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Identification of a subset of trace amine-associated receptors and ligands as potential modulators of insulin secretion

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ABSTRACT

The worldwide prevalence of diabetes has reached 8.5% among adults, and this is characterised by elevated glucose concentrations and failing insulin secretion. Furthermore, most people with type 2 diabetes are either obese or overweight, with the associated dyslipidaemia contributing to the development of insulin resistance and increased cardiovascular risk. Here we incubated INS-1 pancreatic β-cells for 72 h in RPMI-1640 media, or media supplemented with 28 mM glucose, 200 μM palmitic acid, and 200 μM oleic acid as a cellular model of diabetic glucolipotoxicity. Illumina HiSeq gene expression analysis showed the trace amine-associated receptor (TAAR) family to be among the most highly downregulated by glucolipotoxicity. Importantly, MetaCore integrated knowledge database, from Clarivate Analytics, indicated potential TAAR impact on insulin secretion through adenylyl cyclase signalling pathways. We therefore investigated the effect of TAAR ligands on cAMP signalling and insulin secretion, and found that only the branch of the TAAR family tree that is activated by isopentylamine, 2-phenylethylamine, *p*-tyramine, and agmatine significantly increased intracellular cAMP and resulted in increased insulin secretion from INS-1 cells and primary mouse islets under normal conditions. Crucially however, this enhancement was not evident when the receptor family was downregulated by glucolipotoxic conditions. This data indicates that a subset of TAARs are regulators of insulin secretion in pancreatic β-cells, and that their downregulation contributes to glucolipotoxic inhibition of insulin secretion. As such they may be potential targets for treatment of type 2 diabetes.

1. Introduction

Over 80% of patients with type 2 diabetes (T2D) are either overweight or obese, and typically this is associated with insulin resistance. Consequently, these patients have both elevated blood glucose and free fatty acid concentrations [1]. It is known that chronic hyperglycaemia inhibits stimulated insulin secretion, but it is the combination of both high sugar and high fat that has the largest detrimental effect on β-cell function [2]. We previously employed microarray technology to investigate the effect of glucolipotoxicity upon INS-1 pancreatic β-cell gene expression, and found a wide spectrum of damaging effects including diminished insulin secretion, chronic inflammation, increased apoptosis, biological oxidation, and dysregulated nucleic acid processing and repair [3]. Furthermore, based on ranked statistical significance of enrichment following MetaCore integrated knowledge database analysis (https://clarivate.com/products/metacore) of that dataset, we also demonstrated the presence of disease association with both endocrine and metabolic disorders, which indicates the presence of pathways in INS-1 cells with known T2D and obesity aetiology in man [3].

Although microarray technology has proven successful, the development of next generation sequencing (RNAseq) technology offers a number of advantages over microarray approaches. For example, microarray analysis is limited by the need to rely upon existing knowledge of genome sequence, they have high background levels due to cross-hybridization, and a limited dynamic range of detection due to both background and saturation of signals. By contrast, next generation sequencing strategies analyse total amount of reads that map to the exons of a gene, normalised by the length of the exons, and do not have an upper limit for quantification [4]. The presence and amount of each RNA can therefore be calculated and subsequently compared with the amount in any other sequenced sample. In addition, RNAseq data also have

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high levels of reproducibility for both technical (repeated measurements of the same sample) and biological (parallel measurements of biologically distinct samples) replicates.

Building from our previous work [2,3], we utilised Illumina HiSeq gene expression analysis to more accurately define the pathophysiology of type 2 diabetes, with a view to identifying potential new targets for therapeutic intervention. Data presented here indicate that downregulation of trace amine-associated receptor signalling contributes to the failure of insulin secretion that results from chronic exposure of pancreatic β-cells to high concentrations of glucose and fatty acids. Furthermore, under control conditions, only stimulation of family members associated with one specific arm of the TAAR phylogenic tree enhanced insulin secretion. We propose that future studies focus on this specific subset of family members as potential new therapeutic targets for treatment of type 2 diabetes.

2. Materials and methods

2.1. Materials

Antibodies were obtained from Abcam (Cambridge, UK) and Agilent Technologies (Santa Clara, CA, USA). Unless otherwise stated, all other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) or VWR International Ltd (Lutterworth, UK).

2.2. Islet isolation and INS-1 β-cell culture

Islets were isolated from male CD1 mice by collagenase injection into the pancreatic duct. Digested pancreas was washed with MEM-2279 and separated from exocrine tissues by centrifuging through a Histopaque 1.077 g/ml gradient. After washing, islets were picked and incubated at 37 °C in RPMI-1640 (supplemented with 10% [vol/vol] fetal calf serum, 2 mM glutamine and 100U/ml penicillin/ 0.1 mg/ml streptomycin) for 24 h prior to further analysis. INS-1 rat pancreatic β-cells were cultured in RPMI-1640 media, or RPMI media supplemented with 28 mM glucose, 200 μM oleic acid, and 200 μM palmitic acid (GLT media), for 72 h as detailed previously [2]. All animal procedures were approved by the King's College London Ethics Committee and carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

2.3. Cell viability

INS-1 cells were cultured in either RPMI 1640 or GLT media for 72 h. Media was aspirated and cells washed three times in modified Krebs-Ringer solution (KRB) (125 mM NaCl, 1.2 mM KH_2PO_4, 5 mM KCl, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 1.67 mM glucose, 0.1% BSA, 25 mM HEPES, pH7.4). Cells were then incubated in KRB media supplemented with a final concentration of 5 μM Calcein AM Cell Viability Dye (ThermoFisher) for 1 h, before being washed in KRB for a final time. Cell viability was measured via fluorescence, with excitation and emission at 490 nm and 520 nm respectively.

2.4. RNA isolation

Cells were incubated in the appropriate conditions for 72 h, harvested and RNA extracted using an RNA isolation kit (Life Technologies, UK). RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA), with all samples analysed in this study having a RIN score >8. Samples for transcriptome analysis were prepared using a Truseq™ RNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's recommendations. Briefly, mRNA was isolated from >5 μg of total RNA using oligo (dT) magnetic beads. mRNA was cut into short fragments by adding fragmentation buffer. First-strand cDNA was synthesized using random hexamer-primers, taking these short fragments as templates. RNaseH, buffer, dNTPs, and DNA polymerase I was used to synthesize second-strand cDNA. Short fragments were purified with Takara's PCR extraction kit (Takara Bio, Inc.). Sequencing adapters were ligated to short fragments and resolved by agarose gel electrophoresis. Fragments were then selected, purified, and subsequently PCR amplified to create the final cDNA library template.

