\mu$ M LH-21 in the presence of 25 mM  
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41 glucose increased levels of phosphorylated CREB (P-CREB) (Figure 5A), and these ligands also  
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43 promoted increases in Akt phosphorylation (P-Akt) in mouse islets (Figure 5B). Abn-CBD and LH-21  
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45 also increased P-AKT in human islets (Figure 5D), but in experiments with four different batches of  
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47 human islets they had no effect on P-CREB (Figure 5C).  
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## 51 **4- DISCUSSION**

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53 Despite confirmation of the expression of CB1 and GPR55 by both rodent and human  $\beta$ -cells [10, 14,  
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55 18, 19, 47, 48], the role played by the endocannabinoid system in the modulation of islet secretory  
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3 activity is still far from being completely understood. For example, while the majority of studies  
4 suggest that activation of CB1 receptors increases insulin secretion [47, 49-51], others point to the  
5 opposite effects [19, 20]. The reasons for the controversies are likely to lie in the experimental  
6 models used, the selectivity of the ligands and the difficulties in addressing the intracellular coupling  
7 where cannabinoid receptors signal via more than one heterotrimeric G-protein [15, 48]. Indeed,  
8 various factors such as differences in the concentrations of the agonists and antagonists used,  
9 diversities in the experimental design and impact of circulatory factors such as incretins in the *in vivo*  
10 studies [19], can all contribute to the variabilities between studies. In the current study we  
11 measured insulin release from isolated mouse and human islets under dynamic conditions, where  
12 the continuous flow of perfusing buffers minimises autocrine and paracrine effects of secreted  
13 products that may occur in static incubation protocols [52]. In terms of ligand selectivity, it has been  
14 demonstrated that some CB1 antagonists such as AM251 and rimonabant can also act as agonists for  
15 GPR55 [23], which is expressed by islet  $\beta$ -cells and whose activation increases insulin secretion from  
16 both human and mouse islets [10, 11, 13, 14], and this may have led to differences in interpretation  
17 of earlier studies.  
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36 Since LH-21, an inverse agonist/neutral antagonist of CB1, shows structural similarities with AM251  
37 and rimonabant [24, 25, 27-29], we investigated whether its activation of islet GPR55 could underlie  
38 at least some of its reported positive effects on glucose management [24, 27-29]. An earlier study  
39 used a static incubation protocol to show that the CB1 antagonists AM251 and JD-5037 increased  
40 insulin release from human islets [19], and in the current study we employed perfusion experiments  
41 to demonstrate that LH-21 also promoted insulin secretion from human islets. However, we found  
42 that the potentiation of insulin release by lower concentrations of LH-21 (1 nM and 0.1  $\mu$ M) was lost  
43 in islets isolated from GPR55<sup>-/-</sup> mice demonstrating that the insulinotropic effects were not  
44 secondary to antagonism of islet CB1 receptors, but were a consequence of LH-21 binding to islet  
45 GPR55. Thus, these data indicate that caution should be exerted when concluding that the effects  
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3 evoked by LH-21 are mediated exclusively by CB1, especially when it is used at nanomolar  
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5 concentrations. Nevertheless, we found that 1  $\mu\text{M}$  LH-21 was able to stimulate insulin secretion  
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7 following GPR55 deletion, indicating signalling in a GPR55-independent manner at higher  
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9 concentrations of LH-21. This stimulatory effect most likely occurs via an undefined receptor, as we  
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11 previously observed for 10  $\mu\text{M}$  AM251 in human islets [17].  
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15 As a parallel investigative strategy we studied the requirement of GPR55 for insulin secretion  
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17 induced by Abn-CBD, a cannabinoid that is reported to be at least 10-fold more selective for GPR55  
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19 than for CB1 or CB2 [16]. It has previously been demonstrated that Abn-CBD increases insulin  
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21 secretion from BRIN-BD11 insulin-secreting cells and mouse islets [11]. In the current study we  
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23 confirmed that Abn-CBD potentiated glucose-induced insulin secretion from mouse islets and we  
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25 also demonstrated, for the first time, that it has similar stimulatory effects in human islets. However,  
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27 despite its reported selectivity as a GPR55 agonist, Abn-CBD stimulated elevations in insulin release  
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29 in islets from GPR55<sup>-/-</sup> mice, indicating that at concentrations of 1  $\mu\text{M}$  and above it exerts GPR55-  
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31 independent effects in islets.  
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36 The insulin secretion potentiating properties of Abn-CBD and LH-21 were accompanied by increases  
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38 in  $[\text{Ca}^{2+}]_i$  in both human and mouse islets. The elevation in  $[\text{Ca}^{2+}]_i$  evoked by 0.1  $\mu\text{M}$  LH-21 required  
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40 the expression of GPR55, in agreement with the insulin secretory response to that low concentration  
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42 of the drug and confirming that LH-21 acts as a GPR55 agonist in islets. **However, Abn-CBD induced**  
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44 **increased  $[\text{Ca}^{2+}]_i$  in islets from GPR55<sup>-/-</sup> mice, consistent with the insulin secretion data and**  
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46 **suggesting that it acts through another receptor. A possible candidate is GPR18, which is activated**  
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48 **by Abn-CBD [53], expressed by islets [54], and it has been reported that GPR18 activation is**  
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50 **associated with transient elevation of  $[\text{Ca}^{2+}]_i$  [55].** Nothing is known about the functional role of  
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52 GPR18 in islets, but it has been reported that the GPR18 agonist N-arachidonylglycine promotes  
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54 elevations in  $\beta$ -cell  $[\text{Ca}^{2+}]_i$  and potentiates insulin secretion from rat islets [56].  
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5 There is a growing body of evidence suggesting that the endocannabinoid system has a role in the  
6 regulation of cell proliferation and apoptosis [57-59]. We have previously reported that CB1 and CB2  
7 agonists protect mouse islets from apoptosis [60], and we recently demonstrated that LH-21 delivery  
8 to pre-diabetic mice has anti-inflammatory and cytoprotective effects [29]. In the current study we  
9 showed that LH-21 has direct anti-apoptotic effects in isolated human and mouse islets and the use  
10 of islets from GPR55<sup>-/-</sup> mice indicated that, as for stimulation of insulin secretion and [Ca<sup>2+</sup>]<sub>i</sub>, this was  
11 GPR55-mediated. Abn-CBD also possessed anti-apoptotic effects in islets but, in contrast to its  
12 GPR55-independent effects on insulin secretion, it failed to significantly protect against cytokine-  
13 induced apoptosis in GPR55<sup>-/-</sup> islets. In addition, incubation of isolated islets for 5 days in the  
14 presence of 1 μM Abn-CBD or 0.1 μM LH-21 stimulated islet β-cell proliferation. The beneficial  
15 effects of LH-21 were strictly dependent on GPR55. **Abn-CBD-stimulated BrdU incorporation was**  
16 **reduced, but not abolished, in GPR55<sup>-/-</sup> islets, indicating that GPR55 is required for some of its pro-**  
17 **proliferative effects but that it acts via another receptor to fully stimulate β-cell mass expansion.**  
18 Analysis of the untreated islets also indicated that endogenous GPR55 is required for normal  
19 maintenance of β-cell mass, since vehicle-treated islets from GPR55<sup>-/-</sup> mice had fewer proliferating β-  
20 cells, which was associated with decreased numbers of β-cells per islet and reduced islet area.

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41 In terms of identifying signalling cascades by which LH-21 and Abn-CBD can regulate β-cell  
42 proliferation and apoptosis, it has been reported that the cannabinoid system can activate the  
43 serine/threonine kinase AKT and the transcription factor CREB [61, 62]. **Our observations that LH-21**  
44 **and Abn-CBD stimulated AKT phosphorylation in mouse and human islets support a role for GPR55**  
45 **signalling via this kinase in islets. However, increased CREB phosphorylation was only observed in**  
46 **mouse islets, suggesting either species-specific signalling downstream of GPR55 or it might reflect**  
47 **the capacity of the ligands to activate multiple receptors, which may be differentially expressed in**  
48 **mouse and human islets.**

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5 In conclusion, the cannabinoid ligands LH-21 and Abn-CBD increase insulin secretion from human  
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7 and mouse islets, most likely via  $\text{Ca}^{2+}$ -regulated intracellular pathways, protect  $\beta$ -cells from  
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9 apoptosis and they foster increased  $\beta$ -cell proliferation. The use of islets isolated from  $\text{GPR55}^{-/-}$  mice  
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11 allowed the elucidation of the contribution of GPR55-mediated signalling in the functional effects of  
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13 LH-21 and Abn-CBD and our work provides the first evidence of LH-21 acting as a GPR55 agonist in  
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15 islets. Furthermore, our data clearly demonstrate that Abn-CBD cannot be considered to be a  
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17 selective GPR55 agonist, at least in islets. The ability of LH-21 to potentiate glucose-induced insulin  
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19 secretion at nanomolar concentrations, its capacity to protect islets from apoptosis and stimulate  $\beta$ -  
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21 cell proliferation, and its protected profile at the CNS level in terms of inducing anxiety/depressive  
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23 traits [28] suggest that a re-evaluation of this molecule as an active tool for the regulation of glucose  
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25 management in diabetic patients is warranted.  
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### 49 **Author contributions**

50  
51 S.J.P., F.J.B.S. and I.R.M. conceived, designed and supervised the study. I.R.M., A.P., L.B., and P.A.,  
52  
53 performed the experiments. D.B. provided the GPR55 null mice and G. C. H. provided the isolated  
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3 human islets of Langerhans. I.R.M., S.J.P., A.P., F.J.A and F.J.B.S. analysed the results. S.J.P. and I.R.M.  
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5 wrote the paper. All the authors revised the manuscript.  
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## 9 References

