Trace Amine-Associated Receptor 1 Displays Species-Dependent Stereoselectivity for Isomers of Methamphetamine, Amphetamine, and Para-Hydroxyamphetamine

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ABSTRACT

The synthetic amines methamphetamine (METH), amphetamine (AMPH), and their metabolite para-hydroxyamphetamine (POHA) are chemically and structurally related to the catecholamine neurotransmitters and a small group of endogenous biogenic amines collectively referred to as the trace amines (TAs). Recently, it was reported that METH, AMPH, POHA, and biogenic amines collectively referred to as the trace amines (TAs) are chemically and structurally related to the catecholamine neurotransmitters and a small group of endogenous biogenic amines collectively referred to as the trace amines (TAs). The trace amines (TAs) octopamine (OCT) and β-phenylethylamine (PEA) are bona fide neurotransmitters that act to modulate metabolism and skeletal muscles (Axelrod and Saavedra, 1977; Roeder, 2005), but the role of TAs in mammalian physiology is unclear. Some evidence suggests that decreased levels of TYR, OCT, and PEA are found in patients with clinical depression, and elevated levels of TYR, OCT, and PEA are found in patients with schizophrenia (Sandler et al., 1979; Davis and Boulton, 1994). In the early 1930s, AMPH was marketed as both a decongestant and a bronchial dilator. However, its ability to promote wakefulness and vigilance was used to treat narcolepsy (Prinzmetal and Bloomberg, 1935). Soon thereafter, a study demonstrated that administration of benzedrine (racemic dl-AMPH) could improve the academic performance of children with behavior disorders (Bradley, 1937). This laid the foundation for the use of psychostimulants in the treatment of attention-deficit hyperactivity disorder (ADHD). Clinically, both AMPH and METH (Anglin et al., 2000) have been used as anorectic agents for controlling obesity.

Methamphetamine (METH) and its congener amphetamine (AMPH) are potent psychostimulants that can lead to abuse and often to addiction. METH, AMPH, and the monoamine neurotransmitters dopamine (DA) and norepinephrine (NE) share a similar chemical structure with the endogenous trace amines (TAs) β-phenylethylamine (PEA) and para-tyramine (TYR). In invertebrates, the TAs octopamine (OCT) and TYR are bona fide neurotransmitters that act to modulate metabolism and skeletal muscles (Axelrod and Saavedra, 1977; Roeder, 2005), but the role of TAs in mammalian physiology is unclear. Some evidence suggests that decreased levels of TYR, OCT, and PEA are found in patients with clinical depression, and elevated levels of PEA exist in patients with schizophrenia (Sandler et al., 1979; Davis and Boulton, 1994).

In the early 1930s, AMPH was marketed as both a decongestant and a bronchial dilator. However, its ability to promote wakefulness and vigilance was used to treat narcolepsy (Prinzmetal and Bloomberg, 1935). Soon thereafter, a study demonstrated that administration of benzedrine (racemic dl-AMPH) could improve the academic performance of children with behavior disorders (Bradley, 1937). This laid the foundation for the use of psychostimulants in the treatment of attention-deficit hyperactivity disorder (ADHD). Clinically, both AMPH and METH (Anglin et al., 2000) have been used as anorectic agents for controlling obesity.

The few medically recognized therapeutic benefits of METH or AMPH consumption are derived from low-dose exposure over time. In contrast, rapidly administered high doses of either drug produce short-lived feelings of intense elation or euphoria, stimulated libido, enhanced self-confi-
ence, heightened motivation, and increased initiative, resulting in a significant potential for abuse. Because of the development of tolerance, long-term users of METH and AMPH can eventually resort to taking gram quantities per day (Derlet et al., 1989). Because of the high bioavailability and low protein-binding characteristics of METH and AMPH (Drummer and Odell, 2001), peak free drug in human blood can reach high-micromolar to low-millimolar concentrations (Logan et al., 1998), often resulting in death from hyperthermia or stroke. Other adverse health consequences include violent mood swings, aggressive behavior, anxiety, confusion, psychotic ideation, paranoia, delusions, hallucinations, and convulsions. In rodents, a single high dose of METH or AMPH is reported to cause neurotoxic effects on DA and 5-hydroxytryptamine (serotonin) (5HT)-producing neurons (Metzger et al., 2000; Jeng et al., 2006). Presently, there is no known antidote for METH or AMPH overdose or pharmacotherapy for dependence. Currently, the molecular mechanisms by which METH and AMPH are thought to alter the distribution of DA, NE, and 5HT levels in the central nervous system (CNS) include interfering with reuptake by monoamine transporters, vesicular storage, and inhibition of deamination by monoamine oxidase (Sulzer et al., 2005; Partilla et al., 2006).

