Identification of Anti-diabetic Signaling Mechanisms of TAAR1 in Pancreatic β-cells

A thesis submitted by

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In partial fulfillment of the requirements for the degree of

PhD

in

Biochemistry

Tufts University

Sackler School of Graduate Biomedical Sciences

August 2019

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Abstract

Type 2 diabetes is a growing problem in the United States and across the world, as rates of metabolic diseases continue to climb. Finding treatments that can stimulate insulin production and promote the maintenance of functional b-cell mass are of particular interest in the treatment of the disease. Pancreatic β-cell failure in type 2 diabetes mellitus is a serious challenge that results in an inability of the pancreas to produce sufficient insulin to properly regulate blood glucose levels. Trace amine-associated receptor 1 (TAAR1) is a G protein-coupled receptor expressed by β-cells that has recently been proposed as a potential target for improving glycemic control and suppressing binge eating behaviors. We discovered that TAAR1 couples to Gαs-signaling pathways in insulin-secreting β-cells to stimulate PKA/Epac mediated and glucose-dependent release of insulin, activation of Raf/MAPK signaling, induction of CREB-Irs-2, and increased β-cell proliferation. Interestingly, TAAR1 triggers cAMP-mediated calcium influx and release from internal stores, both of which were required for activation of a MAPK cascade utilizing calmodulin-dependent protein kinase II (CaMKII), Raf, and MAPK/ERK kinase 1/2 (MEK1/2). Together, these data identify TAAR1/Gαs-mediated signaling pathways that promote insulin secretion, improved β-cell function and proliferation, and highlight TAAR1 as target worthy of further investigation for improving β-cell health in type 2 diabetes mellitus.

Acknowledgements

I would like to thank my advisor, Athan Kuliopulos, without whose boundless enthusiasm for science and tireless optimism (even when faced with the ugliest of data), this project would not have come to fruition. I am truly grateful for the relationships developed and the knowledge gained during this experience. I would like to thank Lidija Covic, who has been almost a co-mentor to me. You are a voice of reason in trying times, and an incredible source of both knowledge and support. I would like to thank my other two committee members—Martin Beinborn and Daniel Cox—for their time, continued patience, and practical guidance over the span of several years and projects. I would like to thank Dr. John Sondek for taking time to travel here from UNC Chapel Hill in order to participate in my thesis defense. I would like to thank all lab members (present and past) who have contributed to my success and the preservation of my sanity: Chris Garzia, Nga Nguyen, Elizabeth Fletcher, Susan Turner, Rajashree Rana, Srijoy Guha, Jim Perkins, and others I may have missed have been wonderful friends and coworkers. A particular thank you to my former classmate and labmate Andrew Shearer, with whom I shared almost my entire graduate school experience, certainly for the betterment of it. I would like to thank loved ones—family and friends—for supporting me in this journey as well. My mother Jeanne Ranker and sister Sarah Michael, in particular, as well as my fiancée Paul Estrada have been invaluable sources of love and encouragement for me during this exhausting process. I would like to thank the everyone who has inspired me, whether by encouraging my development as a scientist (Susan Tsunoda) or as a human being, or even both (George Perides). To all of you, to Tufts University, the staff and programs who have supported me, as well as anyone I may have forgotten to name-a sincere thank you.

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List of Abbreviations

5-HT	5-hydroxytryptamine; serotonin
AC	adenylyl cyclase
AGEs	advanced glycation endproducts
AHD	alpha helical domain
AT	adenosine triphosphate
β_1-AR	β_1-adrenergic receptor
β_2-AR	β_2-adrenergic receptor
CaMKKII	CaM Kinase Kinase II
cAMP	cyclic AMP
CNS	central nervous system
CREB	cAMP responsive element binding protein
CT	carboxy terminus; C terminus
DA	Dopamine
DAG	diacylglycerol
DIO	diet-induced obese
DPP-4	Dipeptidyl peptidase-4
ECL	extracellular
Epac	Exchange protein directly activated by cAMP
ER	endoplasmic reticulum
FAs	fatty acids
FBS	Fetal Bovine Serum
FFA	free fatty acids
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine-nucleotide-exchange factor
GHS-R	Growth hormone secretagogue receptor
GIP	gastric inhibitory polypeptide
GIP	glucose-dependent insulinotropic/gastric inhibitory polypeptide
GIPR	gastric inhibitory polypeptide receptor
GLP-1	glucagon-like polypeptide-1
GLP-1R	GLP-1 receptor
GPCR	G protein coupled receptor
GRK	G protein receptor kinase
GSIS	glucose-stimulated insulin secretion
GTP	guanosine-5'-triphosphate
GTP	guanosine 5’-triphosphate
ICL	intracellular
IL-1β	interleukin-1β
IP	intraperitoneal
IP_3	(1,4,5) triphosphate
IP_3R	(1,4,5) triphosphate receptor
IR	insulin resistance
K^+	potassium ion
K_{ATP}	ATP-regulated potassium channels

$Kir_{6.2}$	inward-rectifying potassium channel 6.2
LDL	low-density lipoprotein
MAO	monoamine oxidases
NT	amino terminus; N terminus
OE	olfactory epithelium
PEA	phenylethylamine
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PLC	phospholipase C
PP	pancreatic polypeptide
PPARγ	peroxisome proliferator-activated receptor γ
PYY	peptide YY
RGS	regulator of G protein signaling
RHD	ras-like homology domain
ROS	reactive oxygen species
SAR	structure-activity relationship
Ser	serine
SRIF	somatostatin
SSTR2	somatostatin receptor 2
SUR1	sulfonylurea receptor 1
T_1AM	3-iodothyronamine
T4	3,5,3',5' tetraiodothyronine
TA	trace amine
TAAR	Trace amine associated receptor
TBST	tween-buffered saline with 0.05% tween
TH	thyroid hormone
Thr	threonine
TM	transmembrane
Tyr	tyramine
TZDs	thiazolidinediones
VTA	ventral tegmental area

Chapter 1: Introduction

1.1 G protein coupled receptors and downstream signaling

1.1.1 Structural aspects of GPCR and G protein signaling

G protein coupled receptors (GPCRs) are seven transmembrane receptors that represent the largest class of cell surface receptors and one of the most popular drug targets of modern day, accounting for approximately 35% of drug targets as of 2018 (Sriram and Insel, 2018). To date, over 800 GPCRs have been identified, which respond to a diverse array of ligands including hormones, light, neurotransmitters, chemokines, odorants, metabolism intermediates, and more to initiate intracellular signaling responses to extracellular stimuli (Erlandson et al., 2018; Mahoney and Sunahara, 2016). There are 5 basic groups of vertebrate GPCRs: rhodopsin (family A), secretin (family B), glutamate (family C), adhesion, and Frizzled/Taste (Fredriksson et al., 2003; Schioth and Fredriksson, 2005). The rhodopsin family is the largest and perhaps best researched of all the groups, although all GPCRs share certain structural similarities.

GPCRs are comprised of a bundle of seven hydrophobic transmembrane (TM1-7) α-helices connected by 3 intracellular (ICL1-3) and 3 extracellular loops (ECL1-3), flanked by an extracellular amino (N) and intracellular carboxy (C) terminus (Krebs et al., 2003; Palczewski et al., 2000; Rosenbaum et al., 2009; Venkatakrishnan et al., 2013). Many also have an 8^{th} helix, found on the intracellular C terminal side of the receptor, which is oriented parallel to the membrane and plays critical roles in receptor activation (Erlandson et al., 2018; Palczewski et al., 2000). The majority of non-class A GPCRs have large extracellular domains on the N terminus that are involved in ligand binding but are not found in Class A Rhodopsin family members. Activation of GPCRs by

extracellular ligand binding—whether to the N terminal or ECL domains, or both—stimulates heterotrimeric G protein-dependent and-independent signaling cascades to bring about specific cellular responses, achieved through differential coupling of receptors with a highly regulated network of downstream effectors (Benovic et al., 1989; Gilman, 1987; Lohse et al., 1990; Luttrell et al., 2018). Some receptors even exhibit differential coupling with downstream effectors exists in response to different agonists or modulators of that receptor—a phenomenon referred to as biased agonism—adding further mechanisms of GPCR signaling regulation (Rahmeh et al., 2012; Rankovic et al., 2016). Further differential control and activity can be achieved by receptor homo- and hetero-dimerization, allowing one receptor to influence the activity of other(s) by direct physical interactions between receptors (Braunig et al., 2018; Levoye et al., 2006; Milligan, 2009; Ugur et al., 2018).

There exists a high degree of variability within the extracellular domains of GPCRs; diversity is achieved by a variation in both the actual length as well individual amino acid sequences and resulting secondary structure, crosslinking patterns and electrostatic properties of the domains that make up the N terminus and extracellular loops, thus providing a mechanism of generating specific ligand binding domains for each receptor (Chung, 2013). Ligand binding domains—or binding pockets—exhibit a high degree of specificity for a receptor's ligand(s); in some receptors, the extracellular binding pocket is also completely sealed off by a tight "lid" formed by the extracellular loops and/or N terminus, which actually covers the binding pocket in some receptors—the rhodopsin and sphingosine-1-phosphate (S1P1) receptors are two such examples (Hanson et al., 2012; Palczewski et al., 2000). This structural feature is not ubiquitous,

and in many other GPCRs, the ligand binding domain is freely accessible to extracellular solvents (Wheatley et al., 2012).

Some of this variability can be seen in Fig. 1.1 (Rosenbaum et al., 2009), where three different GPCR structures are shown, aligned with that of the human β_2-adrenergic receptor (β_2-AR; blue). Overall, these structures reveal the basic folding of GPCR proteins, highlighting the conservation of certain GPCR structural features, particularly in terms of the general arrangement of their TM domains in relation to each other and to what would be the plasma membrane. The three GPCRs aligned here with the β_2-AR (blue) from left to right are bovine rhodopsin (purple), avian β_1-adrenergic receptor (β_1-AR; orange), and the human α_{2A}-adenosine (Adenosine A_{2A}; green). This group of alignments highlight some of the greater degree of variability that has been discussed in terms of GPCR

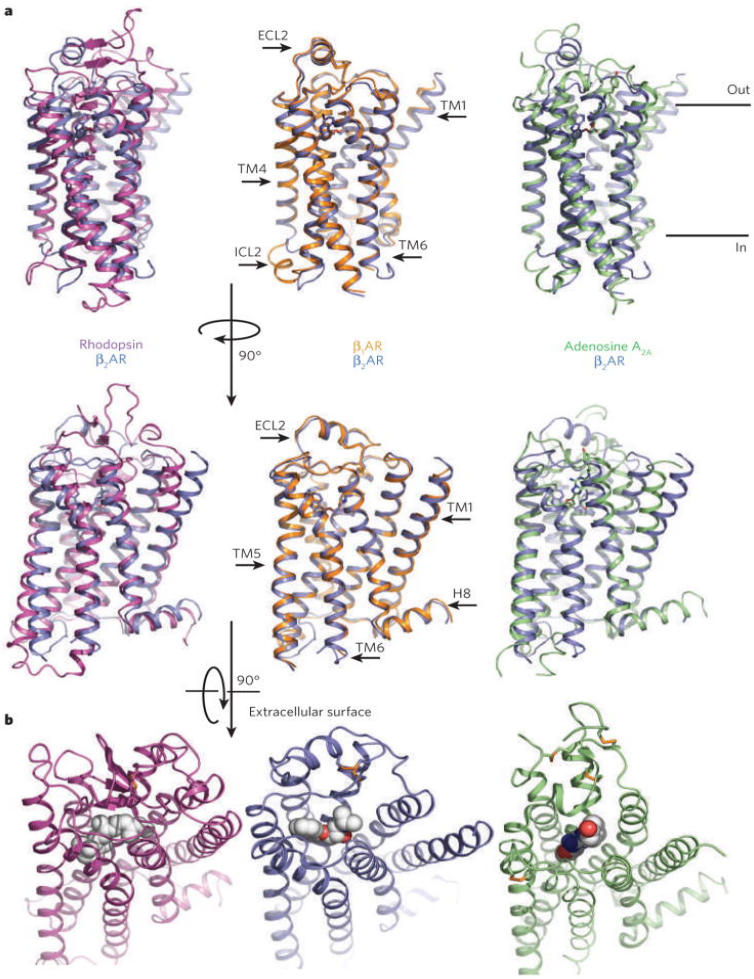

Figure 1.1: GPCR Alignments

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structures thus far—in particular within the extracellular loops/N terminal regions. The presence of the "lid" over the ligand binding pocket can be seen in the rhodopsin structure but is clearly absent in the β_2-AR; this is particularly clear in the view from the top/extracellular surface, where the ECL/NT ligand binding domains are highlighted in gray/red spheres (lower panels) (Rosenbaum et al., 2009). Advances in crystallography over the years have produced structures of different receptors in both inactive and active conformations, and each new structure produced continues to highlight the variable, complex nature of GPCR proteins and their movements, and inform upon the ways that these movements contribute to receptor mediated signaling (Cherezov et al., 2007; Krebs et al., 2003; Palczewski et al., 2000; Rosenbaum et al., 2007).

Primary (or orthosteric) binding sites for a receptor's endogenous ligand can also be regulated by the presence of allosteric sites; non-ligand molecules bind to other/secondary ("allosteric") sites on the extracellular domain to alter the receptor's behavior and/or affinity for ligand binding at the orthosteric site (Kruse et al., 2012; Thal et al., 2016). A wide array of allosteric modulators have been identified, exhibiting a range of both positive and negative effects on receptor activity, providing another important mechanism of GPCR regulation (Weis and Kobilka, 2018).

Upon ligand binding, there are conformational shifts induced within both the TM and ICL domains of the receptor that activate heterotrimeric G protein signaling (Nygaard et al., 2009; Rasmussen et al., 2011b). Within each class of GPCR, it is believed that there are generally conserved residues found in different transmembrane domains that are involved in driving the conformational shifts observed following ligand binding. A basic 2D GPCR caricature highlights the different conserved domains (often

referred to as molecular switches) that will be discussed in terms of their structure and function (Fig. 1.2). These molecular switches have been extensively studied in the Class A family, where there is a conserved DRY ((d/e)/RY) motif in TM3 that is critical for holding the receptor in its inactive and constrained state via intramolecular interactions between the charged residues of the (D/E)RY motif and certain other residues in TM6 (Palczewski et al., 2000). The release of this "ionic lock" and neutralization of the charged arginine residue involves a counterclockwise, rotational movement of the cytoplasmic end of TM6 away from this DRY motif (Ballesteros et al., 2001; Rasmussen et al., 1999). The rotational movement of TM 6 is one of the largest movements yet observed with GPCR activation; TM6 of Rhodopsin, for example, moves 14 angstroms away from the center of the helical bundle (Rasmussen et al., 2011a), although the magnitude of this shift does vary between receptors. A dissolution of the salt bridge between neighboring charged residues (D/E and R) is also believed to be essential in disruption of this ionic lock mechanism (Vogel et al., 2008). As always, there are

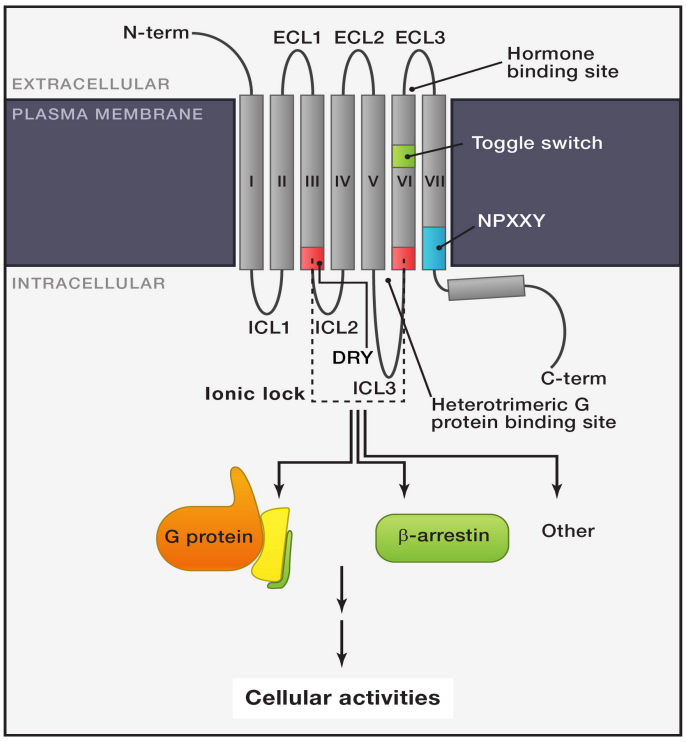

Figure 1.2: Important Conserved GPCR Molecular Switches

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exceptions—more than one crystal structure of an active conformation GPCR has been produced that shows this salt bridge still intact (Rasmussen et al., 2011a; Xu et al., 2011).

Another well studied molecular switch found in Class A GPCRs involves the "NPxxY" motif, found on the cytoplasmic end of TM7; the tyrosine residue found here is conserved in 92% of GPCRs (Katritch et al., 2013), and its high degree of conservation emphasizes its often critical role in receptor activation. In an inactive conformation, the side chains of this tyrosine residue point towards helices 1, 2, or 8—if an 8^{th} helix is present (Park et al., 2008a; Stenkamp et al., 2005). When the receptor is activated, however, a marked kink forms at this tyrosine residue as the side chain rotates, pointing instead toward the center of the entire transmembrane bundle, where it can interact with side chains of amino acid residues found within both TM6 and TM3, stabilizing the receptor in its active state (Lebon et al., 2011; Nygaard et al., 2009; Scheerer et al., 2008; Xu et al., 2011; Zhang et al., 2015). Overall, these movements of the transmembrane domains are generally thought to facilitate the rearrangement of a space on the intracellular face of the receptor that promotes G protein interaction and allows for activation of downstream signaling cascades by the receptor. In many cases, these movements on the cytoplasmic ends of transmembrane domains also correspond to movements in the opposite direction on the extracellular side that actually induce a contraction of the ligand-binding pocket (Katritch et al., 2013; Latorraca et al., 2017).

Also generally conserved within GPCR activation dynamics, but not yet discussed—transmembrane domains 5 and 7 exhibit a slight inward motion on the cytoplasmic face, allowing the interaction of certain key residues found within these two helical domains, stabilizing the receptor in an active state (Huang et al., 2015; Rasmussen

et al., 2011a; Rosenbaum et al., 2009). New crystal structures of receptors along with different stabilizing nanobodies and ligands—endogenous and otherwise—continue to inform on our understanding of the complex physical mechanisms that underlie GPCR activation and downstream signaling, although we should keep in mind that each of these crystal structures only provide single static snapshots of what are in reality dynamic proteins in continually shifting conformations.

Regardless, the adaptation of an active conformation by a GPCR, as mentioned, promotes interactions of the intracellular domains of the receptor with G proteins—this is a complex interaction in which it seems that ligand binding to a GPCR increases its affinity for G protein, and G protein binding to the receptor can also increase its affinity for ligand, again highlighting the complex nature of GPCR structure and activational dynamics (Latorraca et al., 2017). As there are many more diverse ligands than G proteins, the intracellular face of the receptor exhibits markedly less variability than the extracellular. There are in fact only four families of Gα proteins—Gαs, Gαi/o, Gαq, and Gα12/13, encompassing 18 different G proteins in humans—and in terms of understanding the mechanics that govern the selectivity of GPCR/G protein coupling, much remains to be uncovered (Masuho et al., 2015).

Gα proteins are part of a heterotrimeric G protein signaling complex, made up of Gα, β and γ subunits, the latter two of which form a single complex that is not known to dissociate (the Gβγ dimer) (Simon et al., 1991). This heterotrimeric G protein complex associates with guanine nucleotides in an activation-state dependent manner. The inactive complex associates with guanosine diphosphate (GDP) via direct binding of GDP to the Gα subunit; upon activation of the GPCR by ligand binding, the receptor catalyzes

exchange of GDP for guanosine-5'-triphosphate (GTP) at the nucleotide binding site of the Gα subunit (Higashijima et al., 1987). Gα also dissociates from the βγ subunit, and both Gα and the βγ dimer are capable of initiating distinct downstream signaling pathways (Oldham and Hamm, 2006). It has become apparent that in an inactive state, receptor and G proteins can (but do not always) form a preassembled complex, even in the absence of agonist binding; this preassembled complex can in some cases prime the receptor for activation by increasing the receptor's affinity for ligand binding (Hilger et al., 2018). As is often the case however, these complex interaction dynamics seem to vary between receptor and G protein pairings, as well as in a cell-type specific manner (Syrovatkina et al., 2016).

To better understand how the conformational changes of the GPCR/G protein complex occur, we must also understand the structure of Gα itself, which has been found to exhibit a fair degree of conservation across families. As highlighted in the structure of Gα shown in Fig. 1.3, all Gα proteins are made up of two domains: the Ras-homology domain (RHD; gray), which exhibits the GTPase activity, and the α-

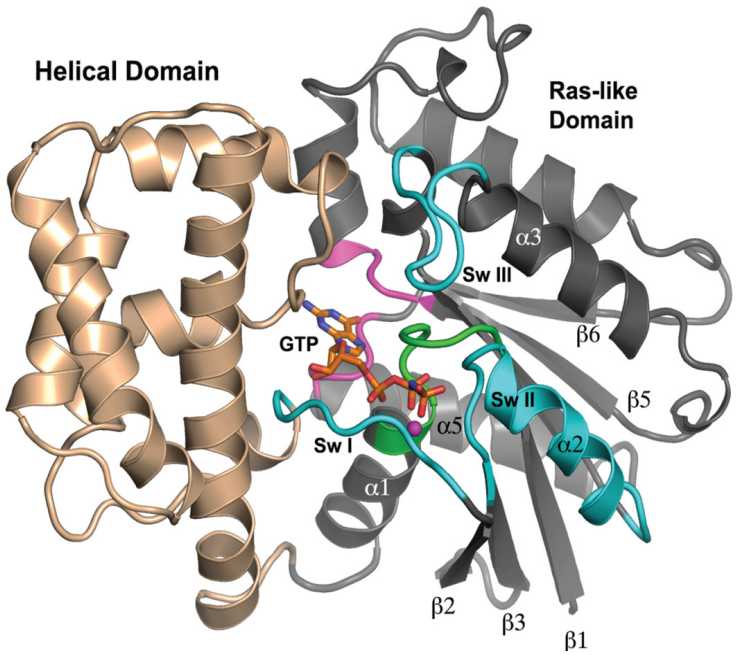

Figure 1.3: Crystal Structure of Gα in its Active, GTP-Bound Form

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helical domain (AHD; brown) (Sprang, 2016). Gα in this figure is depicted in its active conformation, in which the two domains have physically separated to reveal the nucleotide-binding pocket. The RHD is comprised of a six-stranded β-sheet (β1-6) surrounded by 5 α helices (α1-5), while the AHD, situated between α1 and β2 of the Ras domain is made up of six α helices (αA-αF) (Erlandson et al., 2018). The nucleotide-binding domain is found at the interface of these two domains, and is surrounded by four flexible regions (p-loop, switch I, switch II, and switch III; switch regions teal, Fig. 1.3) that are key for the activation state of the G protein and its interactions with various effectors (Chung, 2013; Sprang et al., 2007). Switch regions, in an inactive conformation, are also shielded by the physical presence of βγ, preventing these regions from interacting with downstream effectors, but this is no longer the case following βγ dissociation.

Both the AHD and RHD are involved in binding to GDP; within the Ras domain, the P-loop, found between β1 and α1, interacts with the β-phosphates of GDP molecules, while the β5-α4 and β6-α5 loops make contact with the guanine ring. (Mahoney and Sunahara, 2016). Based on the tight shielding of the nucleotide-binding domain between the AHD and RHD domains, it is not surprising that a rearrangement of the two, with respect to each other is required to allow for nucleotide exchange. The conformational changes involved promote the release of GDP from the p-loop and switch region interactions (Goricanec et al., 2016); it is interesting to wonder if targeting the freely accessible switch regions of activated Gα (with antibodies against switch regions, for example) could in theory represent a novel method for measuring receptor activation for

any GPCR. The important stabilizing effects of GTP binding on these flexible switch regions will also be discussed at a later point.

GPCRs themselves have an important role in driving G protein conformational changes via direct interactions between receptor and Gα (Rasmussen et al., 2011b). The CT of Gα is actually inserted into the cytoplasmic pocket of the receptor, where critical interactions are formed between the CT α5 helix of Gα and the transmembrane helices and intracellular loops of the receptor (Rasmussen et al., 2011b; Scheerer et al., 2008). Interestingly—and in further support of a critical role for important interactions between the CT of Gα and GPCRs—it has been reported that the last three residues of Gα-CT are actually important for selectivity of receptor/G protein interactions (Conklin et al., 1993).

In its activated, receptor-bound but nucleotide-free state, crystal structures have revealed that the end of the C-terminus of Gα rotates and actually moves upwards into the core of the receptor, thought to disrupt previous interactions between Gα and GDP (Carpenter et al., 2016; Dror et al., 2015). Within Gα, this movement disrupts interactions between the α5 and α1 helices; the α1 helix of the Ras domain interacts with the AHD and GDP, so the breakage of interactions between α1 and α5 helices is thought to be essential in the domain separation and nucleotide release observed with Gα activation (Flock et al., 2015; Oldham and Hamm, 2006). This "domain separation" is actually a movement of tens of angstroms—quite dramatic in terms of absolute distance, and almost resembles a clamshell opening (Van Eps et al., 2011). With the opening of these domains, Gα AHD becomes much more flexible, no longer held in rigid conformation by stabilizing interactions with GDP and the Ras domain (Westfield et al., 2011). Further conformational shifts induced in Gα with activation involve a

rearrangement of the β6-α5 loop in a manner that reduces the affinity of the G protein for GDP nucleotides (Mahoney and Sunahara, 2016).

The N-terminus of Gα is also believed to play a role in inducing nucleotide exchange upon receptor activation (Herrmann et al., 2006; Swift et al., 2010). According to crystal structures and receptor modeling, the N terminal helix (αN) and αN/β-1 junction of Gα directly interact with ICL2 of GPCRs (Mnpotra et al., 2014; Rasmussen et al., 2011b). Through interactions with the activated receptor and resultant restructuring of the NT, αN is involved in physical disruption of the bond between the P-loop and the β-phosphate of GDP, promoting nucleotide exchange and Gα activation (Chung et al., 2011; Duc et al., 2015). The NT of Gα also engages in direct interactions with the βγ subunit; the presence of the bound βγ subunit may be important for helping to hold the NT of Gα in a conformation that promotes its interaction with the intracellular face of the receptor (Herrmann et al., 2006; Mahoney and Sunahara, 2016). Much of the job of the N terminus of Gα actually seems to relate to correctly orienting the G protein with respect to the receptor and cell membrane, as the N terminus of the G proteins are known to be palmitoylated and/or myristoylated, generally a sign that the region is involved in the docking of that protein at the cell membrane (Bohm et al., 1997; Milligan and Grassie, 1997; Wedegaertner et al., 1995). Interestingly, palmitoylation (rather than myristoylation) of this NT has been specifically linked to the Gαi/o family of G proteins, and myristoylation of Gαi/o drastically alters its affinity for various downstream effectors (Linder et al., 1991).

Overall, the release of GDP from Gα induces conformational changes that actually stabilize the G protein in its active, receptor-bound state and reduce the affinity

of Gα for GDP (Huang et al., 2015; Kruse et al., 2012; Rasmussen et al., 2011b). Additionally, intracellular concentrations of GTP are high enough ensure that GTP will bind quickly to the Gα nucleotide-binding domain, inducing a conformational shift in the G protein that includes a restructuring of the β6-α5 loop. Cycling of Gα to a more rigid and ordered conformation with GTP binding facilitates a disruption of the interactions between the α5 helix of Gα-CT and the receptor core that result in an uncoupling of the GPCR-G protein complex (Dror et al., 2015; Oldham and Hamm, 2006). This is not always the case with GPCR/G protein activation, as a tighter complex between (rather than dissociation of) Gαi and PAR1 has been documented following receptor activation with pepducin agonists. Switch regions, flexible domains mentioned in brief previously, are also important in the different activation states of Gα. With GTP binding, the Gα switch II region is adopts a more rigid conformation, driven at least in part by interactions between the γ phosphate of GTP and the amide groups at its N terminus (Sunahara et al., 1997). Increased order and structure within switch II region then allow it to form the base of a binding scaffold for G protein effector interactions, along with two other helices of Gα (Chen et al., 2008; Noel et al., 1993). GTP binding in general stabilizes other switch regions as well (Luttrell, 2008), locking these regions in a more rigid conformation that promotes their interactions with important downstream effectors (Li and Cerione, 1997).

The energy of GTP binding also disrupts the interaction between Gα and the βγ dimer, allowing each to then freely interact with downstream signaling partners, although whether this disruption leads to a rearrangement in the interaction, or a full dissociation of subunits has been the subject of much discussion and may even vary between receptor-G protein pairs (Frank et al., 2005; Medkova et al., 2002; Sprang et al., 2007).

Deactivation of signaling is brought about by the intrinsic GTPase activity of Gα itself, a process which has been studied already for decades. The catalytic sites of Gα are very conserved, and also share a high degree of conservation even with the Ras superfamily proteins. In terms of Gα, two residues that are crucial for the GTPase activity of Gα have been identified thus far (Sprang, 2016). There is a glutamate (Glu) residue near the NT of switch II (Glu 204), as well as a key arginine residue in switch I—a functional analogue of the "arginine finger" found in the catalytic domain of Ras-GTPase-activating protein "RAS-GAP"(Mann et al., 2016)—which together are involved in properly orienting and stabilizing GTP for a nucleophilic attack by a water molecule and hydrolysis of the γ phosphate (Coleman et al., 1994; Kleuss et al., 1994). There are specific ions and other signaling molecules (RGS, or regulators of G protein signaling proteins, for example) that are also involved, functioning generally to increase the rate and efficacy of GTP hydrolysis through their interactions with Gα, but overall, with GTP hydrolyzed back to GDP, the cycle resets as Gα adopts again its GDP-bound, inactive state where it can again reform the heterotrimeric complex with βγ subunits and contact membrane receptors (Sprang, 2016).

1.1.2 Classical GPCR activated signaling cascades

Now that the some of the main mechanisms governing GPCR and G protein dynamics have been covered, an exploration into classical downstream signaling pathways should follow—after all, these complex and dynamic movements of GPCRs are carried out for the purpose of transmitting signals from the outside of the cell to the inside, allowing the cell to respond to extracellular stimuli. As mentioned previously, there are four families of Gα proteins, and each of these utilizes different downstream

signaling cascades (Luttrell, 2008). While the full extent of signaling is subject to outside regulation and can vary in a cell-type, receptor, and ligand-dependent manner, the interactions of G proteins with their direct effectors are quite well established in most cases (Liu et al., 2015). Gαs and Gαi proteins both target adenylyl cyclase (AC), while Gαq activates β isoforms of phospholipase C (PLC-β). Gα12/13 is the most poorly understood of the different Gα family members, although a few signaling partners are known (Neves et al., 2002).