- 10  
11 [1] Baker D, Pryce G, Davies WL, Hiley CR. In silico patent searching reveals a new cannabinoid  
12 receptor. Trends in pharmacological sciences. 2006; **27**: 1-4  
13 [2] Kotsikorou E, Madrigal KE, Hurst DP, *et al.* Identification of the GPR55 agonist binding site  
14 using a novel set of high-potency GPR55 selective ligands. Biochemistry. 2011; **50**: 5633-5647  
15 [3] Oka S, Nakajima K, Yamashita A, Kishimoto S, Sugiura T. Identification of GPR55 as a  
16 lysophosphatidylinositol receptor. Biochemical and biophysical research communications. 2007; **362**:  
17 928-934  
18 [4] Lauckner JE, Jensen JB, Chen HY, Lu HC, Hille B, Mackie K. GPR55 is a cannabinoid receptor  
19 that increases intracellular calcium and inhibits M current. Proceedings of the National Academy of  
20 Sciences of the United States of America. 2008; **105**: 2699-2704  
21 [5] Waldeck-Weiermair M, Zoratti C, Osibow K, *et al.* Integrin clustering enables anandamide-  
22 induced Ca<sup>2+</sup> signaling in endothelial cells via GPR55 by protection against CB1-receptor-triggered  
23 repression. Journal of cell science. 2008; **121**: 1704-1717  
24 [6] Henstridge CM, Balenga NA, Ford LA, Ross RA, Waldhoer M, Irving AJ. The GPR55 ligand L-  
25 alpha-lysophosphatidylinositol promotes RhoA-dependent Ca<sup>2+</sup> signaling and NFAT activation.  
26 FASEB journal : official publication of the Federation of American Societies for Experimental Biology.  
27 2009; **23**: 183-193  
28 [7] Oka S, Toshida T, Maruyama K, Nakajima K, Yamashita A, Sugiura T. 2-Arachidonoyl-sn-  
29 glycerol-3-phosphoinositol: a possible natural ligand for GPR55. Journal of biochemistry. 2009; **145**:  
30 13-20  
31 [8] Kargl J, Brown AJ, Andersen L, *et al.* A selective antagonist reveals a potential role of G  
32 protein-coupled receptor 55 in platelet and endothelial cell function. The Journal of pharmacology  
33 and experimental therapeutics. 2013; **346**: 54-66  
34 [9] Sylantsev S, Jensen TP, Ross RA, Rusakov DA. Cannabinoid- and lysophosphatidylinositol-  
35 sensitive receptor GPR55 boosts neurotransmitter release at central synapses. Proceedings of the  
36 National Academy of Sciences of the United States of America. 2013; **110**: 5193-5198  
37 [10] Romero-Zerbo SY, Rafacho A, Diaz-Arteaga A, *et al.* A role for the putative cannabinoid  
38 receptor GPR55 in the islets of Langerhans. The Journal of endocrinology. 2011; **211**: 177-185  
39 [11] McKillop AM, Moran BM, Abdel-Wahab YH, Flatt PR. Evaluation of the insulin releasing and  
40 antihyperglycaemic activities of GPR55 lipid agonists using clonal beta-cells, isolated pancreatic islets  
41 and mice. British journal of pharmacology. 2013; **170**: 978-990  
42 [12] Ligresti A, De Petrocellis L, Di Marzo V. From Phytocannabinoids to Cannabinoid Receptors  
43 and Endocannabinoids: Pleiotropic Physiological and Pathological Roles Through Complex  
44 Pharmacology. Physiological reviews. 2016; **96**: 1593-1659  
45 [13] McKillop AM, Moran BM, Abdel-Wahab YH, Gormley NM, Flatt PR. Metabolic effects of  
46 orally administered small-molecule agonists of GPR55 and GPR119 in multiple low-dose  
47 streptozotocin-induced diabetic and incretin-receptor-knockout mice. Diabetologia. 2016; **59**: 2674-  
48 2685  
49 [14] Liu B, Song S, Ruz-Maldonado I, *et al.* GPR55-dependent stimulation of insulin secretion from  
50 isolated mouse and human islets of Langerhans. Diabetes, obesity & metabolism. 2016; **18**: 1263-  
51 1273  
52 [15] Liu B, Song S, Jones PM, Persaud SJ. GPR55: from orphan to metabolic regulator?  
53 Pharmacology & therapeutics. 2015; **145**: 35-42  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 [16] Tuduri E, Imbernon M, Hernandez-Bautista RJ, *et al.* GPR55: a new promising target for  
4 metabolism? *Journal of molecular endocrinology*. 2017; **58**: R191-R202
- 5 [17] Li C, Bowe JE, Huang GC, Amiel SA, Jones PM, Persaud SJ. Cannabinoid receptor agonists and  
6 antagonists stimulate insulin secretion from isolated human islets of Langerhans. *Diabetes, obesity &*  
7 *metabolism*. 2011; **13**: 903-910
- 8 [18] Li C, Bowe JE, Jones PM, Persaud SJ. Expression and function of cannabinoid receptors in  
9 mouse islets. *Islets*. 2010; **2**: 293-302
- 10 [19] Gonzalez-Mariscal I, Krzysik-Walker SM, Kim W, Rouse M, Egan JM. Blockade of cannabinoid  
11 1 receptor improves GLP-1R mediated insulin secretion in mice. *Molecular and cellular*  
12 *endocrinology*. 2016; **423**: 1-10
- 13 [20] Nakata M, Yada T. Cannabinoids inhibit insulin secretion and cytosolic Ca<sup>2+</sup> oscillation in  
14 islet beta-cells via CB1 receptors. *Regulatory peptides*. 2008; **145**: 49-53
- 15 [21] Henstridge CM, Balenga NA, Schroder R, *et al.* GPR55 ligands promote receptor coupling to  
16 multiple signalling pathways. *British journal of pharmacology*. 2010; **160**: 604-614
- 17 [22] Ryberg E, Larsson N, Sjogren S, *et al.* The orphan receptor GPR55 is a novel cannabinoid  
18 receptor. *British journal of pharmacology*. 2007; **152**: 1092-1101
- 19 [23] Kapur A, Zhao P, Sharir H, *et al.* Atypical responsiveness of the orphan receptor GPR55 to  
20 cannabinoid ligands. *The Journal of biological chemistry*. 2009; **284**: 29817-29827
- 21 [24] Pavon FJ, Serrano A, Perez-Valero V, *et al.* Central versus peripheral antagonism of  
22 cannabinoid CB1 receptor in obesity: effects of LH-21, a peripherally acting neutral cannabinoid  
23 receptor antagonist, in Zucker rats. *Journal of neuroendocrinology*. 2008; **20 Suppl 1**: 116-123
- 24 [25] Chen RZ, Frassetto A, Lao JZ, *et al.* Pharmacological evaluation of LH-21, a newly discovered  
25 molecule that binds to cannabinoid CB1 receptor. *European journal of pharmacology*. 2008; **584**:  
26 338-342
- 27 [26] Jagerovic N, Hernandez-Folgado L, Alkorta I, *et al.* Discovery of 5-(4-chlorophenyl)-1-(2,4-  
28 dichlorophenyl)-3-hexyl-1h-1,2,4-triazole, a novel *in vivo* cannabinoid antagonist containing a 1,2,4-  
29 triazole motif. *Journal of medicinal chemistry*. 2004; **47**: 2939-2942
- 30 [27] Alonso M, Serrano A, Vida M, *et al.* Anti-obesity efficacy of LH-21, a cannabinoid CB(1)  
31 receptor antagonist with poor brain penetration, in diet-induced obese rats. *British journal of*  
32 *pharmacology*. 2012; **165**: 2274-2291
- 33 [28] Pavon FJ, Bilbao A, Hernandez-Folgado L, *et al.* Antiobesity effects of the novel *in vivo*  
34 neutral cannabinoid receptor antagonist 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-hexyl-1H-1,2,4-  
35 triazole--LH 21. *Neuropharmacology*. 2006; **51**: 358-366
- 36 [29] Romero-Zerbo SY, Ruz-Maldonado I, Espinosa-Jimenez V, *et al.* The cannabinoid ligand LH-21  
37 reduces anxiety and improves glucose handling in diet-induced obese pre-diabetic mice. *Scientific*  
38 *reports*. 2017; **7**: 3946
- 39 [30] Razdan RK, Dalzell HC, Handrick GR. Hashish. A simple one-step synthesis of (-)-delta1-  
40 tetrahydrocannabinol (THC) from p-mentha-2,8-dien-1-ol and olivetol. *Journal of the American*  
41 *Chemical Society*. 1974; **96**: 5860-5865
- 42 [31] Jarai Z, Wagner JA, Varga K, *et al.* Cannabinoid-induced mesenteric vasodilation through an  
43 endothelial site distinct from CB1 or CB2 receptors. *Proceedings of the National Academy of Sciences*  
44 *of the United States of America*. 1999; **96**: 14136-14141
- 45 [32] Whyte LS, Ryberg E, Sims NA, *et al.* The putative cannabinoid receptor GPR55 affects  
46 osteoclast function *in vitro* and bone mass *in vivo*. *Proceedings of the National Academy of Sciences*  
47 *of the United States of America*. 2009; **106**: 16511-16516
- 48 [33] Andradas C, Caffarel MM, Perez-Gomez E, *et al.* The orphan G protein-coupled receptor  
49 GPR55 promotes cancer cell proliferation via ERK. *Oncogene*. 2011; **30**: 245-252
- 50 [34] Pineiro R, Maffucci T, Falasca M. The putative cannabinoid receptor GPR55 defines a novel  
51 autocrine loop in cancer cell proliferation. *Oncogene*. 2011; **30**: 142-152
- 52  
53  
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57  
58  
59  
60