2.5. Next generation sequencing

Library was generated from the fragmentation of mRNA in small pieces using divalent cations under elevated temperature, with the fragments converted to first strand cDNA using reverse transcriptase and random primers. Subsequently the RNA template was removed and a replacement cDNA strand synthesized, generating a double strand-cDNA. The fragments were then end-repaired, in a process that converts the overhangs into blunt ends, using T4 DNA polymerase and Klenow DNA polymerase. Adapters were then ligated to the DNA fragments, preparing them to be hybridized to a single read flow cell. cDNA samples were purified on a gel to select a size range of templates (around 200 bp) for downstream enrichment. The purified cDNA templates were enriched using PCR to amplify the cDNA in the library, using primers that anneal to the ends of the adapters. The library was validated by performing a quality control analysis to quantify DNA concentration using Agilent Bioanalyzer. Library was then used to perform paired end sequencing over one lane of a flow cell on an Illumina HiSeq™ 2000 instrument at a read depth of ~65 million reads per sample.

2.6. Expression analysis

In the sequencing process, the Illumina instrument produced quality-scored base calls. The sequencing output files (compressed FASTQ files) were then used for the secondary analysis.

Reads were aligned to an annotated reference genome using Top Hat v 2.0.9: http://tophat.cbcb.umd.edu. Reads aligned to exons, genes and splice junctions are counted using the reference genome "rn4", extracted from UCSC (http://genome.ucsc.edu/goldenPath/credits.html#rat_credits). Data visualisation and interpretation, calculating gene as well as transcript expression, then reporting differential expression, was done using the HTseq-count program:

(http://www-huber.embl.de/users/anders/HTSeq/doc/count.html). Normalization, which corrects for in-sample distributional differences within the read counts was performed using the program DEseq:

(http://www.bioconductor.org/packages/devel/bioc/html/DESeq.html).

Statistical significance of fold changes was calculated by comparing the experimental read values to the control samples, with p values adjusted using the Bonferroni formula.

2.7. Pathway identification and visualization

For comprehensive analysis of biological pathways in which transcripts were differentially expressed we combined expression data from the next generation sequencing analysis with information in Reactome version 29, (http://www.reactome.org), a knowledgebase of biological pathways [5]. In order to visualize the networks we used MetaCore version 6.34, build 69200 from Clarivate Analytics (http://genego.com), an integrated knowledge-based platform for pathway analysis. Data analysis tools were used to generate high-resolution images of networks, with details of network objects and interactions provided in the Metacore legend.

2.8. Quantitative RT-PCR

INS-1 cells were trypsinised, washed with cold phosphate-buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 1 mM Na_2HPO_4, 0.2 mM KH_2PO_4, and pH7.4) and lysed in RNAeasy QTL lysis buffer. Total RNA was prepared from lysed cells using RNeasy kit (Qiagen, Hilden, Germany) according to manufacturers recommended procedures (Qiagen). cDNA was generated from RNA using a standard RT kit (Promega, Madison, WI, USA). qRT-PCR reactions were performed using Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Scientific, Loughborough, UK) and the following primers: *TAAR1 forward* GAAGGAGTTGAGGAGCAGTATC; *TAAR1 reverse* AAGACGTCATGAATGCCAGTA; *TAAR2 forward* AATGACTATTCCGGTCGTCAAG; *TAAR2 reverse* CCATCAGCATAAGCCTCTGAA; TAAR3 forward GGTATGCAGAGCTACGAGATTC; *TAAR3 reverse* CATGATGGAGCCAGGAGTAAA; *TAAR4 forward* CATGATCGGAGCGATAGTGATG; *TAAR4 reverse* GTGGTAGCCATGGAGAGAATAAG; *TAAR5 forward* CTCCAAGTTCACAGTCAGGATAG; *TAAR5 reverse* GTAGAGAAAGAAGGCAGTGTAGG; *TAAR6 forward* CAGGAATTTGCATCAGCATCTC; *TAAR6 reverse* CTCCAGGCCATCAGCATAAA; *TAAR7b forward* TCTGCTCTCAGCCTCATCTA; *TAAR7b reverse* CCAAAGCCAAAGACTGCATAAA; *TAAR9 forward* CCAAGTTCACCATTTCGGTTTC; *TAAR9 reverse* CTCGTTGGCTCCTGTGTAAA; *GAPDH forward* CATCTCCCTCACAATTCCATCC; *GAPDH reverse* GAGGGTGCAGCGAACTTTAT. Due to the high homology between TAAR7 and TAAR8 only one primer pair (TAAR7) was designed to amplify these family members [6]. Target gene mRNA was quantified and normalised for GAPDH using an ABI7700 bioanalyzer (Applied Biosystems, Foster City, CA, USA). Data were analysed using the $\Delta\Delta_{CT}$method.

2.9. Western blotting

INS-1 cells were lysed, protein separated by SDS-PAGE, then transferred to nitrocellulose as described previously [2]. Protein was detected using anti-TAAR1 (Abcam, Cambridge, UK) primary antibody and polyclonal goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Agilent Technologies, Santa Clara, CA, USA).

2.10. cAMP determination

INS-1 cells were treated with TAAR ligands (either isopentylamine, *p*-tyramine, 2-phenylethylamine, agmatine, *N,N*-dimethylcyclohexylamine, *N,N*-dimethyloctylamine, or the inverse agonist EPPTB) suspended in KRB plus 0.5 mM IBMX for half an hour prior to cAMP determination. Cells were then lysed in 0.1 M HCl according to manufacturer's protocol, and cAMP accumulation level determined using cAMP Select ELISA kit (Cayman Chemical Company, Ann Arbour, MI, USA).