Gαs is ubiquitously expressed and quite well characterized in terms of its effectors; the Gαs family also includes the olfactory G protein, Gα-olf, a Gαs-like protein that exhibits much more restricted tissue expression (Baltoumas et al., 2013). The "s" of Gαs actually stands for stimulatory, which accurately describes its actions towards its effector protein, adenylyl cyclase (AC). There are a number of AC isoforms, all of which are activated by Gαs but are differentially expressed between cell types (Harry et al., 1997; Sunahara et al., 1996). AC is an enzyme that catalyzes the conversion of adenosine triphosphate (ATP) into 3',5'-cyclic adenosine monophosphate (cAMP), leading to the initiation of cAMP dependent signaling. Increased intracellular [cAMP] stimulates both protein kinase A (PKA) and the exchange protein directly activated by cyclic AMP (Epac) (Holz et al., 2006; Luttrell, 2008).

PKA is a tetramer made up of two regulatory and two catalytic subunits; the catalytic subunits are responsible for the kinase activity of the protein, while the regulatory units anchor the protein in the cytoplasm (Taylor et al., 2013). PKA is activated by the binding of four molecules of cAMP (two molecules to each regulatory subunit), inducing conformational changes that lead to the release of the catalytic

(PKA_{cat}) from the regulatory subunits, thus leaving PKA_{cat} free to phosphorylate its various intracellular targets both in the cytoplasm and nucleus (Hamuro et al., 2004; Turnham and Scott, 2016). Epac is a guanine-nucleotide-exchange factor (GEF) that contains a cAMP binding site and GEF domain that is homologous to that of Ras and Rap1; when activated by cAMP binding, Epac acts as a GEF for Rap1, to stimulate a myriad of downstream signaling pathways (de Rooij et al., 1998; Kawasaki et al., 1998). Epac and PKA are the primary transducers of Gαs signaling, although intracellular cAMP accumulation can also activate certain membrane channels (Syrovatkina et al., 2016; Wettschureck and Offermanns, 2005). This G protein family will be the focus of much discussion later in terms of its demonstrable salutary effects in diabetes therapeutics.

Gαi proteins are also expressed in all cell types; this family actually includes the greatest amount of diversity between members, encompassing also Gαo, Gαt (transducin; involved in phototransduction), Gαz (primarily expressed in platelets and neuronal cells), and Gα-gust (gustatory, involved in taste), all of which couple to AC, along with a more variable list of other effectors (Casey et al., 1990; Coleman et al., 1994; Hollmann et al., 2005; Jiang and Bajpayee, 2009; Wong et al., 1992; Yang et al., 2000). Gαi—for inhibitory—functions in direct opposition of Gαs to inhibit AC and prevent the generation of intracellular cAMP and downstream signaling (Luttrell, 2008; Van Eps et al., 2018). There are quite a few AC isoforms, and while all of them can be activated by Gαs, not all can be inhibited by Gαi (Sunahara et al., 1996).

The Gαq family—including Gα11, Gα14, and Gα15/16 in addition to Gαq itself—all activate PLC-β to stimulate inositol lipid signaling, although this family of G

proteins is also known interact with a number of other effector proteins (Hubbard and Hepler, 2006). PLC-β is an enzyme that catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG). The generation of IP_3 activates IP_3 receptors (IP_3R) and causes intracellular calcium release from endoplasmic reticulum (ER) stores. DAG has its own cellular activities, and induces activation of protein kinase C (PKC) and further downstream signaling (Berridge, 1987; Mizuno and Itoh, 2009).

Gα12/13 finally then stimulates RhoA GTPase activity through its interactions with a small subset of specific RhoGEFs, although both Gα12 and Gα13 have been

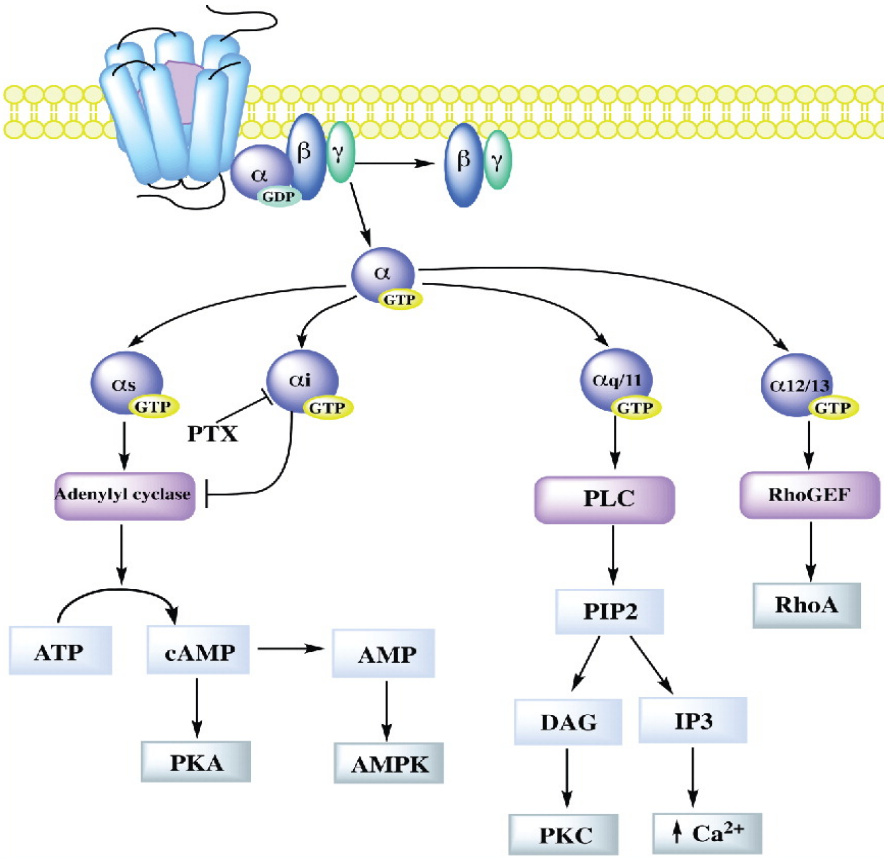


Figure 1.4: G protein Signaling Effector Pathways

Reprinted with permission from: Gonzalez-Mariscal, L., Raya-Sandino, A., Gonzalez-Gonzalez, L., and Hernandez-Guzman, C. (2018). Relationship between G protein coupled receptors and tight junctions. Tissue Barriers *6*, e1414015.

shown to interact with a variety of signaling proteins including kinases, integrins, GTPase Activating proteins (GAPs) and more (Suzuki et al., 2009; Wettschureck and Offermanns, 2005). The basic effector pathways of each Gα protein family member are summarized in Fig. 1.4. Interestingly, receptors studied thus far do not seem to couple only to Gα12/13—those that can activate Gα12/13 always seem to couple to other G proteins as well (Hollmann et al., 2005). While it is not uncommon for a receptor to be capable of interacting with more than one type of G protein, they will generally exhibit preferential coupling to one type of G protein with reduced efficacy at another, or will only display alternate couplings under special circumstances, such as in heterologous overexpression systems (Wang et al., 2018).

While much of GPCR signaling research initially focused on the roles of Gα, the Gβγ subunit actually exhibits its own range of cellular activities in response to receptor/G protein activation. Initially, the βγ dimer was thought to only play a role in the negative regulation of Gα through its physical interactions and sequestration of Gα away from other signaling partners (Neer, 1995). This would make sense, as Gα exhibits significant structural changes in response to its interactions with βγ; βγ dimers engage with Gα subunits at Gα switch regions I and II—which as discussed previously, exhibit critical GTP/GDP nucleotide-dependent structural rearrangements—as well as at its N terminal helix (Bohm et al., 1997). A relatively wide range of signaling effectors have been documented for the βγ dimer upon its dissociation from the α subunit, which includes PLC-β (Boyer et al., 1994; Camps et al., 1992), certain adenylyl cyclases (Chen et al., 1995; Federman et al., 1992; Tang and Gilman, 1991), membrane calcium channels (Herlitze et al., 1996; Ikeda, 1996; Ruiz-Velasco and Ikeda, 2000), and membrane

potassium channels (Peng et al., 2003; Sadja et al., 2001). Gβγ interacts with other non-canonical effectors as well, including some that are not even found at the plasma membrane. These activities have indirectly been linked to the regulation of cellular transcription, cytoskeletal rearrangement, mitochondrial activity, and other cellular processes (Senarath et al., 2018). While the β and γ are always found as a dimer rather than individual monomers, there are actually 5 different Gβ isoforms, and 12 different Gγ isoforms (Clapham and Neer, 1997; Simon et al., 1991). The particular pairings of dimers and expression of specific isoforms varies between different cells and signaling complexes, offering one mechanism of regulation by which the Gβ γ dimer can interact with wide variety of downstream effectors in a highly specialized and selective manner (Khan et al., 2016; Mirshahi et al., 2002; Samaradivakara et al., 2018).

There are two other well studied families of proteins that should be discussed with respect to GPCRs—β-arrestins and G protein coupled receptor kinases (GRKs) which are recruited to activated GPCRs and are involved in three basic activities: receptor silencing, receptor trafficking, and G protein-independent signaling (Lefkowitz and Shenoy, 2005). There are 7 different members of the GRK family (GRK1-7), which show differential tissue expression, membrane localization, and signaling properties but share certain key features (Pitcher et al., 1998; Premont et al., 1995). Generally, GRKs regulate GPCR signaling by targeting and phosphorylating activated GPCRs at serine and threonine (Ser/Thr) residues found within the C terminus and/or ICL3 (Reiter and Lefkowitz, 2006). This is an important step in initiating signal termination and membrane internalization of activated GPCRs, a process referred to as “homologous desensitization” (Shenoy and Lefkowitz, 2003). β-arrestins then induce receptor desensitization, as these

proteins bind with high affinity to agonist-bound receptors displaying these key Ser/Thr phosphorylations (Ferguson, 2001). In most (but not all) cases, this β-arrestin recruitment and binding disrupts G protein binding and further signaling by physical steric inhibition (Freedman et al., 1997; Liggett et al., 1993; Shetzline et al., 1998).

Recruitment of β-arrestins to GPCRs induces receptor sequestration by one of two distinct receptor trafficking pathways—internalization of the receptor into either clathrin coated pits or endosomal compartments—based on certain functional characteristics of receptors (Zhang et al., 1999). Class A receptors lack a conserved serine/threonine rich cluster found within the CT tail region of other classes of GPCRs that has been shown to—when present—greatly increase the affinity of β-arrestin/receptor binding (Oakley et al., 2001). β-arrestin binding to class A receptors induces their internalization into clathrin-coated pits, but the β-arrestin/GPCR complex dissociates near the plasma membrane, ensuring internalization, sorting, and trafficking of the receptor by itself, often resulting in a relatively rapid recycling of the receptor to the plasma membrane (Bahouth and Nooh, 2017; Fernandez et al., 2008; Lefkowitz and Shenoy, 2005; Oakley et al., 2000). Receptors that do carry the Ser/Thr rich cluster interact with β-arrestin with much greater affinity; in these cases, the β-arrestin/GPCR complex is instead trafficked into endosomal vesicles without dissociation of the two proteins (Moore et al., 2007; Shenoy and Lefkowitz, 2003). From here, these complexes are trafficked and sorted generally into late endosomes which are targeted for degradation rather than recycling (Zhang et al., 1999). Swapping of CT tails between GPCRs of different classes and/or removal/mutation of the phosphorylation domains has been shown to alter the stability of the receptor at the membrane as well as its trafficking after internalization, supporting the

hypothesis that these interactions between β-arrestin and the C terminal tail of GPCRs are key in determining receptor fate (Anborgh et al., 2000; Kara et al., 2006; Moore et al., 2007; Shiina et al., 2000; Vaughan et al., 2006). There are certainly other points of regulation as well, and other mechanisms by which GPCRs can be phosphorylated, desensitized, and trafficked, but this is one of the most ubiquitously known.

β-arrestins, once thought of as merely scaffolding proteins believed to serve only to regulate GPCR signaling and trafficking, have since been found to participate in their own g-protein independent signaling cascades. β-arrestins actually have been shown to have an incredibly diverse array of potential protein interacting domains, used to facilitate the activation of a number of intracellular signaling pathways (DeFea, 2011). β-arrestin recruitment has been linked to the activation of signaling proteins such as nonreceptor tyrosine kinases, mitogen-activated protein kinases (MAPKs or MAP kinases), lipid kinases, protein phosphatases, transcription factors, ubiquitin ligases and deubiquitinating enzymes and more (Peterson and Luttrell, 2017; Pierce et al., 2001); interestingly, β-arrestin has even been found in the nucleus of cells, indicating that its functions are certainly not limited to peripheral actions within the cell at the cellular membrane (Scott et al., 2002; Song et al., 2006). Not surprisingly, based on these many varied interactions, β-arrestin has been implicated in diverse cellular functions including growth/proliferation, survival, differentiation, apoptosis, gene transcription, chemotaxis, cellular adhesion, and inflammation, and more (Cleghorn et al., 2015; Hunton et al., 2005; Luttrell and Lefkowitz, 2002; Ma and Pei, 2007; Santos-Zas et al., 2013; Zhuang et al., 2011). Our continually developing understanding of G protein coupled receptors and the signaling pathways they initiate reveal their complex regulation and important

contributions to both health and disease; there remain many GPCRs that we know little to nothing about, and unraveling the secrets of these lesser known GPCRs and their functions will undoubtedly bring about changes in our understanding of health and disease, while elucidating exciting new drug targets.

1.2 Trace Amine Associated Receptor 1 (TAAR1)

1.2.1 Trace Amine Associated Receptor Family

One receptor is of particular interest, within the confines of this manuscript: Trace amine associated receptor 1 (TAAR1), a member of the family of trace amine receptors (TAARs), a group of GPCRs that have been experiencing a renaissance in understanding and appreciation as new tools emerge to examine these under-studied receptors.

TAARs, referred to by a number of names/abbreviations when first identified— TA, TAR, and TRAR— are rhodopsin-like, type A, G protein-coupled receptors (Borowsky et al., 2001; Bunzow et al., 2001). The current nomenclature was suggested to unify a group of phylogenetically and structurally related receptors into the TAAR family, which is currently made up of 9 subfamilies, TAARs 1-9 (Hashiguchi and Nishida, 2007; Lindemann et al., 2005). This group of receptors of this family are named for their endogenous agonists—amines found at "trace" levels in the body, with physiological concentrations falling under 100 ng/g tissue (Borowsky et al., 2001; Boulton, 1974). The presence of circulating "trace amines" (monoamines including tyramine, β-phenylethylamine (β-PEA), and octopamine) in the body had been documented well before the discovery of these receptors, and had been of interest in particular to neuroscientists studying other biogenic amine neurotransmitters—i.e. dopamine (DA), noraderenaline (NE), and serotonin (5-HT).

These classical neurotransmitters bear structural similarities to trace amines (TAs), but have physiologic concentrations roughly hundreds of times that of TAs (Berry, 2004). Classical trace amines such as β-phenylethylamine (β-PEA) and para-tyramine (p-

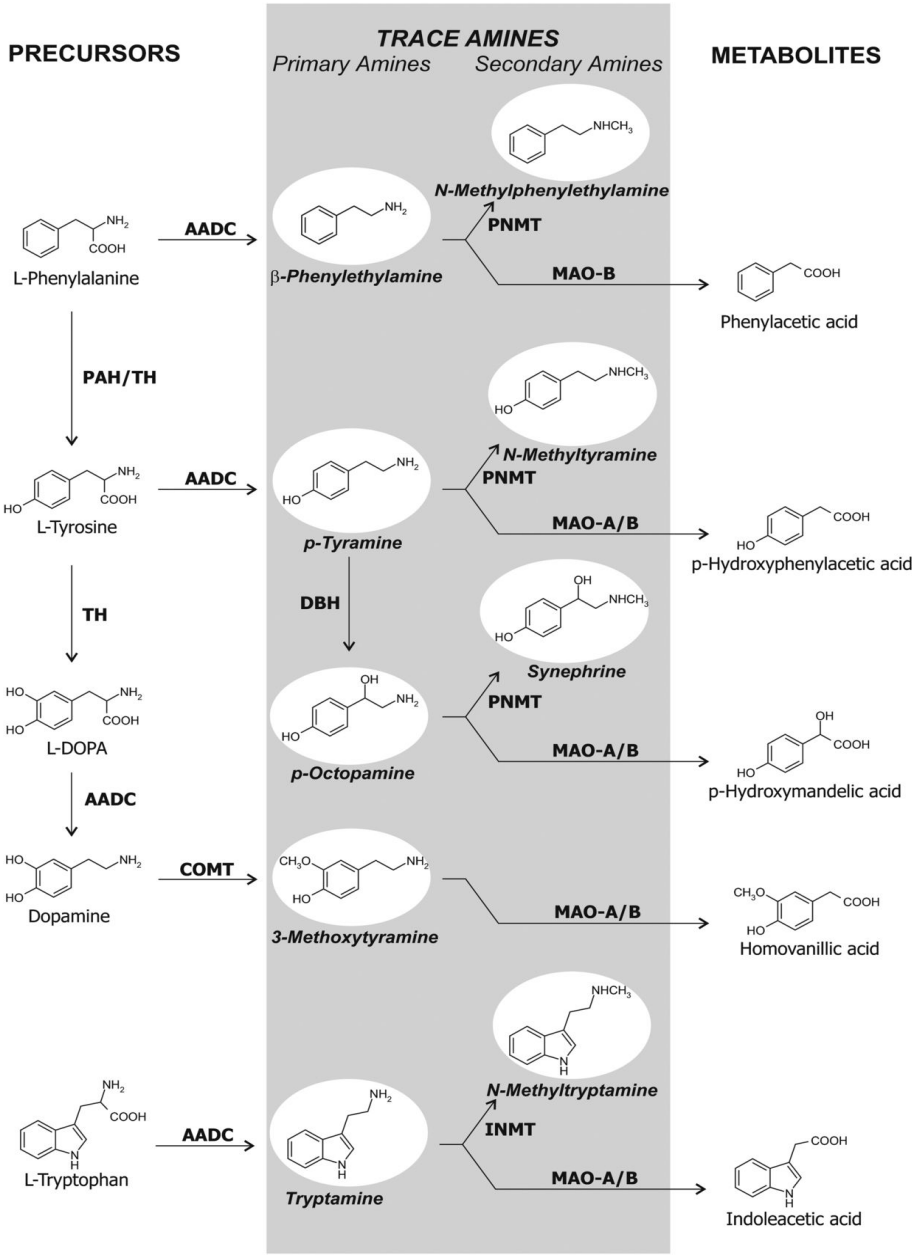


Figure 1.5: Common Biosynthesis Pathways for Endogenous Trace Amines
Reprinted with permission from: Gainetdinov, R.R., Hoener, M.C., and Berry, M.D. (2018). Trace Amines and Their Receptors. Pharmacological Reviews *70 (3)*, 549-620.

tyr) are produced from an enzymatic decarboxylation of amino acid precursors L-phenylalanine and L-tyrosine, respectively, and tyramine can be further converted to octopamine by dopamine β-hydroxylase, as shown in Fig. 1.5 (Berry et al., 2017).

Despite the disparity in the concentrations of trace amines and neurotransmitters, the two are actually synthesized at the same rate, but TAs have much higher turnover rates. Trace amine breakdown for the most part occurs through the actions of monoamine oxidases (MAOs), which are so effective that the average half-life of TAs in the body is approximately only 30 seconds (Baker et al., 1976; Berry, 2004). It has become clear that in addition initiating traditional GPCR signaling pathways, there exists a complex interplay of regulation between trace amines, TAARs and the GPCRs that mediate responses to classical neurotransmitters (Gainetdinov et al., 2018; Lewin, 2006).

The activity of trace amines is not restricted however to the nervous system. Platelets, for example, store and release trace amines into the bloodstream upon activation (D'Andrea et al., 2003), and a role for differential levels of platelet-released trace amines has been suggested in migraine susceptibility (D'Andrea et al., 2004). Regardless, it is certainly clear that much remains to be understood in terms of this diverse and complex family of receptors.

This nomenclature eventually agreed upon for TAARs—trace amine *associated* receptors, rather than simply trace amine receptors—was suggested because not all family members are activated by the classic monoaminergic trace amines, although authentic agonists of many of the receptors have yet to be identified (Gainetdinov et al., 2018; Lindemann et al., 2005). Generally however, TAARs 1-4 are activated by primary amines, while TAARs 5-9 have been found to respond to tertiary amines (Ferrero et al.,

2012). The TAAR genes are localized within a single cluster on one chromosome—6q23.2, in humans, specifically (Borowsky et al., 2001; Bunzow et al., 2001), and each TAAR is made up of one single exon, except TAAR2, which contains 2 (Lindemann et al., 2005). TAAR homologues are not found in invertebrate species, despite the presence of endogenous trace amines in those species, and while TAARs are widely expressed within vertebrates, the receptors of this family actually exhibit a high degree of variation between species, with real functional consequences. Of the nine family members, TAARs 3, 4, and 7 appear to be pseudogenes in humans but not necessarily in other species (Eyun et al., 2016; Hashiguchi and Nishida, 2007). Interestingly, TAAR1 is a pseudogene in dogs, but remains intact in the majority of other vertebrates investigated thus far, including humans and other primates (Vallender et al., 2010). Furthermore, while humans only seem to express 6 TAAR isoforms, some fish have been found to express over 100. The high degree of speciation and recent divergence between species noted within this family suggests significant and perhaps ongoing evolutionary pressure upon different species to differentially detect and respond to various trace amines (Gloriam et al., 2005; Hashiguchi and Nishida, 2007; Hussain et al., 2009; Syed et al., 2015; Tan et al., 2009).

TAAR expression has been notoriously difficult to reliably detect, leading to some disagreements about which tissues express which family members. However, consistent with their ability to detect volatile amines, there is general consensus that all family members except TAAR1 are expressed within the olfactory epithelium (OE) (Carnicelli et al., 2010; Liberles and Buck, 2006; Zhang et al., 2013). Many of the odorants found to activate TAARs tend to have aversive behavioral responses; TAAR5, found in the OE, and is activated by trimethylamine (TMA) in humans and other species;

TMA is a bacterial degradation product and a compound known for its fishy odor (Li et al., 2013; Wallrabenstein et al., 2013). In rodents in particular, TAARs have been found to be involved in behavioral stress and fear responses—many TAAR agonists can actually be found in urine, offering a TAAR mediated mechanism of predator detection and avoidance in these animals (Dewan et al., 2013; Ferrero et al., 2011; Liberles, 2009). Certain TAARs (TAARs 6 and 8 in humans) have been suggested to be activated by polyamine "necromones", or "death hormones" including cadaverine and putrescine (Izquierdo et al., 2018; Li, 2018), lending further support to the fact that TAAR based olfaction may have evolved to allow for the detection and resulting behavioral avoidance responses to averse and/or potentially dangerous odorant stimuli (Pacifico et al., 2012; Voznessenskaya, 2014).

These trace amine associated receptors are also found in several tissues outside of the olfactory system, although their generally low levels of expression have made it difficult to reliably determine the expression of TAARs in many tissues. Widespread but heterogenous expression of TAAR1 (and some of the other TAARs) has been revealed throughout the brain and central nervous system (CNS), where TAAR1 in particular is believed to modulate a variety of monoamine neurotransmitter signaling pathways (Gainetdinov et al., 2018); these TAAR1 specific effects will be covered in greater detail in subsequent chapters. TAAR6 also exhibits robust expression within the brain, although less is known about its roles there (Duan et al., 2004). In terms of other tissues, leukocytes have been shown express a number of TAAR family members, at least some of which may be capable of stimulating chemotaxis of white blood cells, leading to speculation about the involvement of TAARs in mediating inflammatory responses to

injury (Babusyte et al., 2013; Nelson et al., 2007). Expression of TAAR1, TAAR2, TAAR4, and TAAR8 mRNA been identified in heart tissue (Chiellini et al., 2012; Fehler et al., 2010). TAAR8 expression has been documented in a variety of tissues, although there remain some ongoing inconsistencies between labs in their detection of TAAR8 in some of these cases (Babusyte et al., 2013; Borowsky et al., 2001; Chiellini et al., 2012). The expression of several TAARs (TAAR1, TAAR2, TAAR6, and TAAR9) has also been documented within the gastrointestinal tract (Ito et al., 2009), which is perhaps not surprising, as a number of trace amines do have dietary sources. Trace amines such as tyramine and β-phenylethylamine, for example, are breakdown products produced by gut bacteria during the digestion of foods such as chocolate, cheese, and wine (Marcobal et al., 2012; Ohta et al., 2017). Another significant dietary source of trace amines is seafood, as certain types-such as mollusks and crustaceans- have high concentrations of TAAR agonists cadaverine, octopamine, PEA, putrescine, tyramine and more (Biji et al., 2016); octopamine, in fact, is so named because it was originally identified as an amphetamine-like chemical found in octopus salivary gland secretions (Erspamer, 1952). Many fermented foods (including wine, certain sausages, soybean products, and others) also contain high levels of trace amines, produced by the microbial decarboxylation of precursor amino acids during their fermentation (Byun and Mah, 2012; Ferreira and Pinho, 2006; Gardini et al., 2016; Suzzi and Gardini, 2003; Toro-Funes et al., 2015). It is certainly clear however that our understanding of these receptors is still in its infancy, as we continue to refine our knowledge about the differential expression and function of various TAARs in different cell types and species.

One particular stumbling block that has slowed progress in the TAAR field is what many believe is the primarily intracellular localization of the receptor, which has been documented by several labs, particularly in the case of human TAAR1 (Barak et al., 2008; Lindemann et al., 2005; Xie et al., 2008). The actual subcellular compartment in which TAARs might reside is not known, but it should also be noted that the struggle to induce expression of TAARs specifically at the plasma membrane is an issue that has previously plagued researchers attempting to express a number of odorant and chemosensory receptors in heterologous systems (Saito et al., 2004; Zhuang and Matsunami, 2007). As such, these findings may not truly represent the actual localization of TAAR1 in native tissues. The lack of specific, effective, and commercially available antibodies against these receptors has made it difficult to accurately determine the subcellular localization of receptors in their native state. It also—not surprisingly—has contributed to a difficulty in characterizing TAARs at the level of protein expression at all (Berry et al., 2017).

1.2.2 Identification and cloning of TAAR1

TAAR1 is a class A rhodopsin like GPCR of the Rhodopsin-like family; as such, it does possess many of the hallmarks of this GPCR family, including the (D/E)RY motive at the end of TM3, as well as the NPxxY motif in TM7. TAAR1 also contains a highly conserved aspartic acid residue found within TM3 that is considered a hallmark of classic biogenic amine receptors (Ferrero et al., 2012). TAAR1 does have an unusually short N-terminal region that has a very small number of glycosylation sites compared to other GPCRs; some have speculated that this may play a role in its intracellular localization (Borowsky et al., 2001; Reese et al., 2007).

The identification of trace amines actually occurred long before the identification of their receptors. In 2001, two separate groups actually identified TAAR1 as a G protein coupled receptor that could be activated by trace amines (β-PEA, p-tyr, and octopomine), as well as a number of amphetamine psychostimulants (Borowsky et al., 2001; Bunzow et al., 2001). Bunzow et al. were the first to publish their findings, and within this first manuscript, they identified a group of 15 GPCRs (including some pseudogenes, as well as some TAARs not found in humans) that they classified as belonging to (what is now) the trace amine associated receptor family.

In what was initially a search for human serotonin$_1$ (5-HT$_1$)–like GPCRs, they identified a gene product that exhibited nearly 50% amino acid identify to other serotonin, dopamine, and adrenergic receptor sequences, but had not yet been entered into the GenBank database. After sequencing of the full-length cDNA corresponding to this gene product, which would be TAAR1, the authors continued their search, identifying several other receptors that shared significant homology to this newly identified receptor, thus providing the first identification of TAAR1 and related TAAR family members (Borowsky et al., 2001).

Borowsky et al. identified a significantly higher number of TAAR variants in rodents, compared to humans—15 vs 4, in fact. A phylogenetic tree based on the sequence homologies of TAARs of different species, related receptors, as well as invertebrate TA receptors is shown in Fig. 1.6 and highlights the close relationships between TAARs and other classic biogenic amine neurotransmitter receptors, as well as the divergent evolution of vertebrate and invertebrate systems for volatile amine recognition. The separate evolution of vertebrate and invertebrate trace amine sensing

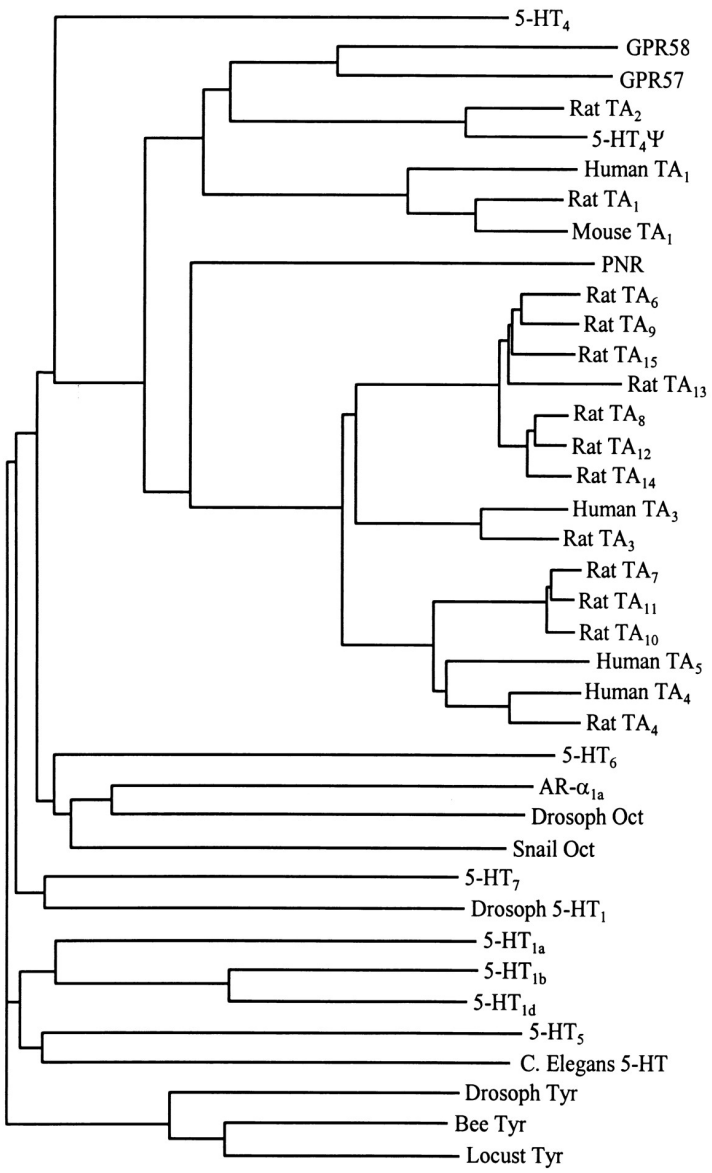


Figure 1.6: Phylogenetic Tree of TAARs and Related GPCRs

Reprinted with permission from: Borowsky, B., Adham, N., Jones, K.A., Raddatz, R., Artymyshyn, R., Ogozalek, K.L., Durkin, M.M., Lakhlani, P.P., Bonini, J.A., Pathirana, S.*, et al.* (2001). Trace amines: identification of a family of mammalian G protein-coupled receptors. Proc Natl Acad Sci USA *98*, 8966-8971. Copyright 2001 National Academy.

systems, along with an unusually high degree of variation in amino acid sequences between TAARs of different species, are suggestive of more recent or even ongoing evolutionary pressures on TAARs and amine recognition in different species (Borowsky et al., 2001; Eyun et al., 2016).

