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Pharmacological Characterization of Membrane-Expressed Human Trace Amine Associated Receptor 1 (TAAR1) by a Bioluminescence Resonance Energy Transfer (BRET) cAMP Biosensor

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d) Abbreviations:

β2-AR, β2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; DA, Dopamine; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, Hemagglutinin epitope tag; IBMX, isobutylmethylxanthine ; MDMA, methylenedioxymethamphetamine; TA, trace amine; TAAR, trace amine associated receptor; TAAR1, Trace amine associated receptor 1; hTAAR1, human TAAR1; βTAAR1, hTAAR1 with the first 9 amino acids of the human β2-adrenergic receptor (β2-AR) inserted at the N-terminus , β-PEA, β-phenylethylamine; BRET, bioluminescence resonance energy transfer; EPAC, exchange protein activated by cAMP.

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Abstract

Trace amines are neurotransmitters whose role in regulating invertebrate physiology has been appreciated for many decades. Recent studies indicate that trace amines may also play a role in mammalian physiology by binding to a novel family of G protein-coupled receptors (GPCRs) that are found throughout the central nervous system. A major obstacle impeding the careful pharmacological characterization of trace amine associated receptors (TAARs) is their extremely poor membrane expression in model cell systems, and a molecular basis for this phenomenon has not been determined. In the present study we show that the addition of an asparagine-linked glycosylation site to the N-terminus of the human trace amine associated receptor 1 (TAAR1) is sufficient to enable its plasma membrane expression, and thus its pharmacological characterization with a novel cAMP EPAC (exchange protein directly activated by cAMP) protein based BRET (bioluminescence resonance energy transfer) biosensor. We applied this novel cAMP BRET biosensor to evaluate the activity of putative TAAR1 ligands. This study represents the first comprehensive investigations of the membrane-expressed human TAAR1 pharmacology. Our strategy to express TAARs and to identify their ligands using a cAMP BRET assay could provide a foundation for characterizing the functional role of trace amines *in vivo,* and suggests a strategy to apply to groups of poorly expressing GPCRs that have remained difficult to investigate in model systems.

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Trace amines (TAs) are biogenic amines that include tyramine, tryptamine, octopamine, and β-phenylethylamine (β-PEA). TAs are found in the mammalian central nervous system at substantially lower levels than classical transmitters like norepinephrine, serotonin, and dopamine (Berry, 2004; Boulton, 1980; Branchek and Blackburn, 2003; Premont et al., 2001; Grandy, 2007). TA dysregulation has been linked to various psychiatric and neurological disorders including schizophrenia and Parkinson's Disease. As such, the TAs offer tempting targets for ameliorating the symptoms of central nervous system diseases when used in combination with standard therapies, or for reducing doses and hence the side effects that accompany the chronic use of some currently prescribed medications. This is particularly relevant for Parkinson's Disease where chronic L-DOPA therapy often produces drug related dyskinesias (Fahn, 2003; Hornykiewicz, 2002; Mercuri and Bernardi, 2005).

Trace amines are structurally related to several psychotropic molecules that naturally occur in plants, including amphetamine-like compounds. Amphetamines are recognized inhibitors of inward monoamine transport (Jones et al., 1998), and it was generally proposed that TAs also affect the monoamine system indirectly via interaction with plasma membrane transporters, such as the dopamine transporter (Sotnikova et al., 2004). In this view, the TAs function simply as neuromodulators of classical mammalian brain amine transmitters, and at physiological levels TAs generally should have only minor effects on neuronal excitability in the absence of classical monoamines. However, binding sites for tryptamine, tyramine and β-PEA have been reported in rat brain and a family of trace amine G-protein coupled receptors (GPCRs) has been recently identified in rodents and humans (Borowsky et al., 2001; Bunzow et al., 2001; Lindemann and Hoener, 2005; Grandy, 2007). Thus solid support exists for a potential direct behavioral mechanism in which trace amines influence neuronal activity.

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In humans, six trace amine associated receptors (TAARs) and three pseudogenes have been identified (Lindemann and Hoener, 2005). Similar to their mouse counterparts on chromosome ten, human TAARs are coded for by intronless genes clustered tightly on chromosome six. Trace amine associated receptor 1 (TAAR1) is the best characterized of them (Borowsky et al., 2001; Bunzow et al., 2001; Grandy, 2007). TAAR1 couples to G_s, and when assessed using cAMP, is activated by TAs such as β-PEA in addition to metabolites of catecholamines, amphetamines (including MDMA), and other compounds known to affect monoaminergic transmission (Borowsky et al., 2001; Bunzow et al., 2001; Grandy, 2007; Kim and von Zastrow, 2001; Lindemann and Hoener, 2005; Miller et al., 2005; Reese et al., 2007; Scanlan et al., 2004; Wainscott et al., 2007; Wolinsky et al., 2006; Xie and Miller, 2007; Xie et al., 2007).

In the brain, the mRNA for TAAR1 is distributed throughout the limbic system and in regions containing catecholaminergic cell bodies and their projections (Borowsky et al., 2001; Bunzow et al., 2001; Lindemann and Hoener, 2005; Xie et al., 2007). Thus, TAAR1 is well-positioned to modulate locomotor, emotional and motivated behaviors that are traditionally associated with monoaminergic activity. Interestingly, recent evidence indicates that at least some TAARs can also serve as a new class of chemosensory receptors in mammals (Liberles and Buck, 2006). Since an imbalance in the function of TAs might have important implications in the pathology of several disorders, finding selective small molecule agonists and antagonists for these receptors could provide novel approaches of disease management. However, a major obstacle in studying and screening TA receptors is their extremely poor plasma membrane expression in heterologous cell systems (Borowsky et al., 2001; Bunzow et al., 2001; Grandy, 2007; Lindemann and Hoener, 2005; Miller et al., 2005; Wainscott et al., 2007; Wolinsky et al.,

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2006), a scenario remarkably similar to a large GPCR subfamily of chemosensory odorant receptors (Saito et al., 2004; Zhuang and Matsunami, 2007).