- 1  
2  
3 [35] Burns CJ, Squires PE, Persaud SJ. Signaling through the p38 and p42/44 mitogen-activated  
4 families of protein kinases in pancreatic beta-cell proliferation. *Biochemical and biophysical research*  
5 *communications*. 2000; **268**: 541-546
- 6 [36] Jhala US, Canettieri G, Sreaton RA, *et al.* cAMP promotes pancreatic beta-cell survival via  
7 CREB-mediated induction of IRS2. *Genes & development*. 2003; **17**: 1575-1580
- 8 [37] Bowe JE, King AJ, Kinsey-Jones JS, *et al.* Kisspeptin stimulation of insulin secretion:  
9 mechanisms of action in mouse islets and rats. *Diabetologia*. 2009; **52**: 855-862
- 10 [38] Huang GC, Zhao M, Jones P, *et al.* The development of new density gradient media for  
11 purifying human islets and islet-quality assessments. *Transplantation*. 2004; **77**: 143-145
- 12 [39] Pingitore A, Chambers ES, Hill T, *et al.* The diet-derived short chain fatty acid propionate  
13 improves beta-cell function in humans and stimulates insulin secretion from human islets in vitro.  
14 *Diabetes, obesity & metabolism*. 2017; **19**: 257-265
- 15 [40] Gey GO GM. The maintenance of human normal cells and tumor cells in continuous culture:  
16 I. Preliminary report: cultivation of mesoblastic tumors and normal tissue and notes on methods of  
17 cultivation. . *Am J Cancer* 1936; **27:45–76**:
- 18 [41] Jones PM, Salmon DM, Howell SL. Protein phosphorylation in electrically permeabilized islets  
19 of Langerhans. Effects of Ca<sup>2+</sup>, cyclic AMP, a phorbol ester and noradrenaline. *The Biochemical*  
20 *journal*. 1988; **254**: 397-403
- 21 [42] Ramracheya RD, Muller DS, Squires PE, *et al.* Function and expression of melatonin receptors  
22 on human pancreatic islets. *Journal of pineal research*. 2008; **44**: 273-279
- 23 [43] Barbosa-Sampaio HC, Liu B, Drynda R, *et al.* Nupr1 deletion protects against glucose  
24 intolerance by increasing beta cell mass. *Diabetologia*. 2013; **56**: 2477-2486
- 25 [44] Barbosa-Sampaio HC, Drynda R, Liu B, *et al.* Reduced nuclear protein 1 expression improves  
26 insulin sensitivity and protects against diet-induced glucose intolerance through up-regulation of  
27 heat shock protein 70. *Biochimica et biophysica acta*. 2015; **1852**: 962-969
- 28 [45] Mackie K. Cannabinoid receptors as therapeutic targets. *Annual review of pharmacology and*  
29 *toxicology*. 2006; **46**: 101-122
- 30 [46] Stone VM, Dhayal S, Smith DM, Lenaghan C, Brocklehurst KJ, Morgan NG. The cytoprotective  
31 effects of oleoylethanolamide in insulin-secreting cells do not require activation of GPR119. *British*  
32 *journal of pharmacology*. 2012; **165**: 2758-2770
- 33 [47] Bermudez-Silva FJ, Suarez J, Baixeras E, *et al.* Presence of functional cannabinoid receptors in  
34 human endocrine pancreas. *Diabetologia*. 2008; **51**: 476-487
- 35 [48] Li C, Jones PM, Persaud SJ. Role of the endocannabinoid system in food intake, energy  
36 homeostasis and regulation of the endocrine pancreas. *Pharmacology & therapeutics*. 2011; **129**:  
37 307-320
- 38 [49] Matias I, Bisogno T, Di Marzo V. Endogenous cannabinoids in the brain and peripheral  
39 tissues: regulation of their levels and control of food intake. *International journal of obesity*. 2006;  
40 **30 Suppl 1**: S7-S12
- 41 [50] Getty-Kaushik L, Richard AM, Deeney JT, Krawczyk S, Shirihai O, Corkey BE. The CB1  
42 antagonist rimonabant decreases insulin hypersecretion in rat pancreatic islets. *Obesity*. 2009; **17**:  
43 1856-1860
- 44 [51] Bermudez-Silva FJ, Romero-Zerbo SY, Haissaguerre M, *et al.* The cannabinoid CB1 receptor  
45 and mTORC1 signalling pathways interact to modulate glucose homeostasis in mice. *Disease models*  
46 *& mechanisms*. 2016; **9**: 51-61
- 47 [52] Li C, Jones PM, Persaud SJ. Cannabinoid receptors are coupled to stimulation of insulin  
48 secretion from mouse MIN6 beta-cells. *Cellular physiology and biochemistry : international journal*  
49 *of experimental cellular physiology, biochemistry, and pharmacology*. 2010; **26**: 187-196
- 50 [53] Console-Bram L, Brailoiu E, Brailoiu GC, Sharir H, Abood ME. Activation of GPR18 by  
51 cannabinoid compounds: a tale of biased agonism. *British journal of pharmacology*. 2014; **171**: 3908-  
52 3917
- 53  
54  
55  
56  
57  
58  
59  
60

- [54] Rajaraman G, Simcocks A, Hryciw DH, Hutchinson DS, McAinch AJ. G protein coupled receptor 18: A potential role for endocannabinoid signaling in metabolic dysfunction. *Molecular nutrition & food research*. 2016; **60**: 92-102
- [55] Kohno M, Hasegawa H, Inoue A, *et al.* Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18. *Biochemical and biophysical research communications*. 2006; **347**: 827-832
- [56] Ikeda Y, Iguchi H, Nakata M, *et al.* Identification of N-arachidonylglycine, U18666A, and 4-androstene-3,17-dione as novel insulin Secretagogues. *Biochemical and biophysical research communications*. 2005; **333**: 778-786
- [57] Fernandez-Ruiz J, Romero J, Velasco G, Tolon RM, Ramos JA, Guzman M. Cannabinoid CB2 receptor: a new target for controlling neural cell survival? *Trends in pharmacological sciences*. 2007; **28**: 39-45
- [58] Mukhopadhyay P, Rajesh M, Pan H, *et al.* Cannabinoid-2 receptor limits inflammation, oxidative/nitrosative stress, and cell death in nephropathy. *Free radical biology & medicine*. 2010; **48**: 457-467
- [59] Horvath B, Magid L, Mukhopadhyay P, *et al.* A new cannabinoid CB2 receptor agonist HU-910 attenuates oxidative stress, inflammation and cell death associated with hepatic ischaemia/reperfusion injury. *British journal of pharmacology*. 2012; **165**: 2462-2478
- [60] Vilches-Flores A, Hauge-Evans AC, Jones PM, Persaud SJ. Chronic activation of cannabinoid receptors in vitro does not compromise mouse islet function. *Clinical science*. 2013; **124**: 467-478
- [61] Kim W, Doyle ME, Liu Z, *et al.* Cannabinoids inhibit insulin receptor signaling in pancreatic beta-cells. *Diabetes*. 2011; **60**: 1198-1209
- [62] Janiak P, Poirier B, Bidouard JP, *et al.* Blockade of cannabinoid CB1 receptors improves renal function, metabolic profile, and increased survival of obese Zucker rats. *Kidney international*. 2007; **72**: 1345-1357

### Figure Legends

#### FIGURE 1: Effects of Abn-CBD and LH-21 on dynamic insulin secretion from human and mouse islets.

Dynamic profiles of insulin secretion from islets isolated from human donors (A-D) and from GPR55<sup>+/+</sup> and GPR55<sup>-/-</sup> mice (E-K) in response to Abn-CBD and LH-21. The horizontal arrows indicate the duration of exposure to 2 mM and 20 mM glucose and the period of perfusion with Abn-CBD and LH-21. Data are expressed as mean + SEM of experiments with islets from 6 separate human donors or 5 independent experiments with mouse islets. ##p<0.01 and ###p<0.001: response of islets to 20 mM glucose vs. 2 mM glucose; \*\*\*p<0.001 and \*\*\*\*p<0.0001 vs. secretion in the presence of vehicle (experiments with human islets) ###p<0.01 and ####p<0.001 response of islets to 20 mM glucose vs. 2 mM glucose; \*\*p<0.01 and \*\*\*p<0.001 secretion from GPR55<sup>+/+</sup> vs. GPR55<sup>-/-</sup> islets (experiments with mice islets). Data were analysed by unpaired Student's t tests.

**FIGURE 2: Effects of Abn-CBD and LH-21 on  $[Ca^{2+}]_i$  and cAMP in mouse and human dispersed islets.**

Dynamic profiles of  $[Ca^{2+}]_i$  in Fura-2-loaded dispersed GPR55<sup>+/+</sup> (A, C), GPR55<sup>-/-</sup> (B, D) and human (E, F) islets. The horizontal arrows indicate the duration of exposure to 2 mM and 20 mM glucose and the period of perfusion with Abn-CBD, LH-21 and the sulphonylurea tolbutamide. Data of the 340/380 fluorescence ratios are expressed as mean + SEM; 30  $\beta$ -cells from 5 independent experiments with mouse islets and 8  $\beta$ -cells from 3 independent experiments with human islets. Intracellular cAMP levels in GPR55<sup>+/+</sup> (G, I), GPR55<sup>-/-</sup> (H, J) and human (K, L) islets. Data are expressed as mean + SEM of 6 replicates within individual experiments using one batch of human islets and islets from 2 GPR55<sup>+/+</sup> and 2 GPR55<sup>-/-</sup> mice. \*\*\*\*p<0.0001 extendin-4 vs. basal; #p<0.1 and ##p<0.01 clonidine vs. forskolin. Data were analysed by One-way ANOVA, Dunnett's multiple comparisons post test.

**FIGURE 3: Effects of Abn-CBD and LH-21 on mouse and human islet apoptosis.**

Effects of Abn-CBD and LH-21 on apoptosis of GPR55<sup>+/+</sup> (A) and GPR55<sup>-/-</sup> (B) islets and human (C) islets that had been maintained for 20 h in the absence or presence of a cytokine cocktail. Apoptosis was detected by luminescence assay of caspase 3/7 activities. Data are expressed as mean + SEM of 3 independent experiments for both mouse and human islets, each of 8-10 replicates. \*\*p<0.01, \*\*\*p<0.001, ns p>0.05. Data were analysed by one-way ANOVA with repeated measures followed by Tukey's multiple comparison post tests.