In addition to establishing the TAAR family, Borowsky et. al. characterized the activity of human, rat, and mouse TAAR1 in response to different trace amines. Activity was established on the basis of Gαolf/Gαs coupling, leading to increases in intracellular cAMP in response to TAAR1 agonism. TAAR2 also was activated by some of these ligands, with similar Gαs coupling, although difficulties in effectively driving the heterologous expression of hTAAR2 at the plasma membrane in Cos7 cells made it difficult to fully characterize the extent of TAAR2 activation (Borowsky et al., 2001).

All of the trace amine associated receptors were also found relatively close together at the human chromosome locus 6q23.2, which is actually near a susceptibility locus for schizophrenia. This led the authors to suggest a possible link between the TAARs and schizophrenia, which further research by several groups has actually supported since then (Borowsky et al., 2001; John et al., 2017; Revel et al., 2013; Wolinsky et al., 2007). *Taar1* expression could be detected in certain peripheral tissues, but was highest in the CNS, particularly within the amygdala. In fact, the authors found that 3 out of the 4 human TAARs they had identified were expressed within the amygdala, and suggested, based on this, a potential role for TAARs in mediating fear responses in humans. The authors do note that in general, expression levels of TAARs were notably lower than that of classical monoamine neurotransmitter receptors, but could still be reliably detected (Borowsky et al., 2001).

Bunzow et al published their own independent cloning and characterization of rTAAR1 later that same year; their identification of TAAR1 resulted from a search for receptors exhibiting homology to catecholamine (dopamine, norepinephrine, epinephrine) receptors, with the goal of identifying other GPCRs that might be responsive to DA and

similar biogenic amines. Their search, using cDNA isolated from pancreatic tumor cell lines and rat cerebellum led them to identify DNA fragments corresponding to that of TAAR1, which had just been entered into GenBank databases earlier that same year. Identification of *rTaar1* led to them to identify a human orthologue as well, and the identification of a chromosomal location of *hTaar1* that was consistent with Borowsky et al.'s findings. The authors again suggested potential links between TAARs, schizophrenia, affective disorders, and psychosis based on the chromosomal locus of TAAR1 (Bunzow et al., 2001).

Also consistent with Borowsky et al., the authors detected TAAR1 dependent cAMP accumulation in response to trace amines, but were also able to identify a number of other agonists for the receptor. Novel agonists included amphetamines and several of its analogues (including MDMA, known as ecstasy), as well as a number of dopamine analogues and drugs targeting dopaminergic, serotonergic, and adrenergic receptors (Bunzow et al., 2001). It should be noted again, however, that the potency of many of these compounds at TAAR1 is significantly reduced when compared to that of trace amines. True to their name, TAs activate TAAR1 at nanomolar concentrations, rather than the micromolar levels required for many of these other agonists (Bunzow et al., 2001; Reese et al., 2007).

1.2.3 Characterization of TAAR1 signaling

1.2.3.1 TAAR1 G protein coupling and effector signaling pathways

TAAR1 has generally been shown to couple to Gαs, similarly to what has been documented for other TAARs that couple to the Gαs/olf family. This preferential coupling to Gαs means that receptor activation induces intracellular cAMP accumulation

and corresponding downstream signaling (Borowsky et al., 2001; Bunzow et al., 2001; Liberles and Buck, 2006; Reese et al., 2007). Despite the majority of literature citing TAAR1 as an exclusively Gαs-coupled receptor, there have been two separate reports of alternate G proteins coupling to TAAR1. First, coupling to Gαi by TAAR1 has been suggested in leukocytes; interestingly, this particular coupling and activity of TAAR1 is thought to require heterodimerization between TAAR1 and TAAR2, which if true, may underlie this differential receptor coupling (Babusyte et al., 2013; Malki et al., 2015). As a relevant side note, TAAR1 heterodimerization has also been documented with other receptors (select serotonergic, dopaminergic, and adrenergic GPCRs), where association with TAAR1 induces biased agonism in some and desensitization/silencing of others (Braunig et al., 2018; Dinter et al., 2015; Espinoza et al., 2011; Harmeier et al., 2015).

In the second report of alternative G protein coupling, the overexpression of TAAR1 in heterologous systems was shown to allow receptor coupling alternate G proteins—specifically, the more promiscuous Gαq family member, Gα16—to induce calcium flux (Navarro et al., 2006). It is important to note that this type of coupling has not been documented for endogenously expressed TAAR1 or for that matter, by any other group. Thus, while coupling of TAAR1 to more than one G protein may be possible, it seems clear that the preferred TAAR1 coupling is to Gαs and cAMP dependent pathways. TAAR1 activation of β-arrestin dependent signaling has also been documented (Espinoza et al., 2015a; Harmeier et al., 2015), but this facet of TAAR1 dependent signaling has been far less studied thus far.

1.2.3.2 Trace amine, biogenic amine, and amphetamine agonists of TAAR1

One characteristic of TAAR1 that has made it difficult to fully comprehend its physiological function(s) has been the wide range of agonists. Initial experiments, as mentioned, identified trace and classic biogenic amines as TAAR1 agonists, with the most potent being β-PEA and *p*-tyramine, followed by octopamine, tryptamine, and dopamine. (Borowsky et al., 2001). Since then, the number of compounds identified which exhibit agonistic activity towards TAAR1 has only continued to grow, starting with the inclusion of amphetamine and amphetamine derivatives, but expanding to include quite a more diverse library of compounds.

A very thorough characterization of the activity of over 100 compounds at mouse, rat, and human TAAR1 was recently published (Simmler et al., 2016). The table reveals the binding affinity of each agonist for the receptor, as well as EC_{50}s where possible, breaking down the wide array of compounds tested into the following categories based on their structures. The short list of more potent agonists includes endogenous ligands such as trace amines (β-PEA, tyramine, tryptamine), a number of amphetamines (amphetamine, methamphetamine, MDMA), as well as other compounds belonging to the phenethylamine, aminoindane, phenethylamine, cathinone, and miscellaneous classes (Table 1.1) (Berry, 2017). It is interesting to note, when comparing the binding affinities and EC_{50} values for human, rat, and mouse TAAR1, that the majority of these compounds seem to activate hTAAR1 with reduced potency, when compared what rat and mouse TAAR1 (Simmler et al., 2016).

Table 1.1: Pharmacological Characterization of TAAR1 Agonists

	Rat TAAR1			Mouse TAAR1			Human TAAR1		
TAAR1 agonists	EC_{50}	Eff.	Ki	EC_{50}	Eff.	Ki	EC_{50}	Eff.	Ki
	[μM]	[%]	[μM]	[μM]	[%]	[μM]	[μM]	[%]	[μM]
Endogenous ligands									
2-phenylethylamine	0.11	100	0.24	0.20	102	0.31	0.26	104	
p-Tyramine	0.030	94	0.059	0.28	88	0.38	0.99	91	
(Endogenous ligands, cont)	EC_{50}	Eff.	Ki	EC_{50}	Eff.	Ki	EC_{50}	Eff.	Ki
Tryptamine	0.41	91	0.13	2.7	117	1.4	21	73	
Octopamine	2.1	100		20	100		10	100	
Dopamine	5.1	50		12	50		16	50	
Serotonin	5.2	50		> 50			> 50		
3-Methoxytyramine							0.70	100	
Amphetamine-like									
Amphetamine	0.66	91	0.23	0.53	90	0.089	2.8	91	
Methamphetamine	0.85	73	0.35	0.73	78	0.55	5.3	70	
MDMA	1.0	56	0.37	4.0	71	2.4	35	26	
Cathinone	1.2	28	2.2	1.2	66	2.1	6.9	53	
Methcathinone	8.2	41	4.1	6.8	64	> 10	> 30		
Phenethylamines									
2,5-Dimethoxy-4-bromo-phenethylamine	0.24	57	0.079	2.3	69	2.2	3.3	10	
2,5-Dimethoxy-4-propyl-phenethylamine	0.030	84	0.020	0.56	91	0.28	4.2	72	
Mescaline	3.7	37	3.3	4.8	25	11	> 10		
(-)-Ephedrine	2.5	42	3.7	14	31	> 15	> 10		
Tryptamines									
Psilocin	0.92	85	1.4	2.7	80	17	> 30		
N,*N*-Dimethyltryptamine (DMT)	1.5	81	2.2	1.2	73	3.3	> 10		

	Rat TAAR1			Mouse TAAR1			Human TAAR1		
	EC_{50}	Eff.	Ki	EC_{50}	Eff.	Ki	EC_{50}	Eff.	Ki
Ergolines									
Lysergic acid diethylamide (LSD)	1.4	29	0.45	9.7	13	10	> 20		
Piperazines									
m-Chlorophenylpiperazine	0.15	60	0.054	3.2	40	6.6	> 30		
Aminoindanes									
2-Aminoindane (2-AI)	0.11	90	0.31	0.33	54	2.1	1.5	110	
5-Iodo-2-aminoindane	0.033	96	0.030	0.41	36	1.1	3.2	33	
Miscellaneous compounds									
Apomorphine	0.99	79	0.37	2.5	59	0.37	> 20		0.70
Ractopamine				0.016	100				
3-Iodothyronamine	0.014			0.090	61		1.7	56	
Clonidine	0.21			0.97					
Guanabenz	0.007			0.025					
Idazoxan	0.11			6.7					

Eff.: Efficacy, maximal cAMP levels reached compared to 10 μM PEA. Reprinted with permission from: Berry, M.D., Gainetdinov, R.R., Hoener, M.C., and Shahid, M. (2017). Pharmacology of human trace amine-associated receptors: Therapeutic opportunities and challenges. Pharmacology & Therapeutics *180*, 161-180.

1.2.3.3 Thyroid hormone derivative and TAAR1 agonist, 3-iodothyronamine

Beyond this exhaustive characterization of TAAR1 agonists, there remain a few important ones to be discussed: 3-iodothyronamine (T_1AM), and small molecule agonists of TAAR1. T_1AM, found endogenously within the body at nanomolar concentrations, can in fact be categorized as a trace amine and has been established as an efficacious agonist of TAAR1 (Cöster et al., 2015; Scanlan et al., 2004) and TAAR2 (Babusyte et al., 2013; Cichero and Tonelli, 2017). While some debate exists over the some of the mechanisms, it has been generally agreed upon that T_1AM is a derivative of thyroid hormones, already

suggesting a potential involvement of TAAR1 in the regulation of metabolism (Hart et al., 2006; Hoefig et al., 2016; Rathmann et al., 2015).

Thyroid hormone (TH) is predominantly synthesized in the thyroid as the prohormone 3,5,3',5' tetraiodothyronine (T4, also known as thyroxine), and it is has been hypothesized that further metabolism of T4 within the thyroid (involving its deiodination and decarboxylation by specific enzymes) leads to the production of T_1AM. Structural similarities between T4 and T_1AM, seen in side-to-side comparisons of the two make it easy to picture a shared biosynthesis pathway for these compounds (Fig. 1.7) (Hackenmueller et al., 2012). Furthermore, experimental induction of hypothyroidism in mice significantly reduces T_1AM levels in mouse liver, even after the administration of exogenous T4, seemingly confirming that processing of this thyroid hormones into T_1AM must occur within the thyroid (Hackenmueller et al., 2012). Other studies however have provided contradictory evidence supporting instead a mechanism of extrathyroidal metabolism of T4 into T_1AM; one published study has shown that thyroid cancer patients who have been treated with exogenous T4 exhibited normal serum T_1AM levels, suggesting that some mechanism(s) of extrathyroidal processing of T4 into T_1AM must exist (Hoefig et al., 2011; Hoefig et al., 2015). The differing effects noted by these two groups following exogenous T4 administration has been hypothesized to relate to the

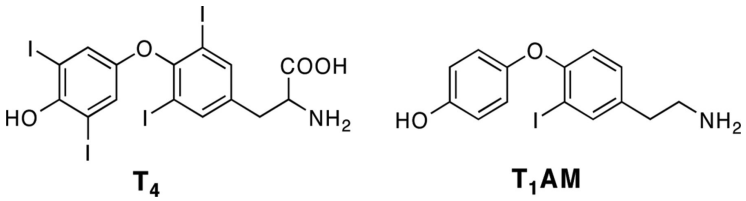


Figure 1.7: Chemical Structures of Thyroid Prohormone T4 and T_1AM

Reprinted with permission from: Hackenmueller, S., Marchini, M., Saba, A., Zucchi, R., Scanlan, T. (2012). Biosynthesis of 3-Iodothyronamine (T_1AM) is Dependent on Sodium-Iodide Symporter and Thyroperoxidase but Does Not Involve Extrathyroidal Metabolism of T4. Endocrinology *153(11)*, 5659-5667.

route of administration—specifically, if intestinal tissues are capable of processing T4, the orally administered T4 may have allowed for full processing of the enzyme in intestinal tissues of cancer patients that could not occur in the mice who were administered T4 instead via intraperitoneal (IP) injection (Hoefig et al., 2016). However, arrival at this conclusion also involves a direct comparison of mice to humans, introducing a host of other significant variables that are not taken into account.

One pitfall of using T_1AM as a TAAR1 agonist is that it also has activity at the α_{2a}-adrenergic receptor (ADRA2A), albeit with lower affinity than it exhibits towards TAAR1. T_1AM has also been shown to exhibit activity towards the serotonin 1b (5-HT-1b) receptor, which are both Gαi coupled receptors that when activated, induce a suppression of cAMP formation. It is interesting to note that both of these receptors, when co-expressed with TAAR1 no longer induce cAMP suppression in response to T_1AM; TAAR1 is proposed to form heterodimers with these receptors to downregulate or completely silence their activity. At least in these cases of heterologous co-expression, the activity of TAAR1/Gαs coupling seems to dominate in response to T_1AM, providing two incredibly relevant examples of biased signaling in GPCRs, a phenomenon discussed previously (Braunig et al., 2018; Dinter et al., 2015).

1.2.3.4 Small molecule TAAR1 agonists

After discussing the list of TAAR1 agonists identified previously, it may not be hard to understand another of the key problems that has slowed progress in terms of unraveling TAAR1 dependent signaling—while there were plenty of compounds that could activate TAAR1, there were few, if any, potent and selective agonists of the receptor. The majority of agonists instead also exhibited activity towards other trace

amine, biogenic amine, and/or adrenergic receptors, and even non-GPCR targets (Gainetdinov et al., 2018). To combat this problem, long devoted researchers within neuroscience research departments at Hoffman La Roche have conducted extensive structure-activity relationship (SAR) studies of TAARs and related receptors with the goal of designing, synthesizing, and screening a library of small molecule compounds for specific TAAR1 activity (Galley et al., 2016; Galley et al., 2012; Revel et al., 2012; Revel et al., 2011; Revel et al., 2013; Simmler et al., 2016).

This group has been the only one thus far to identify a number of potent, efficacious, and selective—even orally active—TAAR1 agonists, as well as one antagonist. TAAR1 agonists identified by this group include two full agonists (RO5166017 and RO5256390) and three partial agonists (RO5203648, RO5073012, RO5263397); the TAAR1 antagonist is EPPTB, also known as RO5212773 (Bradaia et al., 2009; Revel et al., 2012; Revel et al., 2011; Revel et al., 2013; Stalder et al., 2011). In the initial SAR-based design of these small molecule TAAR1 agonists, one of the starting compounds was the ADRA2A agonist S18616, an amino-oxazoline compound, which led to the eventual synthesis of the first identified small molecule TAAR1 agonist, "RO5166017", also known by its chemical name, (S)-4-[(ethyl-phenyl-amino)-methyl]-4,5-dihydro-oxazol-2-ylamine] and depicted in Fig. 1.8 (Revel et al.,

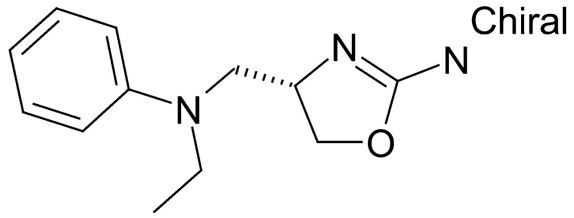
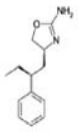
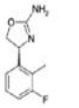


Figure 1.8: RO5166017 Small Molecule TAAR1 Agonist Structure

Reprinted with permission from: Revel, F.G., Moreau, J.-L., Gainetdinov, R.R., Bradaia, A., Sotnikova, T.D., Mory, R., Durkin, S., Zbinden, K.G., Norcross, R., Meyer, C.A., et al (2011). TAAR1 activation modulates monoaminergic neurotransmission, preventing hyperdopaminergic and hypoglutamatergic activity. Proc Natl Acad Sci U S A *108*, 8485-8490.

2011). This discovery has led to the identification of further agonists, whose structures and basic pharmacological properties are shown in Table 1.2 (Revel et al., 2013).

Table 1.2: Characterization of Small Molecule TAAR1 Agonists

Compound	Parameter, assay, preparation	Mouse	Rat	Human	Monkey
RO5256390 	K_i, binding, HEK293 cells[a]	4.4 ± 1.6	2.9 ± 0.8	24 ± 1	16 ± 6
	EC_{50}, cAMP, HEK293 cells[b]	2 ± 0.9 (79 ± 9%)	5.1 ± 1.7 (107 ± 13%)	16 ± 12 (98 ± 13%)	16 ± 13 (100 ± 3%)
	EC_{50}, GIRK, *Xenopus* oocytes [c,d]	18 ± 6.0 (68 ± 7%)	ND	ND	ND
	IC_{50}, patch-clamp, VTA slices[d]	15.2	ND	ND	ND
	IC_{50}, patch-clamp, DRN slices[d]	9.8	ND	ND	
RO5263397 	K_i, binding, HEK293 cells[a]	0.9 ± 0.5	9.1 ± 3	4.1 ± 0.2	24 ± 5
	EC_{50}, cAMP, HEK293 cells[b]	1.3 ± 0.4 (59 ± 4%)	47 ± 20 (76 ± 7%)	17 ± 13 (81 ± 9%)	251 ± 96 (85 ± 8%)
	EC_{50}, GIRK, *Xenopus* oocytes[c,d]	7.5 ± 1.1 (64 ± 2%)	ND	ND	ND
	EC_{50}, patch-clamp, VTA slices[d]	91.0	ND	ND	ND
	EC_{50}, patch-clamp, DRN slices[d]	99.9	ND	ND	ND

Abbreviations: DRN, dorsal raphe nucleus; ND, not determined; VTA, ventral tegmental area.
Values are given as nM (mean ± s.e.m.). The results were obtained from at least three independent experiments. Values in parentheses represent the maximal efficacy with respect to that of β-phenylethylamine (EC_{50}, cAMP) or *p*-tyramine (EC_{50}, GIRK).
[a]Radioligand [^{3}H]RO5166017 for mouse and rat TAAR1, [^{3}H]RO5192022 for human and Cynomolgus monkey TAAR1.
[b]Millipore immunoassay for cAMP.
[c]Current mediated by $K_{ir}3.1$ and $K_{ir}3.2$ co-expressed with TAAR1.
[d]Current at −50 mV holding potential.

Reprinted with permission from: Revel, F.G., Moreau, J.L., Pouzet, B., Mory, R., Bradaia, A., Buchy, D., Metzler, V., Chaboz, S., Groebke Zbinden, K., Galley, G., *et al.* (2013). A new perspective for schizophrenia: TAAR1 agonists reveal antipsychotic- and antidepressant-like activity, improve cognition and control body weight. Mol Psychiatry *18*, 543-556.

Most of the research published thus far characterizing the activity of these compounds—beyond necessary determination of binding affinities and EC50s—has centered on their possible psychiatric applications. A number of beneficial effects have been demonstrated in response to TAAR1 agonism in tests and models of schizophrenia, Parkinson's, narcolepsy, affective/mood disorders, and a number of addiction-related behaviors (Black et al., 2017; Ferragud et al., 2017; Liu et al., 2017b; Pei et al., 2016b; Pei et al., 2015b; Revel et al., 2012; Sukhanov et al., 2019; Thorn et al., 2014). What is

currently understood about the general mechanics by which TAAR1 exhibits its neuromodulatory effects in these models will be summarized in the following section.

1.2.4 Established TAAR1 tissue distribution and functional effects

As mentioned previously, a lack of reliable and specific TAAR1 antibodies has made it difficult to ascertain the distribution of TAAR1 across various tissues. One of the more useful tools instead has been the generation of a TAAR1 knockout mouse in which a *LacZ* reporter gene was inserted in place of the entire *Taar1* gene; this resulted in *Taar1* promoter stimulated *LacZ* expression in tissues that specifically express *Taar1* (Lindemann et al., 2008). Reporter based characterization of TAAR1 distribution was focused on CNS expression, and revealed variable levels of the receptor in different brain regions, with significant expression detected within monoaminergic (dopaminergic/serotonergic) nuclei and their projections within the hypothalamus/pre-optic area, ventral tegmental area (VTA), amygdala, dorsal raphe nucleus, nucleus of the solitary tract, and the parahippocampal region, but surprisingly, not within the olfactory bulb (Lindemann et al., 2008).

Initial studies, once underway, were quick to suggest that TAAR1 plays an inhibitory role in the regulation of dopaminergic signaling (Bradaia et al., 2009; Lindemann et al., 2008; Revel et al., 2011; Xie and Miller, 2007; Xie and Miller, 2009). This has been confirmed, and generally, TAAR1 is thought to downregulate dopamine-based reward circuits through its direct interactions with dopamine transporters and receptors, resulting in the reduction of a variety of addictive and drug related behaviors (Asif-Malik et al., 2017; Espinoza et al., 2011; Jing and Li, 2015; Leo et al., 2014; Xie and Miller, 2007). Activation of TAAR1 signaling within specific subregions of the

mesocorticolimbic system—the VTA and prelimbic cortex in particular—reduces addictive, drug reinforcing, and drug seeking behaviors in rats, including self-administration of cocaine (Liu et al., 2017b). TAAR1's regulation of monoamergic neurotransmitter signaling is not however limited to dopamine. The receptor's targeted localization within key regions of the brain, and its similarities to classic biogenic amine receptors primes TAAR1 for interactions with dopaminergic, serotonergic, and glutamatergic systems, resulting in a range of behavioral effects mediated by TAAR1 that fall into several general classifications in terms of their therapeutic value—including antipsychotic, anti-depressant, anti-addictive, and pro-cognitive. (Babusyte et al., 2013; Miller, 2011; Moore et al., 2018; Pei et al., 2015a; Revel et al., 2011; Revel et al., 2013; Sukhanov et al., 2019; Xie and Miller, 2008). A variety of addiction related behaviors are downregulated by TAAR1 agonist treatment in rodents, including binge eating as well as drug (cocaine, methamphetamine, MDMA) and alcohol related addictive behaviors (Di Cara et al., 2011; Ferragud et al., 2017; Liu et al., 2017b; Lynch et al., 2013; Pei et al., 2016b).

Expression of TAAR1 has been detected within the prefrontal cortex, where TAAR1 activation is associated with reduced impulsivity, brought about by TAAR1 mediated enhancement of NMDA-glutamatergic signaling, one mechanism by which TAAR1 could be involved in regulating addictive behaviors (Espinoza et al., 2015b). TAAR1 agonists have also demonstrated beneficial effects in rodent models of Parkinson's disease, a disease which is associated with a deregulation of dopamine signaling as well as glutamate (Alvarsson et al., 2015; Pei et al., 2016a). As originally predicted, TAAR1 agonists also exhibit beneficial effects in rodent models of

schizophrenia, inducing antipsychotic effects comparable to the traditional antipsychotic olanzapine, without weight gain associated with many antipsychotics; TAAR1 agonism by several measures was even associated with improved cognition and reduced depression and anxiety-associated behaviors (Revel et al., 2013).

TAAR1 expression (with varying success) has also been documented within a variety of peripheral tissues. So far, this has included leukocytes (Nelson et al., 2007; Wasik et al., 2012), which migrate towards trace amines in a TAAR1/TAAR2 dependent manner (Babusyte et al., 2013), as well as heart and aorta (Bunzow et al., 2001) where in rats at least, TAAR1 may be involved in inducing vasoconstriction and elevating blood pressure (Fehler et al., 2010). TAAR1 has been found in breast cancer tissues, where its expression level correlates positively with survival, although this field of research is still in its infancy (Kovacs et al., 2019; Tremmel et al., 2019; Vattai et al., 2017).

Other tissues with notable TAAR1 expression include adipose tissue, small intestine, stomach and pancreas (Borowsky et al., 2001; Raab et al.; Regard et al., 2007; Regard et al., 2008). There has been growing interest in the importance of the "gut-brain axis" within metabolic diseases in recent years (Cabou and Burcelin, 2011; Khamsi, 2016); when considering this particular subset of TAAR1-expressing tissues, in conjunction with TAAR1's additional neural distribution and links to metabolism (for example, agonists include dietary metabolites and thyroid hormone derivative T_1AM) it raises the question of whether TAAR1 might be involved in the regulation of different metabolic processes.

1.3 Type 2 diabetes

1.3.1 Rising prevalence of type 2 diabetes and metabolic diseases

Diabetes mellitus is a serious and increasingly prevalent metabolic disease that afflicted 382 million people worldwide in 2013, with future projections reaching nearly 600 million people by 2035 (Guariguata et al., 2014). Type II diabetes (T2DM)— the cause of approximately 90-95% of all diabetes cases— is generally associated with genetic and environmental risk factors such as advanced age, obesity, overnutrition, and a sedentary lifestyle (Bellou et al., 2018; Dendup et al., 2018; Pal and McCarthy, 2013; Tan et al., 2008). Type I diabetes mellitus (T1DM) however is an autoimmune disease, in which pancreatic β-cells are recognized as foreign by the immune system and destroyed, resulting in a very different etiology of disease than "insulin-independent" diabetes, T2DM (Alberti and Zimmet, 1998).

Prevalence of obesity and metabolic diseases have also been on the rise both in the United States and globally; studies have shown that the global prevalence of obesity nearly doubled from 1980 and 2000, and rates continue to rise (Stevens et al., 2012). Development of obesity and other parameters of metabolic dysfunction significantly increase the risk of T2DM, and in a difficult-to-entangle feed-forward system, diabetes is also associated with the development of other metabolic comorbidities, all of which are associated with increased risk of death (Raghavan et al., 2019). "Metabolic syndrome" in fact is a term that has been created to refer to the clustering of four key risk factors— central/abdominal obesity, insulin resistance (IR), hypertension (high blood pressure), and dyslipidemia (specifically, hypertriglyceridemia, or high cirgulating plasma triglycerides) –which when present, increase a person's risk of developing T2DM by five

times, and increase their risk of developing cardiovascular disease two-fold (Cornier et al., 2008; Grundy, 2016). A staggering roughly 34% of adults in the United States meet the diagnostic criteria for metabolic syndrome (Ervin, 2009); as this syndrome and associated comorbidities are some of the leading contributors to mortality, it is clear that we must identify new targets to tackle these growing epidemics (O'Neill and O'Driscoll, 2015).

1.3.2 Etiology and pathophysiology of Type 2 Diabetes

1.3.2.1 Mechanisms of insulin secretion in healthy β-cells

Insulin, a hormone secreted by specialized cells within the pancreas is essential for the maintenance of proper glucose homeostasis and development of diabetes. The pancreas itself actually controls a variety of functions and is made up of both endocrine and exocrine cells. The exocrine portion of the pancreas accounts for the majority of the organ (95-99% of cells) and is comprised of acinar and ductal cells which produce and transport essential digestive enzymes to the duodenum where they are used in nutrient breakdown and absorption (Bastidas-Ponce et al., 2017; Saito et al., 1978). The remaining cells that make up the pancreas are endocrine cells referred to as islets of Langerhans; these are arranged into generally oval but sometimes irregular shapes consisting of up to several thousand endocrine cells grouped together and dispersed throughout the exocrine tissue (Benitez et al., 2012; Jain and Lammert, 2009). Pancreatic β-cells, which produce and secrete insulin are the largest cellular component of islets, although the specific ratios of β-cells to other endocrine cell types varies between islets, species, and disease states (Steiner et al., 2010). There are several other cell types distributed throughout islet cell clusters and each secretes specific polypeptide hormones.

Alpha (α)-cells, the next most numerous cellular subgroup within islets secrete glucagon, a hormone that more or less acts in opposition to insulin, and is released under conditions of hypoglycemia to raise blood glucose levels (Nadal et al., 1999; Quesada et al., 2008). Glucose stimulates somatostatin release from pancreatic delta (δ) cells, which can be potentiated by other pancreatic polypeptide hormones. By these actions, pancreatic δ-cells and somatostatin participate locally in a negative feedback loop, as somatostatin secretion then reduces electrical excitability of α and β cells to suppress both glucagon and insulin vesicle exocytosis. This downregulation of electrical excitability is brought about through the activation of Gαi-coupled somatostatin receptors found on α and β cells (Braun, 2014; Rorsman and Huising, 2018). PP-cells (sometimes called "F-cells") secrete pancreatic polypeptide (PP) in response to hyperglycemia; PP secretion plays a role in regulating food intake and digestion, and these cells make up generally less than 10% of islet cells (Ekblad and Sundler, 2002; Larsson et al., 1975). The final and least numerous cell group within islets are epsilon cells, which secrete the hormone ghrelin. Ghrelin is a ligand for the growth hormone secretagogue receptor (GHS-R), and it is believed that these cells make up a larger percentage of endocrine pancreas at early stages of development, but account for less than 1% of islet cells in adult humans (Andralojc et al., 2009; Wierup et al., 2002). As the roles of and cross communications between islet subpopulations have been elucidated over the years, we continue to better understand the complex and multi-layered processes regulating glucose homeostasis. Insulin secretion by pancreatic β-cells is the classic and most studied mechanism of glucose regulation, and dysfunctions within this cellular process and cell type in particular are critical in the development of type 2 diabetes.