Discovery of the novel rat G protein–coupled receptor (GPCR) (Borowsky et al., 2001; Bunzow et al., 2001), now referred to as rat trace amine-associated receptor 1 (rTAAR1) (Lindemann et al., 2005), and the demonstration that it is functionally activated by PEA and TYR led Bunzow et al. (2001) to test synthetic amines structurally related to PEA and TYR, such as METH, AMPH, and their metabolite para-hydroxyamphetamine (POHA). Human embryonic kidney (HEK) cells stably expressing rTAAR1 and exposed to AMPH or METH responded by elevating their cAMP content in a concentration-dependent and saturable manner. EC_{50}, the effective concentration of an agonist that produces half of the maximal effect, is often sufficient to activate TAAR1s in vitro and raise study unequivocally demonstrate that amphetamines are accumulation to establish the stereoselectivity of all three isomers of TAAR1 for these compounds in terms of potency and efficacy. Although there is as yet no publication documenting in vivo TAAR1 activation, the results of this study unequivocally demonstrate that amphetamines are sufficiently potent to activate TAAR1s in vitro and raise the possibility that TAAR1 may be activated in chronic abusers of amphetamines.

Materials and Methods

Cloning and Plasmids for Rat, Mouse, and Human Chimeric TAAR1. Cloning of the rat TAAR1 was described previously (Bunzow et al., 2001). Because of difficulties associated with stably expressing the wild-type human TAAR1 in a cell line, modifications were made to the hTAAR1 sequence (Lindemann et al., 2005) that replaced short, selected regions with rat TAAR1 sequences. In brief, stretches of nucleotide sequence coding for the N terminus (residues 1–20), C terminus (residues 305–340), and third intracellular loop (residues 204–258) of the human TAAR1 were replaced with the corresponding rat sequences while retaining all of the transmembrane domains from the human TAAR1. In addition, a hemagglutinin virus signal sequence immediately followed by an M1 FLAG epitope was added to the N terminus of this chimeric sequence as described previously (Lindemann et al., 2005). The human-rat TAAR1 chimera (h-rChTAAR1) expression vector was constructed by inserting the chimeric TAAR1 coding sequence into the expression vector pcDNA3.1/V5/His-TOPO (Invitrogen, Carlsbad, CA). The resulting chimera was then stably expressed in HEK-293 cells. A line of HEK-293 cells stably expressing the human-rat chimera receptor was established by picking colonies from cells grown under Genetin (G-418 sulfite; Invitrogen) selection. Plasmids containing the TAAR1 genes for the wild-type rat or mouse were constructed using the same expression vector. The mouse and human TAAR1 were cloned from genomic DNA. The receptor sequences are reported in anti-1-methylxanthine-treated cell suspension. Cells were incubated with 200 μM 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase. KRH buffer (200 μM) to which was added 4 μM of drug compound at 100 × final concentration or forskolin at 10 μM was then combined with 200 μM of the 3-isobutyl-1-methylxanthine-treated cell suspension. Cells were incubated for 1 h at 37°C in a water bath and then lysed by adding 400 μl of 0.5 mM Na^-acetate, briefly vortexed, and incubated at 100°C for 20 min. Cell lysate debris was pelleted by centrifugation, and 200 μl of supernatant was transferred to labeled tubes. To each sample was added 200 μl of ice-cold ETDA followed by 100 μl of H^3]cAMP and 100 μl of cAMP-binding protein (Diagnostic Products, Los Angeles, CA). After brief mixing, tubes were incubated at 0°C for at least 90 min when 500 μl of freshly prepared charcoal dextran was then added to all tubes. These tubes were vortexed and incubated for 10 min at 0°C, centrifuged for another 10 min at 4°C, and then 800 μl of supernatant from each tube was added to vials containing Scinti-Safe 30% liquid scintillation fluid (Fisher Scientific, Fair Lawn, NJ). The tubes were shaken and counted for 5 min. Data were normalized according to protein content, which was determined using a Bradford Protein Assay reagent (Bio-Rad, Hercules, CA). Concentration-response values were calculated using Excel (Microsoft, Redmond, WA). Nonlinear sigmoidal concentration-response curves were fitted to the data and plotted, and EC_{50} values were calculated using Prism software (GraphPad Software Inc., San Diego, CA).

PEA, TYR, or the S-(+)- or R-(−)-enantiomers of AMPH, POHA, or METH were applied to HEK-293 cells stably expressing rTAAR1 and
the induced cAMP level was measured. Identical experiments were conducted using mTAAR1 and h-rChTAAR1. For any given experiment, all cells were harvested from the same tissue culture, and the reactions were carried out using the same freshly made KRH buffer for all drugs used in a given experiment. Experiments were repeated n times (n = 3–8). The average and S.E.M. of the induced cAMP production by test compounds were normalized to the maximal level produced in response to PEA (PEAmax) in the same experiment. At each concentration of applied drug, the average and S.E.M. of cAMP accumulation from n experiments was calculated. These averages and S.E.M. values from multiple experiments were plotted as a function of the concentration of the drug applied, and a nonlinear sigmoidal curve was fit to the averages using GraphPad Prism software. Although the Hill coefficient was allowed to be variable rather than fixed, the fitted coefficients did not differ significantly from 1. The goodness of fit, as measured by $R^2$, was typically 0.98 to 0.99 or greater.