The human variant of the TAAR1 has remained even more difficult to express in model systems than its poorly expressed rodent analogue (Lindemann and Hoener, 2005; Grandy, 2007) thus impeding measurements of biochemical and pharmacological properties of this receptor. In order to improve our ability to study hTAAR1 we investigated potential reasons for the poor hTAAR1 expression. In this report, we demonstrate that an N-glycosylated variant of the human TAAR1 expresses at the plasma membrane of HEK-293 cells well enough to be studied using antibody labeling. We have determined in this system that hTAAR1 can interact with β-arrestin2. Moreover, we have utilized an N-glycosylated variant of the human TAAR1 for identifying and studying TAAR1 ligands by constructing a novel cell-based screening assay that employs a bioluminescence resonance energy transfer (BRET) EPAC cAMP biosensor.

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Materials and Methods

Materials. Anti-HA antibody was from Roche Applied Sciences and goat anti-mouse Alexa 568 antibody from Molecular Probes. Plasmids containing the cDNA for the human trace amine receptor were obtained from the cDNA resource center at the University of Missouri-Rolla and the ATCC. Sources of cell culture reagents and buffers were from Gibco and Sigma, and fetal bovine serum from JRH Biotech. The enhanced green fluorescent protein plasmid was obtained from Clontech. All compounds used in this study were obtained from Sigma.

Construction of Expression vectors. A cDNA leader sequence containing the triple HA epitope was constructed using the polymerase chain reaction for insertion into the plasmid pcDNA3. Full-length human TAAR1 cDNA without a stop codon was amplified by PCR with 5' and 3' in-frame restriction enzyme sites of EcoR I and Kpn I, respectively. A cDNA encoding GFP in the absence of its initiation start codon was amplified by PCR with 5' (in-frame) and 3' (downstream of stop codon) restriction enzyme sites of Kpn I and Xba I, respectively. Enzyme-digested TAAR1 and GFP PCR products were ligated into EcoR I and Xba I restriction enzymes sites of pcDNA3 vector with N-terminal triple HA tag to generate the triple HA-β2N9-TAAR1-GFP construct. The cDNA corresponding to the first nine amino acids of the β2-adrenergic receptor was inserted in-frame between the triple HA and TAAR1.

For the GFP negative construct, the GFP moiety was removed from the above construct by PCR amplification using 5' T7 promoter sequence primer upstream of triple HA sequence and 3' primer containing stop a codon followed by the restriction enzyme site of Xba I. The PCR product was then digested with Hind III (upstream of triple HA sequence) and Xba I, and

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subcloned into the pcDNA3 vector. Two complimentary sequences, encoding the EcoR V/EcoRI-digested β2N9 DNA fragment were allowed to anneal in 10 mM Tris-EDTA (pH 8.0)-NaCl (50 mM) buffer, and ligated into EcoR V/EcoR I restriction enzyme sites. The cDNA illustrative of these modifications is as follows with the upper case characters corresponding to the three triple HA inserts and the last segment to the β2-adrenergic receptor. The corresponding cDNA sequence is:

[tccaccatggctagctaccca**TACGATGTTCCTGACTATGCG**ggc**TATCCCTATGACGTCCCG**
GACTATGCAggatcc**TATCCATATGACGTTCCAGATTACGCC**agatctgatatc**ATGGGGC**
AACCCGGGAACGGCAGCGCCgaattc**ATGATGCCCTTTTGCC].** The translated sequence is: (s t m a s **Y P Y D V P D Y** a g **Y P Y D V P D Y** a g s **Y P Y D V P D Y** a r s d i **M G Q P G N G S A** e f **M M P F C**).

The BRET-based cyclic AMP biosensor (Holz et al., 2006; Jiang et al., 2007; Ponsioen et al., 2004) was constructed by the modification of ICUE2, an existing FRET-based intramolecular biosensor in which eCFP and citrine flank residues 149-881 of Epac1 (*e*xchange *p*roteins directly *a*ctivated by *c*yclic AMP) (DiPilato et al., 2004; Violin et al., 2007). The eCFP moiety of ICUE2 was removed by B*am*HI and K*pn*I restriction, and replaced with a humanized *Renilla* luciferase gene that was PCR amplified from phRluc-C1 (Perkin Elmer) and was engineered with B*am*HI and K*pn*I restriction sites that preserve the frame of translation. Primers for the PCR amplification of Rluc were: 5'GGATCCATGACCAGCAAGGT-GTACGACC and 5'GGTACCCCCCCTGCTCG-TTCTTCAGCAC.

Cell Culture and Transfection of Cell Lines. Cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and 1:100 penicillin/streptomycin in a humidified

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5% CO2 atmosphere. Transfections were carried out by the calcium phosphate precipitation method. HEK-293T cells or U2OS cells were transfected with aliquots containing 1-2 μg β-arrestin2 cDNA and/or 5 μg receptor cDNA per ml. Cells were plated for immunoflourescence studies into the wells of 35 mm glass bottom Matek dishes containing 2 ml MEM/10% FBS. 125 μl of transfection solution was added for a period between 6 hours to overnight, after which the transfection solution was replaced with fresh serum containing media. For BRET studies the HEK-293T cells were transfected with 5 μg of cDNA in 1 ml of transfection solution in 100 mm plastic dishes overnight and selected to permanently express the EPAC sensor using zeocin (200-400 μg/ml). These cells were transfected as mentioned above with TAAR1 for evaluation of compounds in the screening assay.