Insulin secretion from pancreatic β-cells is mainly controlled by blood glucose levels, but gut hormones secreted postprandially—glucagon like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP)—also play important roles in augmenting this response (Drucker, 2003a; Komatsu et al., 2013). The mechanisms driving glucose stimulated insulin secretion (GSIS) are illustrated in Fig. 1.9 (Castiello et al., 2016). Initially, elevations in blood glucose following nutrient ingestion lead to increased glucose uptake

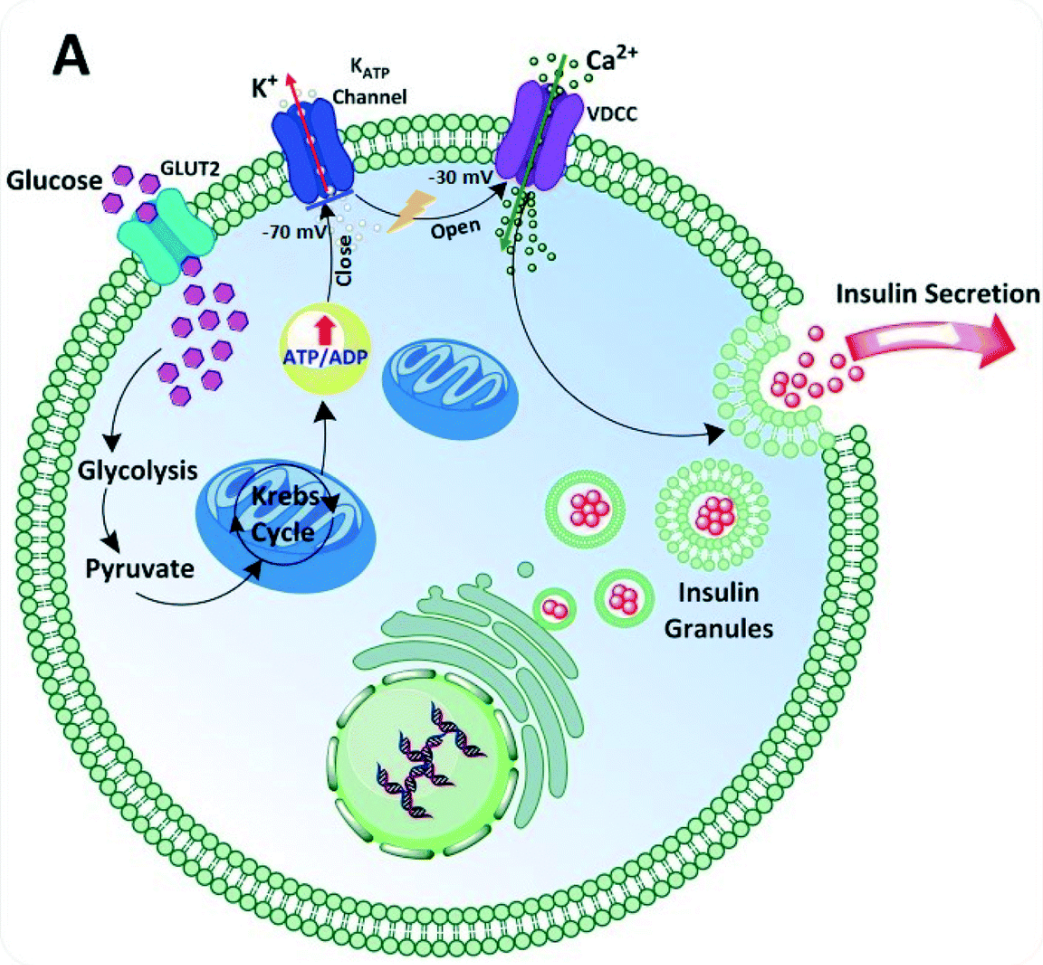


Figure 1.9: Mechanisms of Glucose Stimulated Insulin Secretion (GSIS)
Reprinted with permission from: Castiello, F.R., Heileman, K., and Tabrizian, M. (2016). Microfluidic perfusion systems for secretion fingerprint analysis of pancreatic islets: applications, challenges and opportunities. Lab on a chip *16*, 409-431.

by pancreatic β-cell glucose transporters—specifically, GLUT2 in rodents, GLUT1 and potentially GLUT3 in humans (De Vos et al., 1995; McCulloch et al., 2011; Pingitore et al., 2017).

Following its uptake, glucose is metabolized, beginning with its phosphorylation by glucokinase (GCK), an initiating step of glycolysis and rate limiting step of insulin secretion (Suckale and Solimena, 2008). Completion of glycolysis results in the generation of the intermediary metabolite pyruvate, which is metabolized by mitochondria via the Krebs Cycle to produce ATP (Sugden and Holness, 2011). The resulting increase in the intracellular ATP:ADP ratio induces a closure of ATP-regulated potassium channels (K_{ATP}), an octomeric channel made up of four inward-rectifying potassium channel 6.2 (Kir6.2) pore forming subunits, and four ATP sensitive sufonylurea receptor 1 (SUR1) subunits (Aittoniemi et al., 2009). Closure of K_{ATP} leads to an accumulation of intracellular potassium ions and localized depolarization of the plasma membrane, which stimulates the opening of voltage gated calcium channels and resultant calcium influx, the main trigger for the exocytosis of insulin containing vesicles (Henquin, 2000; Straub and Sharp, 2002). There are a variety of mechanisms by which this process is regulated, both positively and negatively—including differential regulation of insulin secretion by GPCR signaling, which will be discussed within section 1.3.3.

1.3.2.2 T2DM development and the role of β-cell failure in disease

The etiology of T2DM involves a complex interplay between gradually escalating insulin insensitivity and elevated blood glucose and insulin levels. Initially, heightened insulin secretion by pancreatic β-cells effectively compensates for increasing elevations in blood glucose (driven by genetic and environmental risk factors mentioned

previously), but resulting chronic hyperinsulinemia eventually leads to peripheral insulin insensitivity, impairing glucose tolerance (Alberti and Zimmet, 1998). This means that more insulin is required to elicit the same blood glucose-lowering effects, and generally results in impaired glucose tolerance (IGT) and impaired fasting glucose (IFG), key characteristics of prediabetes (Beale, 2013; Edwards and Cusi, 2016). As the disease progresses, hyperglycemia and insulin insensitivity drive in ever-increasing demands for insulin biosynthesis and secretion, placing β-cells under significant ER and oxidative stress (Biden et al., 2014; Evans et al., 2003; Meece, 2007). This, along with increased glucolipotoxicity and other stressors associated with obesity and T2DM (Donath et al., 2009; Esser et al., 2014) drive exhausted β-cells to undergo apoptosis, severely limiting the body's ability to produce insulin and maintain glucose homeostasis (Abdul-Ghani et al., 2006; Bonora, 2008; Campbell, 2009; Gupta et al., 2012).

The death of β-cells is an essential differentiation between prediabetes/insulin resistant states and T2DM itself (Laybutt et al., 2003)—insulin resistance alone does not constitute diabetes in the absence of β-cell dysfunction, although there is certainly a complex interdependency of these factors in disease progression (Abdul-Ghani et al., 2006; Prentki and Nolan, 2006). Early stages of disease progression are generally characterized by decreased insulin sensitivity and impaired glucose tolerance; fasting hyperglycemia is not generally detected at this stage, as β-cells can initially cope with increased demands for insulin, thus maintaining glucose homeostasis (Martin et al., 1992; Porte, 2001). The actual loss of pancreatic β-cells due to apoptosis is a critical step in disease progression, drastically shifting the balance away from somewhat dysregulated but adequate maintenance of glucose homeostasis with associated hyperinsulinemia

towards chronic hyperglycemia with associated hypoinsulinemia in the absence of outside intervention (Campbell, 2009; Fujioka, 2007).

There are a number of contributing factors for β-cell failure in diabetes, many of them intertwined with T2DM itself. In fact, prolonged exposure to high glucose actually has deleterious effects on β-cells, with chronic hyperglycemia causing glucose desensitization and β-cell exhaustion, a process referred to as glucotoxicity (Marshak et al., 1999; Rossetti et al., 1990; Sako and Grill, 1990). Increased demands for insulin biosynthesis in response to prolonged hyperglycemia increase ER stress and reactive oxygen species (ROS) generation, promoting β-cell apoptosis (Robertson et al., 2004; Wang et al., 2005). Furthermore, advanced glycation endproducts (AGEs) are produced under hyperglycemic conditions due to non-enzymatic reactions between glucose and certain cellular proteins and lipids; these glycated proteins behave abnormally and induce significant ER and oxidative stress, another mechanism by which chronic hyperglycemia can be detrimental to β-cell health and function (Lim et al., 2008; Liu et al., 2017a; Nowotny et al., 2015).

Lipotoxicity is another significant source of increased β-cell stress and apoptosis in T2DM development (Unger and Zhou, 2001). In this case, dysregulation of lipid metabolism, commonly associated with obesity and metabolic disease induces not only peripheral insulin resistance, but also β-cell death and dysfunction . While acute increases in free fatty acids (FFA) have beneficial effects, stimulating increases in β-cell mass and insulin secretion, long-term, chronic FFA exposure instead results in lipotoxicity. Chronic palmitic acid treatment of β-cells has been shown to suppress insulin gene transcription (Hagman et al., 2005; Kelpe et al., 2003) while inducing proinflammatory

gene expression (Robertson et al., 2004), ER stress, and mitochondrial dysfunction, all of which promote impaired β-cell function and death (Biden et al., 2014; Cunha et al., 2008; Karaskov et al., 2006). Also relevant, lipotoxic cell death is exacerbated by long-term co-exposure of β-cells to high glucose (Bachar et al., 2009), a phenomenon referred to as glucolipotoxicity (Barlow and Affourtit, 2013; Poitout et al., 2010).

Prolonged exposure to high glucose also causes β-cells themselves to secrete the pro-inflammatory cytokine interleukin-1β (IL-1β). IL-1β has long been implicated in the development of type 1 diabetes, but more recently, has been shown to also play a role in T2DM via its glucose-dependent upregulation (Maedler et al., 2002; Ravussin et al., 2011). IL-1β stimulates NF-kB signaling, FAS upregulation, and DNA fragmentation to induce cellular apoptosis in pancreatic β-cells (Donath et al., 2008). Metabolic diseases are generally associated with a more pro-inflammatory state, and T2DM development in particular has been linked to an increased infiltration of immune cells (particularly macrophages) and cytokines, which promote fibrosis and also induce pancreatic β-cell death (Ehses et al., 2008; Esser et al., 2014; Grant and Dixit, 2013). Keeping in mind the incredible number of overwhelming stressors placed on β-cells during disease development, novel T2DM treatments aimed at the preservation of functional β-cell mass, not just stimulation of insulin secretion are of great therapeutic interest (Bonora, 2008).

1.3.3 Incretin receptors and anti-diabetic cyclic AMP signaling in β-cells

Incretin receptors represent one such promising target for T2DM, as drugs targeting these receptors—the glucagon-like polypeptide 1 receptor (GLP-1R) in particular—have shown significant anti-diabetic therapeutic potential at least in part

through their ability potently stimulate β-cell health and function (Meece, 2007; Nauck et al., 1997a; Verspohl, 2009). Incretins are polypeptide hormones secreted by intestinal cells in response to the nutrient ingestion (including carbohydrates, lipids, and proteins) that significantly increase the glucose-stimulated insulin secretion (GSIS) responses of pancreatic β-cells through the activation of specific Gαs-coupled GPCRs.

The first incretin to be identified was glucose-dependent insulinotropic polypeptide (GIP, also referred to as "gastric inhibitory polypeptide") (Brown et al., 1975), although after a few years, it became clear that GIP was not the sole mediator of the incretin effect (Ebert et al., 1983). This led to the identification of a second incretin hormone, glucagon-like peptide-1 (GLP-1) (Drucker et al., 1987; Kreymann et al., 1987), which, of the two incretin hormones, has actually been the more effective therapeutic target for T2DM treatments (Efendic and Portwood, 2004; Seino et al., 2010). Because these hormones are only secreted when intestinal cells are exposed to glucose/nutrients, administration of glucose via oral routes elicits significantly greater

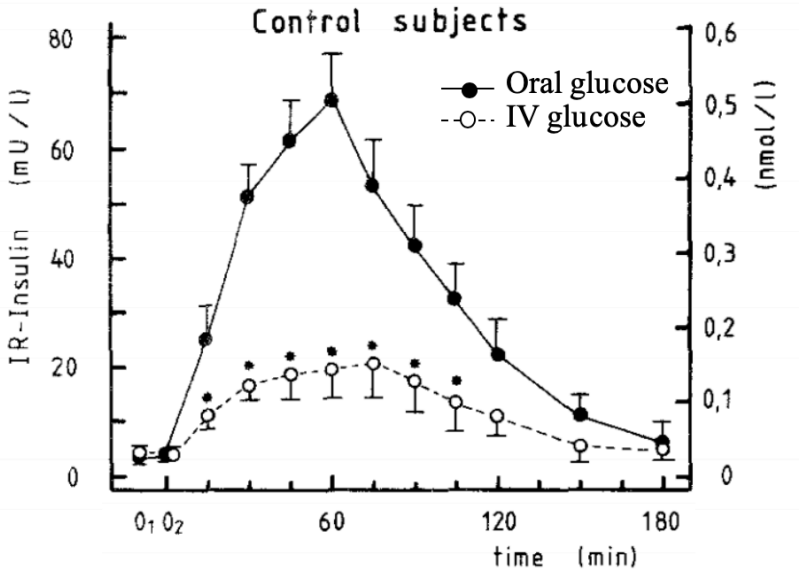


Figure 1.10: The Incretin Effect

Reprinted with permission from: Nauck, M., Stockmann, F., Ebert, R., and Creutzfeldt, W. (1986). Reduced incretin effect in Type 2 (non-insulin-dependent) diabetes. Diabetologia *29*, 46-52.

insulin secretion when compared to intravenously administered glucose challenges, a phenomenon referred to as the "incretin effect" (Baggio and Drucker, 2007)—the magnitude of the incretin effect in healthy individuals is shown in Fig. 1.10 (Nauck, 1986), in which glucose administered via oral routes (closed circles/solid line) induces significantly greater insulin secretion when compared to IV routes (open circles/dotted line).

The fact that incretins potently stimulate insulin secretion from pancreatic β-cells in a glucose dependent manner—in other words, they potentiate GSIS, but will not induce insulin secretion under low glucose conditions—makes incretins and related therapies effective regulators of glucose homeostasis that are not associated with hypoglycemia, even if mis-dosed (Montrose-Rafizadeh et al., 1994; Verspohl, 2009). Remarkably, pancreatic β-cells of type 2 diabetics often lose their responsiveness to GIP but continue to display GLP-1 sensitivity, which may account for at least part of the differing efficacies observed between therapies targeting the two different incretin receptors (Gautier et al., 2008; Nauck and Meier, 2016).

The effects of incretin hormones are not however confined to pancreatic β-cells. GIP is a 42-amino acid hormone secreted by K cells within the upper intestine that (true to its name) suppresses gastric secretions, while GLP-1, a 31 amino acid polypeptide hormone is secreted by intestinal L cells found within the lower intestine, and actually stimulates gastric secretion (Seino et al., 2010). Both GIP and GLP-1 are synthesized in their prohormone forms as prepro-Glucose-dependent Insulinotropic Polypeptide (preproGIP) and proglucagon respectively, after which they can be proteolytically cleaved to produce active peptides. These active peptides bind to and activate distinct

GPCRs found within β-cells—the GIP receptor (GIPR) and GLP-1 receptor (GLP-1R), both Gαs-coupled—to stimulate cAMP-dependent signaling (Dillon et al., 1993; Moens et al., 1996; van Eyll et al., 1994; Volz et al., 1995).

Outside β-cells, GLP-1R expression has been documented in various peripheral tissues including the gut, liver, skeletal muscle, adipocytes, and α-cells of the pancreas, as well as certain regions the brain (the hypothalamus, hindbrain, and mesolimbic regions) (Cork et al., 2015; Heppner et al., 2015; Pyke et al., 2014). GLP-1R agonism across these different tissues has been found to delay gastric emptying, promote satiety, reduce food intake, inhibit glucagon secretion, improve insulin sensitivity, stimulate weight loss, and improve hepatic glucose and lipid handling to suppress steatosis (Cabou and Burcelin, 2011; Chen et al., 2017; Dhir and Cusi, 2018; Rowlands et al., 2018; Skibicka, 2013), highlighting the vast therapeutic potential of targeting GLP-1R in metabolic diseases, not just T2DM. Significant cardiovascular benefits of GLP-1R therapies have also been documented, which involve GLP-1R mediated reductions blood pressure, heart rate and cardiac hypertrophy (Baggio et al., 2017; Kim et al., 2013; Mundil et al., 2012).

Much of the research devoted to establishing incretins as a viable therapeutic target has actually focused on pancreatic β-cells, where both GIPR and GLP-1R are highly expressed (Campbell and Drucker, 2013). A significant component of the anti-diabetic activities of incretins are due to Gαs/adenylyl cyclase induction of cAMP and downstream signaling within pancreatic β-cells (Fujioka, 2007; Furman et al., 2010a). Cyclic AMP potentiation of insulin secretion occurs via both Protein Kinase A (PKA) and Epac2 (exchange protein directly activated by cAMP) dependent pathways (Hussain

et al., 2012; Inada et al., 2004; Kang et al., 2008; Light et al., 2002). While there are two isoforms of Epac, both of which are guanine nucleotide exchange factor (GEF) proteins that act on Rap1 and Rap2 following activation by cAMP, Epac2 is the main isoform expressed by pancreatic β-cells and it is Epac2/Rap1 activity downstream of cAMP that mediates potentiation of glucose stimulated insulin secretion (Alenkvist et al., 2017; Holz et al., 2006; Kashima et al., 2001; Ozaki et al., 2000). Epac2A is involved potentiation of the first-wave insulin response, and it has been theorized that Epac may be involved in the recruitment of insulin granules to the readily releasable pool (Hashiguchi et al., 2006; Shibasaki et al., 2007). PKA-dependent pathways are thought to contribute to the recruitment of insulin granules to the reserve pool, and thus contribute to a potentiation of the second-wave insulin secretion response (Seino et al., 2011). A loss of the first wave response, coupled with a diminished second wave is characteristic of T2DM (Porte, 1991). By these and other mechanisms, GLP-1R agonism leads to the activation of both PKA and Epac dependent signaling to improve β-cell insulin secretion (Holz et al., 1993b; Iltz et al., 2006).

Cylic AMP and downstream signaling not only significantly potentiate GSIS, but also trigger an expansion of β-cell mass and protect cells from inflammatory, ER, and oxidative stress-induced apoptosis (Buteau, 2008; Hui et al., 2003; Jhala et al., 2003; Kashima et al., 2001; Park et al., 2006; Tengholm, 2012). Compensatory β-cell hyperplasia offers a mechanism of replenishing depleted β-cell mass. This potential therapeutic aspect of GLP-1R agonism involves increased β-cell proliferation (Wang et al., 2009; Xu et al., 1999) driven by cAMP initiated signaling and an induction of mitogen-activated protein kinase (MAPK) (Klinger et al., 2008). Activated PKA

phosphorylates the transcription factor cAMP responsive element (CRE)-binding protein (CREB), leading to the coordinated induction of a network of genes that promote β-cell proliferation and viability (Dalle et al., 2011; Hussain et al., 2006; Kim et al., 2006; Liu et al., 2012). Induction of IRS-2 (insulin receptor substrate 2), a known CREB target gene leads to the activation of AKT and other pro-proliferative (as well as anti-apoptotic) signaling pathways (Jhala et al., 2003; Park et al., 2006; Van de Velde et al., 2011; Wang et al., 2004). Cyclin D1 is also a purported CREB target gene, whose induction by GLP-1R activation increases β-cell proliferation (Kim et al., 2006). The potent actions of GLP-1R agonism on β-cell mass have led some to speculate that GLP-1 can act as a growth and differentiation factor for mature β-cells (Drucker, 2003b; Egan et al., 2003).

In addition to promoting cellular proliferation, cAMP-dependent signaling can also protect against β-cell death. GLP-1R agonists reduce ER stress to prevent the induction of cellular apoptosis; these effects are dependent on Gαs/cAMP signaling through PKA (Yusta et al., 2006). Exendin, a GLP-1R agonist has also been shown to effectively prevent cytokine induced β-cell apoptosis (Ferdaoussi et al., 2008; Natalicchio et al., 2010) as well as glucose/palmitate induced cell death (Kwon et al., 2004; Wei et al., 2012). β-cell failure, as mentioned previously, is critical in the development of T2DM, occurring in response to ER, cytokine, and glucolipotoxic stressors, and finding ways—like these Gαs/incretin based therapies—to not only promote the maintenance of functional β-cell mass, but to do so in times of stress is essential in the development of successful T2DM therapeutics.

1.3.4 Currently available T2DM therapeutics

Most currently available drug treatments for T2DM fall into one of a few basic categories: insulin/insulin analogues, incretin mimetic/incretin receptor agonists, sulfonylureas, thiazolidinedione, metformin, and SGLT2 inhibitors, but this list is still growing (Beran et al., 2018). Despite the multitude of available treatments, diabetes and its complications carry a heavy burden both physically and financially (Dall et al., 2010; Derosa and Maffioli, 2012; Gomez-Peralta and Abreu Padin, 2014; Wallia and Molitch, 2014; Waugh et al., 2010); new targets and treatments will be absolutely essential in combating this epidemic, but a basic understanding of the existing groups of diabetes drugs and their mechanisms of actions will also be useful.

Insulin/insulin analogues can be effective in managing the symptoms of T2DM, but are not always effective in blocking disease progression. Self-administration of exogenous insulin in response to blood glucose levels subverts a requirement for insulin synthesis and secretion by the pancreas, and can be an effective way to manage diabetes, but is in fact often associated with weight gain which itself can exacerbate insulin intolerance (Carver, 2006). Because of this, and a few other reasons (such as significant risks of hypoglycemia), insulin based therapies by themselves have actually become a less effective treatment strategy in T2DM, as it becomes increasingly difficult to effectively manage blood glucose levels with exogenous insulin alone in the face of rising insulin resistance (Raccah et al., 2007). Requirements for higher and higher levels of insulin administration can be costly, and hyperinsulinemia itself can have detrimental effects on several different cell types and tissues in the body including the liver, pancreas, muscle, kidneys, and more (Schwartz et al., 2016). Better long-term success has however

been documented for combinatorial treatments of insulin analogues along with other classes of T2DM drugs (Barnett, 2013; Goldenberg and Berard, 2018; Min et al., 2017; Seufert et al., 2013).

Sulfonylureas were the first class of orally bioavailable T2DM drugs to reach the market and have been in use since the 1950s (Seltzer, 1980). This class of insulinotropic drugs induce glucose independent secretion of insulin from β-cells by binding to and inhibiting ATP-regulated potassium channels (K_{ATP}) (Ashcroft, 1996; John et al., 2001). Under unstimulated conditions, these channels in β-cells are open, allowing basal potassium ion (K^+) efflux that is involved in maintaining cellular polarization. Channel closure—which can be induced by sulfonylurea drugs or ATP binding to the channel—blocks this efflux to induce membrane depolarization, the opening of voltage gated calcium channels, and the exocytosis of insulin containing vesicles (de Wet and Proks, 2015; Miki et al., 1999). Interestingly, in more recent years, sulfonylureas have also been found to interact with and stimulate Epac2, which actually mediates some of the anti-diabetic actions of this class of drugs (Herbst et al., 2011; Zhang et al., 2009).

Sulfonylureas can be an effective anti-hyperglycemic treatment and can even improve peripheral insulin sensitivity (Mandarino and Gerich, 1984), but there have been some reports that showing that chronic sulfonylurea treatment can actually lead to β-cell exhaustion and apoptosis (Maedler et al., 2005; Shin et al., 2012). Consistent with this, sulfonylureas have been shown to lose their efficacy over time, reflecting their inability to protect against β-cell damage to prevent a loss of functional β-cell mass (Turner, 1998). Due to the glucose-insensitive nature of insulin secretion induced by these agents, they also carry substantial risks of hypoglycemia. Concerns over these issues, as well as

increased risk of cardiovascular related deaths associated with certain sulfonylureas—potentially even directly linked to increases in severe hypoglycemic episodes (Nunes et al., 2017)—have led to somewhat of a decline in the popularity of this therapy in recent years (Douros et al., 2017; Eriksson et al., 2016).

Thiazolidinediones (TZDs) are another class of anti-diabetic drugs that have in part fallen out of favor in recent years due to serious safety concerns—Rosiglitazone, one formerly popular TZD has even been withdrawn from European markets (Consoli and Formoso, 2013; Rizos et al., 2016). These drugs became popular in the 1990s and are a commonly used secondary therapy, most often used in combination with other anti-diabetic drugs such as sulfonylureas or metformin. Their main mechanism of action is to improve lipid metabolism by activation of the nuclear transcription factor peroxisome-proliferator-activated receptor γ (PPARγ), rather than to act directly on pancreatic β-cells (Hauner, 2002). Coordinated upregulation of specific genes by PPARγ agonism in liver, fat, and other tissues promotes improved lipid and glucose handling, and has been shown to effectively improve glycemic control in T2DM patients when used as a secondary therapy (Mamza et al., 2016; Schwartz, 2008; Stumvoll and Haring, 2002).

Sodium glucose co-transporter type 2 (SGLT2) inhibitors are another class of non-insulin dependent T2DM drugs that do not target pancreatic β-cells to manage hyperglycemia. SGLT proteins (SGLT1 and 2) are active transporters responsible for the reabsorption of glucose within the kidneys. Generally, in a healthy person, nearly all of the glucose that reaches the nephron will be reabsorbed by these two transporters, with little to no glucose being excreted in urine; SGLT2, found in the proximal tubule of the nephron is actually responsible for nearly 90% of glucose reabsorption, following its

filtration in the glomerulus (Hediger and Rhoads, 1994; Vallon, 2015). Under conditions of chronic saturating hyperglycemia or kidney damage, SGLT transporters cannot transport all the glucose that reaches the proximal tubule, leaving some to be excreted in the urine; this is somewhat akin to the effects of SGLT2 inhibitors, which reduce glucose reabsorption by inhibiting SGLT2 specifically in order to promote increased urinary excretion of glucose (Davidson and Kuritzky, 2014). By this mechanism, SGLT2 inhibitors can significantly reduce demands for insulin synthesis and secretion by pancreatic β-cells, which may protect against β-cell failure (Kaneto et al., 2017; Macdonald et al., 2010). SGLT2 inhibitors have had success as both monotherapies and in combination with other T2DM treatments, and are generally effective in improving glycemic control, reducing blood pressure, and stimulating weight loss (Bolinder et al., 2014; Cuypers et al., 2013; Wilding et al., 2014; Zaccardi et al., 2016). One particular SGLT2 inhibitor (Canagliflozin) has been associated with increases in low-density lipoprotein (LDL) cholesterol, raising safety concerns in terms of potential cardiovascular risks (Halimi and Verges, 2014), but otherwise, in terms of serious side effects, this class of drugs is considered relatively safe and well tolerated (Heerspink et al., 2016; Lee, 2017).

Metformin is yet another T2DM therapeutic that does not specifically target pancreatic β-cells to exert its effects. Metformin is a derivative of guanidine, a natural compound that was originally extracted from lilacs and led to the development of metformin and phenformin, both biguanides, in the 1950s (Thomas and Gregg, 2017). The mechanisms by which metformin exhibits its beneficial glucose lowering and improved metabolic profiles are not fully understood, although it is believed at least in

part to occur through AMP-activated protein kinase (AMPK) mediated reductions in hepatic glucose production (Zheng et al., 2015; Zhou et al., 2001). Metformin directly inhibits mitochondrial activity, reducing oxidative phosphorylation and ATP production, which leads to the activation of AMPK, an energy sensor for cells activated by decreased intracellular ratios of ATP relative to AMP and ADP (Foretz et al., 2014; Towler and Hardie, 2007).

In more recent years, quite a number of AMPK-independent mechanisms of action of metformin have been identified (McCreight et al., 2016; Miller et al., 2013; Viollet and Foretz, 2013). Regardless of the lack of complete understanding of metformin's mechanisms of actions, it is clear that metformin exhibits a favorable therapeutic profile as an anti-diabetic agent, eliciting biological responses ranging from reduced blood glucose and glycated hemoglobin levels to increased peripheral and hepatic insulin sensitivity (Hostalek et al., 2015). Metformin is considered relatively safe and is effective for use as both a monotherapy or in combination with other anti-diabetic agents (Bianchi et al., 2017; Maruthur et al., 2016). Unfortunately, metformin has not been shown to improve β-cell functioning or survival, which is reflected by a progressive failure of metformin to maintain adequate glycemic control over time without the addition of secondary therapies (McIntosh et al., 2011; Turner et al., 1999).

Incretin based therapies are one popular class of T2DM therapeutics that do actually target pancreatic β-cells—and others—to exert their effects. Incretin hormones, as discussed previously (section 1.3.3) are potent regulators of insulin secretion secreted by the gut that lead to the activation of G protein coupled receptors expressed in islets and a number of other tissues (including cells in both the CNS and periphery) (Campbell

and Drucker, 2013). Incretin mimetics targeting these $G\alpha_s$-coupled GPCRs (GLP-1R and GIPR) derive many of their therapeutic benefits through the activation of Gαs/cAMP-mediated signaling in pancreatic β-cells (Yang and Yang, 2016). Agonists of GLP-1R/GIPR acutely improve glycemic control in T2DM by acting on glucose-stimulated insulin secretion (Green et al., 2004). GLP-1R agonism in particular initiates a range of finely-tuned signaling cascades that protect and promote β-cell health and survival, a critical component in their longer term efficacy as a T2DM therapeutic (Chon and Gautier, 2016; Drucker, 2003a; Li et al., 2003).

In addition to actual incretin receptor agonists such as Exenatide and Liraglutide (both GLP-1R agonists), therapies targeting the incretin system include inhibitors of dipeptidyl peptidase-4 (DPP-4), the enzyme responsible for the proteolytic cleavage of GLP-1/GIP and the incredibly short biological half-life of these peptides (Rolf et al., 1993; Verspohl, 2009). Prolonging the life of endogenous GLP-1 has been found to be an effective alternative method of increasing incretin hormone signaling to improve glycemic control in T2DM—accordingly, DPP-4 inhibitors have been successfully used as both mono and combinatorial therapies for T2DM treatment (Htike et al., 2017; Min et al., 2017; Scheen, 2010; Seufert et al., 2013).

In addition to promoting the maintenance of functional β-cell mass to halt T2DM progression, incretin hormone based treatments are often associated with weight loss. This may actually confer a significant advantage to these therapies when compared to a number of other T2DM treatments including insulin analogues, sulfonylureas, and thiazolidinediones, which have routinely been associated with weight gain—a particularly undesirable side effect when treating metabolic diseases (Carver, 2006;

Fonseca, 2003; Glass et al., 2008; Hermansen and Mortensen, 2007; Iltz et al., 2006; Lund and Knop, 2012; Mitri and Hamdy, 2009; Moretto et al., 2008; Nauck et al., 1997a; Purnell and Weyer, 2003; Verspohl, 2009; Waugh et al., 2010). The glucose-dependent regulation of insulin secretion by incretin mimetics also elevates the safety profile of these types of drugs over that of sulfonylureas and insulin based therapies, as they do not carry significant risks of hypoglycemia (Schwartz and DeFronzo, 2014). Interestingly, GlP-1R agonists increase the sensitivity of K_{ATP} to channel closure in an adenylyl cyclase (AC)-dependent manner—accordingly, in addition to promoting long-term β-cell survival to improve T2DM outcomes, incretin mimetics can actually improve the sensitivity of β-cells to sulfonylureas (McClenaghan et al., 2006), making them an ideal candidate for secondary therapy in T2DM patients experiencing sulfonylurea failure (Buse et al., 2004).