**Flow Cytometry.** HEK-293 cells heterologously and stably expressing the rTAAR1, mTAAR1, h-rChTAAR1, or empty expression vector were grown to 80% confluence and then harvested by gentle scraping into the plate in KRH buffer. The cells were washed three times and resuspended in KRH buffer 1% bovine serum albumin for 30 min at 37°C. The cells were then exposed to different dilutions of the anti-FLAG M1 monoclonal antibody that had been conjugated to the fluorescent dye Alexa 488 (0.2 μg/ml) for 1 h at 4°C. The cells were then washed three times and resuspended in KRH buffer 1% bovine serum albumin. Propidium iodide was added to stain dead cells, which were then gated out when analyzed on a flow cytometer (BD Biosciences, Franklin Lakes, NJ). The live cell fraction was analyzed, and the geometric mean of fluorescence intensity for 10,000 cells, adjusted for controls, was then used to quantify the relative number of TAAR expressed on the cell surface.

**Drug Compounds.** The S (+) - and R (−)-POHA-HBr were kindly provided by the National Institute on Drug Abuse Drug Supply System (Bethesda, MD). S (+) - and R (−)-AMPH-sulfate and S (+) - and R (−)-METH-HCl and all other compounds were purchased from Sigma-Aldrich (St. Louis, MO). Whereas S and R refer to the absolute configurations of the enantiomers, (+) and (−), respectively, denote the clockwise (dextro, d) and counterclockwise (levo, l) rotation of polarized light through a solution of the compound. Drugs were dissolved in water, diluted to 100 μM at 4°C. Before each experiment, drug samples were thawed and vortexed.

**Statistical Analysis.** Calculated values of cAMP production were performed using Excel (Microsoft) based on standard curves generated from known concentrations of cAMP supplied with the kit purchased from Diagnostic Products and processed according to the manufacturer's instructions. Absolute values of cAMP accumulation per milligram of protein for each experiment also were normalized to the maximal level of cAMP produced in response to PEA for that experiment. The mean and S.E.M. for each concentration of all the applied agonists were calculated using Excel. Tables of cAMP mean and S.E.M. values and concentrations of applied agonists were copied into the Prism 4 graphics and statistics software program (GraphPad Software Inc.). The means and S.E.M.s were graphed using Prism, and sigmoidal curves were generated that fit the data. EC$_{50}$ values and 95% confidence levels were calculated. Differences between two treatments were tested for significance using the two-tailed t test. Differences between three treatments were tested by analysis of variance with a Tukey's post-test for significance. Differences were considered significant when *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$.

**Results**

The synthetic psychostimulants METH and AMPH share the phenylethylamine structure with the TAs PEA, TYR, and OCT as well as with the catecholamine neurotransmitters DA, NE, and epinephrine (Fig. 1). The α carbon of METH and AMPH is methylated, thus making possible the existence of two stereoisomers.

Consistent with the observation of Bunzow et al. (2001), G-418-resistant HEK-293 cells transfected with the empty expression vector did not produce cAMP above background levels when treated with PEA, TYR, AMPH, METH, or POHA (data not shown). However, exposing cells stably expressing recombinant rTAAR1 to PEA or TYR over a range of concentrations, from 3.3 nM to 1 mM, resulted in concentration-dependent increases in cAMP accumulation that saturated between 10$^{-5}$ and 10$^{-3}$ M. PEA and TYR also concentration-dependently stimulated cAMP accumulation in cells stably expressing recombinant mTAAR1 or h-rChTAAR1 (Table 1). Plotted together, the concentration-response curves for PEA of all three species of TAAR1 overlap with EC$_{50}$ values between 0.4 and 0.6 μM (Fig. 2A; Table 1). In contrast, each species of TAAR1 displayed a unique TYR concentration-response profile (Fig. 2B; Table 1). TYR was as efficacious (~99% PEA$_{max}$) but more potent (EC$_{50}$ = 80 nM) than PEA (EC$_{50}$ = 380 nM) in stimulating cAMP accumulation by cells stably expressing rTAAR1 (Fig. 2B; Table 2). In cells expressing either the mouse or chimeric TAAR1, TYR was able to achieve ~90% of the maximal effect of PEA (Fig. 2B; Table 2) with EC$_{50}$ values that were 690 nM and 2.26 μM, respectively (Table 1). Although some reports show normalization of concentration-response data to forskolin, a di-

![Fig. 1. Chemical structures of the trace amines β-phenylethylamine, para-tyramine, and octopamine as well as amphetamine compounds S(+)-amphetamine, S(+)-para-OH-amphetamine, and S(+)-R(-)-methamphetamine and the neurotransmitters dopamine, norepinephrine, and epinephrine.](image-url)
TABLE 1
Rank order of average EC$_{50}$ values for cAMP accumulation in HEK-293 cells stably expressing rTAAR1, mTAAR1, or the h-rChTAAR1 and exposed to the indicated compounds