Fluorescence microscopy. Cells were plated at a density of 20-40,000 per well in 35 mm Matek glass, cover slip bottomed dishes. Anti-HA antibody was diluted 1:400. Alexa 568 goat anti-mouse antibody was used at a 1:1500 dilution. Fixed cells were first washed twice in PBS followed by incubation with 4% paraformaldehyde for 15 minutes at room temperature, rewashed, and then incubated for 40 minutes intervals in PBS with 2% BSA with either anti-HA or subsequently secondary antibody using intervening 3X washes with PBS. Live cells were incubated with the same antibody dilutions in MEM at room temperature or 37 degrees. β-arrestin translocation was assessed as described (Barak et al., 1997). Cells were examined with a Zeiss LSM-510 confocal microscope equipped with a 100x NA 1.4 oil objective using 488 nm excitation for the GFP tag and 514 nm excitation for Alexa 568 antibody, and employing the accompanying fluorescein and rhodamine emission filter sets respectively for the two different chromophores.

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Immunoblotting. HEK-293T cells were transfected with the various HA tagged receptor subtypes and lysed the following day in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100**.** Cell lysates were analyzed by SDS-PAGE and immunoblotted with anti-HA antibody at a dilution of 1:5000.

BRET Screening Assays. HEK-293T cells permanently transfected with the EPAC sensor and transiently with the TAAR1 were split into 96 well plates at 150-200,000 cells per well. On the following day they were washed twice with PBS, and 80 µl of PBS containing calcium and magnesium was added to each well followed by addition of 10 µl of a 50 µM Coelenterazine solution (5 µM final) After 10 minute incubation either 10 µl of vehicle or 10X concentrated solution of drug in PBS was added, and the plate was then placed into a Mithras LB940 instrument (Berthold) that allows the sequential integration of the luminescent signals detected in the 465 to 505 nm and 505 to 555 nm windows using filters with the appropriate band pass and by using MicroWin 2000 software. The BRET signal is determined by calculating the ratio of the light emitted at 505-555 nm over the light emitted at 465-505 nm.

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Results

Expression of TAAR1 receptors in fixed HEK-293T Cells. Receptors of the human trace amine family have been notoriously difficult to express in model cell systems, and are comparable in this regard to odorant receptors (Grandy, 2007; Lindemann and Hoener, 2005; Zhuang and Matsunami, 2007). Our initial attempts at expression were also disappointing, but in the process of evaluating TAAR1 plasma membrane expression we noted a marked absence of glycosylated bands that are common to other rhodopsin-family GPCRs. Moreover, *in-silico* prediction suggested that the N-terminus of TAAR1 should be poorly glycosylated. Therefore, in an attempt to augment TAAR1 glycosylation and the subsequent expression of the receptor at the plasma membrane (Fig.1A), the first 9 amino acids of the human β2-adrenergic receptor (β2-AR) were inserted at the HA–epitope tagged TAAR1 N-terminus HA-βTAAR1 (note that an initial attempt to augment expression using the N-terminal rhodopsin signal sequence proved unsuccessful). This 9 residue segment contains an asparagine glycosylation site at position 6 (Rands et al., 1990). Moreover, for the β2-AR this glycosylation enhances receptor expression at the plasma membrane (Rands et al., 1990). Without the added insert, we observed for a TAAR1-GFP chimera a homogeneous intracellular GFP distribution consistent with degraded TAAR1 (Fig.1B, upper left panel) or receptor trapped in the endoplasmic reticulum (Fig.1B, upper right panel). With addition of the first 9 amino acids of the human β2-adrenergic receptor, we observed a substantial accumulation, at 24 hours, of what appears to be plasma membrane localized receptor at the edge of the cells with loss of the majority of internal staining (Fig.1B, lower panels). Immunoblotting of cell lysates for HA antibody (Fig.1C) demonstrates extended bands for both GFP and non GFP receptor variants characteristic of glycosylated receptor that

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runs above sharper-well defined bands typically characteristic of the immature receptor isoforms (Barak et al., 2001; Sadeghi et al., 1997). Additionally, paraformaldehyde fixation without a permeabilization step of the HA-tagged βTAAR1 transfected cells indicates that the HA tag on the receptor is located at the external face of the plasma membrane (Fig.1D).

Expression of TAAR1 in Live Cells. In order to estimate the expression level of the βTAAR1 and verify its orientation and integrity in the plasma membrane, we performed live cell immunoflourescence labeling with anti-HA antibody. Figure 2A shows the relative expression of HA tagged β2-AR (Fig.2A, left panels) in comparison to the HA-βTAAR1 GFP and non GFP variants (middle and right panels Fig.2A). A more substantial expression of fluorescence associated with the β2-AR is evident as is a smaller expression level for the non βTAAR1-GFP variant in comparison to the GFP version. The ability of GFP tagging to stabilize the expression of the βTAAR1 was also apparent from the immunoblotting in Figure 1B. Labeling N-terminal plasma membrane receptor in live cells with antibody can result in receptor aggregation, and this inhomogeneous distribution can be used to verify the intactness of the receptor construct (Barak et al., 1997). Figure 2B shows that in non-permeabilized live antibody-labeled cells, the C-terminal GFP distribution coincides with the HA epitope distribution, indicating that a fully formed full-length receptor is being expressed at the plasma membrane. Our ability to express βTAAR1 at the plasma membrane is not simply confined to HEK cells as transient labeling of the cell edge by βTAAR1 can also be demonstrated in a U2OS cell line (Fig.2C). To demonstrate receptor functionality, we evaluated whether the βTAAR1 could undergo desensitization to known agonists using a β-arrestin2-GFP translocation assay. However, due to the relatively smaller expression levels of the βTAAR1 we first aggregated the receptors using mouse monoclonal anti-HA and Alexa 568 goat anti-mouse antibodies. As seen in Figure 2D, the

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aggregated βTAAR1 in the presence of *p*-tyramine results in the translocation of a small amount of β-arrestin2-GFP, indicating that βTAAR1 can recruit β-arrestin2 similar to other GPCRs. However, this modest translocation resembles that of dopamine D3 receptor that is known to desensitize relatively poorly in a β-arrestin-dependent manner (Kim et al., 2005; Kim et al., 2001). In addition, the reduced translocation may also be indicative of the lesser expression of βTAAR1 relative to the β2-AR. While the β-arrestin translocation assay can be used to measure receptor activation, and evaluate both agonists and antagonists at many receptors, in this case the weak response precludes using the direct visualization assay for this receptor variant. Therefore, we investigated a novel cAMP signaling sensor that could function well at these smaller, but still greatly improved TAAR1 expression levels.