1.3.5 TAAR1 in β-cells and metabolism: building interest for TAAR1 as a potential T2DM therapeutic

One newly identified β-cells insulinotropic agent is the TAAR1 receptor (Raab et al., 2016). After being identified as a GPCR with "enriched expression in pancreatic β-cells" (Regard et al., 2007), nothing further was published regarding TAAR1's physiological activites in β-cells until nearly a decade later, at which point a group at Hoffman La Roche published a paper describing what they referred to as the "incretin-like" effects of TAAR1 (Raab et al., 2016). They use RO5166017, a novel small molecule TAAR1 agonist previously designed and characterized by Hoffman La Roche (Revel et al., 2011) to characterize TAAR1 activity in this context. The authors make a number of striking discoveries, identifying novel metabolic effects of TAAR1 agonism, and using an in-

house TAAR1 antibody to characterize TAAR1 protein expression in various relevant tissues. The found that pancreatic islets do indeed express TAAR1 (Fig. 11); TAAR1 staining of human pancreatic islets with TAAR1 (green) and insulin (red) reveals significant colocalization (yellow) between the two, indicating that TAAR1 is specifically expressed in insulin-containing β-cells of islets. In terms of pancreatic β-cells, a robust potentiation of glucose stimulated insulin secretion was also documented in response to RO5166017 *in vitro* (Raab et al., 2016), but no examinations of the mechanisms behind this exciting result were made.

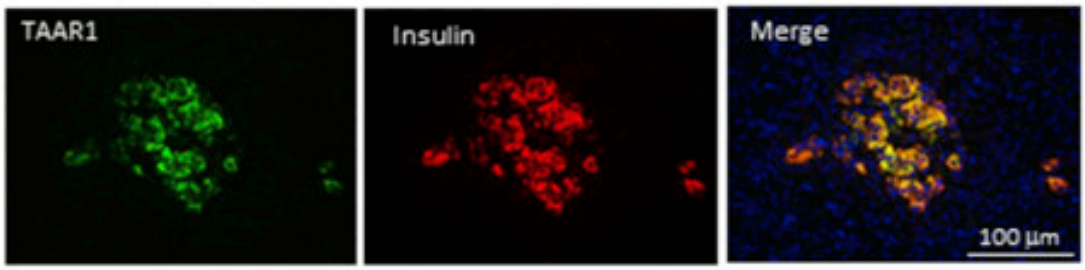


Figure 1.11: TAAR1 Expression in Human Pancreatic Islets

Reprinted here with permission from: Raab, S., Wang, H., Uhles, S. et al. (2016). Incretin-like effects of small molecule trace amine-associated receptor 1 agonists. Molec Metab 5(1): 47-56.

TAAR1 agonism also significantly improved glucose tolerance in oral glucose challenges administered to both healthy WT and "db/db" mice, a commonly used mouse model of T2DM (Raab et al., 2016). Db/db mice are a strain of leptin receptor deficient mice; the leptin receptor plays essential roles in regulating satiety, and thus leptin receptor deficiency is an effective driver of hyperphagic eating behaviors that result in pronounced metabolic dysfunction and a T2DM-like phenotype (Wang et al., 2014). Improved glucose tolerance was also documented in diet-induced obese (DIO) animals acutely pretreated with RO5166017 (Raab et al., 2016). These effects are consistent with

a mechanism of action by which TAAR1 agonists promote efficient glucose clearance via direct potentiation of β-cell GSIS, but do not rule out other potential peripheral effects of RO5166017 that could drive improve glucose tolerance.

TAAR1 expression was also detected within the duodenum, jejunum, and pylorus of the stomach. The researchers found that oral RO5166017 treatment, following a glucose challenge significantly increased plasma levels of both GLP-1 and peptide YY (PYY), both gut hormones that promote satiety. This is likely due to direct effects of TAAR1 expressed within intestinal L-cells, a specific subset of enteroendocrine cells responsible for synthesizing and secreting these important gastric hormones (Raab et al., 2016). Furthermore, TAAR1 agonist treatment was found to delay gastric emptying, an effect also demonstrated by GLP-1R agonists that not only promotes satiety but also prevents sharp and rapid elevations in blood glucose levels after a meal by slowing digestion (Nauck et al., 1997b; Raab et al., 2016).

RO5166017 significantly reduces food intake after both acute and sub-chronic (7 day) dosing of healthy and DIO mice, respectively. Sub-chronic treatments of DIO mice with RO5166017 also stimulate weight loss, reduce fasting insulin concentrations (indicative of improved insulin sensitivity and glucose handling), and significantly reduce liver triglycerides (Raab et al., 2016). Despite the wealth of exciting data contained within the manuscript by Raab et al., there have been no attempts to uncover the signaling mechanisms that underlie these powerful metabolic effects (beyond characterizing these effects as TAAR1-dependent through the use of TAAR1 knockout mice).

In terms of other potentially relevant research, there have been a few interesting reports linking T_1AM administration to significant metabolic shifts and upregulated lipolysis, further raising questions of TAAR1's potential involvement in regulation of metabolism. TAAR1 expression has been reported in adipocytes (Regard et al., 2008), and treatment of rats with T_1AM for 5 days significantly upregulates genes related to lipolysis and beta-oxidation, while downregulating the expression of genes linked to adipogenesis (Mariotti et al., 2014). Daily treatment of mice with low doses of T_1AM stimulates weight loss that persists following discontinuation of treatment and is associated with changes in the respiratory quotients of animals that are indicative of a T_1AM-induced catabolic shift from carbohydrate to lipid breakdown (Haviland et al., 2013). These reports certainly raise an interesting question as to whether TAAR1 might be involved in mediating any of the lipolytic effects observed in response to the known TAAR1 agonist, T_1AM.

Taken together, these findings portray TAAR1 as a target worthy of further investigation for the treatment of metabolic diseases, particularly perhaps for type 2 diabetes. There are quite a number of questions to be answered in order to establish the receptor's therapeutic potential, but considering the TAAR1's tissue distribution and likely Gαs-coupling, we sought to begin by characterizing the induction of potentially anti-diabetic signaling cascades in β-cells by TAAR1.

Chapter 2: Materials and Methods

2.1 Materials

Forskolin, T_1AM, H-89, FK506, and thapsigargin were purchased from Cayman Chemical Company. MDL-12,330A, HJC-0350, Esi-05, and 2-APB were from Tocris. Flag M1 antibody, AZ-628, PD-98049, EGTA, G418, RO5256390 and Esi-09 were obtained from Sigma. Rat and mouse insulin ELISAs were purchased from Mercodia. RPMI-1640, DMEM, Trypsin/EDTA, penicillin/streptomycin, BAPTA, AM and Fura-2, AM were purchased from Invitrogen. T-Per Lysis buffer, HALT protease/phosphatase inhibitors, lipofectamine, Opti-Mem and RPMI-1640 were purchased from Fisher Scientific. Antibodies against phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, and phospho-CREB (Ser133) were from Cell Signaling Technology, and β-Actin was from Santa Cruz Biotechnology. FITC conjugated secondary antibodies were purchased from eBiosciences. siRNAs used were from Santa Cruz Biotechnology. [3H]-thymidine and Steady Lite Luciferase Reporter Gene Assay System kits were purchased from Perkin Elmer. hTAAR1 and mTAAR1 cDNA constructs were purchased from cDNA Resource Center. cAMP ELISA kits were purchased from Enzo Life Sciences.

2.2 Cell culture

Clonally derived Ins-1 (832/3) cells that exhibit robust glucose and incretin responsiveness were provided by Dr. Christopher Newgard, and cultured according to established protocols (Hohmeier et al., 2000). Cells were maintained in a humidified incubator at 37 °C in RPMI-1640 media supplemented with 10% Fetal Bovine Serum (FBS), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin and subcultured

when confluent. Min6 cells were provided by Dr. Melanie Cobb and grown in DMEM supplemented with 10% FBS, 1 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 55 μM mercaptoethanol. HEK293 C34-2BIZZ-CRE cells—HEK293 cells stably expressing a luciferase reporter gene under the control of a cyclic AMP Response Element promoter (CRE-luciferase)—were kindly provided by Dr. Martin Beinborn and cultured according to established protocols. These cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1.25 μg/mL puromycin.

2.3 Generation of hTAAR1 stable cell line

A human-TAAR1 (hTAAR1) cDNA expression construct purchased from cDNA.org was amplified using DH5α *E.coli* and purified using Qiagen DNA plasmid maxiprep kits. Due to a lack of detectable expression in its original form, the hTAAR1 insert was then excised and subcloned into a different backbone vector using unique restriction enzyme sites and sticky end ligation. The modified pcDNA3.1 vector into which hTAAR1 was inserted into contains an N-terminal flag tag and membrane localization sequence designed to drive trafficking of the receptor to the plasma membrane. This construct carries a bacterial resistance gene for ampicillin, and a mammalian resistance gene for G418.

HEK293 cells stably expressing a CRE luciferase construct (under puromycin resistance) were transfected with the modified hTAAR1 construct using lipofectamine transfection reagent, according to manufacturer's instructions. 48 hours after transfection, cells were treated with increasing concentrations of G418 (from 800 μg/mL to 1600 μg/mL) to select for hTAAR1 expressing cells; untransfected HEK293 CRE-luciferase

expressing cells were also treated in parallel, to establish a kill curve. Cells were incubated with G418 for two weeks (media changed every 2-3 days), until all cells in the un-transfected groups had died—at least for some concentrations of G418—while cells transfected with hTAAR1 transfected survived the same treatment. Cells treated with the lowest concentration of G418 for which this was true (1200 µg/mL G418) were then selected for further development.

Cells from this group of hTAAR1-expressing cells were plated into 96 well plates, using a limiting dilution, such that cells were plated at a density of one cell/well to isolate single clones. Clonal populations were grown in complete media supplemented with G418 (with passaging and replating as necessary) until colonies were large enough to assay for TAAR1 expression. TAAR1 activity was assayed by CRE luciferase reporter assays and expression was measured via FACS targeting the N terminal flag tag (see below).

2.4 CRE-luciferase reporter assays

HEK293 cells stably expressing hTAAR1 and a CRE-luciferase reporter gene were seeded into white 96 well plates at 100,000 cells/well with 200 µL of complete media and returned to a humidified incubator at 37^{o}C. On the second day, media was replaced with serum/antibiotic-free DMEM, ± agonists, and returned to the incubator for 6h. Media was then aspirated and replaced with 100 µM DPBS; 100 µL of Steady Lite luciferase reagent was then added, according to manufacturer's instructions, and luminescence of wells was read after approximately 10-15 min incubation in the dark (room temp). Luminescence was read using a Promega Glo-Max microtiter plate reader with a 0.5 ms integration.

2.5 Insulin release

Cells were seeded in 24 well plates and grown until confluent. On the day of the experiment, cells are washed 1X with HBSS and further incubated for 2 h in HBSS (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$, and 0.2% BSA) with 3.5 mM (basal) D-glucose at 37^oC. Supernatants were sampled for basal insulin secretion, after which agonists were added incubated with cells for 2 h at 37^oC. Supernatants were harvested, and insulin secretion was measured via insulin ELISA; relative insulin secretion was calculated as stimulated/basal insulin secretion. Where applicable, antagonists were preincubated with cells for 30 min prior to the addition of agonists.

2.6 Real time quantitative (qPCR) and reverse transcription (RT-PCR)

RNA was isolated from cells using Qiagen RNeasy Mini Kit and reverse transcribed using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real time PCR analysis was carried out using Lightcycler 480 SYBR Green I (Roche Diagnostics), using the following primers to amplify *Irs-2, B-Raf, C-Raf,* and *Gadph*: *Irs-2*: (F): CGCAAGCATCGACTTCTTGTC, (R): GCCCGCAGCACTTTACTCTT; *B-Raf*: (F): GGAGCATAACCCACCGTCAA (R): AACAGCTGCTGCTCTCTCTG *C-Raf:* (F): CTGTCGCTGCACTACGGG (R): TCGTCTTCCAAGCTCCCTGT *Gapdh*: (F): GGCATCGTGGAAGGACTCATGAC, (R) ATGCCAGTGAGCTTCCCGTTCAGC. Relative abundance of *IRS-2, C-Raf, and B-Raf* mRNA was calculated with respect to the housekeeping gene *GAPDH* using ΔΔCT and normalizing to *B-Raf* levels.

2.7 Western blotting

Ins-1 cells were grown to confluence in 12 well plates, at which point complete medium was removed and (unless otherwise indicated) replaced with serum/glucose free RPMI, supplemented with 0.2% BSA. Cells were starved for 2 h at 37ºC and antagonists were preincubated with cells for 30' prior to addition of agonists. Cells were treated with agonists for times specified (37ºC), after which media was removed, cells were washed 1X (cold PBS) and harvested in ice cold T-PER Lysis buffer, supplemented with 1X HALT phosphatase and protease inhibitors. Proteins were resolved in 10% SDS-polyacrylamide gels via gel electrophoresis, transferred to nitrocellulose membranes, and blocked in 5% nonfat milk in Tween-Buffered Saline with 0.05% Tween (TBST) for 30 min. Membranes were nutated overnight at 4ºC with primary antibodies (phospho-ERK1/2, phospho-CREB) at a 1:1000 dilution, washed 3x, followed by an HRP-conjugated secondary antibody incubation (1:5000 dilution) for 1 h at room temp and 3 more TBST washes. After detection of phospho-proteins via chemiluminescence, blots were stripped and reprobed with loading controls (total ERK1/2, β-Actin) for normalization. Several total CREB antibodies were tested (Cell Signaling Technologies) for normalization of total CREB in the Ins-1 cells, but as these antibodies failed to reliably detect CREB, and β-actin was used.

2.8 Intracellular Ca^{2+} flux assays

Ins-1 cells were loaded with the calcium sensitive dye fura-2A/M (2 μM) in phenol red-free RPMI supplemented with 0.5% BSA for 30 min at 37ºC. Cells were then washed and resuspended in the same buffer. Calcium flux was assessed by measuring fluorescence emission at a dual excitation of 340 and 380 nm using an LS-50B

spectrofluorimeter as described previously (Covic et al., 2000). Unless otherwise specified, cells were pretreated with antagonists at room temperature for 30 min before addition of agonists. To quantify data, area under the curve (AUC) was calculated using Prism 5 and standardized to % max AUC, using the AUC for 10 μM T_1AM as 100% max signal.

2.9 [3H]-Thymidine cellular proliferation assays

Ins-1 cells were seeded into 24 well plates at 15 x 10^4 cells/ml and incubated overnight in complete media. After 24 h, media was aspirated and replaced with RPMI + 0.2% BSA + 1 mM glucose to induce senescence, and cells were incubated at 37°C for 24 hours. Media was then aspirated from cells and replaced with RPMI containing 0.2% BSA and 4.5 mM glucose. Agonists were added (where appropriate, antagonists were pre-incubated with cells 30 min prior to addition of agonists), and cells were returned to the incubator for 24 h. During the last 4 h of incubation, 1 μCi of [3H]-thymidine was added to each well. Cells were lifted, washed (2X), fixed with 6% TCA, and centrifuged. The final pellet was resuspended in 0.2N NaOH and added to scintillation fluid and read for ^{3}H-DPM counts.

2.10 FACS analysis

Cells were washed with PBS and lifted using 5 mM EDTA, then washed once in FACS buffer (PBS supplemented with 1% FBS and 0.2 mM sodium azide), before being resuspended in FACS buffer containing α-Flag (M1) antibody (1:100). Cells were incubated on ice for 1 h, washed 3 times in FACS buffer, and incubated with a FITC-conjugated secondary antibody (α-mouse-FITC @ 1:50) on ice for 1 hr. Cells were again washed three times, and resuspended in FACS buffer containing 1% formaldehyde to fix

cells, after which cells were analyzed for FITC fluorescence using a BD FACS Canto II Flow Cytometer.

2.11 Intracellular [cAMP] measurement

Min6 β-cells were plated at 1×10^6 cells/well (6-well plates) in complete media and incubated overnight. After 24 h, media was aspirated and replaced and agonists (T_1AM, PEA) were added. Cells incubated with agonists for 15 min (37ºC), at which point media was removed, and the cells were lysed by incubation with 1 ml of 0.1 M HCl for 20 min at room temperature. Cellular lysates were collected and centrifuged at 3K RPM for 5 min, and the supernatants were collected for measurement of cAMP concentration using a commercially available cAMP ELISA kit, used according to manufacturer's instructions (Enzo Life Sciences). Protein concentration of samples was also measured for normalization of cAMP content.

2.12 Gαi Immunoprecipitation

50 μg of purified membranes (from HEK293 cells transfected with Gai and empty vector or somatostatin receptor 2) were incubated in assay buffer (50 mM triethanolamine, 5 mM MgCl2, 1 mM EDTA, 100μM NaCl, 1 μM DTT, 0-10 μM GDP, and 0-10 μM GTPγS-as labeled) ± agonists (1 μM somatostatin peptide) for 5 min (room temp). Reactions were quenched by placing tubes on ice and adding 5 μL of Gαi-switch (purified antibody)-coated sepharose beads, followed by 10 μM GDP. Tubes were nutated for 1 h at 4ºC to immunoprecipitate active Gαi, after which beads were washed 3X (5 min, 1000RPM, 4ºC), and the bound proteins were eluted from beads by the addition of SDS/β-Mercaptethanol containing sample buffer, along with heating at 95oC for 5 min. Eluted proteins were collected by spinning down the sample and collecting the

supernatants, which were then run on 10% Bis-acrylamide western gels according to standard protocols; resulting membranes were blocked (5% milk in TBST), probed with Gai-CT (1:200) overnight, washed 3X, incubated with secondary (anti-rabbit-HRP conjugated secondary) for 1 hr at room temp, before being developed using chemiluminescence.

2.13 Statistical Analyses

All of the values in the figures are expressed as means ± SE or SD, as indicated in figure legends. Comparisons between experimental and control cohorts were performed by one-way ANOVA (from which a global *P* value is derived) or t-test as labeled, and where applicable, the means of the individual groups were compared using Dunnett's or Newman-Keuls post-hoc correction. Any *P* values noted on graphs are global *P* values for the data set; post-test analyses between individual groups are indicated by asterisks between relevant groups. Analyses were performed using GraphPad Prism (San Diego, CA). Statistical significance was defined as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), or $P < 0.0001$ (****).

Chapter 3: Results

3.1 TAAR1 Agonist Screening

3.1.1 Screening and selection of HEK293 CRE luciferase cell line stably expressing hTAAR1

We initially sought to generate our own line of HEK293 cells expressing both a cyclic AMP response element (CRE)-luciferase reporter gene and hTAAR1 for the purpose of screening agonists for TAAR1 activity. Like many others before us, our initial attempts to express hTAAR1 in heterologous systems failed; previous researchers have in fact commonly resorted to utilizing chimeric human/rat cDNA rather than fully human TAAR1 constructs because of this problem (Bradaia et al., 2009; Lindemann et al., 2005; Reese et al., 2007). We had used a commercially available hTAAR1 cDNA clone without modification in initial transfection experiments, and hypothesized that by changing to backbone vector from a basic pcDNA3.1 vector to a modified one that also encodes an N terminal bovine prolactin signal peptide intended to drive membrane localization of the associated protein, we might create a fully humanized TAAR1 cDNA construct that would exhibit proper membrane localization when expressed in HEK293 cells at the plasma membrane. TAAR1's naturally short N terminus actually lacks multiple glycosylation sites that are commonly found within this region of GPCRs; these N terminal modifications are believed to play a role in docking the protein at the plasma membrane, and their absence is thought to possibly contribute to TAAR1's notoriously poor membrane localization. Our addition of a signal sequence to the N terminus could then supply the required machinery to drive membrane expression of the receptor; the additional inclusion of an (extracellular) N-terminal flag tag within the hTAAR1

construct provides a straightforward method of specifically measuring membrane expression of hTAAR1 (FACS analysis of unpermeabilized cells). Initial experiments revealed robust membrane expression of hTAAR1 in transiently transfected HEK293 cells based on FACS analysis (data not shown), so we set about using this hTAAR1 construct to generate a clonally-derived line of HEK293-CRE luciferase cells stably expressing this novel hTAAR1 construct.

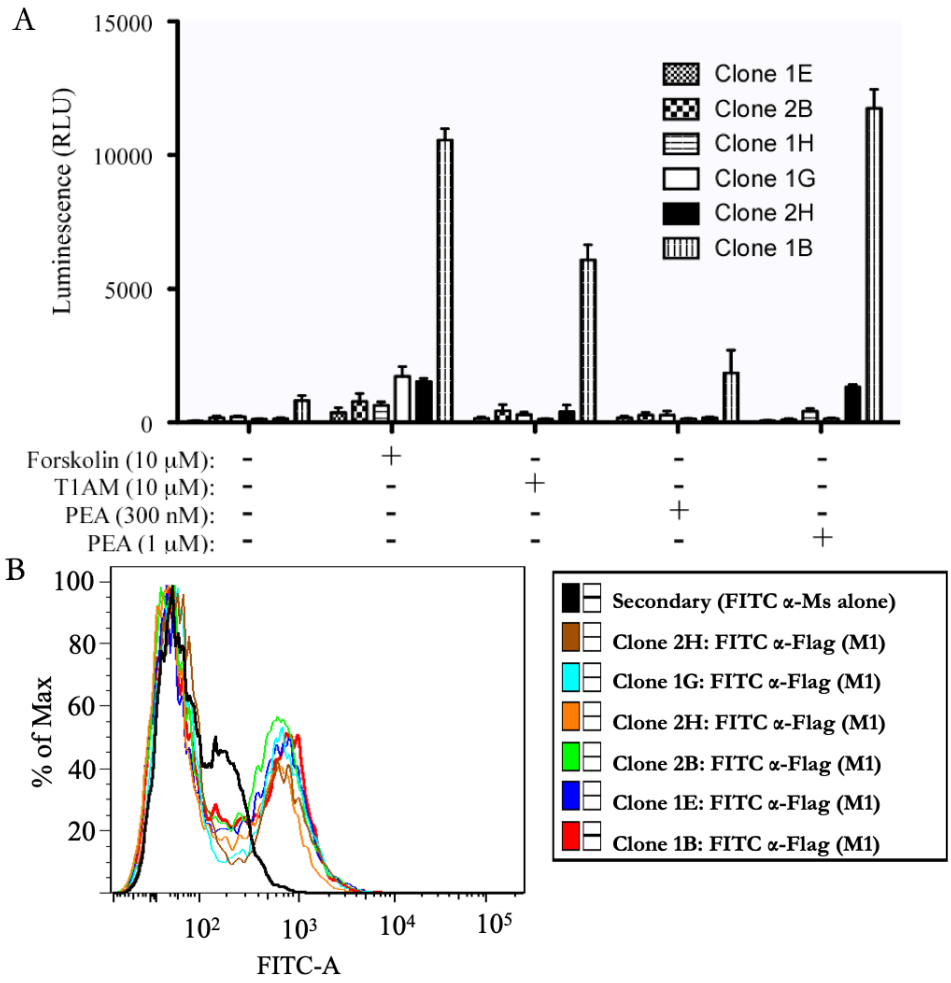


Figure 3.1: Screening of Clonal TAAR1 Stable Cell Lines

Clonal populations of HEK293-CRE Luciferase cells stably expressing hTAAR1 were assayed for (A) TAAR1 activity by measurement of luciferase activity following treatment with T_1AM and PEA (6 h; data expressed as avg. RLU ± SEM), or (B) TAAR1 expression by FACS analysis targeting an N-terminal Flag tag of hTAAR1.

Following transfection of HEK293 cells already stably expressing a CRE luciferase reporter gene with this novel hTAAR1 construct, TAAR1 expressing cells were selected for with G418 antibiotic treatment, after which a limiting dilution was performed to isolate single cells (see methods for further details). Clonal populations were expanded and assayed for both TAAR1 activity (CRE luciferase reporter assays) and expression (FACS analysis targeting NT-flag tag of hTAAR1). Fig. 3.1A shows the results of CRE luciferase reporter assays in which cells were treated with TAAR1 agonists T_1AM and β-PEA, or the positive control forskolin for 6 h. Forskolin induces cAMP generation by direct activation of adenylyl cyclase, and thus serves as a positive control for the CRE luciferase reporter system, in which luciferase production is under the control of a cyclic AMP responsive element.

While all groups displayed increased luciferase activity in response to forskolin, responses to TAAR1 agonists were more varied. Clonally-derived population 1B exhibited the most robust responses to all compounds tested—this was one of the few to actually respond to lower doses of β-PEA. Population 2H exhibited the next highest degree of activity, although responses—particularly to TAAR1 agonists were blunted—when compared to population 1B. Fig. 3.1B shows the expression levels of

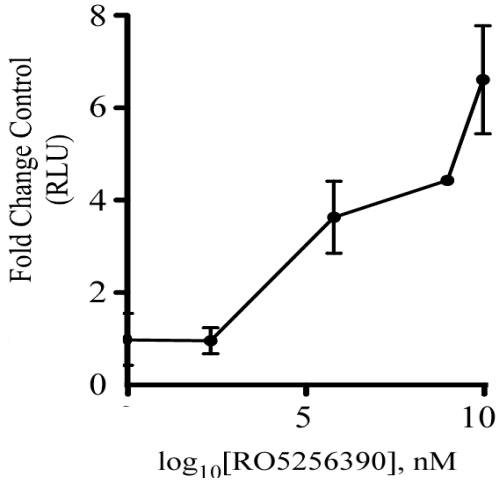


Figure 3.2: Dose Dependent CRE Luciferase Reporter Activation by RO5256390

Luciferase activity in HEK293 cells stably expressing a CRE luciferase reporter and hTaar1, following stimulation with RO5256390 for 6 hours. Data is expressed as avg. fold change control (RLU) ± SEM.

hTAAR1 for the same clonal populations, based on expression of the N terminal flag tag, and it can be seen that clonal population 1B (red) exhibits the highest levels of hTAAR1 expression. This is consistent with the results of CRE luciferase assays, and thus population 1B was selected for further expansion and use in future experiments, when an hTAAR1 expressing cell line was desired.

3.1.2 TAAR1 agonists drive CRE luciferase reporter activity in heterologous systems

In Fig. 3.2, we used our newly generated line of HEK293 cells stably expressing hTAAR1 and a CRE-Luciferase reporter gene to test the activity of small molecule TAAR1 agonist RO5256390, once the compound became commercially available (Sigma). As shown, RO5256390 dose dependently stimulates luciferase production in these cells. Maximal luciferase production in response to small molecule TAAR1 agonist treatment was comparable to that of forskolin, confirming that RO5256390 is an effective agonist of adenylyl cyclase/cAMP signaling.

3.1.3 Activation of endogenous TAAR1 in β-cells drives cAMP accumulation

Results using this novel hTAAR1 construct in our heterologous/overexpression system were consistent with the those of others, supporting Gαs-coupling of TAAR1 signaling. With the long-term goal of examining relevant anti-diabetic

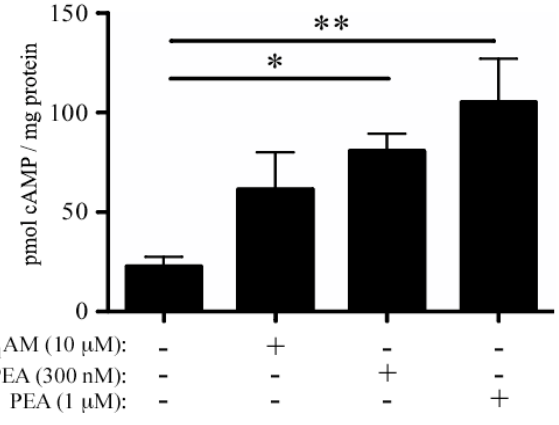


Figure 3.3: TAAR1 Agonists Stimulate cAMP Generation in β-Cells

T_1AM and PEA stimulate cAMP accumulation in Min6 β-cells, as quantified by cAMP ELISA; cAMP content is standardized to protein content. Data is presented as avg ± SEM, and analyzed by ANOVA; global $p<.01$; post-test analysis was conducted using Dunnett's test comparing all values to control; $*P<0.05$, $**P<0.01$.

signaling in pancreatic β-cells, we wanted to confirm whether endogenous TAAR1 might also couple to Gαs/cAMP signaling in cell types of interest. Using Min6 cells, a robust and commonly used glucose-responsive β-cell line derived from mouse insulinoma that express TAAR1 (Regard et al., 2007), we tested whether TAAR1 agonists would induce cAMP production. As shown in Fig. 3.3, TAAR1 agonists T_1AM and β-PEA induced cAMP accumulation in Min6 cells as measured by cAMP ELISA. These results are consistent with β-cell expression of a functional Trace Amine Associated Receptor 1 that couples to Gαs/cAMP signaling. As cyclic AMP is a well-known initiator of anti-diabetic signaling pathways in β-cells, particularly in the case of incretin hormone receptors, we set out to characterize what we hypothesized would be the salutary effects of TAAR1 signaling on β-cell function.

3.2 TAAR1 potentiates glucose stimulated insulin secretion in β-cells

3.2.1 TAAR1 potentiates glucose stimulated insulin secretion through Gαs/cAMP-dependent signaling pathways

An essential function of β-cells is to regulate glucose homeostasis by tightly controlling insulin synthesis and secretion. The secretion of insulin in response to glucose is subject to regulation by multiple inputs, including the activation of G protein signaling, and Gαs in particular is known to potentiate glucose stimulated insulin secretion (GSIS). Based on the results of CRE luciferase assays in HEK293 cells and cAMP ELISAs in β-cells, we hypothesized that TAAR1 would activate Gαs/cAMP signaling pathways to augment insulin secretion. As predicted, T_1AM stimulates potentiation of glucose stimulated insulin secretion (GSIS)—in both Ins-1 (a glucose-responsive, insulin-

secreting, rat insulinoma-derived β-cell line) and Min6 cells (Fig. 3.4A, B), significantly increasing insulin secretion responses to 20 mM glucose.