2.11. Insulin secretion

INS-1 cells were treated with KRB or KRB supplemented with secretagogue cocktail (13.5 mM glucose, 1 μM phorbol 12-myristate 13-acetate, 1 mM isobutyl-methylxanthine, 1 mM tolbutamide, 10 mM leucine, 10 mM glutamine) for 2 h prior to determination. Supernatant was removed and insulin secretion was determined using an ELISA kit as detailed previously [2]. Size-matched islets were pre-incubated for 1 h at 37 °C in buffer containing 2 mM glucose, 2 mM $CaCl_2$ and 0.5 mg/ml BSA, pH 7.4. Islets were further incubated in buffer containing 2 or 20 mM glucose for 1 h ± stated amine concentration at 37 °C with gentle shaking. Insulin secretion was measured by radioimmunoassay with an in-house ^{125}I-labelled insulin tracer as previously detailed [7].

2.12. Statistical analysis

Results are expressed as mean ± standard error of the mean (n = 3 or more independent experiments). Parameters were compared using one-tailed student *t*-test assuming equal variance, with statistical significance determined using an alpha value of 5%. A *p* value below 0.05 was therefore considered to be statistically significant.

3. Results

3.1. Impact of glucolipotoxicity upon β-cell protein classes

In order to better understand the mechanisms by which chronic exposure to high glucose and fatty acids alters cellular function, we incubated INS-1 rat pancreatic β-cells for 72 h in RPMI-1640 media, or media supplemented with 28 mM glucose, 200 μM oleic acid, and 200 μM palmitic acid (GLT media), conditions previously characterised for the study of the effects of glucolipotoxicity upon insulin secretion (2). Total RNA was isolated and sequencing undertaken using an Illumina HiSeq™ 2000 instrument (Illumina Inc., San Diego, CA, USA). Genes differentially expressed in conditions of high glucose/high fat relative to control (with $p < 0.05$) were analysed using PANTHER gene ontology software (http://www.pantherdb.org) in order to determine those that have the greatest differential expression associated with glucolipotoxicity. The program calculates the number of genes that are enriched within a specific pathway, and the associated statistical significance of enrichment. Enrichment is considered statistically significant when there are more genes in the list associated with a particular pathway than would be expected by chance based on the total number of genes associated with that pathway. As can be seen (Table 1), G-protein-coupled receptor (GPCR) signalling was found to be the most significant of any specific protein class differentially expressed. We therefore focussed the remainder of the current study on GPCR families that are differentially expressed within our RNAseq dataset, and which might therefore contribute to β-cell dysfunction caused by glucolipotoxicity.

3.2. Trace amine-associated receptor family downregulation by glucolipotoxicity

Of note, among the GPCR families differentially regulated by high glucose and fatty acids, the trace amine-associated receptor (TAAR) family stands out as all members are downregulated following 72 h incubation in GLT media. Indeed, TAAR3, 4, 5, 6, 7a, 7b, 7c, 7d, 7e, 7g, 7h, 8b, 8c and 9 are downregulated to the level of detection sensitivity (Fig. 1a). As we had incubated cells under control, high glucose,

Table 1
Differential expression of protein family classes.

Protein class	*p* Value
Receptor	1.88 E−38
G-protein coupled receptor	1.67 E−24
RNA binding protein	3.54 E−20
Ribosomal protein	1.54 E−18
Signalling molecule	1.28 E−17
Nucleic acid binding	2.91 E−17
Transporter	8.91 E−16
Immunoglobulin	1.13 E−13
Ion Channel	2.72 E−13
Cell adhesion molecule	4.58 E−11

Comparative analysis of gene expression from INS-1 cells incubated for 72 h in RPMI-1640 media supplemented with or without 28 mM glucose, plus 200 μM oleic acid, and 200 μM palmitic acid. Data is compiled from six independent RNAseq analyses (three per experimental group).