BRET sensor for measuring TAAR1 in real time in Live Cells. cAMP production provides sufficient amplification to measure the functional activity of TAAR1, and to date has been the only practical way to approach the pharmacological study of this very poorly expressed receptor. A significant limitation of current methods to measure cAMP in cells for compound library screening is the extended activation period necessary to accumulate enough of the second messenger product to measure in antibody mediated assays. Here we evaluated a biosensor for real time screening of cAMP production in TAAR1 transfected cells. The cAMP BRET biosensor was generated by modification of the original ICUE2 cAMP FRET biosensor (Violin et al. 2007). The sensor consists of an N-terminal truncated variant of the Exchange Protein Activated by cAMP (EPAC) tagged with the Renilla luciferase and a yellow fluorescent protein variant (citrine) attached at the N- and C-terminus, respectively (Fig.3A). Upon binding cAMP the signal of the biosensor decreases due to a conformational change that increases the distance between the Renilla luciferase donor and the yellow fluorescent protein acceptor. The utility of

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the biosensor for measuring βTAAR1 cAMP signaling in real time is demonstrated in Figure 3B where application of 1 μM β-PEA, a TAAR1 agonist, produces a sustained reduction in the BRET signal for βTARR1 (wild type TAAR1 did not produce changes in the BRET signal, data not shown). Moreover, the addition of IBMX to the incubation media resulted in a significant increase of the BRET signal to β-PEA (Fig. 3B) and isoproterenol (not shown), without changing the EC50s of responses to either ligands (data not shown). The enhancement of the BRET signal with IBMX is consistent with its role as a phosphodiesterase inhibitor that prevents cAMP degradation. Figure 3C shows that by increasing the β-PEA concentration a concomitant increase in the βTAAR1 BRET signal occurs, and that the resulting signal amplitude remains stable for at least 20 minutes following induction of cAMP. In contrast, in these HEK-293 cells isoproterenol stimulation of the endogenous β2-AR produces a transient change in the BRET signal that is maximal at 400 seconds and then returns slowly towards baseline (Fig.3D), an indication of a desensitization of the signaling apparatus and a reduction in cAMP concentration (Violin et al 2007).

Taken together, the β-arrestin/βTAAR1 translocation and the cAMP BRET data indicate that the βTAAR1 has poorer affinity for β-arrestin2 than does the β2-AR. To confirm a βTAAR1/ β-arrestin desensitization response by BRET, we transiently over-expressed both proteins in the same cell (Fig.3E). In the presence of over-expressed β-arrestin2 a return to baseline, i.e. desensitization, of the β-PEA mediated BRET signal is apparent after five minutes as well as a reduction in the magnitude of the BRET signal.

To demonstrate the general utility of the EPAC BRET sensor for determining cAMP concentrations, we co-transfected the sensor with the G_i-coupled D2 Dopamine (DA) Receptor (D2R), expecting that pre-stimulation of the cells by DA would dampen their cAMP

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responsiveness. Fig. 3F demonstrates that increasing concentrations of DA diminish the magnitude of an isoproteronol induced BRET signal towards control levels without significantly changing the time it takes the signal to desensitize.

Specificity of BRET signaling of βTAAR1 in HEK-293 cells. The sensitivity of the BRET EPAC assay enables measurement of the signaling of endogenous receptors (Fig.3D). To verify the absence of TAAR1 signaling in HEK/EPAC cells not containing transfected βTAAR1 cDNA, we compared the isoproterenol and β-PEA response of non-receptor transfected (Mock) cells to cells transfected with βTAAR1 cDNA. Isoproterenol signaling was the same in both cell populations (Fig.4A) whereas only the βTAAR1 transfected cells produced an EPAC signal significantly above baseline. The very small rise in the β-PEA signal in Mock containing cells at high agonist concentrations may be due to activation of endogenous adrenergic receptors.

Activation of the βTAAR1 by TAAR1 agonists β-PEA and *d*-amphetamine. The endogenous trace amine β-PEA is the best established agonist of TAAR1 (Borowsky et al., 2001; Bunzow et al., 2001; Grandy, 2007; Lindemann and Hoener, 2005; Miller et al., 2005; Wainscott et al., 2007; Wolinsky et al., 2006). The effects of amphetamine on behavior have historically been attributed to the release of internal stores of dopamine (Jones et al., 1998). However recent observations indicated that amphetamine derivatives can also activate directly TAAR1 (Bunzow et al., 2001; Grandy, 2007; Miller et al., 2005; Reese et al., 2007; Wainscott et al., 2007; Wolinsky et al., 2006; Xie and Miller, 2007; Xie et al., 2007). Therefore, by using the EPAC cell line the ability of these two compounds to induce cAMP production through the βTAAR1 was tested (Fig.4B). Moreover, to confirm the results, we also measured the cAMP response in HEK cells using the standard cAMP column assay developed by Salmon (Salomon et al., 1974) (Figure 4C). The results of the column and BRET measurements are shown in Table 1. They are

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qualitatively similar, with the BRET assay yielding EC50 values for cAMP activation that are 3 to 4 fold shifted to the left.

