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Cloning, Expression and Functional Analysis of Rhesus Monkey Trace Amine-Associated Receptor 6: Evidence for Lack of Monoaminergic Association

Zhihua Xie¹, Eric J. Vallender¹, Naichen Yu², Shelli L. Kirstein³, Hong Yang¹, Mary E. Bahn¹, Susan V. Westmoreland⁴, and Gregory M. Miller^{1,*}

¹ Division of Neurochemistry, New England Primate Research Center, Harvard Medical School, Southborough, Massachusetts, USA

² ACEA Biosciences Inc., 6779 Mesa Ridge Road, Suite 100, San Diego, California, USA

³ EPIX Pharmaceuticals, 4 Maguire Road, Lexington, Massachusetts, USA

⁴ Division of Comparative Pathology, New England Primate Research Center, Harvard Medical School, Southborough, Massachusetts, USA

Abstract

Several recent studies report an association between trace amine-associated receptor 6 (TAAR6) and susceptibility to schizophrenia and bipolar affective disorder (BPAD) in humans. However, endogenous TAAR6 agonists and the receptor signaling profile and brain distribution remain unclear. Here, we clone TAAR6 from the rhesus monkey and use transfected cells to investigate whether this receptor interacts with brain monoamines and a psychostimulant drug to trigger cAMP signaling or ERK phosphorylation, while investigating its expression profile in the rhesus monkey brain. Unlike TAAR1, rhesus monkey TAAR6 did not alter cAMP levels in response to 10 μ M of monoamines (dopamine, norepinephrine, serotonin, β -PEA, octopamine, tryptamine and tyramine) or methamphetamine in stably transfected cells in vitro. RT-CES analysis indicated that the receptor did not alter cell impedance or change the effect of forskolin on cell impedance at exposure to 20 μ M of each monoamine, suggesting a lack of either Gs or Gi-linked signaling. Whereas κ opioid receptor activation led to ERK phosphorylation at exposure to 1 μ M U69593, rhesus monkey TAAR6 had no such effect at exposure to 10 μ M of monoamines or methamphetamine. Membrane and cell surface localization of TAAR6 was confirmed using immunocytochemistry, biotinylation and Western blotting with a TAAR6 antibody in the transfected cells. Real time RT-PCR amplification showed that TAAR6 mRNA was undetectable in selected rhesus monkey brain regions. Together, the data reveal that TAAR6 is unresponsive to brain monoamines and is not expressed in rhesus monkey brain monoaminergic nuclei, suggesting TAAR6 lacks direct association with brain monoaminergic neuronal function.

Keywords

common biogenic amines; trace amines; methamphetamine; schizophrenia; bipolar disorder

*Correspondence to Gregory M. Miller, New England Primate Research Center, Harvard Medical School, One Pine Hill Drive, Southborough, MA 01772, Tel: (508) 624-8023, Fax: (508)786-3317, email: gmiller@hms.harvard.edu.

INTRODUCTION

Trace amine-associated receptors (TAARs), a structurally and functionally distinct subfamily of novel G protein-coupled receptors, have been cloned from various mammalian species (Borowsky et al., 2001; Bunzow et al., 2001; Miller et al., 2005). Human TAAR genes, including six functional and three pseudo genes are located in tandem in the chromosomal area of 6q23.2 adjacent to regions in which susceptibility loci have been identified for schizophrenia, bipolar affective disorder (BPAD) and other neuropsychiatric disorders/diseases (Kohn, 2005; Levi et al., 2005). Among TAARs, trace amine-associated receptor 1 (TAAR1) has been extensively investigated with respect to its potential agonists, cellular expression, signaling cascades, brain regional distribution, and modulatory function in monoaminergic systems (Borowsky et al., 2001; Bunzow et al., 2001; Miller et al., 2005; Hart et al., 2006; Grandy, 2007; Lindemann et al., 2007; Reese et al., 2007; Wainscott et al., 2007; Xie and Miller, 2007; Xie et al., 2007b; Xie and Miller, 2008; Xie et al., 2008), however, the functionality of other members of this receptor family remain elusive. Several recent studies reported that the TAAR6 (also referred to as TA4 or TRAR4) locus is potentially associated with susceptibility to schizophrenia and bipolar affective disorder (Duan et al., 2004; Abou Jamra et al., 2005; Vladimirov et al., 2007; Pae et al., 2008). However, the endogenous or exogenous agonists or antagonists for TAAR6 and the receptor signaling pathway are unidentified. So far, it remains largely unknown whether TAAR6 is expressed in brain and whether this receptor is functionally related with brain monoaminergic systems.