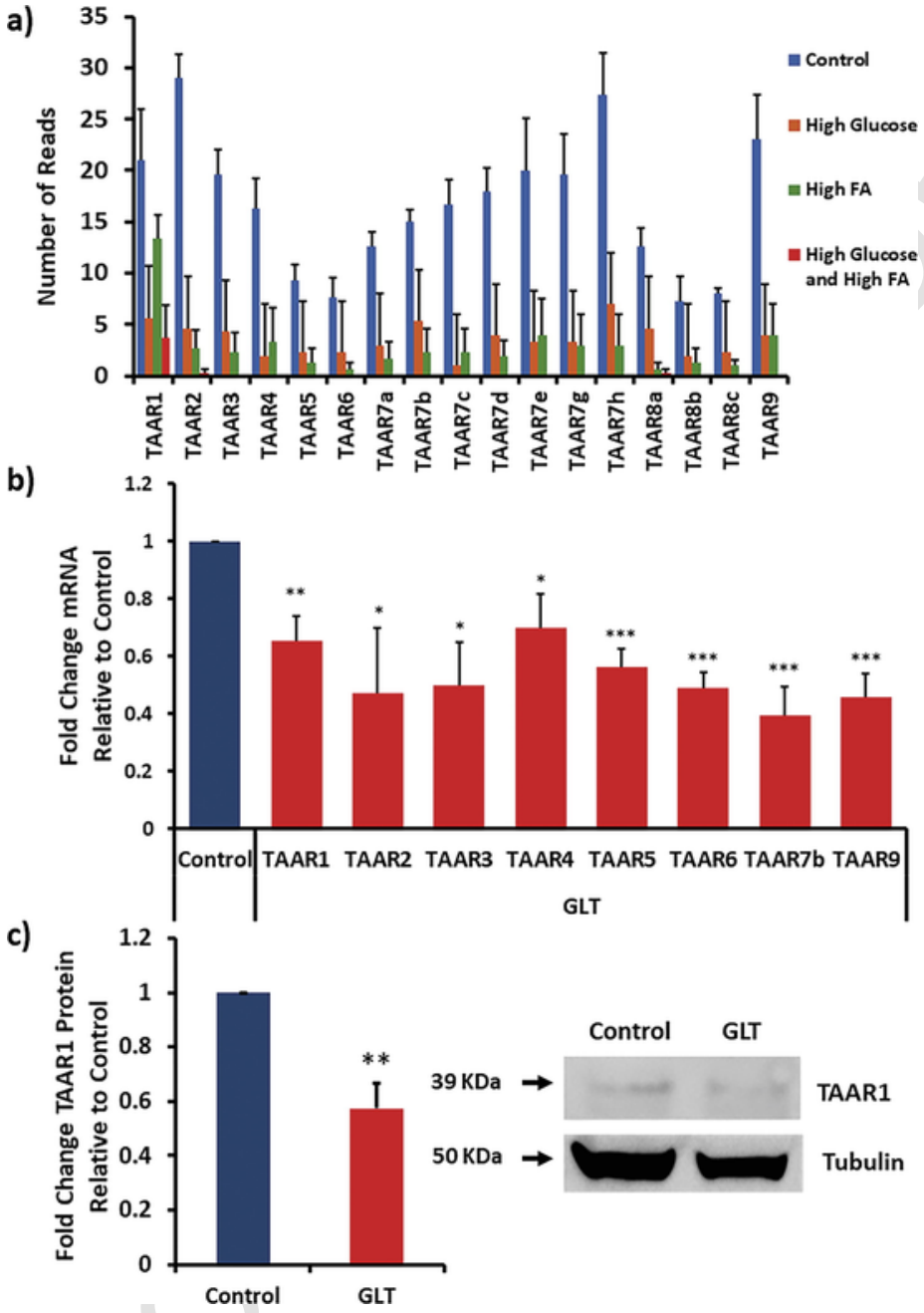

Fig. 1. Effect of glucolipotoxicity on TAAR family expression. a) INS-1 cells were cultured in RPMI-1640 media, or media supplemented with 28 mM glucose, 200 μM oleic acid and 200 μM palmitic acid, or 28 mM glucose plus 200 μM oleic acid and 200 μM palmitic acid (GLT) for 72 h. RNA was extracted and Illumina HiSeq analysis undertaken. Data is expressed as RNA read counts per condition. b) INS-1 cells were cultured in RPMI-1640, or GLT media for 72 h. RNA was extracted and qRT-PCR undertaken using the primers and cycle conditions detailed in the Methods section. c) INS-1 cells were grown in RPMI-1640, or GLT media for 72 h. Protein was extracted and separated by SDS-PAGE, transferred to nitrocellulose, and detected using anti-TAAR1 primary antibody. In all cases data are expressed as mean ± SEM from 3 or more independent experiments. $^{*}p < 0.05$ $^{**}p < 0.005$ $^{***}p < 0.0005$.

high fatty acid, and high glucose plus high fatty acid conditions we were also able to determine the extent to which glucose and fatty acids drive down regulation both individually and together. Glucose and fatty acids each drive down expression significantly ($p < 0.05$ in all cases, except for TAAR1 in high fatty acid). Importantly however, combination treatment with high glucose and high fat (GLT media) had the largest down-regulation on all TAAR members.

In order to validate these results, we next performed independent qRT-PCR analysis of the TAAR family members TAAR1, 2, 3, 4, 5, 6, 7b and 9 (Fig. 1b) from cells incubated +/− GLT media for 72 h. In all cases TAAR expression was significantly downregulated by GLT media, with values ranging from 30.2% ± 12.0% ($p = 0.033$) for TAAR4, to 60.8% ± 10.2% ($p = 0.002$) for TAAR7b. RNA does not always correspond to protein expression however, so it is also important to quantify protein expression between control and GLT conditions. Therefore, INS-1 β-cells were again cultured +/− GLT media for 72 h, cells lysed, and immunoblotted using antibody against TAAR1 (Fig. 1c), the only TAAR family member for which a commercial antibody was available (Abcam, Cambridge, UK). Densitometry analysis indicated that TAAR1 protein expression was downregulated by 42.6% ± 9.1% in comparison to GLT media ($p = 0.0017$). Furthermore, these data are not the result of altered β-cell viability resulting from exposure to glucolipotoxic media, as INS-1 cells incubated for 72 h in GLT media had 103.7 ± 3.4% cell viability relative to RPMI-1640 media control (data not shown).

3.3. Impact of glucolipotoxicity upon the TAAR1 interactome

In order to better understand how TAAR family downregulation might impact upon pancreatic β-cell function, non-biased network analysis was performed using the MetaCore integrated knowledge database of pathways (https://portal.genego.com). Expression data from the RNAseq analysis files was used as the input list for generation of biological networks using the Analyze network algorithm with default settings. By so doing we were able to generate a bespoke network of molecules predicted to interact with the most extensively characterised member of the TAAR family, TAAR1 (Fig. 2). Those genes upregulated by GLT in our experiments are indicated by an adjacent red circle in the interactome, whereas those genes that are downregulated are indicated by a blue circle. Our data indicate that when TAAR1 (shown as TAR1 in Fig. 2) is downregulated by glucolipotoxicity there are