BRET Measurement of TAAR1 for Screening. The above results indicated that the TAAR1 BRET assay should be sensitive and specific enough for general screening of TAAR1 ligands using a higher throughput format. Therefore we measured the dose response of about 40 more compounds that could be potential ligands of the TAAR1 in antagonist and agonist assays. Some examples of these studies are plotted in Figure 4D. While among these compounds we did not detect any antagonist based on their ability to counteract β-PEA induced BRET signal (data not shown), we found several compounds that induced relatively potent agonist activity (Table 2) supporting previous reports on activity of these compounds at TAAR1 receptors (Bunzow et al., 2001; Miller et al., 2005; Reese et al., 2007; Wainscott et al., 2007; Wolinsky et al., 2006; Xie and Miller, 2007; Xie et al., 2007). A list of compounds that did not exert any activity at βTAAR1 in the BRET assay is presented in Table 3.

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Discussion

Trace amine associated receptors, like olfactory receptors, are notoriously difficult to express in heterologous cell systems. This poor cell surface expression has been a major limitation for understanding TAAR1 biology and evaluating TAAR1 selective ligands. In fact, up to this point there has been no compelling direct evidence in the literature to suggest that the intact human TAAR1 could be expressed at sufficient amounts in a cell system, particularly at the plasma membrane compartment (Borowsky et al., 2001; Bunzow et al., 2001; Grandy, 2007; Lindemann and Hoener, 2005; Miller et al., 2005; Reese et al., 2007; Scanlan et al., 2004; Wainscott et al., 2007; Wolinsky et al., 2006; Xie and Miller, 2007; Xie et al., 2007). Our data with HA tagged and GFP modified human TAAR1 provides evidence that glycosylation stabilized expression of the receptor can occur in cells at levels sufficient for characterizing receptor biology and developing reliable *in vitro* cellular assays without modifying intracellular loops, developing human-rat chimeras (Grandy, 2007; Lindemann and Hoener, 2005) or co-expressing human TAAR1 with rat $G\alpha_s$ (Wainscott et al., 2007). Furthermore, we devised and validated a novel BRET cAMP biosensor cellular assay that permits high throughput screening for TAAR1 agonists and antagonists. Though βTAAR1 expression was five to tenfold lower than what can be achieved with the β2-adrenergic receptor, we observed that an N-β2-AR peptide modified TAAR1 enables observable plasma membrane expression for which limited β-arrestin translocation occurs.

The ability of the human TAAR1 to activate adenylyl cyclase with previously identified agonists was assessed by classical methodology using column chromatography and by a novel BRET method employing an EPAC biosensor. The two distinct techniques were in agreement

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that human TAAR1 in HEK cells responds to β-PEA and *d*-amphetamine, similar to what has been reported for rodent receptors or rodent modified human variants (Borowsky et al., 2001; Bunzow et al., 2001; Lindemann and Hoener, 2005; Miller et al., 2005; Reese et al., 2007; Wainscott et al., 2007; Wolinsky et al., 2006). While the measurement of cAMP using chromatography is not well suited for high throughput screening, we observed that the BRET method described here can be applied to large scale screening for GPCR ligands. In fact, in our initial BRET cAMP screen we confirmed agonistic activity at human TAAR1 of several other compounds, including the trace amines octopamine and tryptamine, the amphetamine derivatives *l*-amphetamine, *d*-methamphetamine, (+)-MDMA, phentermine and the catecholamine metabolites 3-MT and 4-MT (Bunzow et al., 2001; Lindemann and Hoener, 2005; Reese et al., 2007; Wainscott et al., 2007; Wolinsky et al., 2006; Xie and Miller, 2007; Xie et al., 2007). It has been noted previously that activity of these compounds at TAAR1 can differ significantly between different species (Grandy, 2007; Lindemann and Hoener, 2005; Wainscott et al., 2007). Accordingly, we observed that while these compounds were active at human TAAR1 with potencies generally similar to that observed in rodent, *rhesus* monkey and chimeric human-rodent receptors (Bunzow et al., 2001; Grandy, 2007; Lindemann and Hoener, 2005; Wainscott et al., 2007; Wolinsky et al., 2006; Xie and Miller, 2007; Xie et al., 2007), several important differences were also noted. In particular, the most efficacious compound at human TAAR1 appeared to be *l*-amphetamine, followed by *d*-methamphetamine with activities at least equal or exceeding β-PEA. It is interesting also that trace amines octopamine and tryptamine showed partial agonist activity at human TAAR1 with potencies that were somewhat higher than those observed in previous studies with human-rat chimeric receptors (Grandy, 2007; Lindemann and Hoener, 2005; Wainscott et al., 2007). Finally, these studies also confirmed activity of

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catecholamine metabolites 3-MT and 4-MT at human TAAR1 (Bunzow et al., 2001; Grandy, 2007; Wainscott et al., 2007), raising an intriguing question on physiological roles that may be mediated by these metabolites via activation of TAAR1 receptors. By assessing the capability of compounds to reduce β-PEA – induced BRET signaling, we also sought to identify antagonists of TAAR1; but in a small list of compounds tested no such activity was found. Clearly, tests of larger libraries of compounds for TAAR1 activity by using this assay are warranted and are being performed currently in our lab.