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rat EC$_{50}$ (95% CI)</th>
<th>p Value</th>
<th>Rank Order</th>
<th>Mouse EC$_{50}$ (95% CI)</th>
<th>p Value</th>
<th>Rank Order</th>
<th>Human-Rat Chimera EC$_{50}$ (95% CI)</th>
<th>p Value</th>
<th>Rank Order</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEA</td>
<td>0.38 (0.18, 0.82)</td>
<td>5</td>
<td></td>
<td>0.56 (0.47, 0.67)</td>
<td>3</td>
<td></td>
<td>0.38 (0.31, 0.49)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TYR</td>
<td>0.08 (0.06, 0.12)</td>
<td>2</td>
<td></td>
<td>0.69 (0.54, 0.88)</td>
<td>4</td>
<td></td>
<td>2.26 (1.86, 2.50)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>S(+)-AMPH</td>
<td>0.81 (0.41, 1.64)</td>
<td>6</td>
<td></td>
<td>&lt;0.0001</td>
<td>7</td>
<td></td>
<td>1.12 (0.85, 1.48)</td>
<td>&lt;0.0001</td>
<td>2</td>
</tr>
<tr>
<td>R(-)-AMPH</td>
<td>0.29 (0.18, 0.49)</td>
<td>4</td>
<td></td>
<td>4.96 (4.32, 5.69)</td>
<td>6</td>
<td></td>
<td>3.09 (2.82, 3.37)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>S(+)-METH</td>
<td>0.89 (0.69, 1.14)</td>
<td>7</td>
<td></td>
<td>0.0001</td>
<td>5</td>
<td></td>
<td>4.44 (3.47, 5.69)</td>
<td>0.002</td>
<td>5</td>
</tr>
<tr>
<td>R(-)-METH</td>
<td>1.19 (0.72, 1.96)</td>
<td>8</td>
<td></td>
<td>2.44 (1.77, 3.36)</td>
<td>6</td>
<td></td>
<td>9.83 (6.60, 14.62)</td>
<td>0.002</td>
<td>5</td>
</tr>
<tr>
<td>S(+)-POHA</td>
<td>0.19 (0.06, 0.43)</td>
<td>8</td>
<td></td>
<td>&gt;5.65 not saturated</td>
<td>5</td>
<td></td>
<td>&gt;5.42 not saturated</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>R(-)-POHA</td>
<td>0.06 (0.02, 0.26)</td>
<td>1</td>
<td></td>
<td>&gt;8.54 not saturated</td>
<td>7</td>
<td></td>
<td>&gt;8.54 not saturated</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.A., not applicable.

EC$_{50}$ is calculated from concentration-response curves and is the concentration of the applied compound that results in a response equal to half of the maximal response.

Statistical significance of the difference in EC$_{50}$ values between two isomers is indicated by the p value calculated from a two-tailed t test. p values relate to the comparison of EC$_{50}$ values for enantiomeric pairs.

Many GPCRs, such as opioid receptors, display stereoselectivity for their cognate ligands. Furthermore, some of the behavioral literature indicates that the S(+)-isomer of AMPH is more potent and efficacious than the R(-)-isomer in eliciting locomotor activation in rodents (Angrist et al., 1971) and in producing activation and antidepressant effects in humans (Smith and Davis, 1977). Consequently, it was of interest to determine whether the different species of TAAR1 display preferences for either isomer of METH, AMPH, or POHA.

In agreement with Bunzow et al. (2001), the EC$_{50}$ of the R(-)-isomer of AMPH seemed to be slightly lower and statistically different (p = 0.0152) from the EC$_{50}$ of the S(+)-isomer at stimulating cAMP in cells stably expressing rTAAR1 (Table 1). However, relative to PEA, the S(+)-AMPH isomer was a full agonist, whereas the R(-)-AMPH was a 30% less efficacious partial agonist (Fig. 3A; Table 2).

When HEK-293 cells stably expressing mTAAR1 were exposed to AMPH, both isomers were found to be nearly full agonists (~89% PEA$_{max}$; Table 2) but displayed an order of magnitude difference in potencies (p < 0.0001) as measured by EC$_{50}$; S(+)-AMPH displaying an average EC$_{50}$ of 210 nM compared with 4.96 μM for R(-)-AMPH (Fig. 3B; Table 1).

In cells stably expressing the h-rChTAAR, S(+)-AMPH was not only significantly more potent than R(-)-AMPH (EC$_{50}$ = 1.12 versus 3.09 μM, p < 0.0001) with respect to stimulating the accumulation of cAMP (Fig. 3C; Table 1) but also it was a full agonist (Table 2).