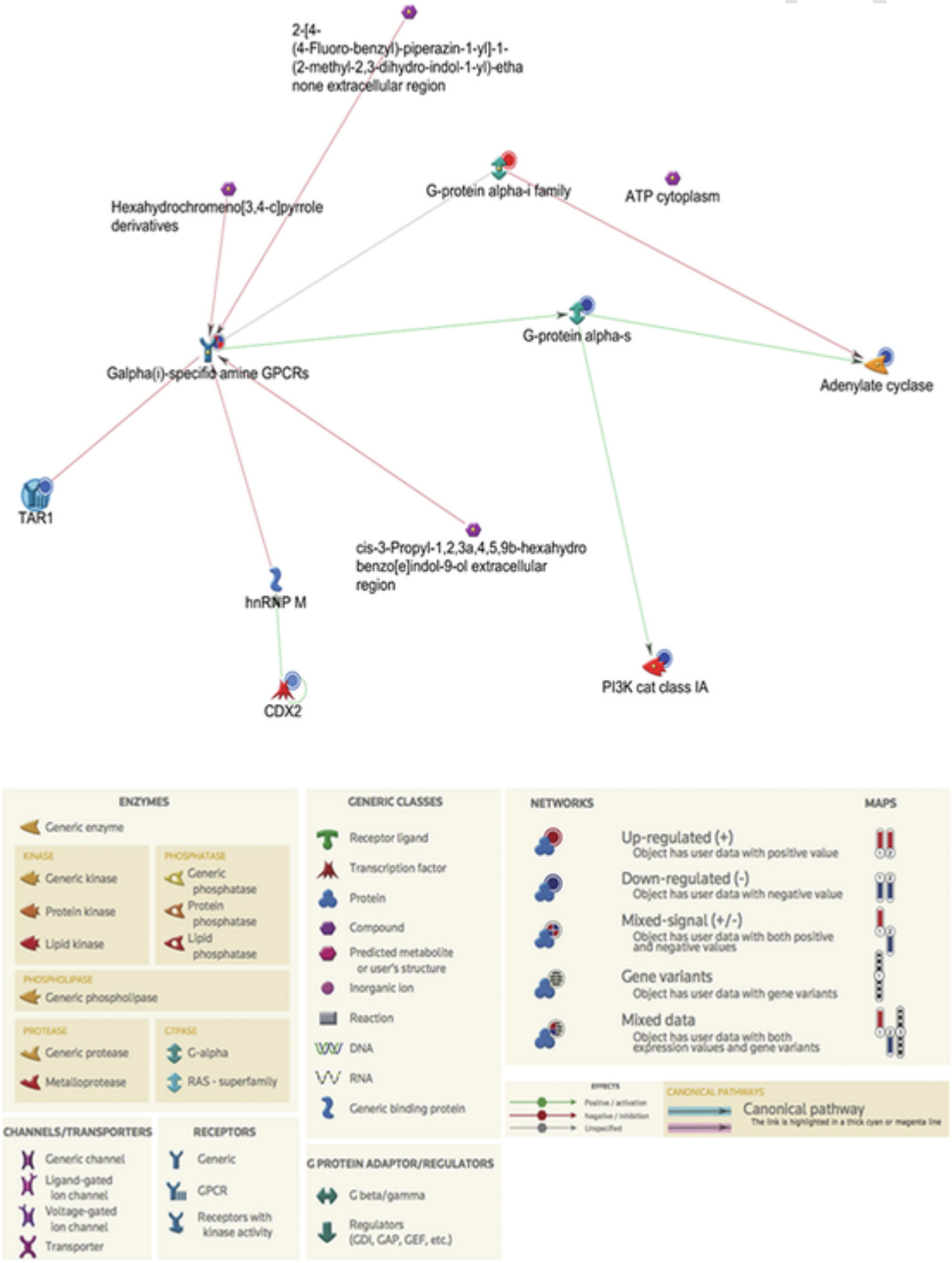

Fig. 2. Network analysis of TAAR1 signalling. INS-1 cells were cultured in RPMI-1640 media, or media supplemented with 28 mM glucose, 200 μM oleic acid and 200 μM palmitic acid, (GLT) for 72 h. RNA was extracted and Illumina HiSeq analysis undertaken. MetaCore software (http://genego.com) was utilised to construct an interactome linked to TAAR1 (TAR1). Significantly upregulated genes (Bonferroni corrected p value < 0.05) are indicated by an adjacent red circle, whereas downregulated genes are indicated by an adjacent blue circle.

both positive and negative downstream effects on GPCR signalling through G_{α} pathways. Specifically, stimulatory $G_{\alpha s}$ protein is downregulated, whereas inhibitory $G_{\alpha i}$ protein is upregulated. The consequence of these complementary effects would be a concerted negative downstream action upon adenylyl cyclase. Furthermore, such an effect would reduce ATP conversion to cAMP, a molecule known to amplify insulin secretion from pancreatic β-cells [8].

3.4. Effect of TAAR ligands upon cAMP level and insulin secretion

The TAAR1 interactome is consistent with previous literature that has indicated an association between TAAR1 signalling and adenylyl cyclases via $G_{\alpha s}$ proteins [9]. We therefore sought to determine whether physiological TAAR ligands could enhance insulin secretion via cAMP pathways in β-cells. INS-1 cells were grown in RPMI-1640 media, then washed and incubated in KRB and 0.5 mM IBMX, or KRB and 0.5 mM IBMX supplemented with the trace amine ligands, isopentylamine (TAAR3), *p*-tyramine (TAAR1 and TAAR4), 2-phenylethylamine (TAAR1 and TAAR4), agmatine (putative TAAR6 and TAAR8 ligand), *N,N*-dimethylcyclohexylamine (TAAR7, TAAR8 and TAAR9 isoforms), and *N,N*-dimethyloctylamine (TAAR7 isoforms) at a single final concentration of 10 μM for direct comparison, or the TAAR1 inverse agonist EPPTB at a concentration of 10 nM, for 30 min. Cells were lysed in 0.1 M HCl and cAMP determined via select ELISA. Isopentylamine was found to significantly increase the level of cAMP by 3.5 ± 0.6 fold ($p = 0.02$) relative to control, whilst p-tyramine increased cAMP accumulation by 3.8 ± 0.5 fold ($p = 0.01$), 2-phenylethylamine by 4.1 ± 0.7 fold ($p = 0.02$) and agmatine by 2.8 ± 0.3 fold ($p = 0.005$) respectively. By contrast, incubation with EPPTB decreased the cAMP level to 0.78 ± 0.04 ($p = 0.004$), whilst *N,N*-dimethylcyclohexylamine and *N,N*-dimethyloctylamine also resulted in a modest decrease in cAMP level to 0.90 ± 0.01 ($p = 0.003$) and 0.83 ± 0.05 ($p = 0.01$) respectively (Fig. 3a).

In order to determine how this might relate to insulin secretion, INS-1 cells were cultured to 70% confluency, washed, and then incubated with either KRB or KRB secretagogue cocktail (supplemented with 13.5 mM glucose, 1 μM phorbol 12-myristate 13-acetate, 1 mM isobutyl-methylxanthine, 1 mM tolbutamide, 10 mM leucine, 10 mM glutamine) along once more with, where indicated, isopentylamine, *p*-tyramine, 2-phenylethylamine, agmatine, *N,N*-dimethylcyclohexylamine, or *N,N*-dimethyloctylamine at a final concentration of 10 μM, or the inverse agonist EPPTB at a concentration of 10 nM, for 2 h. Insulin secretion was determined by ELISA (Mercodia, Uppsala, Sweden) and normalised to cellular protein content. Data is presented as fold change relative to stimulated control from the corresponding experiment (Fig. 3b). Consistent with our cAMP data, *N,N*-dimethylcyclohexylamine had no significant effect upon secretagogue-stimulated insulin secretion, whilst *N,N*-dimethyloctylamine and the TAAR1 inverse agonist, EPPTB, decreased secretion by 13.2% ± 5.0% ($p = 0.029$) and 18.2% ± 4.0% ($p = 0.005$) respectively. By contrast, isopentylamine, *p*-tyramine, and 2-phenylethylamine significantly increased secretagogue-stimulated insulin secretion by 16.8% ± 5.1% ($p = 0.0149$), 23.3% ± 8.7%, ($p = 0.045$), and 26.5% ± 7.1% ($p = 0.0155$) respectively. Agmatine also caused a modest increase in stimulated insulin secretion by 16.0% ± 6.9%, albeit this was just outside statistical significance ($p = 0.056$). There was, however, no significant change in unstimulated insulin secretion for all treated ligands compared to control.