Notably, the BRET method has been used for high-throughput screening of chemokine receptor CCR5 antagonists using a variant of a BRET-sensitive β-arrestin2 recruitment assay (Hamdan et al., 2005). During the course of this work, a similar BRET sensor for cAMP, CAMYEL (cAMP sensor using YFP-Epac-RLuc) was developed independently for characterizing cAMP synthesis stimulated via a sphingosine 1-Phosphate/G13 pathway (Jiang et al., 2007). Moreover, the modified EPAC cAMP sensor, described here, has shown recently great utility to monitor dynamics of β2AR desensitization in live cells by fluorescence resonance energy transfer (Violin et al., 2007). The BRET data in our study indicates that the TAAR1-induced cellular concentration of cAMP is stable over an extended period. In contrast, under similar assay conditions, β2AR cAMP production decreases relatively rapidly presumably as a result of more pronounced receptor desensitization (Jiang et al., 2007; Violin et al., 2007). The TAAR1 behaves in this regard similarly to the dopamine D3 receptor that also shows no discernable desensitization in HEK 293 cells (Kim et al., 2005; Kim et al., 2001).

In conclusion, we expressed human TAAR1 receptor in HEK cells at the plasma membrane at levels enabling biologic characterization and the development of a BRET signaling cellular assay for large scale screening of TAAR1 ligands. Identification of selective ligands of

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the TAAR1 would be critical for investigating the functional role of this receptor in mammalian physiology in vivo and/or managing human disorders where abnormalities in TA physiology may occur. The use of the BRET EPAC biosensor described here may become an effective approach to identify selective agonists and antagonists of a wide range of GPCRs in general. In comparison to other available cAMP assays the described BRET assay provides a user friendly opportunity for real-time dynamic assessment of cAMP production rather than single end-point analysis of total accumulated cAMP. In addition, the strategy applied to express TAAR1 in HEK cells may be applicable to other GPCRs such as olfactory receptors that exhibit very limited expression at the plasma membrane in model systems.

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Footnotes

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c) Numbered footnotes:

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Legends for Figures

Figure 1. Expression of the TAAR1 in HEK-293 Cells. A. Pictured are the two variants of the human trace amine receptor that were used throughout the study. Insertion of the 9 amino acid proximal portion of the human β2-adrenergic receptor into the N-terminus of the TAAR1 was a key to stabilizing it at the plasma membrane. B Confocal micrograph of the fluorescence distribution of GFP in HEK-293 cells transfected with 5 μg cDNA of the GFP variants of the TAAR1 without and with (βTAAR1) the β2-adrenergic receptor insert. C. Western Blot for the HA epitope of the triple HA tagged, β2-adrenergic receptor (Amino acids 1-9) modified TAAR1 variants transiently expressed in HEK-293 cells. Note that right image represents the same blot with a lower exposure period. D. Immunostaining using mouse anti-HA antibody followed by goat anti-mouse alexa 568 secondary antibody of fixed, non-permeabilized HEK cells transiently expressing either the βTAAR1 (upper panels), or the βTAAR1 conjugated to GFP (lower panels).

Figure 2. Expression levels, distribution, and function of the βTAAR1 in live HEK Cells. A. Immunofluorescence and transmitted light images of alexa 568 immuno-stained HA-β2AR (leftmost images), βTAAR1-GFP (middle images), and βTAAR1 (rightmost images). Images using live HEK-293 cells expressing labeled receptors were acquired on a Zeiss LSM 510 confocal microscope using a 100x NA (1.4) oil objective. B. Overlay of Alexa 568, anti-HA immuno-staing and GFP imaging of the βTAAR1-GFP receptor. Images were acquired as above in live HEK cells plated and transfected in 35 mm glass bottomed dishes (Matek). C. Imaging of βTAAR1-GFP receptor in live U2OS cells transfected using calcium phosphate protocol with

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βTAAR1-GFP receptor as in (B). Receptor can be seen as enhanced fluorescence at the edge of the cells. D. Translocation of β-arrestin2-GFP was measured in cells transfected with β2AR (leftmost images) or the βTAAR1 (rightmost images). Live cells were imaged after labeling with monoclonal anti-HA antibody followed by alexa 568 goat anti-mouse antibody (upper panels), and treatment for 30 minutes at 37 degrees with either 20 μM isoproterenol (β2AR) or 20-50 μM of *p*-tyramine for the βTAAR1. β-arrestin2-GFP translocation is shown in the lower panels (green).

Figure 3. Evaluation of cAMP response in HEK-293 cells using an EPAC BRET biosensor. A. Cartoon of the postulated molecular rearrangement of the full length EPAC protein with and without cAMP present. B. Time course of the BRET response computed as the ratio of YFP/Rluc emissions (see *Materials and Methods*) in HEK-293 cells transfected with the βTAAR1 and permanently expressing the BRET biosensor (clone 8). To generate the lower β-PEA curve the cells were exposed to 10 μM compound in PBS containing calcium and magnesium (see Materials and Methods), and 5 μM coelanterezine at room temperature. The lower β-PEA curve reflects the increased levels of cAMP in the presence of IBMX (0.5 mM). C. Time course of the BRET sensor response to cAMP accumulation after exposure to various doses of β-PEA in clone 8 cells expressing βTAAR1 receptor. D. An analogous time course graph is generated for cAMP production secondary to isoproterenol exposure of endogenous β2-adrenergic receptors in the clone 8 HEK cells. E. The desensitizing effects of β-arrestin2 (βarrest2) co-transfection on the cAMP response of 10 μM β-PEA treated βTAAR1 results in an upwards deflection of the BRET response curve. F. The G_i coupled D2 Dopamine Receptor was co-expressed in HEK-293 cells expressing the EPAC BRET biosensor and endogenous β-adrenergic receptor. Increasing

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concentrations of dopamine preceding isoproterenol stimulation of the β-adrenergic receptors results in decreased cAMP production and deflection of the BRET response upwards towards the measured basal cAMP response profile.