**FIGURE 4: Effects of Abn-CBD and LH-21 on  $\beta$ -cell proliferation in GPR55<sup>+/+</sup> and GPR55<sup>-/-</sup> mouse islets.**

Immunofluorescence of paraffin-embedded sections of islets from GPR55<sup>+/+</sup> or GPR55<sup>-/-</sup> mice after 5 days of exposure to 1mg/ml BrdU *in vitro* in the presence or absence of Abn-CBD or LH-21, and probed with antibodies directed against insulin (red) and BrdU (green). A) Images of representative immunostained islets; scale bar = 50  $\mu$ m. Post-acquisition analyses were performed

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3 with Image J® and are shown in panels B-E: B) number of BrdU and insulin positive cells per islet; C)  
4 number of  $\beta$ -cells (insulin positive cells) per islet; D) mean islet area ( $\mu\text{m}^2$ ); E) individual  $\beta$ -cell area,  
5 calculated by dividing the mean islet area ( $\mu\text{m}^2$ ) by the number of  $\beta$ -cells per islet. \* $p < 0.05$ , \*\* $p < 0.01$   
6 and \*\*\* $p < 0.001$ . In panel B and C, # $p < 0.05$  and ### $p < 0.001$  for Abn-CBD treated GPR55<sup>-/-</sup> islets vs. their  
7 vehicle counterparts. Data were obtained from multiple acquisitions of 45-75 islets per condition,  
8 each with a minimum of 6 paraffin sections for analysis. Data were analysed by one way ANOVA  
9 followed by Dunnett's (B) and Newman-Keuls (C-E) multiple comparison post tests.  
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20 **FIGURE 5. Effects of Abn-CBD and LH-21 on CREB and AKT phosphorylation in mouse and human**  
21 **islets.**  
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24 Blotting of mouse (A, B) and human (C, D) islet lysates with antibodies directed against phospho-  
25 CREB (P-CREB), CREB, phospho-AKT (P-AKT) and AKT, with the relative densitometric analyses of the  
26 immunoreactive proteins normalised as ratios between the phosphorylated and non-phosphorylated  
27 forms. Data in the graphs are shown as mean + SEM of 4 independent experiments with mouse and  
28 human islets, each with 200 islets per condition. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ . Results were  
29 analysed by one-way ANOVA with repeated measures followed by Dunnett's Multiple Comparison  
30 post test.  
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38 **SUPPLEMENTARY FIGURE 1. Structures of LH-21 (A) and Abn-CBD (B).**  
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Figure 1 (A-D)

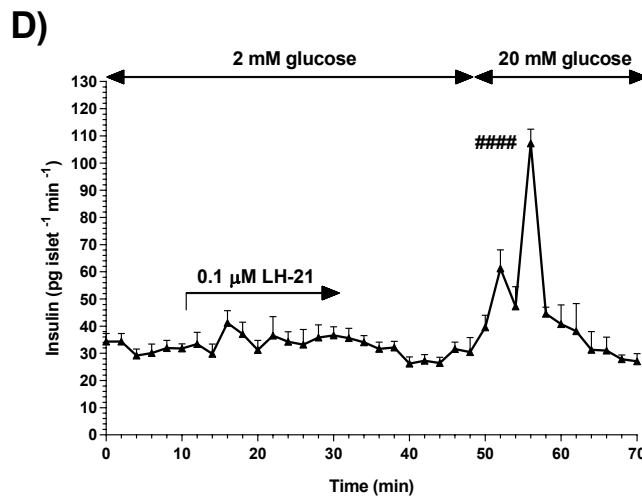
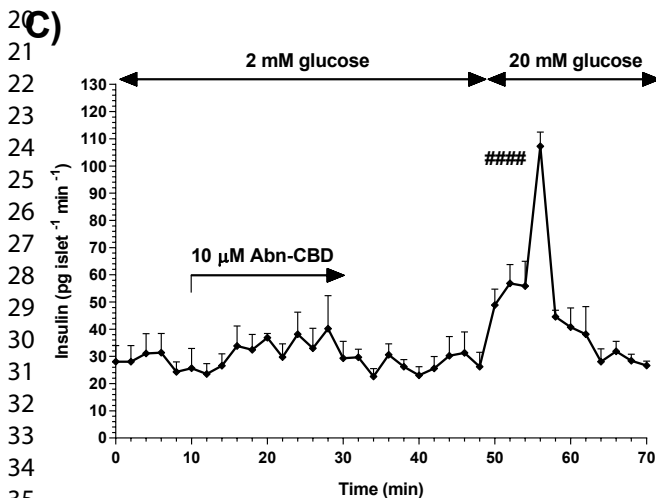
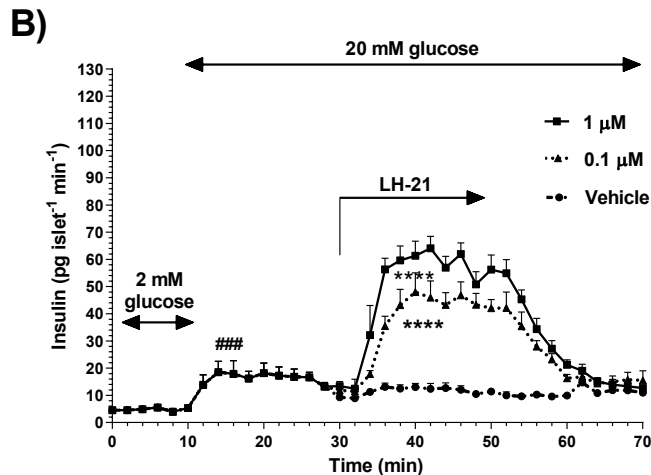
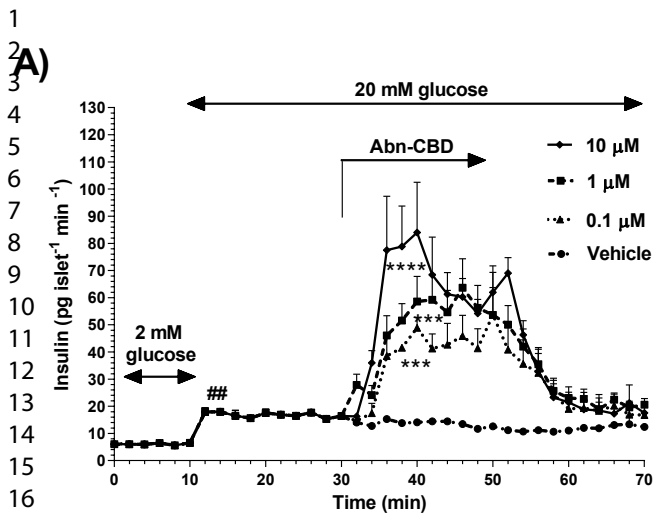
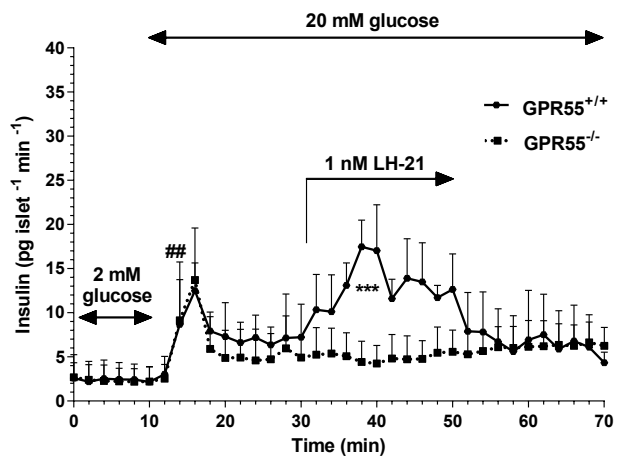




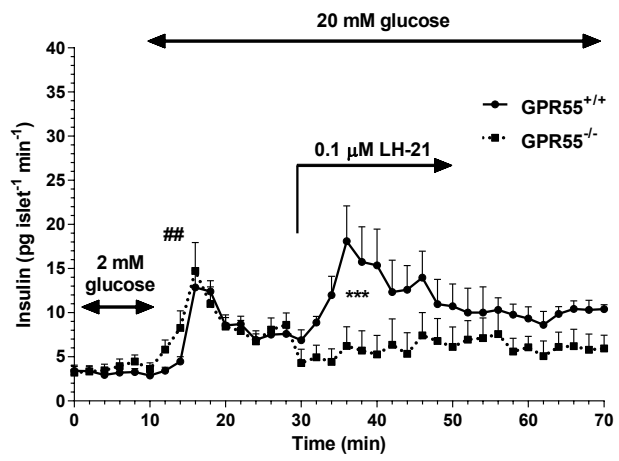
Figure 1 (H-K)