An identical analysis was performed using the S(+)- and R(-)-isomers of METH. When either S(+)-METH or R(-)-METH was applied to cells in culture expressing the recombinant rTAAR1, both isomers concentration-dependently stimulated cAMP production until a maximal accumulation was reached at drug concentrations of 10$^{-4}$ M and higher (Fig. 3D). Both isomers of METH had similar EC$_{50}$ values (~890 nM to 1.19 μM; Table 1) but different efficacies, with S(+)-METH seeming more efficacious than R(-)-METH (~86% PEA$_{max}$ versus ~75%, but with a nonsignificant p value of 0.1191). Plotting the cAMP accumulation data collected from mTAAR1-expressing cells following treatment with either S(+)- or R(-)-METH (Fig. 3E) revealed that, as with AMPH, the S(+)- and R(-)-isomers were equally efficacious full agonists, achieving ~92 and 91% PEA$_{max}$, respectively (Table 2), but that the S(+)-isomer was signifi-

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Fig. 2. Normalized cAMP response to PEA. Data shown are the increases in cAMP production in response to drug treatments compared with the baseline response to treatment by vehicle alone. Data are the percentage of the response at each concentration normalized to the maximal level of cAMP induced by PEA. A, superimposed concentration-dependent responses of rat (●), mouse (▲), and human-rat chimera TAAR1 (●) to PEA. B, concentration-response curves for rat (●), mouse (▲), and human-rat chimera TAAR1 (●)expressing HEK-293 cells for stimulation by TYR. Concentrations of applied drug compounds along the x-axis are given in units of log M, where M is molarity. Cells stably expressing the empty expression vector and selected by G-418 produced no cAMP accumulation in response to drug treatments beyond the endogenous background level.

rect stimulator of adenyl cyclase, we chose to normalize all subsequent drug-response data to PEA$_{max}$ because it acts as a full and equipotent agonist at all three species of heterologously expressed TAAR1s, stimulating cAMP accumulation concentration-dependently and in a saturable manner.
cantly more potent [EC$_{50}$ of S-(-)-METH = 920 nM versus 2.44 μM for R-(-)-METH; $p < 0.0001$; Table 1]. When the cAMP accumulation following incubation with a range of METH concentrations was determined and plotted for cells stably expressing the h-rChTAAR1, both S-(-)-METH and (R)-(-)-METH were full agonists with S-(-)-METH about twice as potent as its R-(-)-enantiomer (Fig. 3F), with an EC$_{50}$ value 4.44 μM compared with 9.83 μM ($p = 0.002$; Table 1). Of the compounds evaluated in this study, both isomers of METH and PEA seemed to be the most efficacious in terms of activating h-rChTAAR1 (Table 2).

The para-hydroxylated form of AMPH (POHA), also known as 4-hydroxyamphetamine, is a major metabolite of both AMPH and METH (Law and Moody, 2000; Kanamori et al., 2005). Produced by the cytochrome P450 detoxification system, POHA is reported to have biological effects of its own (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002) is reported to have biological effects of its own (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002). Following up on the observation reported by Bunzow et al. (2001) that a racemic mix of POHA (Kaminskas et al., 2002).

This display of the data also revealed that the S- (+)-AMPH (Fig. 4A) seemed more potent in stimulating cAMP accumulation in HEK-293 cells expressing mTAAR1 (EC$_{50}$ mTAAR1 = 210 nM) than either tTAAR1- (EC$_{50}$ tTAAR1 = 810 nm) or h-ChrTAAR1-expressing cells (EC$_{50}$ hChrTAAR1 = 1.12 μM), but it seemed less efficacious (≈97% PEA$_{max}$) in producing cAMP than in cells expressing tTAAR1 (-105% PEA$_{max}$). Cells expressing either rodent TAAR1 also seemed more sensitive to the stimulating effects of (+)-METH (Fig. 4B; average EC$_{50}$ rodentTAAR1 of ~905 nM) than cells expressing the h-ChrTAAR1 (EC$_{50}$ hChrTAAR1 = 4.44 μM). Interestingly, although R-(-)-AMPH (Fig. 4D) seemed significantly more potent in stimulating tTAAR1-expressing HEK-293 cells than cells expressing either mTAAR1 or h-ChrTAAR1 (EC$_{50}$ = 290 nm versus 4.96 μM and 3.09 μM, respectively), the difference in efficacy seemed less (≈68% PEA$_{max}$) compared with h-ChrTAAR1 (≈78% PEA$_{max}$) or mTAAR1 (≈91% PEA$_{max}$). R-(-)-METH (Fig. 4E) was least potent at h-rTAAR1 (EC$_{50}$ = 9.83 μM) compared with either rodent TAAR1 (EC$_{50}$ mTAAR1 = 2.44 μM versus EC$_{50}$ h-ChrTAAR1 = 1.19 μM), but it was a full agonist and as efficacious in stimulating h-ChrTAAR1 (≈91% PEA$_{max}$) as mTAAR1 (≈91% PEA$_{max}$) compared with tTAAR1 (≈75% PEA$_{max}$).