Figure 4. EPAC Response in HEK-293 Cells with Stimulation of Endogenous and Transfected Receptors. A. Clone 8 HEK-293 cells permanently expressing Rluc-EPAC-YFP and endogenous receptors only (Mock), or expressing in addition transfected βTAAR1, were exposed to differing concentrations of isoproterenol or β-PEA for five and 15 minutes respectively at room temperature. Data is presented as the relative change ± SEM from the baseline level BRET measurements, and results are the average of 2 (iso Mock) to 4 independent experiments. B. Clone 8 cells expressing the βTAAR1 were exposed to varying concentrations of β-PEA and *d*-amphetamine for 15 minutes at room temperature. Results are presented as mean ± SEM change form baseline BRET signaling and represent two independent experiments performed in duplicate. C. Dowex and Alumina column chromatography was used to measure [3H]-cAMP accumulation in HEK-293 cells transfected with the βTAAR1 receptor and treated with the concentrations of compounds shown in the Figure for 15 minutes at room temperature. Results are the mean ± SEM of two (*d*-amphetamine) or three (β-PEA) independent experiments performed in duplicate. D- Example of TAAR1 BRET assay screening test for TAAR1 ligands. 20 compounds were tested in this experiment at a single concentration (10 μM). Measurements were performed 10, 20 and 30 min after addition of compounds. b - vehicle baseline, Compound 11 - β-PEA; Compound 13 - *l*-amphetamine, Compound 18 - *d*-methamphetamine. Stippled area around the baseline represents 95% confidence interval for vehicle response. From the data presented in this report we estimated Z-score as 0.6.

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Table 1. Comparison of activity of β-PEA and *d*-amphetamine at TAAR1 as measured by column cAMP assay and BRET assay.

Compounds	EC50 in column assay	Efficacy (% of β-PEA) in column assay	EC50 in BRET assay	Efficacy (% of β-PEA) in BRET assay
β-PEA	5.8 +/-0.58E-07	100	1.08+/-0.29E-07	100
d-amphetamine	9.3+/-2.79E-07	73.9+/-12.6	1.36+/-0.14E-07	62.6+/-1.7

Values are estimated from experiments presented in Figs. 4B and 4C.

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Table 2. List of TAAR1 agonists identified in BRET assay.

Compounds	EC50	Efficacy (% of β-PEA)
p-Tyramine	3.24+/-0.39E-07	101.7+/-2.3
Octopamine	1.66+/-0.74E-06	63.6 +/- 1.9
Tryptamine	1.95+/-0.49E-06	82.6 +/- 4.7
l-amphetamine	2.45+/-0.14E-07	127.9 +/- 4.1
(+)-MDMA	3.70+/-0.54E-07	89.6+/-6.3
d-Methamphetamine	1.31+/-0.19E-06	107.7 +/- 5.1
Phentermine	1.08+/-0.04E-06	87.7 +/- 1.1
3-MT	2.81+/-0.24E-06	63.7+/-5.4
4-MT	2.87+/-0.04E-06	74.4 +/- 3.74

Dose-response curves were generated as in Fig. 4B by measuring responses from 10^{-4} – 10^{-10} M of each compound. Results of at least two independent experiments are presented. Note that no response was observed in mock cells not transfected with βTAAR1.

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Table 3. List of compound that did not show activity (agonist or antagonist) at TAAR1 in EPAC BRET assay.

Compounds
L-DOPA
D-DOPA
Bupropion
Spermidine
Spermine
Haloperidol
α-methyl-serotonin
Morphine
Naltrexone
Naloxone
Methadone
L-tyrosine
Methylphenidate
Phentolamine
3-hydroxy-4-methoxy-phenethylamine
MPEP
Zonisamide
Lamotrigine
Modafinil

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D-phenylalanine
5-methoxy-NN-dimethyltryptamine
3-methoxy-L-tyrosine
L-DOPS
Pergolide
Propranolol
Atropine
Capsaicin
Capsazepine

Dose-response curves were generated as in Fig. 4B by measuring responses from 10^{-4} – 10^{-10} M of each compound. In antagonist assay the ability of compounds to counteract β-PEA (10 μM) induced BRET signal was evaluated. At least two tests were performed.

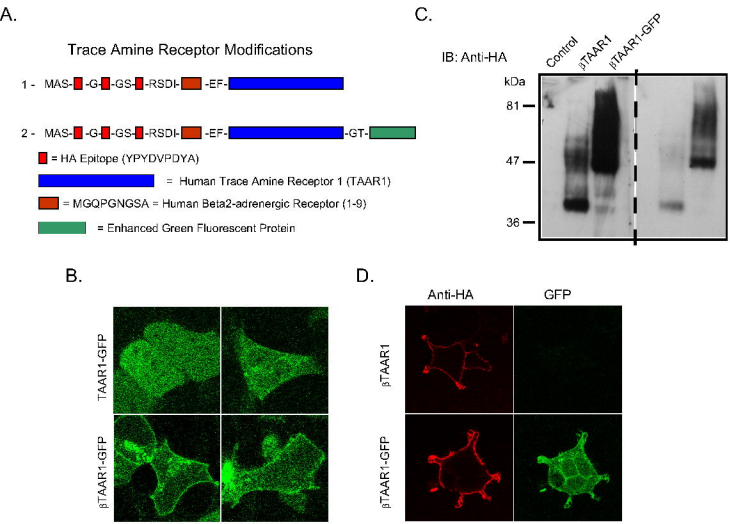
A.
Trace Amine Receptor Modifications
1 - MAS- -G- -GS- -RSDI- -EF-
2 - MAS- -G- -GS- -RSDI- -EF- -GT-
= HA Epitope (YPYDVPDYA)
= Human Trace Amine Receptor 1 (TAAR1)
= MGQPGNGSA = Human Beta2-adrenergic Receptor (1-9)
= Enhanced Green Fluorescent Protein
C.
IB: Anti-HA
Control
βTAAR1
βTAAR1-GFP
kDa
81
47
36
B.
TAAR1-GFP
βTAAR1-GFP
D.
Anti-HA
GFP
βTAAR1
βTAAR1-GFP