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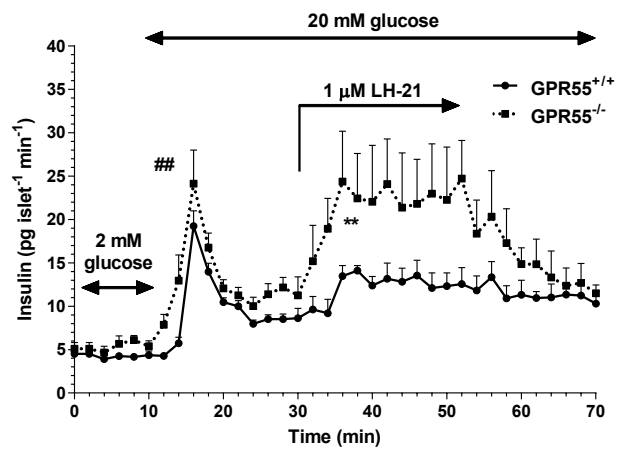
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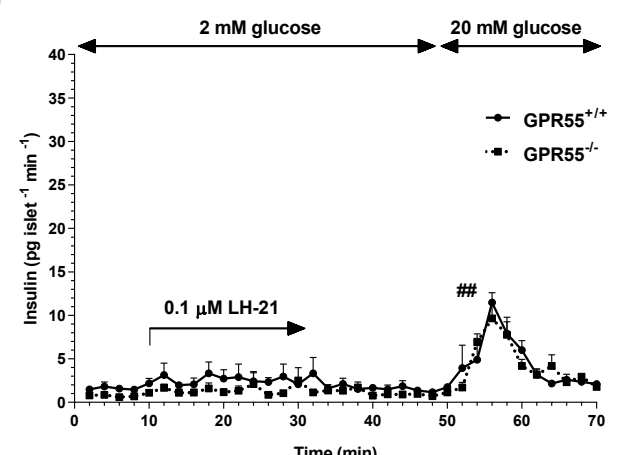
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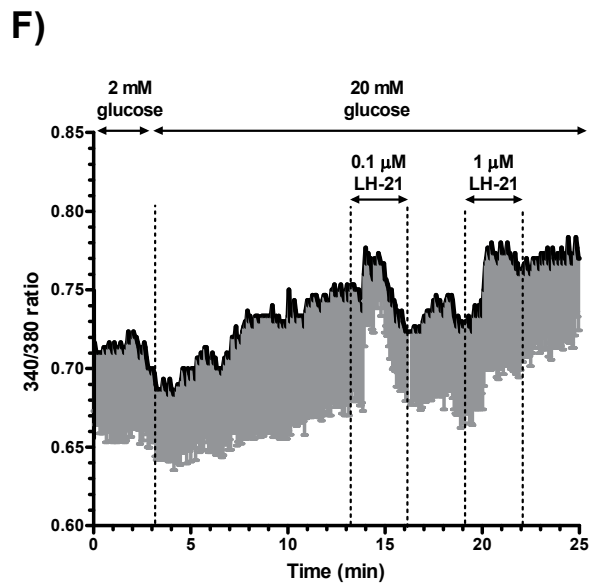
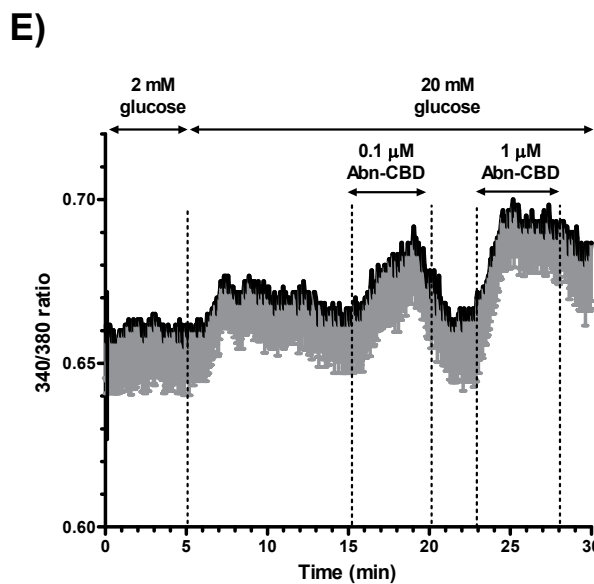
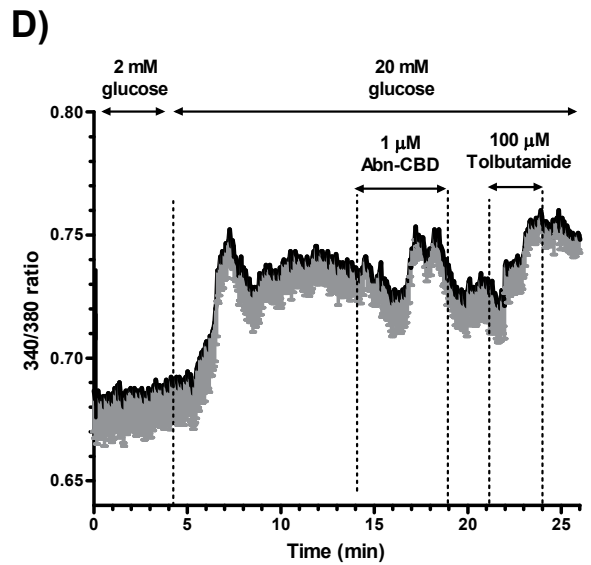
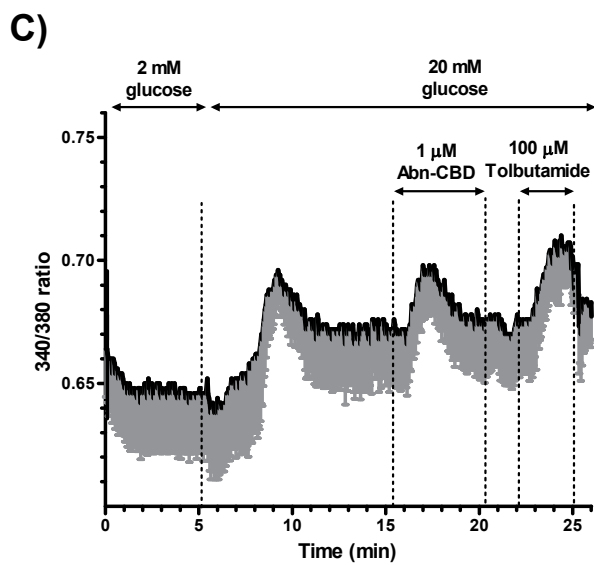
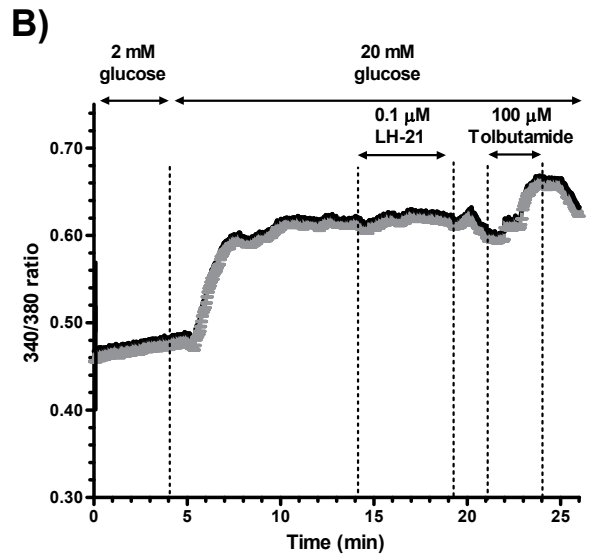
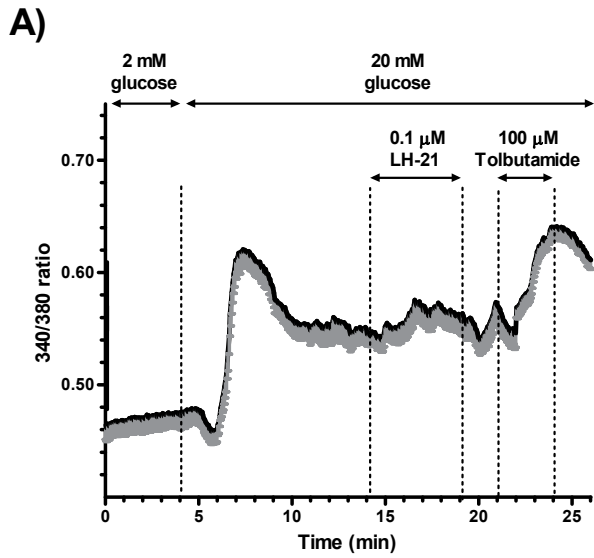
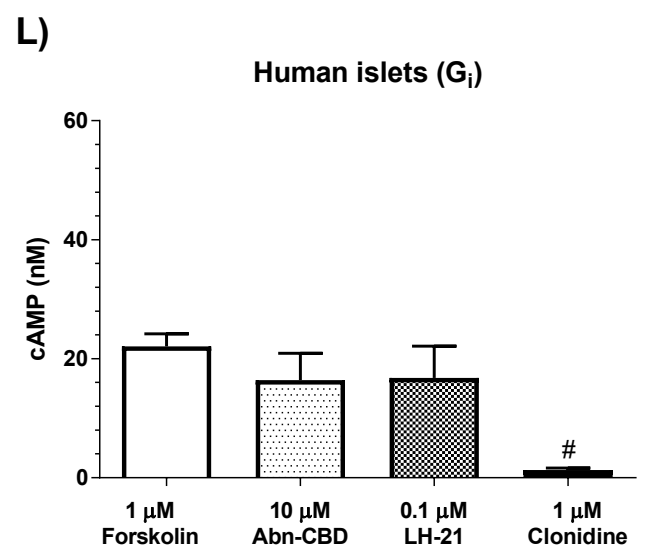
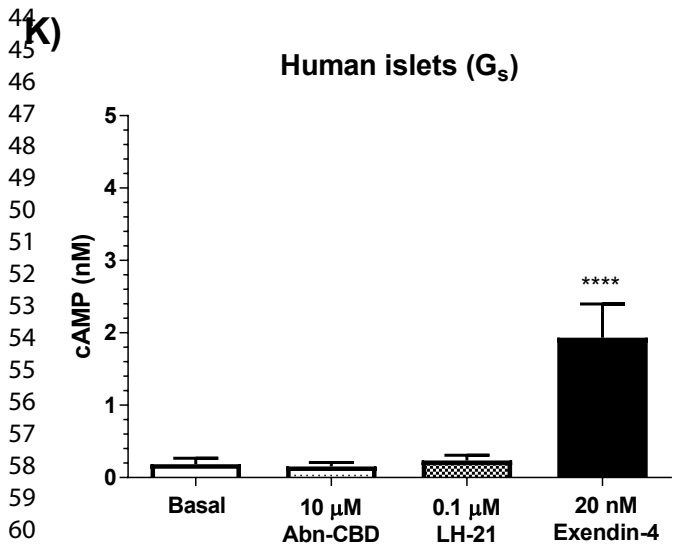
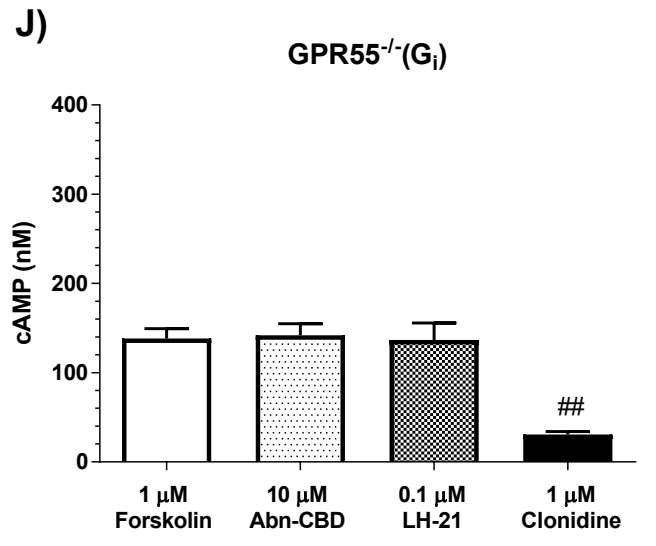
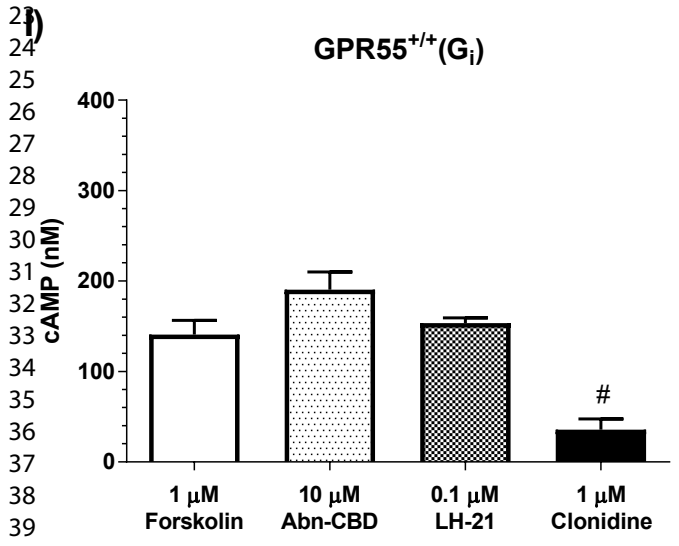
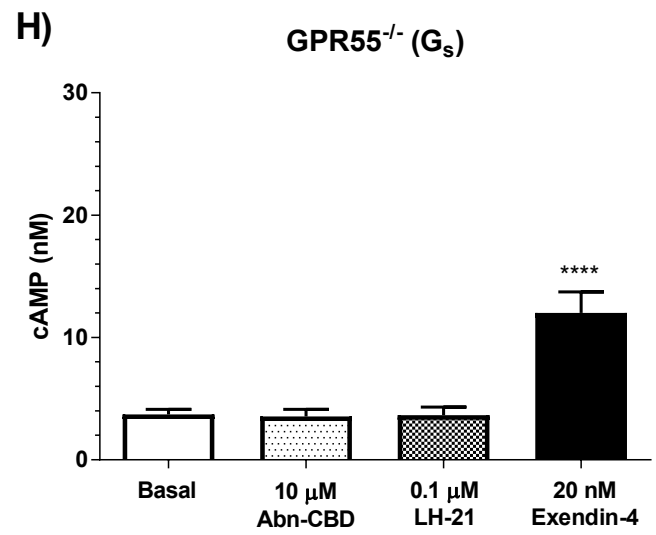
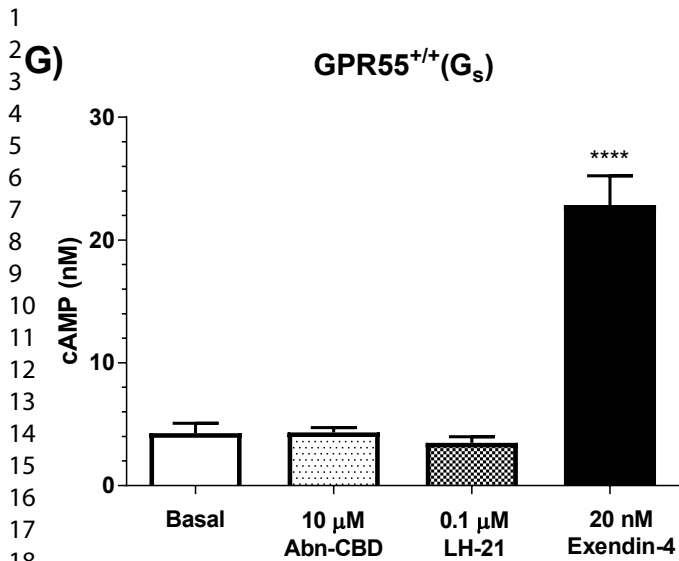
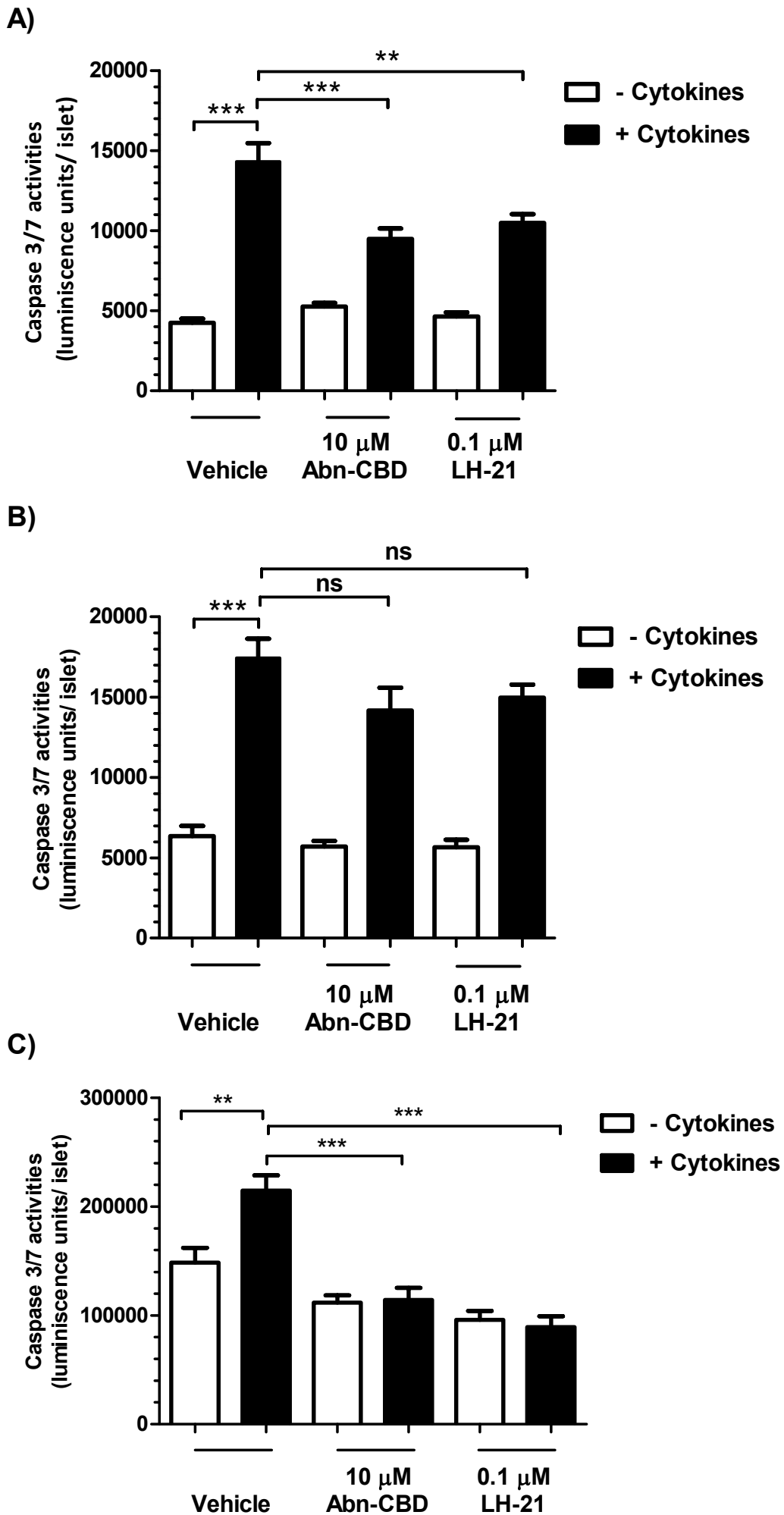