Because it has been shown that the level of receptor and G protein expression can influence potency and efficacy, a saturation binding assay would have been useful to experimentally determine the total number of receptors per milligram

### TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%PEA$_{max}$ (95% CI)</th>
<th>$p$ Value*</th>
<th>%PEA$_{max}$ (95% CI)</th>
<th>$p$ Value</th>
<th>%PEA$_{max}$ (95% CI)</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEA</td>
<td>97.88 (86.54, 109.2)</td>
<td></td>
<td>97.29 (94.23, 100.4)</td>
<td></td>
<td>97.44 (93.51, 101.4)</td>
<td></td>
</tr>
<tr>
<td>TYR</td>
<td>95.71 (91.11, 106.3)</td>
<td></td>
<td>90.67 (85.28, 96.07)</td>
<td></td>
<td>87.88 (84.89, 90.87)</td>
<td></td>
</tr>
<tr>
<td>S- (+)-AMPH</td>
<td>105.5 (93.57, 117.3)</td>
<td>0.0001</td>
<td>89.35 (85.92, 92.79)</td>
<td>0.9890</td>
<td>90.95 (85.92, 95.98)</td>
<td>0.2937</td>
</tr>
<tr>
<td>R-(-)-AMPH</td>
<td>68.36 (62.8, 73.93)</td>
<td>***</td>
<td>90.58 (87.68, 93.47)</td>
<td></td>
<td>77.72 (76.11, 79.34)</td>
<td></td>
</tr>
<tr>
<td>S- (+)-METH</td>
<td>86.20 (81.99, 90.41)</td>
<td>0.1191</td>
<td>91.76 (87.48, 96.03)</td>
<td>0.9751</td>
<td>96.71 (91.16, 102.3)</td>
<td>0.2034</td>
</tr>
<tr>
<td>R(-)-METH</td>
<td>75.05 (68.3, 82.27)</td>
<td></td>
<td>91.33 (85.42, 97.23)</td>
<td></td>
<td>91.55 (83.25, 99.84)</td>
<td></td>
</tr>
<tr>
<td>S- (+)-POHA</td>
<td>119 (94.12, 143.8)</td>
<td>0.0251</td>
<td>87.42 (81.93, 92.90)</td>
<td>N.A.</td>
<td>76.01 Not saturated</td>
<td></td>
</tr>
<tr>
<td>R-(-)-POHA</td>
<td>91.04 (70.73, 111.3)</td>
<td>*</td>
<td>74.79 Not saturated</td>
<td>43.20</td>
<td>Not saturated</td>
<td></td>
</tr>
</tbody>
</table>

N.A., not applicable.

* $p$ values relate to the comparison of values for enantiomeric pairs. Statistical significance is indicated as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. 

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**Fig. 3**

- **G**: HEK-293 cells responded to both isoforms of POHA (S- (+)-POHA, R-(-)-POHA) (Fig. 3G); however, availability of both POHA enantiomers, rTAAR1-expressing rodent TAAR1s responded to low nanomolar concentrations (EC$_{50}$ = 190 nM; EC$_{50}$ mTAAR1 = 280 nM) of the S- (+)-isomer of POHA. This isomer also exhibited full agonism at rTAAR1 (~119% PEA$_{max}$ compared with mTAAR1 (~87% PEA$_{max}$), as noted previously (Figs. 3G and 4C). In contrast, cells expressing the h-rChTAAR1 only responded at much higher concentrations of S- (+)-POHA. Interestingly, cells expressing mTAAR1 seemed relatively insensitive to R-(-)-POHA, responding in a manner similar to cells harboring h-ChrTAAR1. In contrast, cells expressing tTAAR1 responded to R-(-)-POHA by achieving ~91% PEA$_{max}$ with the lowest EC$_{50}$ of any compound studied (EC$_{50}$ rodentTAAR1 = 60 nM).