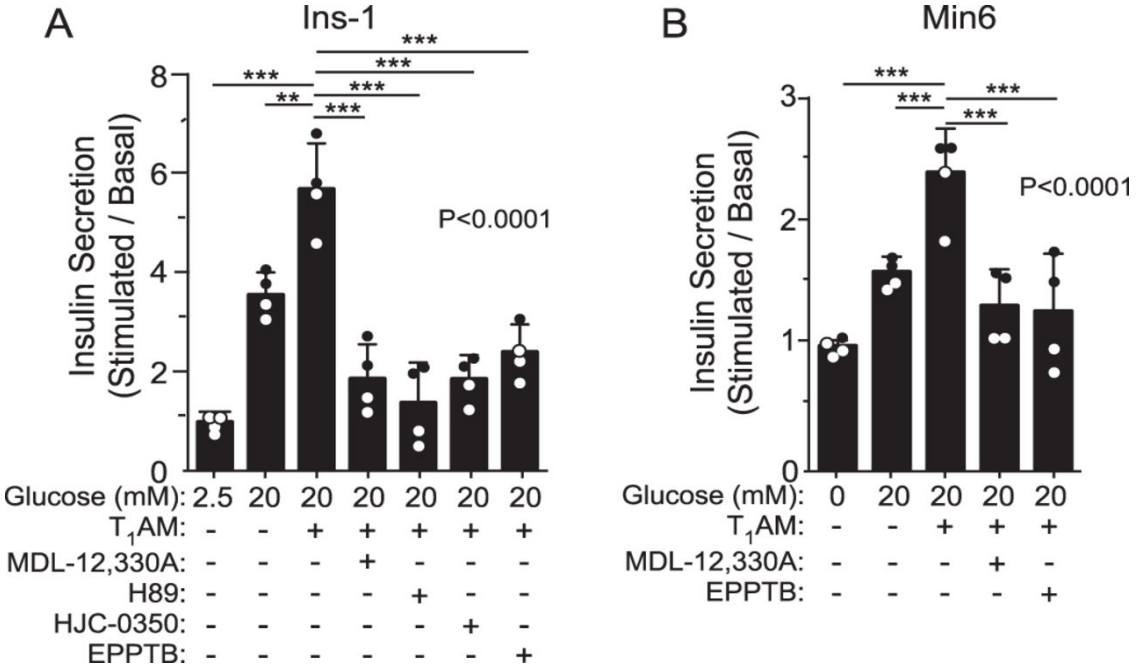


Figure 3.4: TAAR1 Potentiates GSIS via cAMP/Gαs Signaling

TAAR1 dependent potentiation of glucose stimulated insulin secretion was measured ± MDL-12,330A (10 μM; inhibits adenylyl cyclase), H89 (10 μM; inhibits PKA), HJC-0350 (10 μM; inhibits Epac) and EPPTB (10 μM; inhibits TAAR1) in (A) Ins-1 and (B) Min6 pancreatic β-cell lines. Insulin secretion was measured by ELISA (mean ± SD) and analyzed by one-way ANOVA (global P<0.0001), using Dunnett's multiple comparison post hoc test (n=4), ***P*<0.01, ****P*<0.001. Reprinted with permission from: Michael, E.S., Covic, L., and Kuliopulos, A. (2019). Trace amine-associated receptor 1 (TAAR1) promotes anti-diabetic signaling in insulin-secreting cells. The Journal of Biological Chemistry *294*, 4401-4411.

To probe the mechanisms by which TAAR1 potentiates glucose stimulated insulin secretion, we tested the effects of different Gαs signaling inhibitors. TAAR1 mediated potentiation of GSIS requires cAMP and associated downstream signaling, as treatment of cells with 10 μM MDL-12,330A, an inhibitor of adenylyl cyclase (AC), significantly (*P*<0.001) reduces TAAR1 potentiation of GSIS in both β-cell lines (Fig. 3.4A, B). EPPTB, a selective TAAR1 antagonist (Bradaia et al., 2009) also significantly (*P*<0.001)

inhibit potentiation of GSIS by T_1AM (Fig. 3.4A, B), confirming the effects of T_1AM are in fact specifically mediated by TAAR1.

PKA and Epac are both downstream effectors of adenylyl cyclase-cAMP signaling and are known to modulate granule exocytosis in β-cells. Both H89, and inhibitor of PKA, and HJC-0350, an inhibitor of Epac, caused a significant (P<0.001) reduction of TAAR1 mediated potentiation of GSIS (Fig. 3.4). All together, this data supports a mechanism of Gαs/cAMP-dependent potentiation of GSIS by TAAR1 that requires both PKA and Epac. The effects of Gαs/TAAR1 signaling in these cells are consistent with what has been shown for other similarly coupled receptors in β-cells, such as the popular anti-diabetic target GLP-1R.

3.3 TAAR1 stimulates activation of the CREB transcription factor in β-cell lines

3.3.1 TAAR1 induces glucose-independent CREB phosphorylation

CREB is a cAMP-responsive transcription factor whose activation by GLP-1R agonists plays critical roles in stimulating β-cell health (Dalle et al., 2011; Hussain et al., 2006; Jhala et al., 2003; Liu et al., 2012). Accordingly, we sought to determine if we could detect any activation of CREB in response to agonists of the similarly Gαs-coupled Trace Amine Associated Receptor 1, as indicated by phosphorylation of Ser133 of CREB. Glucose itself induces CREB phosphorylation, so a time course for CREB phosphorylation in response to T_1AM was established in Ins-1 β-cells in the presence and absence of 11 mM glucose (Fig. 3.5A). The direct adenylyl cyclase activator, forskolin, was also included as a positive control. Rapid CREB phosphorylation is induced by T_1AM at early time points (5 min) in both the presence and absence of glucose, but this phosphorylation reaches its maximum by 15 min has entirely disappeared by 30 min (Fig.

3.5A). The positive control forskolin initiates a comparable timeline of CREB phosphorylation, although the magnitude of the response differs.

3.3.2 TAAR1 induces CREB phosphorylation via Gαs/adenylyl cyclase dependent signaling in β-cells

CREB is known to be activated by Gαs/adenylyl cyclase signaling, and we hypothesized that this would be the case here. Treatment of β-cells with 10 μM MDL-12,330A effectively attenuates downstream activation of CREB in response to both T_1AM and the positive control forskolin (Fig. 3.5B), confirming that adenylyl cyclase activation is necessary for TAAR1 mediated CREB phosphorylation (and actually sufficient for the induction of CREB phosphorylation in general).

Ser133 of CREB is actually a known phosphorylation target of the cAMP-regulated kinase PKA; accordingly, inhibition of PKA with H89 effectively blocked phosphorylation of the CREB transcription factor (Fig 3.5C). Knockdown of the catalytic subunit of PKA with siRNA also caused a reduction in CREB phosphorylation when compared to cells treated with control siRNA (Fig. 3.5D). These results are consistent with those generated using H89, as well as MDL-12,330A, overall supporting a mechanism by which TAAR1 agonism leads to Gαs/AC-dependent activation of PKA and downstream CREB signaling.

3.3.3 TAAR1 induces expression of CREB target gene *Irs-2* in β-cells

A key CREB target gene, *insulin receptor substrate-2* (*Irs-2*), shown to promote pro-proliferative and anti-apoptotic signaling in β cells was significantly ($P<0.001$) upregulated by 50% in β-cells in response to the TAAR1 agonist T_1AM (Fig. 3.5E). Induction of *Irs-2* was completely blocked by MDL-12,330A (AC inhibitor; $P<0.001$),

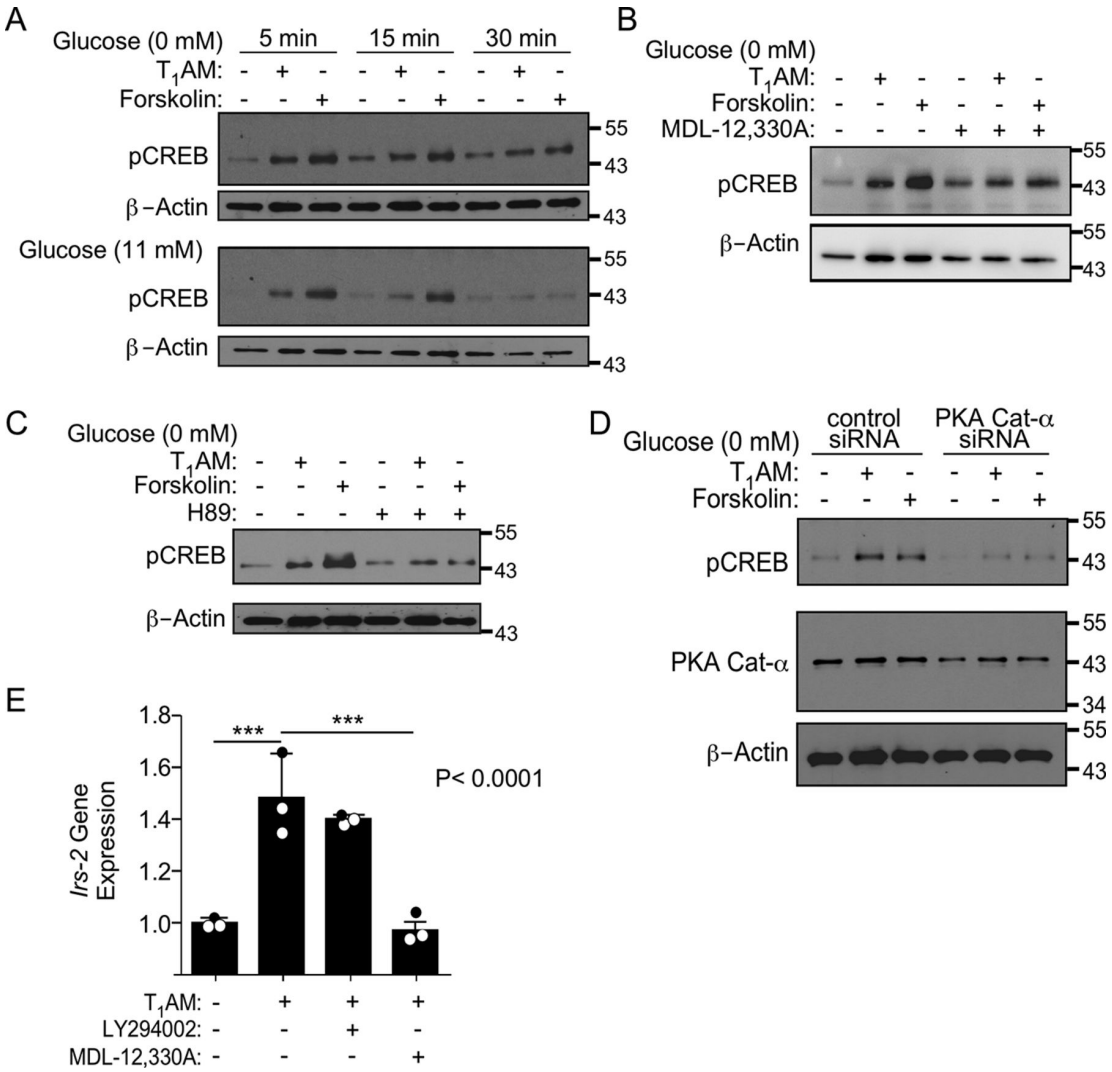


Figure 3.5: TAAR1 Stimulates Gαs Dependent CREB Phosphorylation and Irs-2 Induction

(A-D) Western blots of CREB phosphorylation in response to T_1AM (10 μM) and forskolin (0.3 μM), in the presence (11 mM) or absence of glucose as labeled. Addition of 10 μM MDL-12,330A (B), 10 μM H89 (C), or 150 nM PKA Cat-α siRNA (D) inhibits CREB phosphorylation (10 min) induced by T_1AM and forskolin. Representative blots of pCREB from one of at least three independent experiments are shown; blots have been stripped and reprobed with β-Actin as a loading control. (E) qPCR of *Irs-2* gene expression (1 h) induced by 10 μM T_1AM ± MDL-12,330A (10 μM) or LY294002 (10 μM; inhibits PI3K) pretreatment. Data is expressed as mean ΔΔCT (± SD) of *Irs-2* using *Gapdh* as the housekeeping gene, and then analyzed by one-way ANOVA (global P<0.0001), using Dunnett's multiple comparison test, comparing all columns to 10 μM T_1AM treatment; (n=3), ***P*<0.01, ****P*<0.001. Reprinted with Permission from: Michael, E.S., Covic, L., and Kuliopulos, A. (2019). Trace amine-associated receptor 1 (TAAR1) promotes anti-diabetic signaling in insulin-secreting cells. The Journal of Biological Chemistry 294, 4401-4411.

but not LY294002 (a phosphatidylinositol-4,5-bisphosphate 3-kinase inhibitor), confirming that *Irs*-2 induction by TAAR1 is mediated by Gαs/adenylyl cyclase signaling, as other signaling pathways such as those associated usually with Gαq signaling do not appear to be involved. *Irs-2* induction was also noted in response to the cAMP stimulating positive control, forskolin. These results are consistent with a role for *Irs-2* as a target of the transcription factor CREB.

3.4 TAAR1 increases β-cell proliferation by activation of Gαs-dependent Raf-MEK-ERK1/2 signaling

3.4.1 TAAR1 activates Gαs/AC dependent MAPK signaling in β-cells

We also determined that TAAR1 agonism leads to MAPK signaling in β-cells, as revealed by increased ERK1/2 phosphorylation. T_1AM treatment of Ins-1 cells—either in the presence and absence of glucose—stimulated robust ERK1/2 phosphorylation (Fig. 3.6A). ERK1/2 phosphorylation in Ins-1 cells follows a similar time course to that of pCREB, although unlike CREB, ERK1/2 phosphorylation is still detectable at 30 min. Despite the fact that glucose exposure itself is known to activate ERK1/2 phosphorylation (like CREB phosphorylation, again), TAAR1-stimulated ERK1/2 phosphorylation occurs in both the presence and absence of glucose (Fig. 3.6A).

Forskolin, the direct activator of AC was also a potent stimulator of ERK1/2 phosphorylation, suggesting a potential mechanistic link between AC and MAPK induction. Indeed, we found that ERK1/2 phosphorylation in response to T_1AM (as well as positive control forskolin) was effectively blocked by MDL-12,330A (Fig. 3.6B), supporting a mechanism in which adenylyl cyclase is a required effector of TAAR1-mediated ERK1/2 phosphorylation.

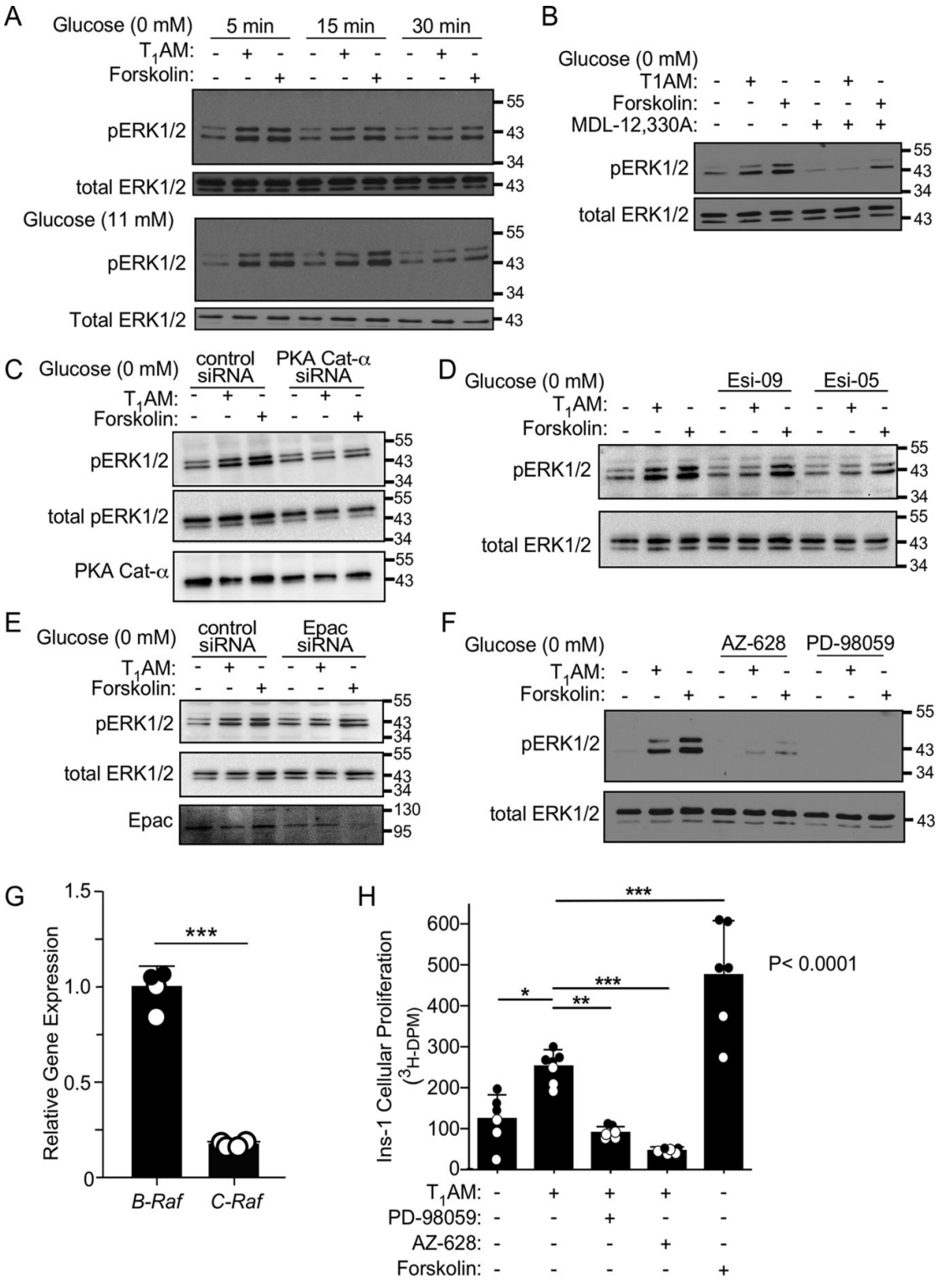


Figure 3.6: TAAR1 Increases Proliferation via Gαs-dependent Raf/MEK/ERK Signaling in Ins-1 Cells.

(A-F) Western blots of ERK1/2 phosphorylation in response to T_1AM (10 μM) and forskolin (0.3 μM), ±11 mM glucose. Addition of (B) 10 μM MDL-12,330A, (C) 150 nM PKA Cat-α siRNA, (D) 10 μM Esi-09 and 30 μM Esi-05, (E) 150 nM Epac siRNA, or (F) 10 μM AZ-628 (Raf inhibitor) and 50 μM PD98059 (MEK1/2 inhibitor) inhibits ERK1/2 phosphorylation (10 min) induced by T_1AM and forskolin. Representative blots of pERK1/2 from one of at least three independent experiments are shown; blots have been stripped and reprobed with total ERK1/2 as a loading control. (G) Relative expression of *B-Raf* and *C-Raf* in Ins-1 β cells (H) T_1AM (10 μM) and forskolin (0.5 μM) increase [^{3}H]-thymidine incorporation into Ins-1 cells (4 h), which is blocked by PD-98059 (50 μM) and AZ-628 (10 μM). Data was analyzed using one-way ANOVA (global $P< 0.0001$), with Dunnett's multiple comparison post hoc test to determine significance between relevant groups (n=6), $*P<0.05$, $**P<0.01$, $***P<0.001$. Reprinted with permission from: Michael, E.S., Covic, L., and Kuliopulos, A. (2019). Trace amine-associated receptor 1 (TAAR1) promotes anti-diabetic signaling in insulin-secreting cells. The Journal of Biological Chemistry 294, 4401-4411.

3.4.2 TA AR1 mediated ERK1/2 phosphorylation requires both PKA and Epac signaling.

AC/cAMP signaling activates downstream signaling partners PKA and Epac, both of which have been linked to MAPK signaling in several systems; as such, we sought to identify which of these proteins might be involved in transducing TAAR1 mediated activation of ERK1/2. Interestingly, we found that TAAR1 mediated ERK1/2 phosphorylation was reduced by siRNA knockdown of the catalytic subunit of PKA (PKA Cat-α), when compared to cells treated with control siRNA (Fig. 3.6C). Additionally, however, two Epac inhibitors—Esi-09 (Epac1/2 inhibitor) and Esi-05 (specific Epac 2 inhibitor)—both attenuated ERK1/2 phosphorylation downstream of T_1AM (Fig. 3.6D), revealing that Epac is also involved in TAAR1 mediated ERK1/2 phosphorylation. A critical role for Epac in activating ERK1/2 phosphorylation downstream of TAAR1 was further confirmed by siRNA knockdown of Epac. When compared to cells treated with control siRNA, cells treated with Epac targeting siRNA exhibited reduced TAAR1 and forskolin mediated ERK1/2 phosphorylation (Fig. 3.6E).

Together, these results support a mechanism of TAAR1-Gαs/AC dependent activation of MAPK that requires both PKA and Epac.

3.4.3 TAAR1 utilizes Raf/MEK/ERK1/2 signaling to drive increased β-cell proliferation

We also probed the involvement of other MAP kinases in TAAR1 mediated activation of ERK1/2 signaling. Both the MAP Kinase Kinase Kinase (MAPKKK) Raf and the MAP Kinase Kinase (MAPKK) MEK1/2 were identified as upstream mediators of ERK1/2 phosphorylation, as the pan-Raf inhibitor AZ-628 and the MEK1/2 inhibitor PD98059 completely prevent ERK1/2 phosphorylation in response to both T_1AM and forskolin (Fig. 3.6F). As ERK1/2 phosphorylation also requires Epac/PKA activation, these findings are actually consistent with what has been shown in several other systems where the MAPKKK Raf has been shown to be regulated by both Epac and PKA in a very cell specific manner (Duan and Cobb, 2010; Emery et al., 2013). Quantitative RT-PCR did confirm the expression of both *B-Raf* and *C-Raf* in Ins-1 β cells, although *B-Raf* expression is approximately 5-fold higher than that of *C-Raf* (Fig. 3.6G).

As ERK1/2 activation is frequently associated with increased rates of cellular proliferation, we wanted to determine whether TAAR1 agonism would alter cellular proliferation rates of Ins-1 β-cells. Treatment of cells with both T_1AM and the positive control forskolin significantly increased radiolabeled thymidine incorporation (Fig. 3.6H), while pretreatment of cells with AZ-628 and PD98059 completely blocked these increases in response to T_1AM. As AZ-628 is a pan-Raf inhibitor, and both *B-Raf* and *C-Raf* expression could be detected in these cells, it cannot be said without further study which Raf isoform mediates these pro-proliferatory effects of TAAR1 agonists. It is clear

however that TAAR1 agonism induces β-cell proliferation in a Raf/MEK-dependent manner.

Unlike short term, long term incubation of β-cells with MDL-12,330A (AC inhibitor) caused the death of the majority of cells in that treatment group, preventing the inclusion of this inhibitor in these assays. However, adenylyl cyclase is necessary for the activation of MAPK signaling by TAAR1, and MAPK signaling is necessary for TAAR1 mediated increases in cellular proliferation; while not conclusive, these two findings do support a mechanism by which TAAR1/Gαs signaling stimulates increased cellular proliferation in β-cells, via PKA/Epac-dependent activation of Raf/MEK/ERK1/2 signaling. The ability of the positive control forskolin to effectively induce MAPK signaling and cellular proliferation is also consistent with a mechanism of adenylyl cyclase-initiated signaling to that stimulates MAPK-dependent proliferation in β-cells.

3.5 TAAR1-MAPK signaling in β-cells requires intracellular calcium flux

3.5.1 TAAR1 mediated activation of ERK1/2 requires calcium influx and intracellular calcium release

Calcium is also a critical second messenger for signaling in β-cells; previous research has in fact revealed that cAMP-elevating agents such as forskolin and GLP-1 also stimulate intracellular calcium flux in β-cells (Kang et al., 2001; Prentki et al., 1987; Tengholm, 2012). This led us to ask whether Ca^{2+} could be an effector of TAAR1 in our Ins-1 cells, despite the established Gαs-coupling of the receptor. Interestingly, we found that addition of the extracellular calcium chelator EGTA to Ins-1 cells completely blocked ERK1/2 phosphorylation in response to both T_1AM and forskolin (Fig. 3.7A). These results indicate that calcium influx is necessary for the activation of MAPK

signaling by TAAR1/adenylyl cyclase. Release of calcium from intracellular stores is also likely involved, as reloading of intracellular calcium stores was partially required for activation of pERK1/2—blockade of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump with thapsigargin (TG) reduced ERK1/2 phosphorylation in response to TAAR1 agonism as well as forskolin (Fig. 3.7A).

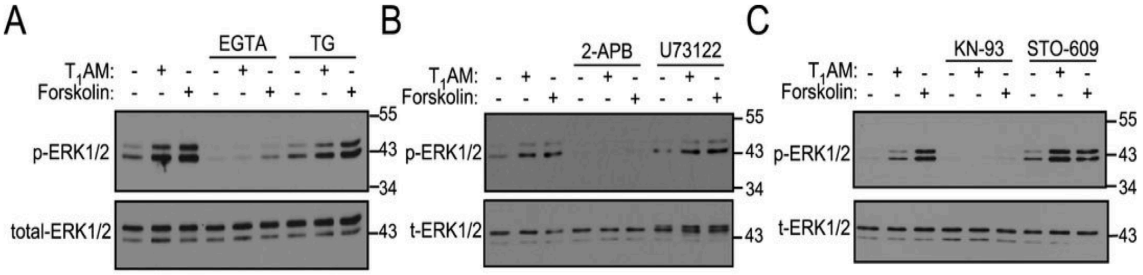


Figure 3.7: TAAR1 Mediated ERK1/2 Phosphorylation Requires Calcium Flux

(A-C) Western blot of ERK1/2 phosphorylation (10 min) induced by T_1AM (10 μM) and forskolin (0.3 μM) in the presence and absence of EGTA (1 mM), thapsigargin (5 μM; inhibits SERCA calcium pump), 2-APB (50 μM; inhibits IP_3R), U73122 (20 μM; inhibits PLC-β), KN-93 (10 μM; inhibits CaMKII) and STO-609 (25 μM; inhibits CaMKKII). Representative blots of pERK1/2 from one of at least three independent experiments are shown, and blots have been stripped and reprobed with total ERK1/2 as a loading control. Reprinted with permission from: Michael, E.S., Covic, L., and Kuliopulos, A. (2019). Trace amine-associated receptor 1 (TAAR1) promotes anti-diabetic signaling in insulin-secreting cells. The Journal of Biological Chemistry 294, 4401-4411.

3.5.2 ERK1/2 phosphorylation induced by TAAR1 agonism requires IP_3R mediated calcium release but not PLC-β

Consistent with this new evidence supporting calcium being a downstream effector of TAAR1 signaling, we found that inositol trisphosphate receptor (IP_3R) activation was also required for ERK1/2 phosphorylation. Activation of IP_3R stimulates calcium release from internal stores, and we found that 2-APB (an IP_3R antagonist) completely blocks ERK1/2 phosphorylation in response to both T_1AM and forskolin (Fig. 3.7B). Phospholipase C-β (PLC-β), a common mediator of calcium signaling that when

activated (generally by Gαq and Gαi) results in IP_3 production, was surprisingly not involved in TAAR1-mediated ERK1/2 phosphorylation, as the PLC-β inhibitor U73122 had no effect on T_1AM or forskolin-mediated ERK1/2 phosphorylation (Fig. 3.7B).

U73122 did however effectively inhibit calcium signaling in response to LIGRLO, an agonist of a known Gαq-coupled receptor expressed in pancreas (Michael et al., 2013), Protease Activated Receptor 2 (PAR2), confirming the efficacy of the compound (Fig. 3.8) in these insulin-secreting cells. This supports an alternate mechanism of calcium release induced by TAAR1 in β-cells in which calcium flux is not dependent on classical Gαq/PLC-β but instead Gαs/AC mediated activation of IP_3R.

3.5.3 TAAR1 mediated ERK1/2 phosphorylation requires CaMKII

Ca^{2+}/Calmodulin (CaM) kinase-dependent signaling has previously been shown to regulate Raf/MEK/ERK pathways (Illario et al., 2003), providing a potential link between intracellular calcium and downstream ERK1/2 phosphorylation. Accordingly, we found that treatment of Ins-1 β-cells with KN-93, a potent inhibitor of CaM kinase II (CaMKII), effectively blocked ERK1/2 phosphorylation in response to T_1AM and forskolin (Fig. 3.7C). In contrast, a divergent calmodulin-regulated kinase, CaM Kinase Kinase II (CaMKKII),

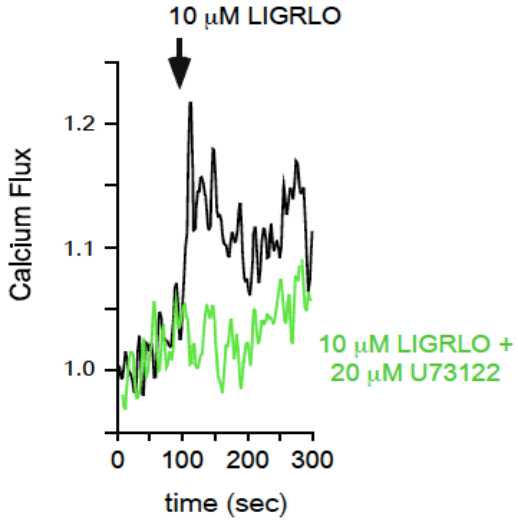


Figure 3.8: U73122 Blocks PAR2 Mediated Ca2+ Release
Calcium signaling in Ins-1 cells induced by LIGRLO (10 μM; black) ± 20 μM U73122 (green). Reprinted with permission from: Michael, E.S., Covic, L., and Kuliopulos, A. (2019). Trace amine-associated receptor 1 (TAAR1) promotes anti-diabetic signaling in insulin-secreting cells. The Journal of Biological Chemistry 294, 4401-4411.

may even play a suppressive role, as STO-609, a CaMKKII inhibitor enhanced both the basal and agonist-stimulated ERK1/2 phosphorylation in Ins-1 cells (Fig. 3.7C).

3.6 TAAR1 agonism stimulates Gαs/cAMP mediated calcium flux in β-cells

3.6.1 TAAR1 activation triggers calcium influx and intracellular calcium release

Consistent with the powerful effects of restricting calcium signaling on TAAR1-stimulated ERK1/2 phosphorylation, we were able to detect a significant, rapid, intracellular calcium flux signal in response to T_1AM, as well as the cAMP-stimulating positive control, forskolin in Ins-1 β-cells (Fig. 3.9A, E). The magnitude of calcium influx was dependent on both extracellular and intracellular calcium, as a reduction in extracellular calcium from physiologic levels (1.5 mM) to 1 mM, as well as the further introduction of EGTA caused significant reductions in calcium influx. Specifically, the restriction of extracellular calcium and the introduction of EGTA caused up to a 75% reduction ($P<0.001$) in calcium flux (Fig. 3.9A, B). The residual 25% of the original calcium signal could be completely blocked by the further addition of thapsigargin (TG; Fig. 3.9A, B), which inhibits reloading of calcium into ER stores. These results indicate that the full TAAR1-stimulated calcium flux signal is derived from both extracellular calcium influx and the release of internal calcium stores, although a larger portion of the signal is derived from extracellular sources.