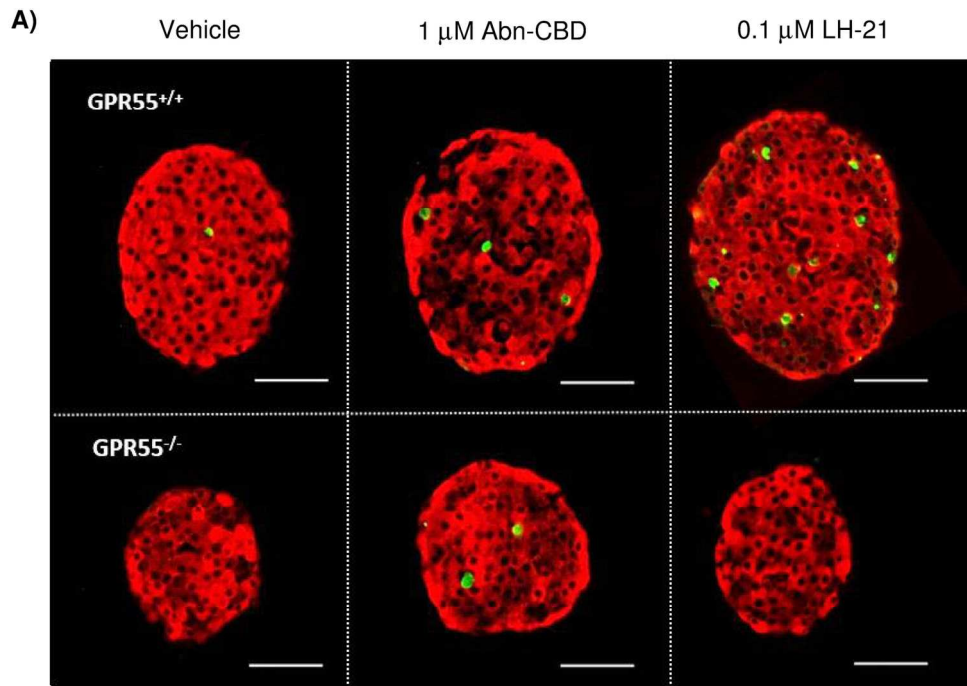
Figure 2 (G-L)





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Figure 4 (A)