In order to determine whether those ligands that enhance cAMP and insulin secretion under standard conditions might make suitable drug candidates for the treatment of T2D, we then repeated the insulin secretion experiments in glucolipotoxic conditions. Our results show that in the absence of ligands, GLT media decreased insulin secretion

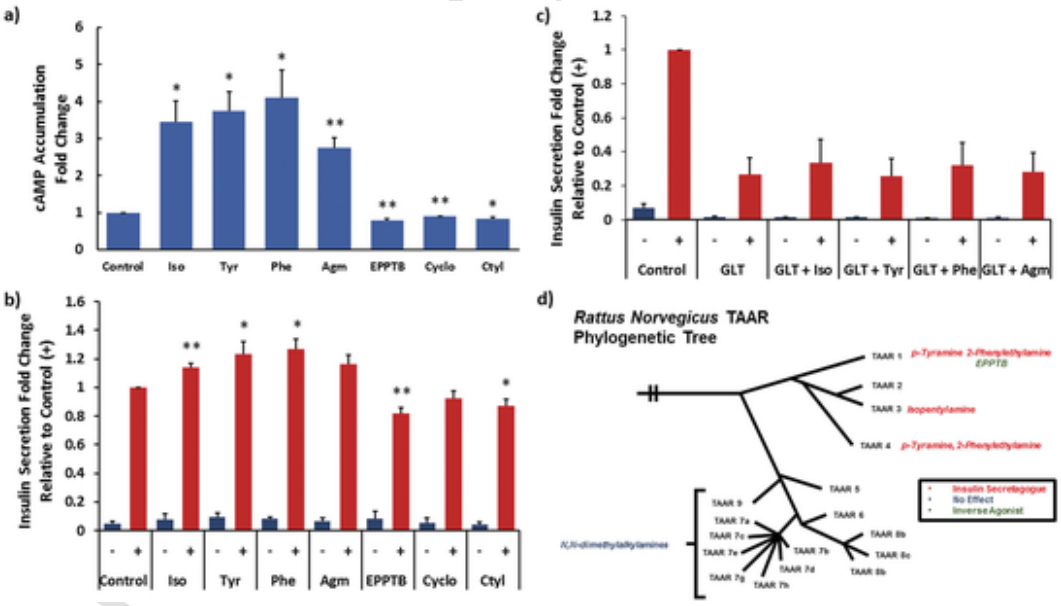

Fig. 3. Effect of trace amines on cAMP and insulin secretion. a) INS-1 cells were grown in standard growth media before being washed and incubated in KRB and 0.5 mM IBMX or KRB and 0.5 mM IBMX supplemented with either isopentylamine (Iso), *p*-tyramine (Tyr), 2-phenylethylamine (Phe), agmatine (Agm), *N,N*-dimethylcyclohexylamine (Cyclo), or *N,N*-dimethyloctylamine (Ctyl) at a final concentration of 10 μM, or the inverse agonist EPPTB at a concentration of 10 nM, for 30 min. Cells were lysed in 0.1 M HCl and cAMP determined via select ELISA. b) INS-1 cells were cultured in standard growth media to 70% confluency, washed, then incubated in either KRB [(−) blue] or KRB secretagogue cocktail [(+) red] (containing 13.5 mM glucose, 1 μM phorbol 12-myristate 13-acetate, 1 mM isobutyl-methylxanthine, 1 mM tolbutamide, 10 mM leucine, 10 mM glutamine), minus or plus trace amine ligands as indicated: isopentylamine (Iso), *p*-tyramine (Tyr), 2-phenylethylamine (Phe), agmatine (Agm), *N,N*-dimethylcyclohexylamine (Cyclo), *N,N*-dimethyloctylamine (Ctyl) at a final concentration of 10 μM, or TAAR1 inverse agonist EPPTB at a concentration of 10 nM for 2 h. Insulin secretion was then determined by ELISA (Mercodia, Sweden), and normalised to cellular protein content. c) INS-1 cells were cultured in RPMI-1640 media, or RPMI-1640 GLT media for 72 h. Insulin secretion was determined by ELISA assay following incubation ± secretagogue cocktail [(−) blue, (+) red], minus or plus trace amine ligands as indicated: isopentylamine (Iso), *p*-tyramine (Tyr), 2-phenylethylamine (Phe), or agmatine (Agm) at a final concentration of 10 μM for 2 h, with data normalized to cellular protein content. d) TAAR family phylogenetic tree, with trace amine ligand responsiveness mapped alongside. In all cases data are expressed as mean ± SEM from a minimum of 3 independent experiments. $^{*}p < 0.05$ $^{**}p < 0.005$ compared to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

to 26.7 ± 9.6% of control stimulated secretion values (Fig. 3c). Crucially, however, all of the previously observed increases in insulin secretion that had been seen under standard media conditions were no longer observed when cells had been incubated in GLT media, where a decrease in TAAR expression is seen.

Given the clear split in cellular response to the different amine ligands under standard conditions, between those that enhance cAMP signalling and insulin secretion and those that do not, we then sought to determine whether this might be explained by evolutionary divergence within the TAAR family. The resulting phylogenetic tree, complete with ligand responsiveness mapped alongside this, is shown in Fig. 3d.

The above data is fully consistent with a role for TAAR family members 1–4 in insulin secretion. However, transformed β-cell lines are not always fully representative of primary β-cell biology, and so it is important to determine whether these findings are indicative of whole animal physiology. In order to address this point pancreatic islets were isolated from CD1 mice, then incubated with the monoamine oxidase inhibitor pargyline (in order to prolong the half-life of administered tyramine) and the indicated concentration of p-tyramine, and insulin secretion determined (Fig. 4). *p*-Tyramine increased glucose-stimulated insulin secretion by 33.2% ± 20.3% ($p = 0.199$), 83.2% ± 11.8% ($p = 0.004$), 41.2% ± 23.4% ($p = 0.156$) and 47.8% ± 26.8% ($p = 0.067$) at concentrations of 0.1 μM, 1 μM, 10 μM and 100 μM respectively. This U-shaped curve and decrease in tyramine effect likely represents a loss of selectivity seen at concentrations of tyramine greater than 10 μM, where there are known to be competing mechanisms from interaction with other pharmacological targets.