The data presented in Fig. 3 were replotted so that the accumulation of cAMP could be easily viewed as a function of isomer and species of TAAR1 (Fig. 4). From this representation of the data, several observations can be made. First, in general, the three receptors responded in similar ways to the S- (+)- and R-(-)-isomers of all compounds tested with the exception of POHA (Fig. 4, C and F). Over the limited range of POHA concentrations evaluated, each species of TAAR1 responded in a unique and characteristic way. Cells that expressed either of the rodent TAAR1s responded to low nanomolar concentrations (EC$_{50}$ tTAAR1 = 190 nM; EC$_{50}$ mTAAR1 = 280 nM) of the S- (+)-isomer of POHA. This isomer also exhibited full agonism at rTAAR1 (~119% PEA$_{max}$ compared with mTAAR1 (~87% PEA$_{max}$), as noted previously (Figs. 3G and 4C). In contrast, cells expressing the h-rChTAAR1 only responded at much higher concentrations of S- (+)-POHA. Interestingly, cells expressing mTAAR1 seemed relatively insensitive to R-(-)-POHA, responding in a manner similar to cells harboring h-ChrTAAR1. In contrast, cells expressing tTAAR1 responded to R-(-)-POHA by achieving ~91% PEA$_{max}$ with the lowest EC$_{50}$ of any compound studied (EC$_{50}$ rodentTAAR1 = 60 nM).
of protein per cell line. Unfortunately, a viable binding assay is not currently available due to the lack of a labeled ligand with high affinity for the TAAR1 receptor and low nonspecific binding. Consequently, the relative amounts of cell surface TAAR1 protein expression in the three different cell lines were estimated by flow cytometry. Fluorescence measurements were taken of samples containing HEK-293 cells that expressed vector alone or epitope-tagged and fluorescent anti-FLAG M1 antibody-labeled mTAAR1, rTAAR1, or h-rChTAAR1. Analysis of the fluorescence corresponding to rTAAR1 and mTAAR1 normalized to the fluorescence of h-rChTAAR1, which had the least number of receptors, gave relative cell surface receptor expression levels of approximately 2.8:1.25:1 (data not shown).

Discussion

Here, we have characterized the potency and efficacy of AMPH, METH, and POHA to stimulate the accumulation of cAMP in HEK-293 cells stably expressing recombinant TAAR1 sequences cloned from mouse and human sources, in addition to the previously described rTAAR1 (Bunzow et al., 2001). The chimeric receptor h-rChTAAR1 was engineered to

In this study, we normalized cAMP results to the maximal cAMP level produced in response to PEA, a convention also adopted by Wainscott et al. (2007), because PEA is a full agonist for each species of TAAR1. The normalized concentration-response curves for PEA for each species were virtually coincident across all the applied concentrations. However, receptor density and G protein abundance can both affect apparent agonist potency and efficacy in an in vitro assay (Kenakin and Morgan, 1989). Using a mathematical model, it was shown that increasing concentrations of receptors cause an increase in both potency and efficacy (Kenakin and Morgan, 1989). Consequently, caution should be exercised whenever comparing results from two or more cell lines heterologously expressing different species of GPCR, that in all probability have different receptor densities, as in the present study where flow cytometry revealed that the rTAAR1-expressing HEK-293 cells expressed on average two to three times as many receptors on their surface as either the mTAAR1 or h-rChTAAR1.

With the above-mentioned caveat in mind, our results demonstrated a species-specific response to the compounds tested. The TAs PEA and TYR concentration-dependently stimulated each species of TAAR1 stably and heterologously expressed in HEK-293 cells (Fig. 2). In cells expressing the h-rChTAAR1 chimera, PEA had the lowest rank order of potency and was a full agonist, which suggests that it could be an endogenous TAAR1 ligand in humans.

More than 30 years ago, it was shown that the physiological and behavioral responses of an animal to S- (+)-isomers of AMPH and METH are more potent and efficacious than their optical antipodes at inducing motor hyperactivity (Angrist et al., 1971; Segal, 1975). Although both isomers were full agonists at mTAAR1 and h-rChTAAR1 (Table 2), the potencies of the S- (+)-isomers of METH and AMPH were significantly greater than the potencies of the R- (+)-isomers (Table 1). In cells expressing rTAAR1 both enantiomers of METH were approximately equipotent but only partial agonists (Fig. 3).

The stereoselectivity of mTAAR1 was especially apparent for the isomers of POHA. S- (+)-POHA was apparently the more potent of the two isomers (Fig. 3H), given that the R- (+)-POHA response did not reach a saturated maximum. For rTAAR1-expressing cells, the R- (+)-isomers of AMPH and POHA showed significantly (p = 0.0001 and 0.025, respectively) lower efficacy than the S- (+) forms in terms of stimulating cAMP accumulation (Fig. 3, A and G). At the concentrations tested, the h-rChTAAR1 was less responsive to POHA than either of the rodent receptors.

The physiological and behavioral effects of AMPH and METH are generally accepted to be mediated by their actions as substrates for the dopamine, norepinephrine, and vesicular monoamine transporters. The main mechanism for the
termination of monoamine neurotransmitter signaling is by reuptake of DA, NE, and 5HT via the presynaptic plasma membrane dopamine transporter DAT, norepinephrine transporter, and serotonin transporter. Once in the cytoplasm, these neurotransmitters are either sequestered for reuse in storage vesicles via the vesicular monoamine transporter 2 or are inactivated by deamination by monoamine oxidase. This reuptake process is interfered with by compounds such as AMPH and METH that act as "substrate-type releasers" (Rothman and Baumann, 2006), which promote neurotransmitter release from synaptic storage vesicles into the cytoplasm and reverse the direction of neurotransmitter flow through the transporter (Partilla et al., 2006).