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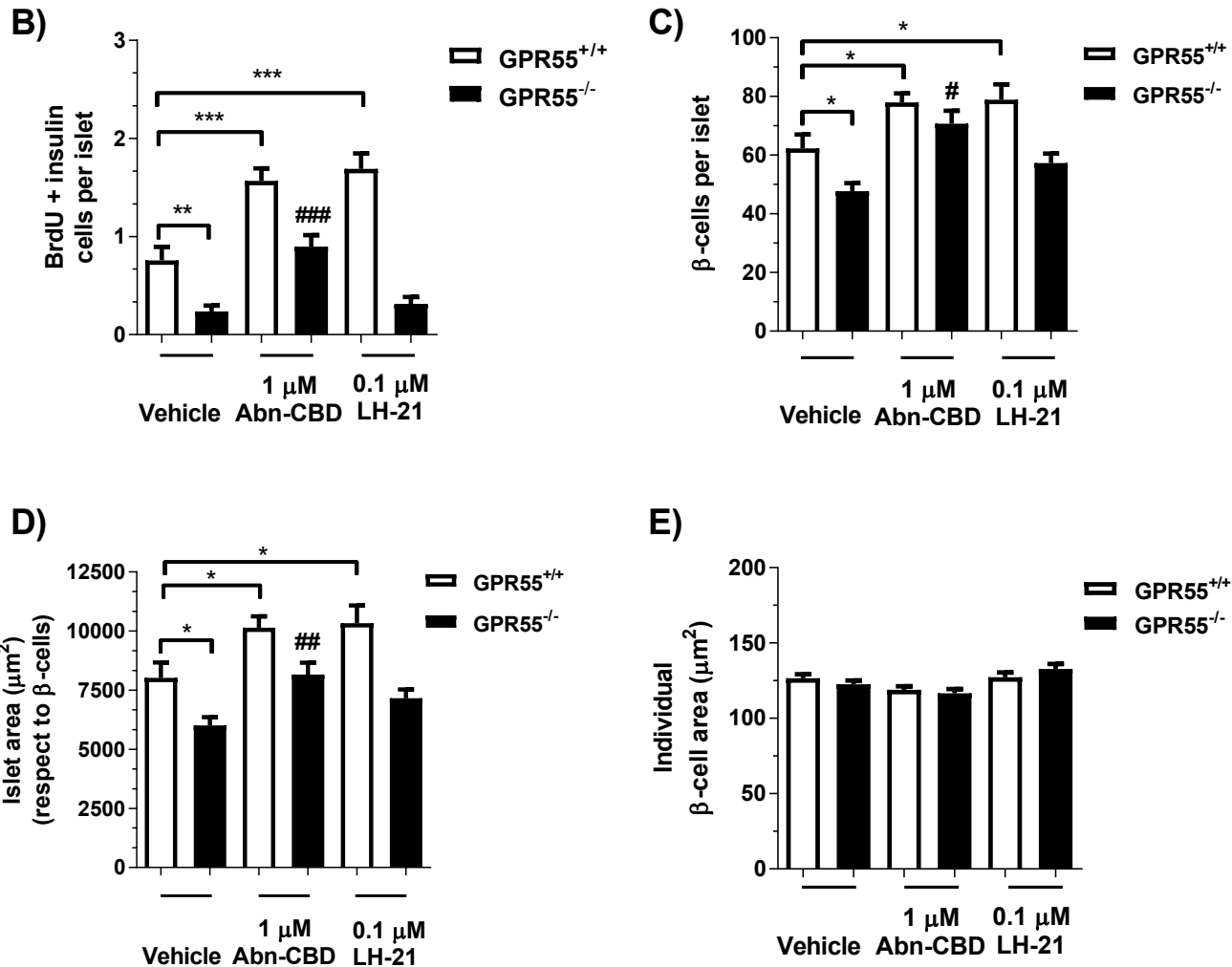
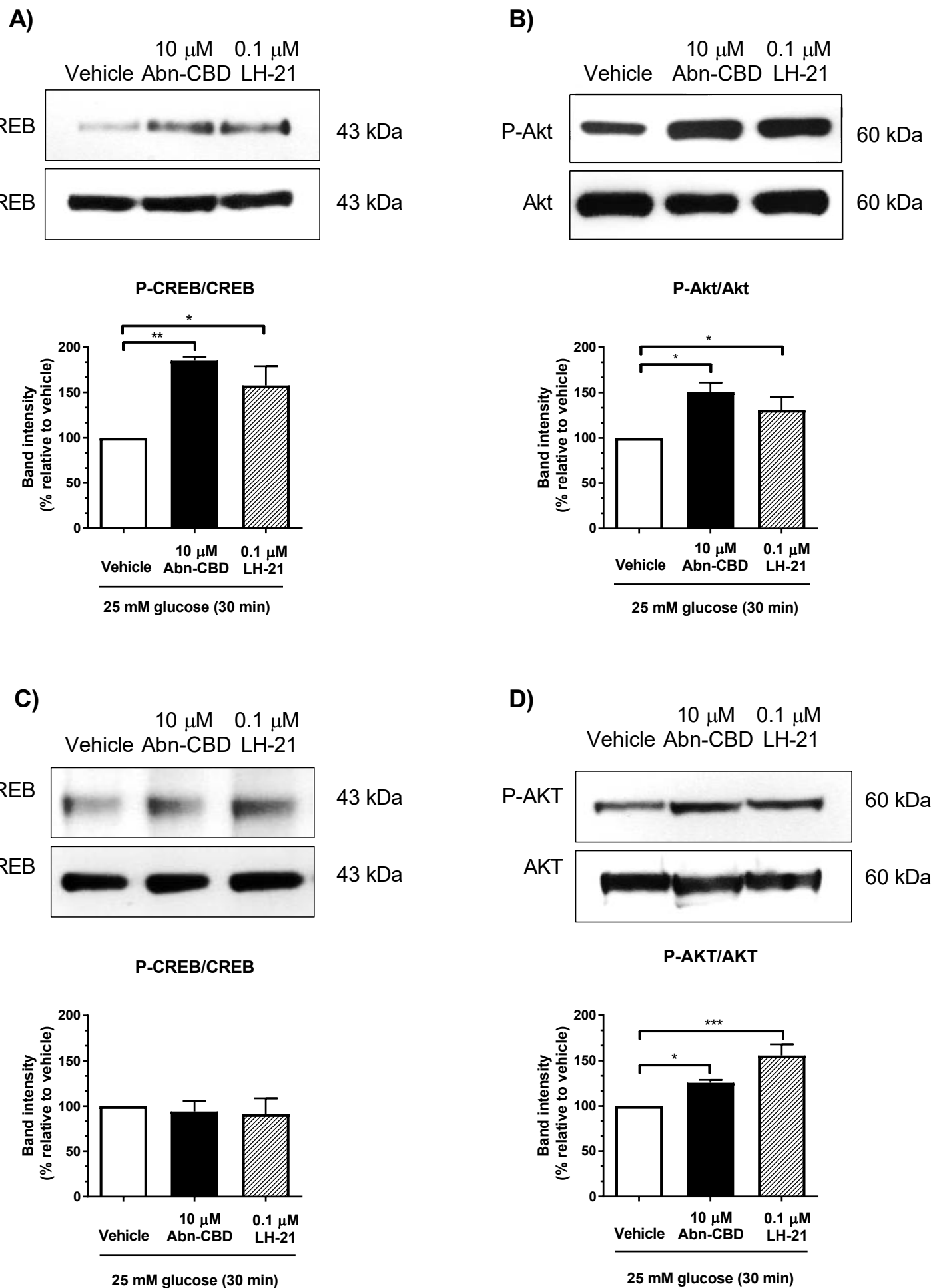