Figure 1

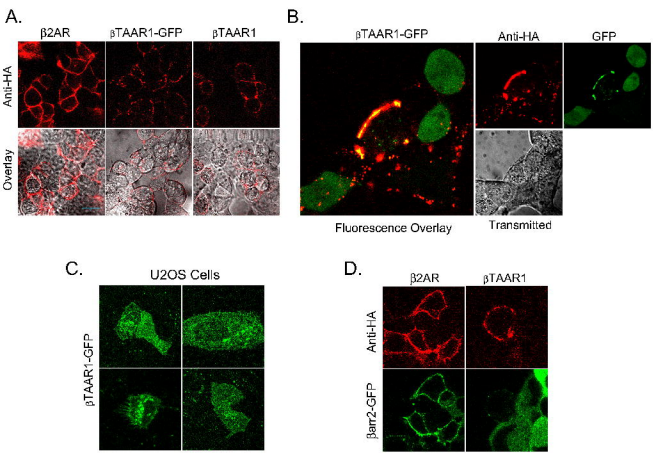
A.
β2AR
βTAAR1-GFP
βTAAR1
Anti-HA
Overlay
B.
βTAAR1-GFP
Anti-HA
GFP
Fluorescence Overlay
Transmitted
C.
U2OS Cells
βTAAR1-GFP
D.
β2AR
βTAAR1
Anti-HA
βarr2-GFP

Figure 2

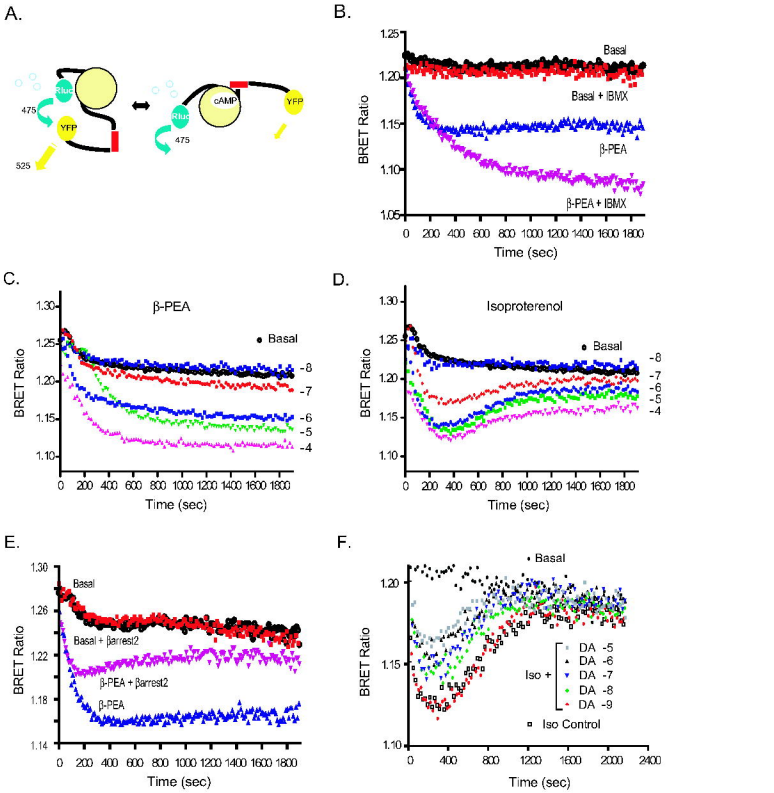
A.
Rluc
475
YFP
525
cAMP
YFP
Rluc
475
B.
BRET Ratio
Time (sec)
Basal
Basal + IBMX
β-PEA
β-PEA + IBMX
C.
β-PEA
Basal
-8
-7
-6
-5
-4
BRET Ratio
Time (sec)
D.
Isoproterenol
Basal
-8
-7
-6
-5
-4
BRET Ratio
Time (sec)
E.
Basal
Basal + βarrest2
β-PEA + βarrest2
β-PEA
BRET Ratio
Time (sec)
F.
Basal
DA -5
DA -6
DA -7
DA -8
DA -9
Iso +
Iso Control
BRET Ratio
Time (sec)

Figure 3

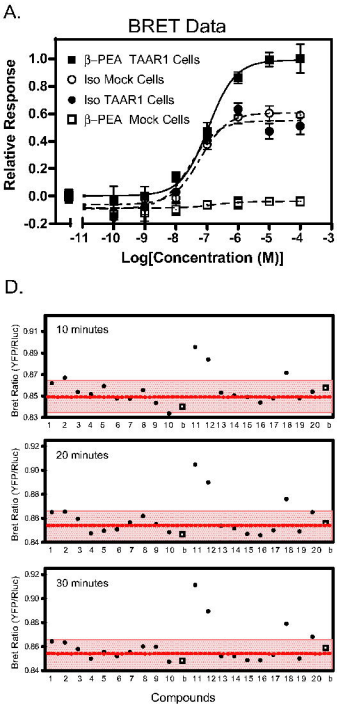
A.
BRET Data
β–PEA TAAR1 Cells
Iso Mock Cells
Iso TAAR1 Cells
β–PEA Mock Cells
Relative Response
Log[Concentration (M)]
B.
BRET Data
β–PEA
Amphetamine
Relative Response
Log[Concentration (M)]
D.
10 minutes
20 minutes
30 minutes
Bret Ratio (YFP/RLuc)
Compounds
C.
Column Data
β–PEA
Amphetamine
[3H]cAMP
Log[Concentration (M)]

Figure 4