3.6.2 TAAR1 stimulates calcium flux via Gαs/AC dependent signaling in β-cells

To confirm whether TAAR1 mediated calcium flux is due to Gαs/AC dependent signaling in Ins-1 β-cells, the effects of AC inhibition were tested. Short term preincubation of cells with MDL-12,330A significantly ($P<0.001$) reduced calcium signaling in response to T_1AM treatment (Fig. 3.9C, F), indicating that TAAR1-mediated

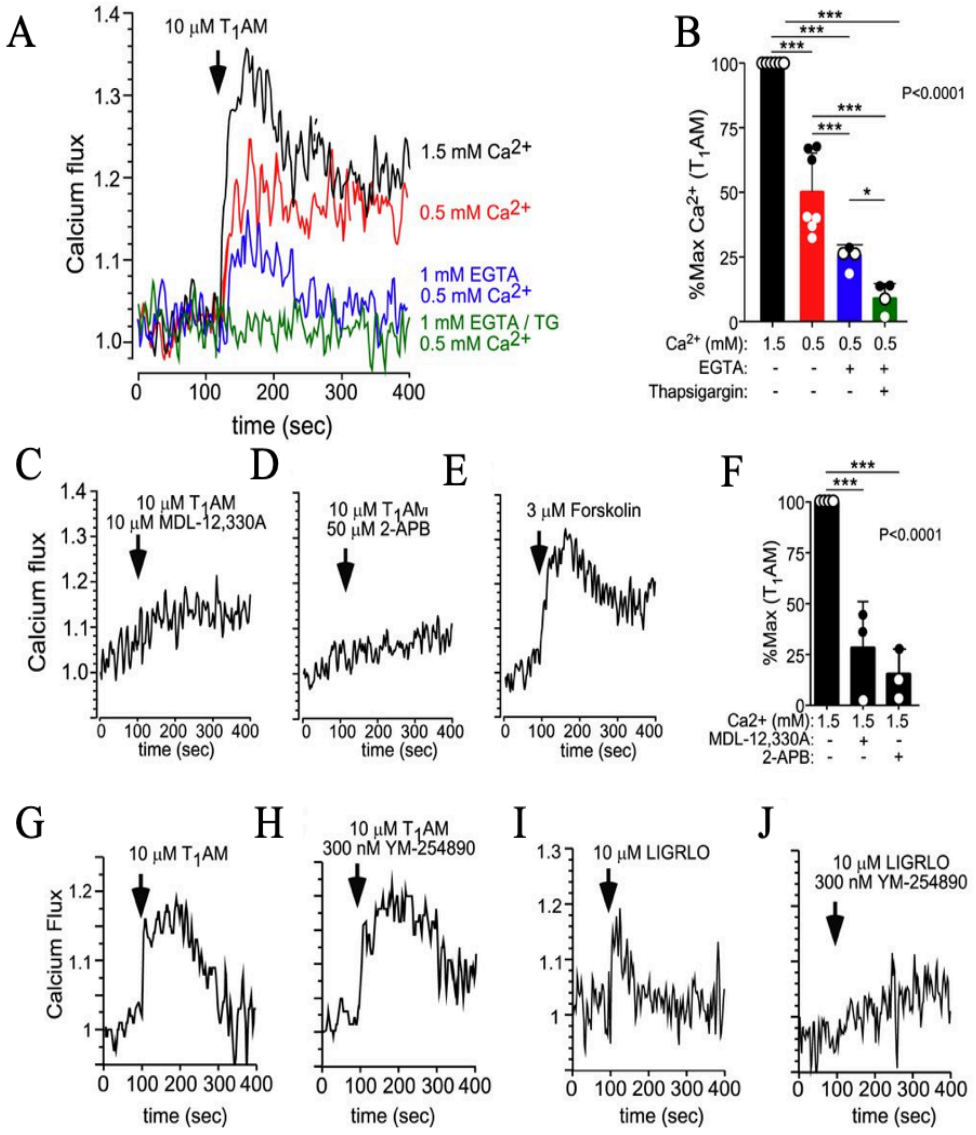


Figure 3.9: TAAR1 Stimulates Ca^{2+} Influx and Release from Internal Stores

(A, C, D) Calcium signaling in Ins-1 β-cells induced by T_1AM (10 μM) was measured in the presence of 1.5 mM (black traces) or 0.5 mM (red, blue, green) extracellular Ca^{2+}, 1 mM EGTA, 5 μM thapsigargin, 10 μM MDL-12,330A or 50 μM 2-APB as labeled. (E) Calcium signaling induced by forskolin (0.3 μM). Representative traces of at least 3 experiments are shown. (B, F) Calcium flux induced by T_1AM ± antagonists was quantified by measuring area under the curve and normalizing data to 100% of the max signal; data is shown as mean ±SD. AUCs were analyzed by one-way ANOVA (global $P<0.0001$) using Newman-Keuls multiple comparison post-hoc test to determine significance between groups; (n=3-6), *$P<0.05$, **$P<0.01$, ***$P<0.001$. (G,H) Calcium signaling induced by T1AM ± 300 nM YM-254890. (I, J) Calcium signaling induced by LIGRLO ± 300 nM YM-254890. Reprinted with permission from: Michael, E.S., Covic, L., and Kuliopulos, A. (2019). Trace amine-associated receptor 1 (TAAR1) promotes anti-diabetic signaling in insulin-secreting cells. JBC 294, 4401-4411.

calcium release occurs through cAMP-dependent pathways. Consistent with its dramatic effects demonstrated previously on ERK1/2 phosphorylation, 2-APB significantly (P<0.001) inhibited calcium flux in response to T_1AM in Ins-1 cells (Fig. 3.9C, F), indicating that the IP_3R activation is necessary for TAAR1-mediated calcium signaling. The ability of forskolin to also potently induce calcium flux in Ins-1 cells (Fig. 3.9E) lends further support to our mechanism in which adenylyl cyclase activation in β-cells stimulates calcium release. While not specifically tested under these conditions, we also would predict that 2-APB would reduce calcium flux in response to the positive control forskolin.

Consistent with this type of Gαs/AC-regulated calcium flux in β-cells, we were also able to further rule out any potential involvement of Gαq-TAAR1 coupling. The potent and selective Gαq inhibitor YM-254890 had no effect on calcium release in response to T_1AM (Fig. 3.9G, H). YM-254890 did however completely block calcium flux in response to LIGRLO, an agonist of the Gαq-coupled Protease Activated Receptor 2 (PAR2; Fig. 3.9I, J). Together, these data all support a mechanism in which TAAR1 stimulates calcium release from internal stores and extracellular calcium influx through purely Gαs-derived signaling.

3.7 Small molecule agonist RO5256390 is an efficacious agonist of TAAR1 signaling in β-cells

3.7.1 RO5256390 stimulates CREB phosphorylation and CREB target gene induction in Ins-1 β cells

RO5256390, the previously discussed small molecule agonist of TAAR1 discovered by Hoffman La Roche, has thus far been characterized in literature for

potential use as a CNS-acting anti-psychotic drug, with potent *in vivo* activity in rodents and non-human primates (Revel et al., 2013). Once this compound became commercially available, we sought to determine whether this new small molecule compound would exhibit the same beneficial signaling properties in β-cells that were observed with the endogenous TAAR1 agonist, T_1AM. Treatment of Ins-1 β-cells with RO5256390 for 10 minutes stimulated CREB phosphorylation (Fig. 3.10A). After 1 hour of RO5256390 treatment, significant induction of the CREB target gene, *Irs-2* could also be detected ($P<0.0001$; Fig. 3.10B).

3.7.2 RO5256390 stimulates ERK1/2 phosphorylation and calcium signaling

Again, consistent with results generated using the endogenous agonist T_1AM, RO5256390 dose-dependently stimulated ERK1/2 phosphorylation (Fig. 3.10C), and intracellular calcium flux in Ins-1 β-cells (Fig. 3-10D). Treatment of cells with RO5256390 (or the positive control forskolin) for 24 h significantly upregulated cellular proliferation rates (Fig. 3.10E), further validating the potentially anti-diabetic signaling pathways initiated by TAAR1. It should be noted that the maximal signal of RO5256390 in several assays is greater than that of T_1AM in these cells, suggesting that RO5256390 may be a more efficacious agonist of TAAR1, although side-by-side testing of these agonists would be necessary to confirm this.

3.7.3 RO5256390 stimulates glucose-dependent insulin secretion

As shown in Fig. 3.10F, RO5256390 induces striking potentiation of glucose stimulated insulin secretion, but did not cause insulin secretion in the absence of high glucose stimulation. Similarly, T_1AM also did not induce insulin secretion under low glucose conditions (Fig. 3.10F). This demonstrates that the insulinotropic functions of the

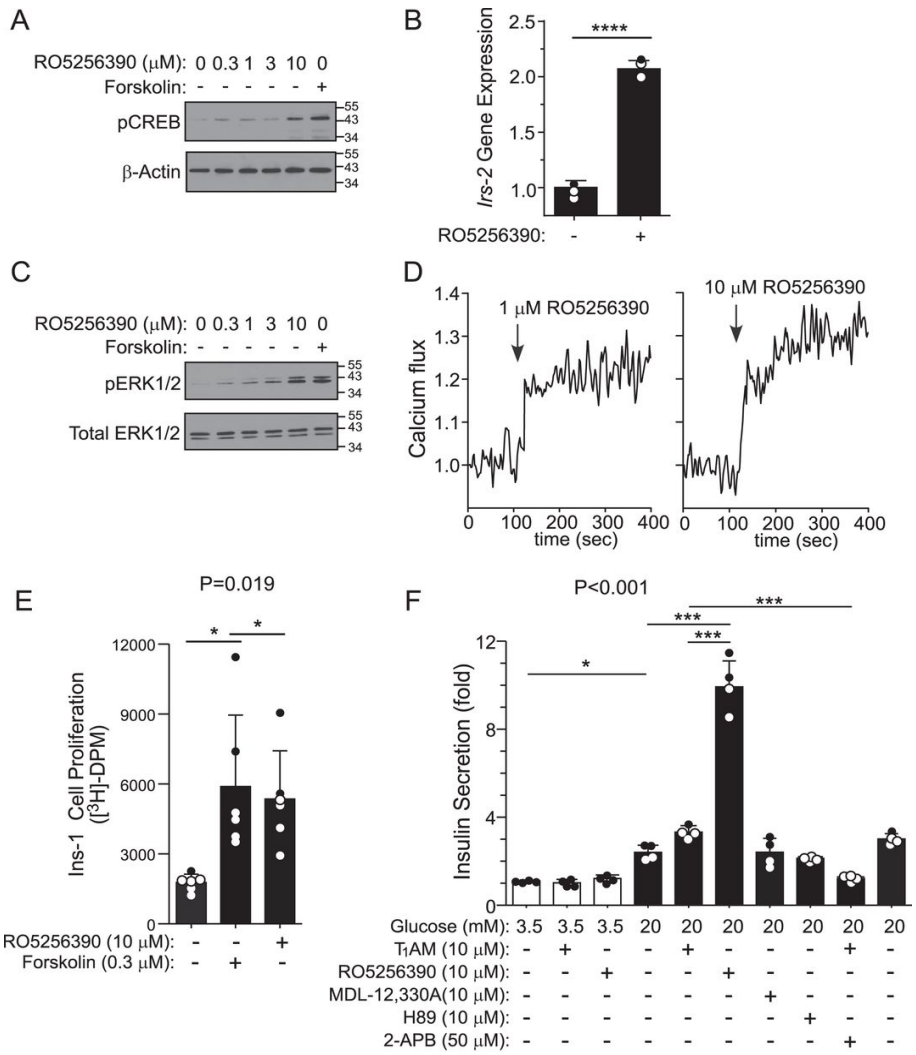


Figure 3.10: RO5256390 is an Efficacious Agonist of β-cell TAAR1

(A, C) Western blots of CREB or ERK1/2 phosphorylation in Ins-1 cells 10' after RO5256390 (0-10 μM) or forskolin (0.3 μM). Representative blots of pCREB or pERK1/2 from one of at least three independent experiments are shown; blots have been stripped and reprobed with either β-actin or total ERK1/2 as a loading control. (B) qPCR of *Irs-2* expression (1 h) induced by RO5256390 (10 μM). Data is expressed as mean ΔΔ CT (± SD) of *Irs-2*, using *GAPDH* as the housekeeping gene, and was analyzed by student's t-test; (n=3), ****P<0.0001. (D) RO5256390 induces calcium flux in Ins-1 cells (1.5 mM extracellular Ca^{2+}). (E) RO5256390 (10 μM) and forskolin (0.3 μM) increase [^{3}H]-thymidine incorporation into Ins-1 cells (4 h). (F) Insulin secretion in response to TAAR1 agonists (RO5256390, T_1AM) or modulators of TAAR1/cAMP dependent signaling were added to cells at either 3.5 (low) or 20 mM (high) glucose. Insulin secretion (2 h) was determined by ELISA (mean ± SD) and analyzed by one-way ANOVA (global P<0.0001), using Dunnett's multiple comparison post hoc test, *P<0.05, **P<0.01, ***P<0.001. *P<0.05. Reprinted with permission from: Michael, E.S., Covic, L., and Kuliopulos, A. (2019). Trace amine-associated receptor 1 (TAAR1) promotes anti-diabetic signaling in insulin-secreting cells. JBC 294, 4401-4411.

trace amine associated receptor 1 are in fact dependent on glucose, similar to the actions of peptide incretin receptors GLP-1R and GIPR, which induce glucose dependent secretion of insulin to potentiate GSIS but do not induce insulin secretion in the absence of glucose stimulation. This does indicate that if TAAR1 were to be developed as a T2DM therapeutic, these therapies would be unlikely to carry significant risks of hypoglycemia.

Furthermore, inhibition of cAMP signaling pathways with MDL-12,330A or H89 inhibitors had no significant effect on GSIS in the absence of TAAR1 agonist treatment (Fig. 3.10F). Lastly, as calcium influx is the trigger for vesicle exocytosis, and intracellular [Ca^{2+}]can be modulated by a variety of inputs—including apparently TAAR1—we hypothesized that this calcium flux may have effects on TAAR1 mediated potentiation of GSIS. We determined that 2-APB did in fact significantly reduce TAAR1-dependent insulin secretion in the presence of high glucose (Fig. 3.10F), indicating that calcium signaling activated by TAAR1 plays a role in potentiation of GSIS.

3.8 Preliminary work to identify a novel Gαi activation-specific antibody

As a small but interesting side project relating to GPCR signaling, we also sought to set up a new method of screening for GPCR activation based on the structural dynamics of Gα activation. As mentioned in the introduction, Gα contains three flexible "switch regions", which are physically shielded from interacting with other proteins by βγ binding when the heterotrimeric signaling complex is in its inactive form. Activation of the G protein complex by a receptor stimulates a dissociation of the βγ dimer, unveiling these switch regions; exchange of GDP for GTP also directly stabilizes these regions, priming them for interactions with other proteins. We hypothesized that by

designing antibodies targeting these switch regions, we may be able to produce antibodies against Gα that only recognize the active conformation of the G protein and thus could be used to directly assay receptor activation.

We selected Gαi as our target Gα for initial proof-of-concept assay development, designing an antibody that targets both switch regions 2 and 3 (chosen for their more immunogenic sequences, when compared to that of switch region 1. To accomplish this, we synthesized two separate peptides for this antibody, which were mixed together at 1:1 for antibody generation. As a control, Gα-CT targeting antibodies were also designed, using sequences similar to that of commercially available Gαi antibodies. The specific sequences of these peptides are as follows: switch region 2: GQRSERKKWIHSFEGC; swtich region 3: DLVLAEDEEMNC; Gαi-CT: CKNNLKDCGLF. After synthesis, peptides were coupled to an immunogenic protein (keyhole limpet hemocyanin) and sent to an outside vendor (Pro-Mab) who would use them to produce two polyclonal antibodies in rabbits—one combination antibody targeting both switch regions ("Gαi-switch"), and one antibody targeting the CT region ("Gαi-CT"), which is not predicted to exhibit any activitity dependent antigen recognition.

We confirmed that these antibodies specifically recognize their peptide antigens over negative control peptides via dot blot assays. The ability of the Gαi-switch antibody to recognize both switch region peptides independently was confirmed (Fig. 3.11A), as was the ability of Gαi-CT to recognize the CT antigen peptide (Fig. 3.11B). Antibodies were assayed at several different concentrations, and both Gαi-switch and Gαi-CT selectively bound to their switch/CT antigen peptides even when diluted at 1:10,000. While effective at recognizing both switch peptide antigens, Gαi-swtich displayed

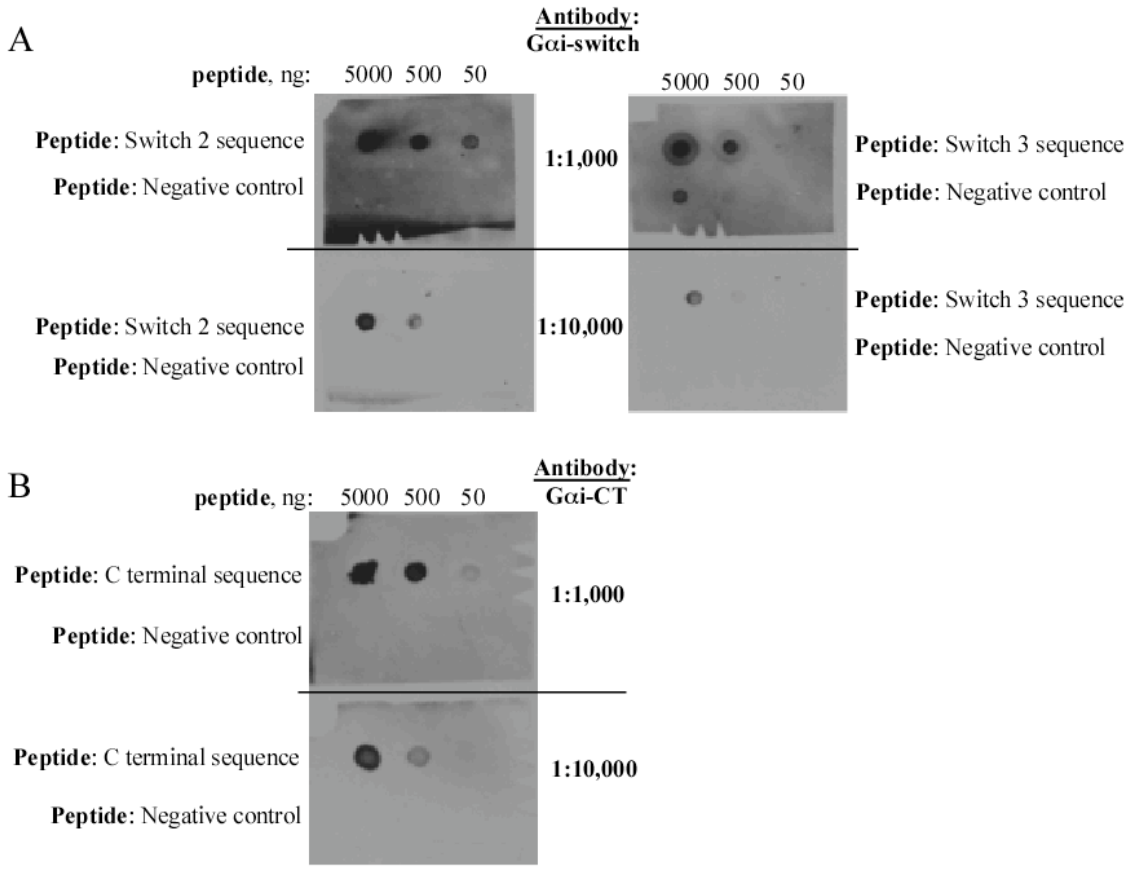


Figure 3.11: Specific Recognition of Peptide Antigens by Gαi antibodies

Dot blots of Gαi-switch 2 and 3 (A) and Gαi-CT (B) antigen peptides (50-5000 ng) on PVDF membranes, incubated with Gαi-switch (A) and Gαi-CT (B) antibodies at 1:1,000 and 1:10,000 dilution.

slightly higher sensitivity to the switch 2 sequence over switch 3, as indicated by its ability to detect lower quantities of switch 2 than switch 3 antigen (50 ng vs 500 ng; Fig. 3.11A). After confirmation of antibody/antigen recognition by these dot blot assays, antibodies were purified for further use via affinity purification using CN-Br Sepharose beads coated with respective peptide antigens.

In terms of designing our assay for measuring receptor activation, we utilized protocols similar to radiolabled GTPγS binding assays but eliminated the use of radiation, using instead an immunoprecipitation of active Gαi with the Gαi-switch antibody as a

readout of GPCR/Gα activation. Immunoprecipitated proteins, after separation by western blotting were detected with the Gαi-CT antibody, in theory allowing first for selection of active G protein during immunoprecipitation and then the quantification of these active Gα proteins via western blotting based densitometry.

Cellular membranes were prepared from HEK293 cells transfected with Gαi and empty vector DNA, or Gαi and a test Gαi-coupled receptor (somatostatin receptor 2; SSTR2). Membranes were then incubated with different concentrations of GDP and GTPγs (along with required ion cofactors) in the presence and absence of somatostain (SRIF) peptide. GTPγs is a "non-hydrolyzable" form of GTP, and thus once bound to Gα, should hold the G protein in its active confirmation by preventing hydrolysis back to

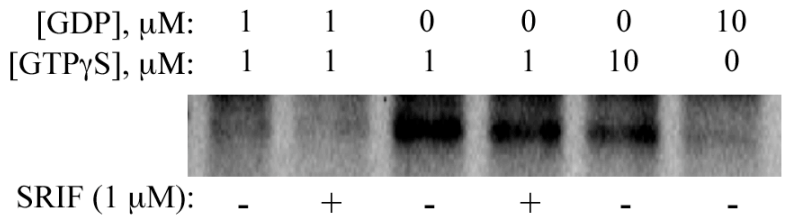


Figure 3.12: Immunoprecipitation of "Active" Gαi from Cellular Membranes

Immunoprecipitation of active Gαi G protein using Gαi-switch agarose beads from HEK293 membranes (purified from cells expressing SSTR2 and Gαi) incubated in the presence of GDP, GTPγs, SRIF as labeled (5 min).

GDP. While this project is still in its very early days, and we have yet to establish entirely agonist dependent effects, we are encouraged to find that removal of GDP from the assay buffer consistently increases Gαi("-active") immunoprecipitation (Fig. 3.12). This is consistent with the known stabilizing effects of GTP binding on the flexible swtich regions of Gαi, which could potentially even stabilize them in a conformation that promotes their recogniztion by our novel Gαi-switch antibody. In the absence of any GDP, significantly higher GTPγS binding to G protein would occur, mimicing G protein

activation in some ways while circumventing the need for receptor mediated activation. Similar results were obtained using membranes purified from HEK293 cells transfected with Gαi only, consistent this effect of GDP removal on Gαi G protein conformation.

Significant further work will be required to identify the correct conditions for optimizing this assay for the specific detection of receptor/agonist dependent immunoprecipitation, which may involve changes in the ratio of receptor:G protein (in transfection), alterations in the buffer recipe, the timing of the assay, the temperature, or quite a number of other important confounding factors, although initial results warrant further investigation.

Chapter 4: Discussion

4.1 Mechanistic insights into TAAR1 mediated GSIS potentiation

4.1.1 Perspective on TAAR1, GSIS, and T2DM

TAAR1 is expressed by both human and rodent β-cells (Raab et al., 2016), although very little mechanistic examinations of TAAR1 signaling have been published in these cells, beyond an initial finding that TAAR1 agonism potentiates GSIS. Type 2 Diabetes (T2DM) is part of a growing and serious worldwide epidemic of metabolic disease. There is no cure for the T2DM, and current treatments do not adequately meet the needs of a continually expanding patient population. Recent strategies to improve glycemic control include enhancing glucose stimulated insulin secretion (GSIS) and islet viability by targeting a small select group of G protein-coupled receptors that couple to Gαs (Drucker, 2003a; Furman et al., 2010b; Gault et al., 2003; Ha et al., 2014; Nakagawa et al., 2009; Nauck et al., 1997a; Yusta et al., 2006). This includes, for example, receptors for incretins such as GIP (Gastric inhibitory polypeptide) and GLP-1 (Glucagon-like polypeptide-1), which stimulate cAMP production via adenylyl cyclase (AC) activation (Drucker and Nauck, 2006; Fortin et al., 2011; Rolin et al., 2002; Yang and Yang, 2016). cAMP is a critical second messenger for β-cells, as it activates a tightly regulated and complex signaling network that can induce potentiation of glucose stimulated insulin secretion, protect β-cells from stress-induced apoptosis and trigger an expansion of β-cell mass to combat β-cell failure (Buteau, 2008; Hui et al., 2003; Jhala et al., 2003; Kashima et al., 2001; Ozaki et al., 2000; Park et al., 2006; Portha et al., 2011; Seino et al., 2009; Shibasaki et al., 2007; Tengholm, 2012; Wei et al., 2012; Yusta et al., 2006).

TAAR1, as a Gαs-coupled receptor expressed in pancreatic beta cells (Regard et al., 2007) that exhibits incretin-like activity (Raab et al., 2016) presents an exciting new possibility for targeting T2DM. One of the biggest hurdles in developing treatments that target Gαs receptors such as GLP-1R and GIPR, is the short half-life of peptide receptor agonists *in vivo* due to degradation by dipeptidyl peptidase 4 (DPP-4) enzymes (O'Harte et al., 2001; Rolf et al., 1993). While longer acting agonists have been somewhat more promising, it could also be useful to find alternative treatments that could stimulate key $G\alpha_s$-coupled signaling cascades in β-cells without being targeted for degradation by DPP-4. Here, we make the case that TAAR1 may be one such receptor, outlining potentially anti-diabetic signaling mechanisms of TAAR1 that we have identified in β-cell lines. To summarize briefly, TAAR1, by coupling to Gαs-signaling in pancreatic β-cell lines causes PKA/Epac-dependent potentiation of glucose stimulated insulin secretion, induction of CREB-*Irs2*, activation of Raf-MAPK signaling, and increased cellular proliferation (Fig. 4.1).

4.1.2 TAAR1/Gαs promotes GSIS in β-cells in a PKA dependent manner

We found that TAAR1 mediated potentiation of GSIS involves adenylyl cyclase/cAMP activation of both PKA and Epac. We are the first to verify that TAAR1 mediated potentiation of GSIS is driven by $G\alpha_s$-initiated signaling, which relies on AC and downstream effectors (Fig. 3.4). While a rise in intracellular calcium—triggered by glucose uptake and metabolism—is the main trigger for insulin vesicle exocytosis, signals such as those from cAMP effectors can modulate this process. PKA is known to modulate insulin secretion on several different levels—significant further work would be required to confirm which of these may be at play downstream of TAAR1. PKA has been

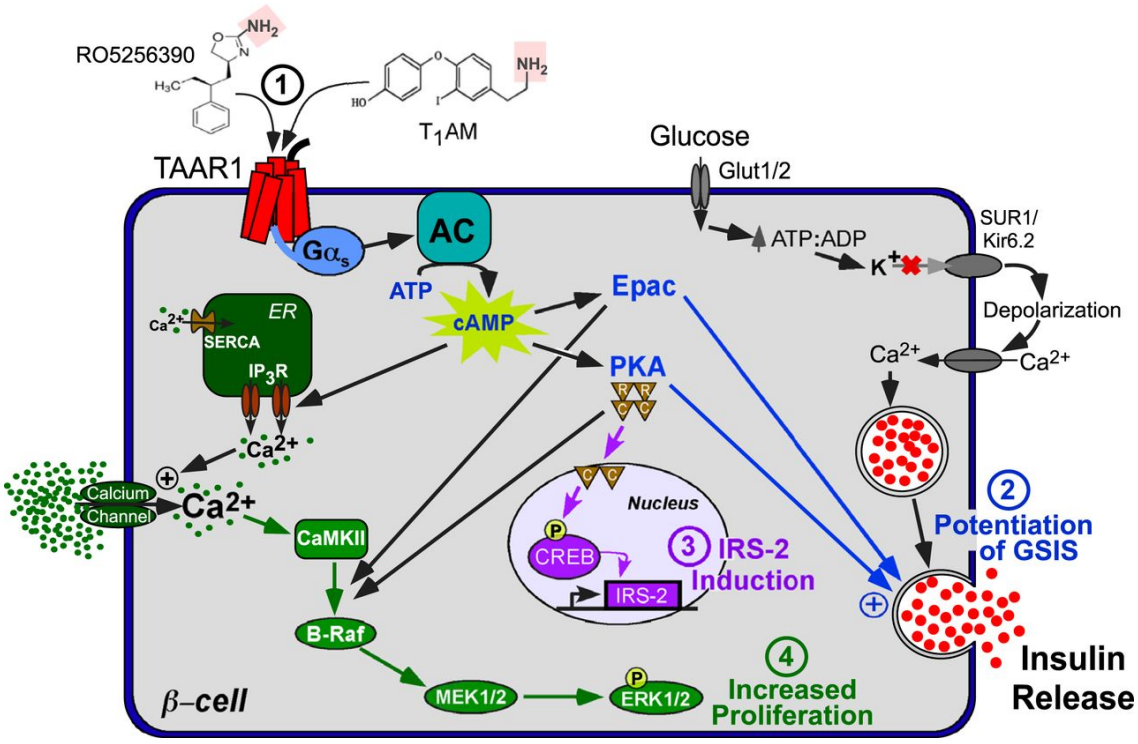


Figure 4.1: Proposed Mechanisms of TAAR1 Signaling in β-cells

(1) Activation of TAAR1-Gαs by amine (pink) ligands leads to generation of cAMP by adenylyl cyclase (AC). (2) cAMP then activates Epac and PKA which are required for potentiation of glucose stimulated insulin secretion by TAAR1. (3) PKA catalytic (c) subunits phosphorylate CREB, leading to the induction of CREB target gene *IRS-2*. (4) TAAR1 stimulates cAMP-dependent calcium flux from internal (IP_3R mediated) stores and influx from extracellular sources, leading to CaMKII-dependent activation of Raf/MEK/ERK signaling and increased cellular proliferation. PKA and Epac also have inputs on Raf/MEK/ERK1/2 signaling and likely proliferation. Reprinted with permission from Michael, E.S., Covic, L., and Kuliopulos, A. (2019). Trace amine-associated receptor 1 (TAAR1) promotes anti-diabetic signaling in insulin-secreting cells. JBC 294, 4401-4411.

shown to both increase recruitment of insulin containing vesicles to the reserve pools (Nagy et al., 2004), as well as facilitate vesicle docking and priming for exocytosis by phosphorylation of key SNARE complex and associated proteins (Chheda et al., 2001; Foster et al., 1998; Hussain et al., 2012; Seino and Shibasaki, 2005; Song et al., 2011; Vikman et al., 2009; Wu et al., 2015). Furthermore, PKA phosphorylates K_{ATP} to reduce

channel activity, contributing to localized membrane depolarization and opening of voltage gated calcium channels (Gromada et al., 1997; Holz et al., 1993a).