4. Discussion

Through Illumina HiSeq analysis of RNA that was extracted from β-cells exposed to conditions of diabetic glucolipotoxicity, we have been able to identify those genes that become dysregulated following chronic exposure to high glucose and fatty acid levels. When analysed for differential expression, receptor signalling showed the highest statistical significance. This indicates that the molecules involved in glucolipotoxicity are likely primarily implicated in signalling pathways, in regulating transcription factors binding, and in molecular transport. The former is consistent with our previous microarray experiments, where we identified a novel role for TNFR5 signalling in initiating NF-κB and STAT1 transcription factor activation and subsequent islet inflammation that leads to β-cell apoptosis (3). However, our current data shows that GPCR signalling was the most significant of any specific protein class differentially expressed. Within this category, the TAAR family were all substantially downregulated, many to the level of our assay detection sensitivity, following 72 h culture in GLT media. This makes the TAAR family prime candidates for participation in insulin stimulus-secretion coupling, which is significantly decreased by glucolipotoxicity.

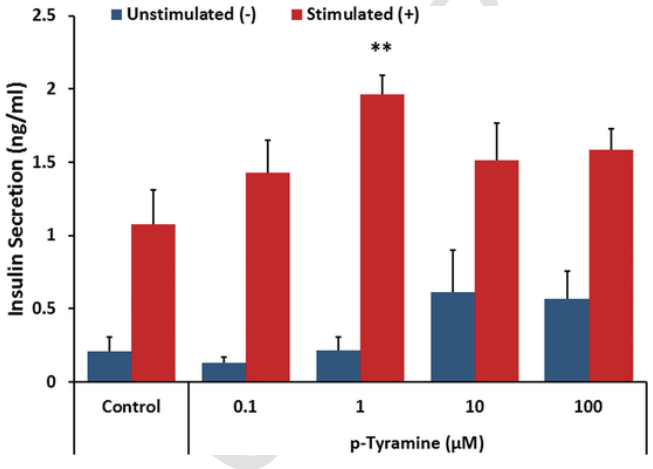

Fig. 4. Dose-dependent effect of p-tyramine upon insulin secretion from mouse islets. Islets were isolated from CD1 mice, then cultured in RPM1-1640 media for 24 h. Islets were then incubated in 2 mM [(−) blue] or 20 mM [(+) red] glucose for 1 h, supplemented with 10 μM paragyline ± p-tyramine at the stated concentration, and insulin secretion determined by radioimmunoassay. Dara are expressed as mean ± SEM from a minimum of 5 independent experiments. **$p < 0.005$ compared to control stimulated (+) samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

TAARs were first identified in 2001 as receptors for endogenous trace amine ligands including *p*-tyramine, 2-phenylethylamine and tryptamine [9,10]. Humans possess six functional TAARs, mice have fifteen, and rat seventeen. All, except TAAR1, have previously been shown to also function as olfactory receptors [11–13]. In the case of TAAR1 the receptor signals via $G_{\alpha s}$ proteins to increase intracellular cAMP concentration [9,14,15]. In the olfactory epithelium however, all TAARs appear to couple to G_{olf}, though this also associates with adenylyl cyclase to increase cAMP [11,16,17]. TAARs have also been implicated in neuropsychiatric disorders, where they modulate monoaminergic neurotransmission [18,19].

Importantly, TAAR1 has previously been shown to be expressed in pancreatic β-cells from both human islets and mouse MIN-6 cells [18,20,21]. Indeed, the discovery of the TAAR family was in part due to screening of pancreatic tumour cell lines [9]. Administration of the potent, although non-selective, TAAR1 ligand 3-iodothyronamine strongly enhanced insulin secretion from MIN-6 cells [20]. More recent studies with highly selective agonists have confirmed a TAAR1-mediated enhancement of glucose-stimulated insulin secretion, an effect observed in both INS-1 cells and isolated human islets [21]. Importantly TAAR1 agonists maintain efficacy *in vivo*, where improved glucose tolerance has been observed, along with increased insulin secretion in response to a glucose challenge [21]. Furthermore, single nucleotide polymorphisms of TAAR1 that result in altered signal transduction were recently reported in a sub-set of patients exhibiting impaired insulin secretion [22]. These studies therefore suggest that TAAR1 may be a novel therapeutic target to treat type 2 diabetes. Crucially however, other TAAR isoforms have not previously been studied in this context.

In order to further explore their potential as possible drug development targets, we investigated the effect of endogenous ligands of the wider TAAR family upon insulin secretion from INS-1 β-cells and primary mouse islets, with cells either incubated under standard conditions or following 72 h incubation in GLT media. Pharmacological studies of human, mouse and rat TAARs have shown that *p*-tyramine and 2-phenylethylamine activate TAAR1 and TAAR4 [10,19,23]. Isopentylamine is a reported endogenous agonist for TAAR3 (11), while agmatine is only currently described as an agonist for TAAR 13c that has currently only been found to be expressed in zebra fish [24]. The diamine binding site through which agmatine interacts with TAAR13c is, however, conserved in both human and rodent TAAR6 and TAAR8 isoforms [24,25] making them potential sites through which agmatine effects might be mediated. The *N*,*N*-dimethylalkylamines, meanwhile, are agonists at TAAR7 and TAAR9 isoforms [16]. Our data show that under control conditions it is not only TAAR1 stimulation that has the ability to influence glucose metabolism, but in fact ligands that activate TAAR1, TAAR3, and TAAR4. By contrast EPPTB, an inverse agonist for TAAR1, and trace amine ligands that bind to TAAR7 and 9 had no positive effect upon secretagogue-stimulated insulin secretion. The positive effect of ligand activation in control conditions is also consistent with our cAMP data, where higher insulin secretion was reflected by higher intracellular cAMP concentrations. This was further validated by the modest, albeit statistically non-significant, increase in insulin secretion following agmatine stimulation, which similarly reflected the smaller effect on cAMP increase. In addition, the inverse agonist EPPTB

decreased cAMP levels and insulin secretion, further supporting the link between cAMP level and secretion data.