Pharmacological data characterizing the inhibition of monoamine uptake and increased neurotransmitter release by various AMPHs was reported by Rothman et al. (2001). The EC\textsubscript{50} of (+)-AMPH to release DA via DAT is 25 nM, with \(K_i\)\textsuperscript{uptake} values in rat synaptosomes for this neurotransmitter of 34 nM at the DAT, concentrations approximately 20- to 30-fold lower than the EC\textsubscript{50} values we calculated for eliciting an in vitro functional response from rTAAR1 (0.8 \(\mu\)M).

Chronic METH abusers can typically consume gram quantities of drug per day (Kramer et al., 1967). Given its high bioavailability, low protein binding, and long half-life, plasma concentrations of both drugs can reach into the high-micromolar range (Drummer and Odell, 2001; Baselt, 2002; Peters et al., 2003). Although the extracellular free concentration of METH around relevant human dopaminergic synapses presumably involved in producing desirable CNS effects is not known with certainty, in rats METH serum levels are typically 1/10 what is found in brain (Riviere et al., 2000).

Forensic evidence indicates that experienced METH users can typically achieve peak blood concentrations of 100 \(\mu\)M (Baselt, 2002; Peters et al., 2003). Both isomers of METH were full agonists of h-rChTAAR1 over a range of EC\textsubscript{50} values from 3.5 to \(~\)15 \(\mu\)M, concentrations often exceeded in the blood of human METH addicts (Derlet et al., 1989). If TAAR1s, whether expressed in the CNS or periphery, are exposed to such concentrations, it is possible they could become functionally activated.

The existence of receptors specific for TAs has revived interest in the possibility that the TAAR gene family could contribute importantly to human mental health, including an individual's response to the psychostimulants METH and AMPH. However, in spite of considerable evidence collected over the years that is consistent with the involvement of TAs in the etiology of several adverse human health conditions, including hypertension (Borowsky et al., 2001), migraine headache (D'Andrea et al., 2006), schizophrenia (Yoshimoto et al., 1987), ADHD (Madras et al., 2005), depression (Carter et al., 1980), bipolar disorder (Abou Jamra et al., 2005), and stress (Paulos and Tessel, 1982), the biological functions of TAs in vertebrates have been difficult to demonstrate with any certainty. This is due in large measure to the difficulties associated with routinely achieving heterologous stable expression of functionally coupled members of this receptor family in tissue culture. A recent report using transient expression claims to have overcome this impediment (Liberles and Buck, 2006) and suggests that several members of the mTAAR receptor family, but not TAAR1, are expressed in mouse nasal epithelia where they respond to volatile amines present in mouse urine that may serve as social cues. Studies involving TAAR1 knockout mice could aid efforts to identify physiological and behavioral effects of METH and AMPH that are TAAR1-mediated. The EC\textsubscript{50} values calculated for the compounds examined in the present study (Table 1) are similar to those reported previously (Bunzow et al., 2001; Lindemann et al., 2005; Miller et al., 2005; Lewin, 2006; Wainscott et al., 2007). For cells expressing rodent TAAR1s, both TYR and PEA were potent and full agonists, supporting the interpretation that either one of these agonists could be an endogenous TAAR1 ligand.

The present study also revealed that different chemical and structural features of AMPH, METH, and POHA are important depending on the species of TAAR1. The para-hydroxyl group on the benzene ring is a functional group that is important for activating rTAAR1, because compounds that contain it, TYR and POHA, displayed the highest potency and efficacy of the compounds tested (Tables 1 and 2). For POHA, rTAAR1 had a strong concentration-response to both isomers, whereas for h-rChTAAR1, both isomers were weak stimulants, results similar to recent findings by Wainscott et al. (2007) who reported higher potency for para-substituted compounds at rTAAR1 compared with the hTAAR1. At mTAAR1, the orientation of the \(\alpha\)-methyl group of the ligand emerged as the most important structural feature of AMPH, METH, and POHA with respect to potency but was of no consequence in terms of efficacy. Future investigations involving site-directed mutagenesis of the putative ligand binding pockets of these receptors should help clarify the structural basis for their stereoselectivities.

In summary, we have established that AMPH, METH, and POHA can be potent and efficacious agonists of TAAR1s heterologously expressed in vitro and that they display concentration-dependent and species-dependent stereospecific pharmacological profiles. Given that the EC\textsubscript{50} values for METH and AMPH activation of the h-rChTAAR1 in vitro are well below the concentrations frequently found in addicts, we suggest that TAAR1 might be a potential mediator of some of the effects of AMPH and METH in humans, including hyperthermia and stroke. In addition, the possibility should be explored that genetic variants in hTAAR1, or other TAAR genes, may be important to several conditions thought to involve TAs, including ADHD, depression, mania, psychosis, and addiction. If TAAR1 is confirmed as a target of METH and AMPH in vivo, then developing novel TAAR1-selective agonists and antagonists could ultimately lead to successful pharmacotherapies for METH and AMPH addiction and overdose as well as contribute to a better understanding of the etiology of several mental disorders.

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