4.1.3 TAAR1/Gαs promotes GSIS in β-cells in an Epac dependent manner

PKA is not the only downstream signaling partner of cAMP—the guanine nucleotide exchange factor activated by cyclic AMP (Epac) is also a target of cAMP that interacts with a network of intracellular proteins to increase GSIS (Eliasson et al., 2003; Holz et al., 2006; Kashima et al., 2001; Ozaki et al., 2000). Epac2 is the predominant isoform of Epac expressed by β-cells (Kawasaki et al., 1998), and thus much of the research involving Epac and GSIS has centered around this isoform. Epac2 is actually recruited to the plasma membrane in response to elevated cAMP in β-cells, where it appears to cluster around secretory vesicles and bind to exocytosis machinery to increase vesicle priming and fusion at docking sites (Alenkvist et al., 2017). Epac2 can interact with Rim2 (Rab3-interacting molecule 2), which together with Rab3, form a GTP dependent complex between plasma membranes and docked synaptic vesicles to regulate exocytosis (Kashima et al., 2001; Ozaki et al., 2000). Epac is also believed to modulate the activity of ATP regulated potassium channels to increase GSIS (Eliasson et al., 2003; Kang et al., 2008; Leech et al., 2010).

Based on these well-established signaling pathways that have been characterized for incretin receptor signaling, we hypothesized that PKA and Epac would play a role in potentiation of GSIS by TAAR1 and were able to confirm that both are required for full TAAR1 mediated potentiation of GSIS. Further studies would be required to determine which of the pathways described above are utilized by PKA and Epac in this system to augment GSIS. Additionally, due to concerns about potential off-target effects of T_1AM,

the specificity of this agonist was confirmed through the use of a small molecule TAAR1 antagonist, EPPTB, verifying that T_1AM acts through TAAR1 activation to potentiate GSIS (Fig. 3.4).

4.1.4 Insulinotropic actions of TAAR1 are glucose dependent

We also found that TAAR1's ability to induce insulin secretion in β-cells are entirely dependent on glucose (Fig. 3.10). In other words, TAAR1 can stimulate insulin secretion in a glucose dependent manner to potentiate GSIS, but under low glucose conditions, treatment of cells with TAAR1 agonists fails to elicit insulin secretion responses. This type of glucose dependent regulation of insulin secretion is similar to what has been documented for similarly Gαs coupled incretin receptors.

In terms of therapeutic value, one of the aspects that differentiates incretin based T2DM treatments from others is this fact that incretins induce β-cell insulin secretion in a glucose-dependent manner. This is important because it means that incretins can reduce T2DM-associated hyperglycemia without significant risk of hypoglycemia. Insulin and sulfonylurea based therapies by comparison are associated with increased occurrences of hypoglycemic episodes, as their blood glucose lowering effects are in no way glucose dependent. Recently published data has even indicated that severe hypoglycemic episodes may actually contribute to increased cardiovascular disease associated with such treatments (Nunes et al., 2017). In the future, if TAAR1 were to be developed as a T2DM therapeutic, it is unlikely that these treatment would carry significant risks of hypoglycemia, which could be advantageous in terms of their safety.

4.2 TAAR1 activates the β-cell transcription factor CREB

4.2.1 TAAR1 utilizes Gαs/AC to stimulate CREB phosphorylation

To investigate whether TAAR1 signaling in β-cells activates Protein Kinase A to induce phosphorylation of the transcription factor CREB and the induction of genes that promote β-cell growth and survival (Dalle et al., 2011; Hussain et al., 2006; Jhala et al., 2003; Liu et al., 2012), we first showed that we could detect phosphorylation of CREB at Ser133, a known target of PKA, in response to both T_1AM (Fig. 3.5) and RO5256390 (Fig. 3.10). Phosphorylation of this key residue is necessary for CREB activation (Brindle and Montminy, 1992), and TAAR1 agonism stimulates CREB phosphorylation in the presence and absence of extracellular glucose, although with slightly enhanced activation dynamics in the presence of 11 mM glucose. This suggests a potential synergistic effect between glucose and TAAR1 agonism, as glucose itself is known to stimulate CREB phosphorylation, although further experiments would be required to confirm whether this is indeed the case.

As mentioned, Ser133 of CREB is a known target of PKA. Unsurprisingly perhaps then, we found that activation of CREB by T_1AM and forskolin is blocked by inhibition of PKA. We were able to confirm this using both genetic (siRNA targeting the catalytic subunit of PKA) or chemical (H89) methods of PKA inhibition (Fig. 3.5C,D). While still a relatively popular inhibitor of PKA, H89 is notorious for its lack of specificity. In fact, H89 is known to inhibit mitogen activated protein kinase kinase 1 (MKK1), MAP Kinase 2 (MAPK2), stress-activated protein kinase 1/c-Jun N-terminal kinase (JNK1/SAPK1), p38, p38-regulated/activated protein kinase (PRAK), phosphoinositide-dependent protein kinase-1 (PDK1), Glycogen synthase kinase 3 beta (GSK3β), and casein kinase 2 (CK2) at 80% or more at just 10 μM, a standard concentration used for targeting PKA. The sheer number of potential confounders makes

the use of a secondary method of PKA inhibition almost necessary, although fortunately, in this case, both chemical and genetic means of PKA inhibition confirmed that PKA is involved in TAAR1 (and forskolin) mediated CREB phosphorylation (Fig. 3.5).

In accordance with these findings, MDL-12,330A (AC inhibitor) also reduced CREB phosphorylation in response to T_1AM and the positive control forskolin (Fig. 3.5B). As the adenylyl cyclase enzyme is directly responsible for cAMP generation, and cAMP then directly activates PKA, these results are consistent with a mechanism of TAAR1 mediated CREB phosphorylation that utilizes classical Gαs signaling effectors AC and PKA.

4.2.2 TAAR1 stimulates *Irs-2* gene induction

Induction of the transcription factor CREB leads to the activation of genes—including *insulin receptor substrate-2* (*Irs-2*) and others—that coordinate the activation of pro-proliferative and anti-apoptotic signaling pathways in β-cells (Dalle et al., 2011; Jhala et al., 2003; Liu et al., 2012; Mohanty et al., 2005; Niessen, 2006; Park et al., 2006; Park et al., 2008b; Yusta et al., 2006). IRS proteins function as scaffolding proteins which are directly phosphorylated by the insulin receptor, leading to the recruitment and activation of other proteins involved in the transduction of downstream insulin signaling. Disruption of IRS-2 has even been linked to T2DM development (Kubota et al., 2000; Withers et al., 1998), and tissue specific IRS-2 knockouts have confirmed its essential role in promoting the maintenance of functional β-cell populations (Kubota et al., 2004). We were able to detect Gαs/AC/PKA dependent phosphorylation of CREB in response to TAAR1 agonism, and gene induction of *Irs-2* (Fig. 3.5E; Fig. 3.10B). As IRS-2 regulates β-cell mass, and, in particular, compensatory responses to stress, it would be of interest in

further studies to determine whether this might translated to a protection of β-cells *in vivo* in models of T2DM.

4.3 TAAR1 stimulates proliferation of β-cells by activation of MAPK

4.3.1 TAAR1 stimulates Gαs/AC dependent ERK1/2 phosphorylation

ERK1/2, much like CREB can be activated by glucose as well as certain GPCR agonists in β-cells, although this activation occurs through distinct mechanisms (Frodin et al., 1995); hormones such as GLP-1 and GIP induce ERK1/2 activation in a Gαs/cAMP dependent manner (Arnette et al., 2003; Ehses et al., 2002; Frodin et al., 1995; Jiang et al., 2001; Lawrence et al., 2007; Vossler et al., 1997). We documented a robust increase in ERK1/2 phosphorylation in response to both TAAR1 agonists and the cAMP stimulating positive control forskolin in Ins-1 β-cells that appears to be glucose-independent (Fig. 3.6A).

MDL-12,330A is an effective inhibitor of ERK1/2 phosphorylation in response to both T_1AM and the positive control forskolin, supporting our mechanism of Gαs/AC-derived signaling by TAAR1 in these cells (Fig. 3.6B). Surprisingly, inhibition of both PKA and Epac suppressed ERK1/2 phosphorylation in response to TAAR1 and forskolin (Fig. 3.6 C, D, E), revealing that both of these effectors are actually involved in the activation of MAPK signaling by TAAR1/AC. The direct connections between these cAMP regulated effector proteins and the initiation of ERK1/2 phosphorylation are still unclear, but the MAP kinase kinase kinase kinase Raf offers one potential link.

4.3.2 Activation of ERK1/2 by TAAR1 involving PKA, Epac, Raf and MEK1/2: potential involvement of isoform specific Raf activation

We determined that the MAPKKK Raf and MAP kinase kinase MEK1/2 are required for TAAR1-mediated activation of ERK1/2, based on the results of AZ-628 (Raf) and PD-98059 (MEK1/2) inhibitor treatment (Fig. 3.6F). As both *B-Raf* and *C-Raf* expression can be detected in Ins-1 β cells, significant further experiments would be required to differentiate the potential roles of the two isoforms here, although there are several possibilities. The involvement of both Raf isoforms in MAPK signaling in β cells has been investigated in response to other stimuli; in the case of both glucose treatment and GLP-1R agonism, B-Raf, but not C-Raf is required for ERK1/2 phosphorylation (Duan and Cobb, 2010; Trumper et al., 2005). Based on other parallels noted between TAAR1 and GLP-1R signaling, this supports B-Raf being the more likely isoform to mediate the effects of TAAR1. Interestingly, C-Raf knockdown actually increases, rather than inhibits, ERK1/2 phosphorylation in response to glucose treatment (Duan and Cobb, 2010). In terms of the roles that PKA and Epac may play, Gαs/cAMP mediated activation of Epac has been shown to induce Rap1/B-Raf and downstream ERK1/2 activation in neuronal, endocrine and other cell types (Emery et al., 2013; Luttrell et al., 2018). In thyroid cells, however, PKA has been shown to directly activate Raf, leading to ERK1/2 phosphorylation (Vuchak et al., 2009). Alternatively, PKA can phosphorylate C-Raf to inhibit its activity (Luttrell et al., 2018), and C-Raf itself has inhibitory effects on ERK1/2 activation in β-cells (Duan and Cobb, 2010), thus it is even possible that in our system, inhibitory phosphorylation of C-Raf by PKA might alleviate some of C-Raf's tonic inhibition of ERK1/2 phosphorylation. Particularly due to the varied and cell-type-

specific signaling differences observed in cAMP/Raf/ERK signal transduction, significant further experiments will be required to fully clarify the detailed mechanisms by which TAAR1stimulates MAPK signaling.

4.3.3 Activation of MAPK by TAAR1 stimulates increased β-cell proliferation

While incretins have been shown to stimulate ERK1/2 activity in β-cells, this activation is not required for or related to their famed actions on insulin secretion (Khoo and Cobb, 1997; Panse et al., 2015) but is instead often linked to increased cellular proliferation rates, which could play a role in compensatory β-cell hyperplasia in T2DM (Fu et al., 2010). Indeed, we observed a Raf/MEK1/2 dependent increases in Ins-1 β-cell proliferation rates in response to TAAR1 agonism (Fig. 3.6H). Increased ERK1/2 phosphorylation and cellular proliferation also occurred in response to our cAMP stimulating positive control, forskolin, further confirming the ability of Gαs-AC signaling to drive MAPK activation and β-cell proliferation. It would be of great interest in the future to determine whether these effects translate to increased β-cell mass *in vivo* using animal models of T2DM. Additionally, ERK1/2 is a transcription factor that can regulate the insulin gene promoter and coordinate responses to ER stress (Khoo et al., 2003; Lawrence et al., 2007), so there are certainly other potential aspects of TAAR1—ERK1/2 mediated anti-diabetic signaling to investigate.

4.4 Implications of TAAR1 mediated calcium signaling

4.4.1 Intracellular calcium flux is necessary for TAAR1 mediated ERK1/2 phosphorylation

Calcium signaling is another essential function of β-cells, particularly due to their secretory nature—calcium influx is the main trigger for the exocytosis of insulin

containing vesicles, one of the most well-known and studied functions of these cells. However, in pathways that diverge away from insulin secretion responses, calcium signaling has also been shown to induce MAPK activation in β-cells. This has been observed for glucose and GLP-1R-Gαs/AC, which both require a rise in intracellular calcium for the initiation of MAPK signaling (Arnette et al., 2003; Frodin et al., 1995; Holz et al., 1995; Jiang et al., 2001).

We were able to determine that calcium signaling is in fact also required for the activation of ERK1/2 by TAAR1, as T_1AM-induced ERK1/2 phosphorylation could be blocked by chelation of calcium with EGTA or poisoning of SERCA pumps (responsible for loading calcium into intracellular ER stores) with thapsigargin (Fig. 3.7A). Some residual ERK1/2 activation in the presence of thapsigargin could still be detected, possibly due to calcium still retained within ER stores. To further probe a potential involvement of intracellular calcium stores, an IP_3 receptor antagonist—2-APB—was tested and found to completely block ERK1/2 phosphorylation by T_1AM (Fig. 3.7B). This indicates that not only is calcium flux a general requirement for MAPK signaling, but, more specifically, that intracellular calcium release from IP_3R mediated stores is required for ERK1/2 activation by TAAR1/AC.

Despite the involvement of IP_3R activation in MAPK induction, we found that this does not involve phospholipase C-β (PLC-β). PLC-β is an enzyme (classically activated by Gαq) that catalyzes the generation of IP_3 and leads to activation of the IP_3R and calcium release from ER stores. We found that U73122—an inhibitor of PLC—did not block TAAR1 mediated ERK1/2 phosphorylation (Fig. 3.7B). As we thus far have

evidence only of Gαs-coupling by TAAR1 in these cells, perhaps this result suggesting an alternate method of IP_3R activation is not so surprising.

Further experiments will be required to ascertain the mechanism of IP_3R activation by TAAR1, although published literature again points to either PKA as a possible culprits. PKA has been reported—in certain cell types, including β-cells—to target IP_3 receptors, phosphorylating them to modulate their sensitivity and channel activity (Ferris et al., 1991; Nakade et al., 1994; Ni et al., 2010; Wagner et al., 2004; Wojcikiewicz and Luo, 1998). This could be the case here, as we found that both PKA and IP_3R are both necessary for activation of ERK1/2 by T_1AM.

4.4.2 Connecting TAAR1 stimulated calcium signaling in β-cells to the MAPK cascade

With the goal of identifying calcium sensitive signaling proteins that might connect TAAR1 mediated calcium flux to activation of ERK1/2, we found that the Ca^{2+}/calmodulin-dependent protein Kinase II (CaMKII; inhibited by KN-93) was necessary for both T_1AM and forskolin induced activation of the MAPK cascade in Ins-1 β-cells (Fig. 3.7C). Such a mechanism would be in keeping with work done by others, showing that both glucose and GLP-1 stimulate ERK1/2 phosphorylation in β-cells in a CaMKII dependent manner. A direct CaMKII association with and activation of Raf, leading to ERK1/2 phosphorylation has actually been documented in thyroid cells (Illario et al., 2003). Raf may even be an important upstream integration point for the generation of MAPK signaling in β-cells that receives input from both calcium-regulated proteins such as CaMKII in addition to the adenylyl cyclase/cAMP-regulated effector proteins PKA and Epac discussed previously.

4.4.3 TAAR1 raises intracellular [Ca^{2+}] through extracellular calcium influx and release of calcium from internal stores

Consistent with the apparent the calcium dependence we observed for TAAR1 mediated ERK1/2 activation (Fig. 3.7), we were able to directly confirm that TAAR1 stimulates calcium release (Fig. 3.9). We were the first to show that endogenously expressed TAAR1 stimulates calcium flux in β-cells, utilizing both endogenous (T_1AM) and small molecule (RO5256390) agonists of TAAR1 (Fig. 3.9, 3.10). For a series of reasons, we believe that TAAR1 mediated calcium flux is achieved by both release from internal stores and by extracellular calcium influx. First, reducing the concentration of extracellular calcium significantly reduces the magnitude of calcium influx. Chelation of calcium with EGTA significantly further reduces the magnitude of calcium flux, and the small but reproducible calcium signal still remaining in the presence of EGTA can be completely blocked by the final inclusion of thapsigargin (Fig. 3.9A, B). This data supports the hypothesis calcium flux induced by TAAR1 is mediated by both calcium channels on the surface of cells, as well as on intracellular stores. Activation of voltage gated calcium channels has been demonstrated in response to elevated cAMP (Lu et al., 1993; Prentki et al., 1987; Rajan et al., 1989), and may be involved in extracellular calcium influx in response to TAAR1 agonists.

Consistent with other literature documenting TAAR1 as a purely Gαs-coupled receptor, the inclusion of MDL-12,330A significantly reduced calcium flux (Fig. 3.9A, C, F). Further experiments would be needed to identify whether PKA and/or Epac are involved in this calcium flux, although both are reasonable candidates. PKA, as mentioned previously for example, could phosphorylate the IP_3R to stimulate channel

activity. Epac has also been shown in β-cells to induce calcium release from internal stores via ryanodine receptor (RyR) activation (Holz et al., 1999; Kang et al., 2001; Tsuboi et al., 2003); future experiments would be required to determine whether this is the case for TAAR1.

In further support of our Gαs/AC-derived calcium hypothesis, TAAR1-calcium signaling was not affected by YM-254890 (Fig. 3.9G, H) a membrane-permeable, potent, and selective Gαq family inhibitor in Ins-1 cells. In keeping with its Gαq targeting, YM-254890 is however an effective blocker of calcium flux signaling induced by the established Gαq-coupled PAR2 (Fig. 3.9I, J). These results are also consistent with the lack of effect of U73122 on induction of MAPK by TAAR1 (Fig. 3.7B), indicating that Gαq is unlikely to be involved, as well as the ability of forskolin, a direct activator of adenylyl cyclase to similarly induce calcium flux (Fig. 3.9E) and calcium-regulated MAPK signaling (Fig. 3.7A-C) in these cells.

4.4.4 A role for TAAR1-mediated calcium release on potentiation of GSIS

These data support the mechanism of Fig. 4.1, whereby the calcium signal induced by TAAR1-Gαs/cAMP is initially triggered by intracellular release of Ca^{2+} through IP_3 receptors, followed by activation of calcium channels on the plasma membrane, which provide the majority of the total observed calcium signal.

Calcium flux has been shown to be a critical second messenger for various Gαs-initiated signaling pathways in β-cells—not just for MAPK signaling as discussed, but also insulin granule vesicle exocytosis, which depends in large part on the activity of CaMKII (Lang, 1999; Prentki et al., 1987; Tengholm, 2012). CaMKII is localized to insulin secretory granules and binds to synapsin-1 and MAP-2 proteins that are involved

in exocytosis (Easom, 1999); as we have found that TAAR1-induced MAPK activity requires CaMKII, it is also possible that Ca^{2+} signaling/CaMKII could be involved in TAAR1's effects on GSIS.

In fact, 2-APB, an IP_3R antagonist does reduce potentiation of GSIS by TAAR1, confirming that that TAAR1-stimulated calcium flux also plays a role in the receptor's potentiation of GSIS. As calcium influx itself is the main trigger for the exocytosis of insulin containing vesicles, and the magnitude of calcium flux directly affects insulin secretion, similar effects for GLP-1R stimulated calcium flux on GSIS have been noted. The restriction of calcium flux to specialized microdomains, particularly in β-cells, is very critical for the activation of specific downstream pathways (i.e., GSIS), and significant further work would be required to track TAAR1-mediated calcium flux at a subcellular level to better understand its roles in these cells.

4.5 RO5256390 is an efficacious agonist of endogenous TAAR1 in β-cells

One of the difficulties in studying TAAR1 has resulted from a lack of widely available and specific agonists of the receptor. T_1AM for example can also activate the adrenergic receptor α-2A (ADRA2A), albeit with a lower affinity (Zucchi et al., 2014);, in our hands however, no additive effects of yohimbine (an ADRA2A antagonist) on top of T_1AM could be detected (data not shown), indicating that the responses to T_1AM in this cell line are likely mediated by TAAR1.

Excitingly, however, Hoffman La Roche has been designing and screening small molecule agonists of TAAR1 for several years now, and a few novel small molecule compounds have been identified as full and partial agonists of TAAR1 (Galley et al., 2016; Galley et al., 2012; Revel et al., 2011). These small molecule agonists developed

by Hoffman La Roche have been used mostly to probe the CNS functions of TAAR1, as the receptor is highly expressed in the brain. TAAR1 agonism reduces monoaminergic signaling in the brain, to reduce drug-seeking, addictive, and impulsive behaviors, via a TAAR1 mediated downregulation of dopamine reward circuits (Asif-Malik et al., 2017; Bradaia et al., 2009; Cotter et al., 2015; Espinoza et al., 2011; Ferragud et al., 2017; Harkness et al., 2015; Harmeier et al., 2015; Leo et al., 2014; Lindemann et al., 2008; Liu et al., 2017b; Pei et al., 2016b; Pei et al., 2015b; Revel et al., 2012; Revel et al., 2011; Thorn et al., 2014; Xue et al., 2018).

Excitingly, one of the compounds (RO5256390) became commercially available recently, allowing us to purchase and test the small molecule to compare its effects to those of T_1AM in our β-cell line. RO5256390 treatment of Ins-1 β-cells results phosphorylation of both CREB and ERK1/2, *Irs-2* induction, and calcium mobilization (Fig. 3.10). The similar results observed for the two different agonists help to confirm that the effects of T_1AM documented here re in fact mediated by TAAR1.

Of note, we generally found that RO5256390 seems to be a more efficacious agonist, as treatment of cells with the same concentration of agonist (10 μM) produced a smaller magnitude of response for T_1AM, when compared to RO5256390. This was particularly evident in levels of *Irs-2* induction, degree of fold change in cellular proliferation rates, and even in potentiation of GSIS, although further side-by-side comparisons would be necessary to draw these conclusions more fully. Regardless, RO5256390 is an efficacious and exciting TAAR1 agonist—it is relatively inexpensive, orally active, and while it has a relatively short half-life *in vivo* (3.7 hours), it does have

good bioavailability and has been shown to be efficacious in various animal models of disease (Ferragud et al., 2017; Revel et al., 2013).

4.6 Future directions and concluding remarks

4.6.1 The short game, the smaller picture: the β-cells and signaling

There are many future experiments that remain concerning the anti-diabetic signaling pathways stimulated by TAAR1 in β-cells. Experiments could be conducted to identify the Raf isoforms involved—Raf kinase assays could be used to identify isoform specific activities of Raf, while other assays (western blotting using phospho-specific antibodies) could identify which, if any PKA sites on B-Raf or C-Raf are phosphorylated in response to TAAR1 agonist treatment, as well as what effects this (these) phosphorylation events have on Raf activity and downstream MEK/ERK1/2 activation. Alternatively, and perhaps more simply, knockout of both B-Raf and C-Raf individually in β-cells using isoform specific siRNAs would reveal which Raf isoform is required for TAAR1 mediated ERK1/2 phosphorylation.

Due to its complex and tightly regulated nature of calcium signaling, particularly in β-cells, there are also many experiments that could be done to further understand both the mechanisms and functional consequences of TAAR1 stimulated calcium flux. Further experiments delineating the involvement of PKA and/or Epac in calcium signaling would be one such example—both have been previously linked to induction of calcium signaling downstream of GLP-1R agonism, and it is possible that this may be the case here. Using both inhibitors and siRNA targeting these proteins, we could identify if either is involved in the cAMP dependent activation of calcium flux. In terms of PKA, phosphorylation sites on IP_3R could also be examined (Nakade et al., 1994), to determine

if PKA phosphorylates the IP_3R to modulate its activity in response to TAAR1 agonists. Additionally, in terms of examining the mechanisms of IP_3 receptor activation, we could further test for any involvement of PLC by performing radiolabeled IP_3 assays, in which we would measure IP_3 production in β-cells, following treatment with TAAR1 agonists in the presence of radiolabeled $^{[3]}$myoinositol. Generation of radiolabeled IP_3 in response to TAAR1 agonist treatment would indicate that PLC-β is involved in IP_3R activation, although based on our previous results in which both YM-254890 and U73122 had no effect on TAAR1 induced signaling (both Ca^{2+} and MAPK), we hypothesize that TAAR1 would not induce IP_3 generation in Ins-1 cells. In these experiments, the use of a good Gαq coupled positive control would be essential for interpretation of results.

Identifying which plasma membrane calcium channels are involved would also be an interesting endeavor—we hypothesize that these are voltage gated channels, but as nifedipine had no detectable effects on the TAAR1-induced calcium flux in our hands, it seems that L-type calcium channels are not the primary transducer of this calcium signal. There are several other types of voltage gated calcium channels, and it has been shown that GLP-1 can directly activate voltage dependent calcium channels in β-cells (Gromada et al., 1998), so these remain a possibility. It is also possible that there are other ion channels involved. Nonspecific cation channels (NSCCs), for example can be activated by cAMP (at certain concentrations) in β-cells and are permeable to calcium (Reale et al., 1995); these might also be activated by TAAR1/Gαs signaling, as has been shown to be the case for the Gαs-coupled GLP-1R and pituitary adenylate cyclase-activating peptide (PACAP) receptor (Leech et al., 1996).

A deeper exploration into the role that calcium plays in TAAR1's potentiation of GSIS is also warranted, based on the effects seen in insulin secretion assays in which 2-APB, an IP_3R antagonist blunted insulin secretion in response to T_1AM. Other reagents could be utilized, such as thapsigargin, to further determine the contribution of ER store calcium release to GSIS, although separating TAAR1-induced calcium flux from the calcium influx induced by glucose would be difficult. Additionally, as CaMKII has been critically linked to regulation of calcium-responsive insulin secretion (Dadi et al., 2014) and has now been implicated within the TAAR1 signaling cascade, it would be interesting to determine if CAMKII is also involved in potentiation of GSIS induced by T_1AM. Of course, there are many other aspects of TAAR1 activity—outside the realm of β-cells—to be explored that might also highlight TAAR1's potential as an anti-diabetic or anti-obesity therapeutic.

4.6.2 The long game, the bigger picture, beyond just β-cells: T2DM and metabolism

Extending these initial findings in β-cell lines to *in vivo* experiments would be a priority, in terms of establishing any therapeutic potential for TAAR1. Experiments utilizing animal models of T2DM and metabolic disease, in combination with RO5256390 treatment should be conducted to determine whether chronic (rather than the 7 day sub-chronic dosing regimen tested for RO5166107) administration of RO5256390 affects fasted glucose and insulin levels, glucose and insulin tolerance, and other parameters of T2DM and metabolism (glycated hemoglobin, respiration rates). Food intake and animal weights would be monitored to determine if RO5256390 affects feeding behavior and/or weight gain, and at completion of the experiment, pancreatic β-cell mass could be assessed, to determine if RO5256390 exhibits any protective effects

on β-cell health, in terms of either promoting compensatory β-cell hyperplasia or preventing β-cell death. Given the effects noted by Raab et al. in liver (significantly reduced liver triglycerides in TAAR1 agonist treated mice), triglycerides and cholesterol levels should also be measured in serum and liver to assess whether TAAR1 might improve lipid as well as glucose handling.

Much like GLP-1R, TAAR1 represents a complex and interesting target—both receptors are expressed in the gut and brain, in addition to pancreatic β-cells (Bullock et al., 1996; Raab et al., 2016; Tornehave et al., 2008; Wei and Mojsov, 1995; Xie and Miller, 2009). Furthermore, a collection of recent research has indicated that outside of the pancreas, TAAR1 may play roles in promoting lipolysis, gut motility, satiety, and weight loss, while reducing food intake (Braulke et al., 2008; Ferragud et al., 2017; Moore et al., 2018; Raab et al., 2016). Trace amines—in addition to being found in foods—bear similarities to neurotransmitters, and it is perhaps not so surprising that this receptor is active in the brain. Its ability to modulate eating behaviors is thought to occur through a down regulation of monoaminergic/dopamine reward circuits (Ferragud et al., 2017), but TAAR1 agonism also slows gastric emptying and stimulates increased PYY secretion from the gut. Both of these actions function to prolong feelings of satiety, representing further mechanisms by which TAAR1 regulate eating behaviors (Raab et al., 2016).

TAAR1 is activated by a relatively wide number of compounds not limited to just trace amines—this relatively promiscuous receptor is activated by T_1AM, trace amines (octopamine, β-phenylethylamine, tyramine), amphetamines, as well as other agonists. It is unclear whether the thyroid hormone derivative T_1AM or these dietary trace amines are

likely the authentic agonists of TAAR1, but it is interesting to note the ties that both have to feeding and metabolism (Bunzow et al., 2001; Lindemann et al., 2005; Reese et al., 2007; Regard et al., 2007; Tan et al., 2009). In the context of metabolic diseases, it is perhaps worth noting that that Lisdexamfetamine dimesylate (LDX)—the first FDA approved drug for binge-eating disorder—is a prodrug that is broken down into the active metabolite d-amphetamine (McElroy et al., 2015; Ward and Citrome, 2018), a known agonist of hTAAR1 (Barak et al., 2008; Bunzow et al., 2001; Lewin et al., 2011; Simmler et al., 2016). In animal studies, inhibition of adrenergic receptors (the intended target of the prodrug's active metabolite d-amphetamine) only partially blocks the effects of LDX (Vickers et al., 2015); it is tempting to speculate as to whether TAAR1 agonism by d-amphetamine could account for some of the effects of LDX on binge eating and weight loss in these studies. Further supporting this idea, LDX is not the only drug currently on the market that could exhibit activity towards the trace amine associated receptor 1: phentermine—currently administered alone or in combination with topiramate as a weight loss drug (Garvey et al., 2014)—is also a TAAR1 agonist (Barak et al., 2008). Phentermine/topiramate therapies suppress appetite and have also been considered for T2DM, as they are associated with lowered glycated hemoglobin levels (HbA1C- a key indicator for blood glucose levels) in obese patients (Garvey et al., 2014).

Phentermine has actually been used as an appetite suppressant since 1959, and was one component of the infamous fen-phen (fenfluramine-phentermine) weight loss supplements that were withdrawn from the market in the 1990s due to serious side effects involving heart valve disease and hypertension (Connolly et al., 1997). Fenfluramine was found to be specifically responsible for these adverse side effects, and phentermine

remains not only available, but actually incredibly popular, accounting for an overwhelming 74% of the anti-obesity drug market between 2012 and 2015 (Thomas et al., 2016). As some reports have indicated that TAAR1 is expressed on adipocytes (Regard et al., 2008) and that administration of T_1AM to rodents induces a metabolic shift from carbohydrate to lipid oxidation (Braulke et al., 2008; Mariotti et al., 2014) and stimulates weight loss that persists even after discontinuation of treatment (Haviland et al., 2013), there are many exciting avenues by which TAAR1 agonists could be pursued for potential use in targeting metabolic dysfunction and disease. It is interesting to note that absolutely no investigations into whether TAAR1 plays a role in phentermine's or LDX's efficacy have yet been published, or whether TAAR1 mediates any of the lipolytic effects documented in response to T_1AM (TAAR1 knockout mice already exist and could be used to test such questions in rodent models). Given recent data regarding TAAR1's effects on eating behavior and weight loss, these may be worthwhile questions to examine, particularly if TAAR1 continues to demonstrate beneficial anti-diabetic signaling activities in β-cells. If TAAR1 indeed stimulates β-cell health, promotes GLP-1 secretion, satiety, and insulin secretion, while also reducing obesity and maladaptive eating behaviors, and improving lipid handling, it could truly represent an incredibly exciting new target for T2DM and metabolic dysfunction.

Chapter 5: Bibliography

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