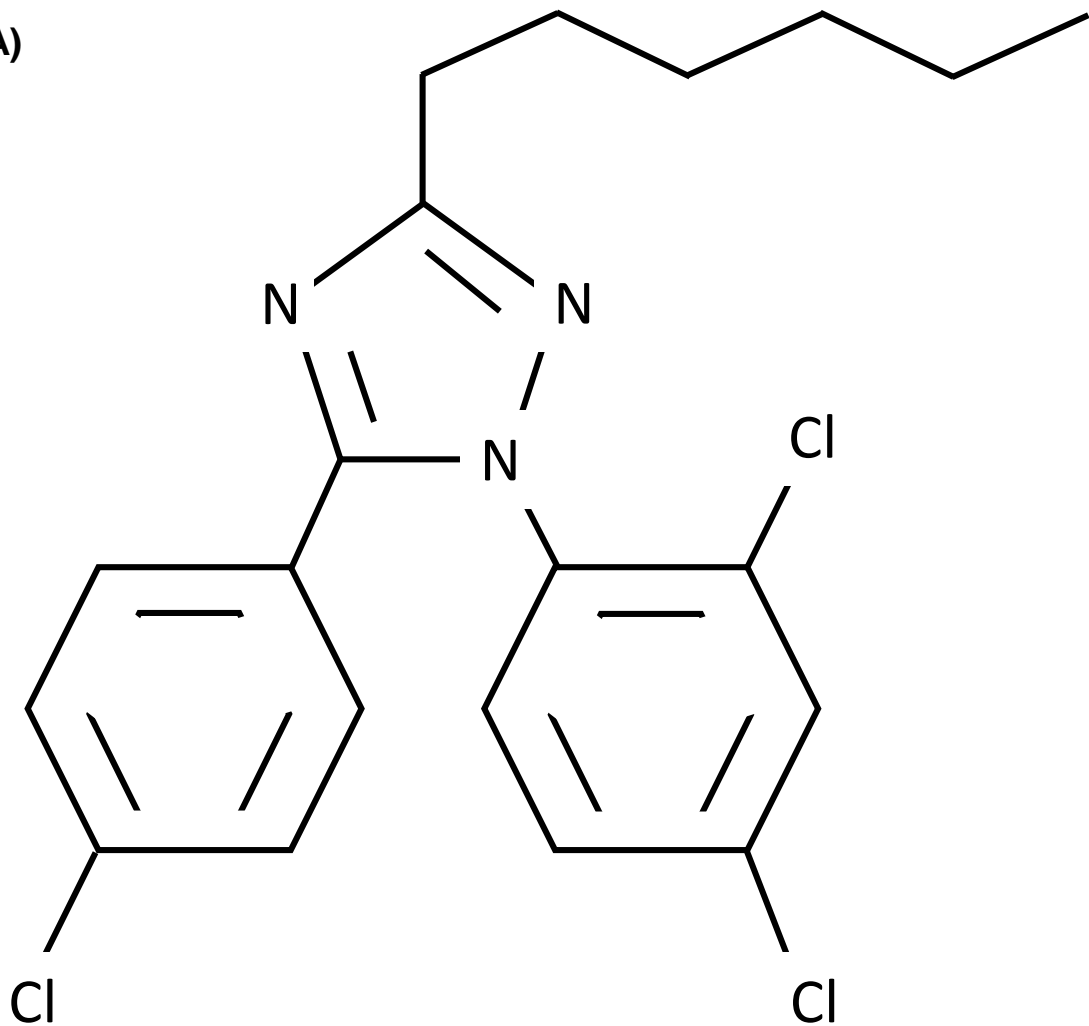


Figure 5

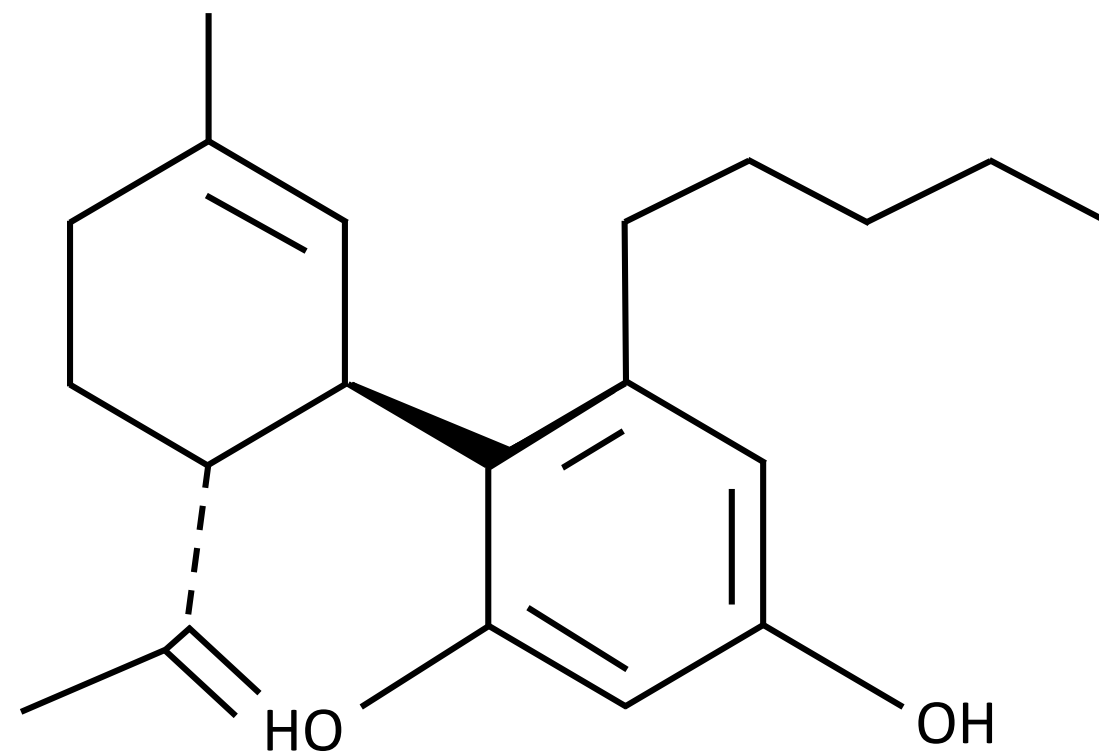


## Supplementary Figure 1

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## Responses to Referees' comments

### Referee: 1

No responses required.

### Referee: 2

We are pleased that the reviewer considered this to be “a comprehensive and carefully conducted pharmacological study on molecules targeting a medically relevant group of G protein coupled receptors”.

In response to the specific comments:

**1. The authors shall better introduce the two molecules. How were they identified and what kind of structure do they have?**

We have now modified the Introduction to provide the requested information on LH-21 and Abn-CBD (pages 4 and 5), and included a supplementary Figure of Abn-CBD and LH-21 structures.

**2. The authors shall explain why they used dispersed islets in calcium measurements.**

We have now provided this information in the Methods section (page 7).

**3. Figure 1: What are the effects of LH-21 and Abn-CBD on insulin release at low glucose concentrations?**

We have now carried out additional experiments to determine the effects of Abn-CBD and LH-21 on insulin secretion from perfused islets at a sub-stimulatory glucose concentration and have included these new data as panels C, D, G and K in Figure 1 of the revised manuscript, and commented on the results obtained (pages 9-10).

**4. Figure 1: The authors shall use a more consistent way to present error bars.**

We have now presented the error bars as +SEM for all 11 panels of perfusion data in Figure 1.

**5. Figure 3: Is it possible to show this Figure as dotplots? Or, alternatively, show SDs rather than SEMs?**

It is usual for measurements of islet caspase activities to be presented as bar charts rather than dot plots, and we think that the data in the three panels of Figure 3 clearly demonstrate the GPR55-dependent anti-apoptotic effects of LH-21 and Abn-CBD. However, we are happy to re-plot the data as dotplots if the Editor thinks that this is necessary.

**6. Figure 3C: Were the effects on human islets also observed in islets from a different human donor? Please indicate whether the results were replicated or not.**

Overall we used islets from 18 human donors for this study. The data shown in Figure 3C were obtained using islets from 3 separate donors. We have now modified the Figure legend to clarify this.

**7. Figure 4A: Please use a scale bar instead of 20x.**

We have now provided scale bars to the panels in Figure 4A, as requested.

**8. Figure 4B-D: Was the image analysis done in a blinded or not blinded manner? Please indicate.**

The image analysis was performed in a non-blinded manner by an experienced researcher. We have now modified the Methods section to clarify this (page 8).

**Referee: 3**

We are pleased that the referee considered that “The performed experiments are coherent with their purpose” and the article “is potentially of interest”. However, we recognise that the referee raised several concerns and we have responded to the comments below.

**The methods are not well described/some experimental procedures need to be clarified.**

We have modified the Methods section to provide some additional detail on the experimental procedures (pages 6-9), but we are limited by including everything in the word count that is available. We have provided reference to our previously published papers with additional detail on the methods used (refs 37, 38, 39, 41, 42, 43 and 44).

**It is unclear when the experiment was done on islets to know if more than one mouse has been used/the number of individual donor for experiments using human islets.**

Experiments were performed on islets from multiple mice and human donors. We have indicated in the Methods section that approximately 200 islets were obtained per mouse pancreas, and that we obtained islets from 18 human donors (page 7). Information has been provided in the Figure legends to clarify the numbers replicates of experiments performed.

**The authors have reported that the GPR55 selective drug Abn-CBD potentiates insulin secretion in mouse islets (fig 1C and D), but there is no explanation for the results obtained from GPR55<sup>-/-</sup> islets. It is unclear why the drug is acting. This result does not strengthen their demonstration about **LH-21.****

We agree that our observation of enhanced glucose-induced insulin secretion in response to 10 $\mu$ M Abn-CBD in islets from GPR55<sup>-/-</sup> mice (Figure 1D) was unexpected and our data have demonstrated that it is not a GPR55-selective drug. We have considered in the Discussion whether the GPR55-independent effects of Abn-CBD are mediated by GPR18, which is also expressed by islets (page 14).

**The results given in figure 2 are obtained using Fura2 fluorescence recording, but it is surprising to get such small variation in signal. Base lines are not always stable and some other, more appropriate, (exendinIV rather than tolbutamide) positive control should have been done.**

The data presented in the panels in Figure 2 show mean-SEM data for Fura-2 fluorescence ratios. The small variation in signal is most apparent when we were recording from numerous islet cells (n=30 cells for panels A and B), with larger variability, as expected when fewer cells were used (n=8 cells for panels E and F). We do not think that exendin-4 is a superior positive control to tolbutamide as the sulphonylurea directly depolarises  $\beta$ -cells through closure of  $K_{ATP}$  channels to elevate  $[Ca^{2+}]_i$  while exendin 4 is G<sub>s</sub>-coupled to promote cAMP elevation.

**In experiments performed in order to analyze b-cell proliferation, the authors have showed that Abn-CBD effect is not fully abolish on GPR55<sup>-/-</sup> mice. Again this point is puzzling. It is not obvious why the authors have chosen to focus on LH-21 instead of the reported GPR55 selective agonist Abn-CBD.**

We are not sure of the precise point being made by the referee here, as we think that we focused equally on Abn-CBD and LH-21. The ability of Abn-CBD to significantly promote BrdU incorporation into  $\beta$ -cells of GPR55<sup>-/-</sup> mice is unexpected for an agonist that is considered to be selective for GPR55, but these are the data that we obtained and they clearly indicate that Abn-CBD has GPR55-independent effects. We have expanded the Discussion section to comment on this (page 15).

**In signaling experiments performed on mice and human islets, the authors have focused on P-**

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3 **CREB and P-Akt. Fig 5B and 5C are puzzling. In fig 5B the vehicle control is not negative, this results**  
4 **may explain the lack of clear activation in mouse. Also in fig 5C it is surprising that P-CREB is not**  
5 **stimulated, a positive control should have been done.**

6 We agree with the referee that the Akt phosphorylation induced by 25mM glucose alone (vehicle  
7 control) in Figure 5B made it difficult to determine whether Abn-CBD or LH-21 had stimulatory  
8 effects. We have carried out four separate experiments using mouse islets and analysis of all of  
9 these experiments has indicated a significant elevation in Akt phosphorylation by Abn-CBD and LH-  
10 21. We have now included a more representative western blot in Figure 5B. However, the lack of  
11 stimulation of CREB phosphorylation by Abn-CBD and LH-21 was evident in experiments using  
12 human islets from four different donors. We think that the experimental protocol was appropriate  
13 since CREB phosphorylation was evident using the phospho-CREB antibody, but enhanced  
14 phosphorylation was not observed with human islets (Figure 5C) as it was for mouse islets (Figure  
15 5A).

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18 **Using a GPR55's selective drug as control, instead of Abn-CBD, should have been a better choice.**

19 One of the aims of the current study was to determine whether Abn-CBD could be considered to be  
20 a selective GPR55 agonist in islets and this would not have been possible if we had used a GPR55-  
21 selective drug. In fact, we have recently reported that O-1602 is a selective agonist for GPR55 (Liu *et*  
22 *al.* Diabetes, Obesity & Metabolism 2016; **18**: 1263-1273; reference 14 in the current manuscript),  
23 and it would not have been appropriate to replicate the experiments that we have already  
24 published.

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28 **Referee: 4**

29 We are pleased that the referee thought that "The present study showed a specific focus" and raised  
30 only minor comments.

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32 In response to the specific comments:

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34 **Calcium-related downstream pathways, such as CAMKII could be studied in LH-21 and Abn-CBD**  
35 **treated GPR55 +/+ or -/- islets.**

36 We agree that it would be interesting to define the pathways downstream of elevations in  
37 intracellular calcium but this is outside of the scope of the current study, which already contains five  
38 multi-panelled figures (and now with additional data as indicated in the responses to all referees).

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40 **What is the intracellular cAMP response to LH-1 and Abn-CBD in GPR55 +/+ and -/- islets?**

41 We have now carried out additional experiments to determine the effects of LH-21 and Abn-CBD on  
42 generation of cAMP in islets from WT and GPR55<sup>-/-</sup> mice and in islets from human donors. These data  
43 have been included as panels G-L of Figure 2, and have been commented on in the text on (page 11).

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45  
46 **It would be better to add "ex vitro" or "isolated islets" in the title. Otherwise, readers may expect**  
47 **the whole body glucose metabolism effect of LH-21 and Abn-CBD, and their relations with GPR55**  
48 **in the present study. In addition, by stating the ex vivo, it will be more clear that the effect is**  
49 **directly on pancreatic islets, but not secondary effect from the improvement of glucose**  
50 **homeostasis.**

51 We agree that it is important to indicate in the title that this work relates to experiments carried out  
52 *ex vivo* so we have now modified the title to reflect that the work was performed using isolated  
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