TAARs 1–4 form one of the two major branches of the TAAR phylogenetic tree known as primary amine detectors. This suggests a conserved genetic ancestry possibly involving the functional ability to regulate cAMP conversion from ATP through primary amine binding [26]. As shown (Fig. 3d), it is only ligands for these particular TAARs that significantly enhance insulin secretion, whilst those that have no positive effect upon insulin secretion act on a separate branch of the phylogenetic tree. Agmatine would appear to be one possible exception to this rule as it has only been previously described as an agonist for TAAR13c, a receptor that has a binding site conserved with TAAR6 and TAAR8. Zebrafish TAAR13c is however nevertheless present on the same branch of the phylogenetic tree as those receptors capable of regulating insulin secretion [27], and while agmatine is known not to interact with TAAR1 [28] it is conceivable that agmatine may activate other TAARs of this conserved mammalian branch of the tree that are currently incompletely characterized. However, this is an area that requires further systematic study, particularly in light of agmatine's known poly-pharmacology. As such the effects we observed in response to agmatine cannot be definitively ascribed to one or more TAAR-family members at this time.

The above studies are consistent with TAAR representing a novel therapeutic target for the treatment of type 2 diabetes. Furthermore, previous work has shown TAAR1 agonists also have beneficial effects on nutrient-induced hormone secretion from the gastrointestinal tract, regulating glucagon-like peptide 1 (GLP-1) and peptide YY secretion from intestinal L-cells [21], and somatostatin secretion from the stomach D-cells [29]. Such potential benefit could be negated, however, by our observation that glucolipotoxicity decreases the expression of the receptors through which such effects are mediated. Indeed, our data shows that the ability of these ligands to enhance insulin secretion was lost when INS-1 cells were cultured in GLT media for 72h. This is in agreement with our expression data, as downregulation of receptor expression following exposure to GLT media would reduce the potential for receptor ligand binding and the resultant signal transduction which leads to downstream increases in secretion. It is worth noting, however, that in other cell types a positive feedback loop has been suggested whereby TAAR1 agonists induce increased expression of TAAR1. As such, the non-selective TAAR1 agonist methamphetamine increases TAAR1 expression in T leukocytes [30], with a similar effect also recently reported in placenta in response to the highly selective TAAR1 agonist RO5203648 [31]. Should such an effect also occur in pancreatic β-cells following a longer-term administration than the 2h treatment used here, this may allow for TAAR agonists to still be effective and overcome the glucolipotoxicity-induced decrease in receptor expression. This is an area for future systematic study. In addition, studies on the status of TAAR gene expression in human diabetic patients are also needed, although as noted earlier signal-modifying TAAR1 polymorphisms have recently been linked to impaired insulin secretion in certain patients [22].

Given that failure of insulin secretion leads to the onset of overt diabetes, this has been a primary target for therapeutic intervention since sulfonylurea compounds were first introduced as oral antidiabetic agents in the 1950s. Indeed, these compounds remain an important pharmacotherapy for the control of glucose homeostasis in patients with T2D to this date. However, safety concerns over sulphonylureas have started to arise in recent years, not least as their use can result in hypoglycemia and weight gain. In addition, over time they also often become less effective. This has therefore resulted in a search for new compounds, and especially those that are able to enhance insulin secretion through different mechanisms. Unlike sulphonylureas, TAAR1 agonists do not appear to promote hypoglycaemia [21]. They do however promote weight loss in models of excessive weight gain [18,21], and moreover may even normalize binge eating phenotypes [32], offering potential additional benefits over current frontline pharmacotherapies. Importantly, TAAR1 has been confirmed in clinical trials as a viable, safe, target to which pharmacotherapies can be targeted [33], and as such TAAR1directed agents may be beneficial prophylactically in pre-diabetic patients.

Sulphoylureas bind to and close pancreatic β-cell K_{ATP} channels, resulting in cell membrane depolarisation and subsequent L_c-type Ca^{2+}-channel opening, Ca^{2+} influx, and exocytosis of insulin-containing secretory granules [34]. More recently incretin mimetics have also been employed to enhance insulin secretion. Their mechanism of action is different to sulphonylureas as, like TAARs, incretin receptors instead activate stimulatory cell membrane GPCRs linked to adenylyl cyclase. This then generates cAMP, which in turn serves to amplify glucose-stimulated insulin secretion [35,36]. Dipeptidyl peptidase 4 (DPP4) inhibitors, or gliptins, also enhance incretin levels, albeit through inhibition of the enzymatic breakdown of incretins [37]. Consequently, they also therefore enhance stimulatory GPCR signalling.

Whilst incretin mimetics are increasingly being advocated as a safer alternative to enhance insulin secretion than sulphonylureas, these too are not without problems. Firstly, hyperglycaemia has been shown to significantly reduce expression of β-cell incretin receptors [38], not unlike our current data showing downregulation of TAARs following exposure to glucolipotoxicity. In addition, these drugs need to be administered through subcutaneous injection, which is often unpopular with patients. In addition, some patients develop irritation at injection sites, further adding to their unpopularity. However, DPP4 inhibitors and TAAR agonists can be administered orally, thereby offering a less problematic way to activate adenylyl cyclase. Therefore, in conclusion, whilst glucolipotoxic downregulation of TAAR family members 1–4 leads to a significant reduction in insulin secretion, and therefore likely contributes to T2D pathophysiology, use of new and existing therapies to augment adenylyl cyclase activity through receptors linked to heterotrimeric $G_{\alpha s}$ signalling pathways might prove effective at offsetting this deficiency.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

MJC performed experiments, analysed data, and helped prepare the manuscript.

MartaB performed experiments, analysed data, and helped prepare the manuscript.

TAJ analysed data, and helped prepare the manuscript.

BWO performed experiments, and analysed data.

SRS performed experiments, and analysed data.

PWC contributed to study design, and analysed data.

KH performed experiments, and analysed data.

MerellB performed experiments, and analysed data.

KF performed experiments, and analysed data.

CN contributed to study design, and helped prepare the manuscript.

RL performed experiments, and analysed data.

GAH contributed to study design, and helped prepare the manuscript.

MDB contributed to study design, and helped prepare the manuscript.

MDT directed the study, analysed data, and prepared the manuscript.

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