Brain monoamines mainly include common biogenic amines and trace amines. Common biogenic amines, including dopamine, norepinephrine and serotonin, are well established as neurotransmitters in brain monoaminergic systems. They are synthesized and packaged in monoaminergic neurons and released into synaptic clefts to interact with presynaptic and postsynaptic receptors, exerting important effects on locomotor activity, motivation and reward, cognition and emotion, and neuroendocrine activity (Giros et al., 1996; Schultz, 2002; Wise, 2004). Trace amines are heterogeneously distributed in mammalian brain tissues, their distribution spatially parallels the origins and terminal projection areas of the monoaminergic neurons, and they are synthesized, packaged and released along with the common biogenic amines (Durden et al., 1973; Boulton, 1976; Philips et al., 1978). Aberrant levels of monoamines in brain caused by inappropriate transporter function or metabolic handicaps are associated with a variety of neuropsychiatric disorders/diseases such as anxiety, depression, Parkinson's disease, schizophrenia, suicide and drug abuse/addiction (Klimek et al., 1997; Arango et al., 2002; Schmitt et al., 2006; Serretti et al., 2006), suggesting the functional importance of brain monoamines in the pathology of these disorders. We have previously shown that rhesus monkey TAAR1 has a wide agonist spectrum which includes both common biogenic amines and trace amines as well as amphetamine-like drugs and is consequently involved in brain monoaminergic regulation (Xie et al., 2007b; Xie and Miller, 2007; Xie and Miller, 2008; Xie et al., 2008). These findings along with the reported potential association of TAAR6 with neuropsychiatric disorders led us to explore whether TAAR6 is similar to TAAR1 in association with brain monoaminergic systems.

In this study, we cloned TAAR6 from rhesus monkey genomic DNA and introduced it into HEK293 cells to evaluate the response of the receptor to monoamines and methamphetamine. To determine whether TAAR6 interacts with these compounds to stimulate cAMP signaling, we employed CRE-Luc assays to measure cAMP variation in the transfected cells following drug challenge. RT-CES analysis was used to further assess whether TAAR6 is linked with either Gs or Gi protein. To investigate whether TAAR6 interacts with monoamines or methamphetamine to trigger ERK phosphorylation, we

evaluated the influence of TAAR6 on ERK phosphorylation-driven luciferase expression. To determine whether TAAR6 was associated with cellular membranes or present on the cell surface plasma membrane of the transfected cells, we performed immunocytochemistry, biotinylation and Western blotting using a TAAR6 antibody. To examine TAAR6 mRNA expression in rhesus monkey brain, we used real time RT-PCR to amplify cDNAs prepared from selected brain regions. The data indicate that similar to rhesus monkey TAAR1, the cloned rhesus monkey TAAR6 has high sequence identity to the human ortholog. But unlike rhesus monkey TAAR1, TAAR6 lacks a direct association with brain monoaminergic neuronal function.

MATERIALS AND METHODS

Materials

Dopamine, norepinephrine, serotonin, β -phenylethylamine, octopamine, tryptamine, tyramine, (+)-methamphetamine, radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor cocktail were purchased from Sigma Aldrich (St. Louis, MO). Water-soluble forskolin was obtained from Tocris Cookson (Ellisville, Mo). Dulbecco's Modified Eagle's Medium (DMEM), 100 $\times$ non-essential amino acids, fetal bovine serum, 100 $\times$ penicillin/streptomycin, geneticin and trypsin/EDTA were obtained from Invitrogen (Carlsbad, CA). Dual-Luciferase[®] Reporter Assay System kit, pGL4.73, RQ1 RNase-free DNase and Passive Lysis Buffer (5 $\times$) were from Promega (Madison, WI). Translucent[™] in vivo Kinase Assay Kit was from Panomics (Redwood, CA). Rabbit anti-human trace amine-associated receptor 1 and rabbit anti-human trace amine-associated receptor 6 antibodies were from ABR Affinity BioReagents (Golden, CO), both of which are targeted against extracellular loop3. Goat anti-rabbit IgG (H+L) was from Chemicon International (Temecula, CA). SuperSignal West Pico Chemiluminescent Substrate was from Pierce (Rockford, IL). RNAlater was from Ambion (Austin, TX). Taqman[®] Master kits and human ProbeLibrary probes were from Roche Diagnostics Corporation (Indianapolis, IN).

Rhesus Monkey Genomic DNA Isolation

Venous blood samples (10 ml) were collected from three rhesus monkeys in Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ) containing EDTA and stored at -80°C . Genomic DNA was isolated from 400 μl of whole blood using a Generation Capture Column Kit (Gentra Systems, Minneapolis, MN). Eluted DNA was then ultrapurified using the Wizard DNA Cleanup system (Promega, Madison, WI).

PCR Cloning

As described for TAAR1 (Miller et al., 2005), the rhesus monkey TAAR6 single exon gene was directly amplified from purified genomic DNA (gDNA). Because this project was initiated prior to publication of the rhesus genome, oligonucleotide primer sets that anneal outside of the TAAR6 coding region were designed based on the human TAAR6 gene (AL513524) using Oligo Primer Analysis Software (Molecular Biology Insights, Cascade, CO), and then custom synthesized by AlphaDNA (Montreal, QC, Canada). One set of primers that resulted in a PCR product of the expected size was selected for cloning: TAAR6-f, 5'-TACAGCGGTGCTGTGTTCTAC-3' and TAAR6-r, 5'-CCTATACAGTTCAGGGCATCAGA-3'. All amplification reactions were carried out in a total volume of 30 μL solution containing 1 μL of Elongase Enzyme Mix (Invitrogen, Carlsbad, CA), 6 μL of 5 $\times$ buffer B (300 mM Tris-SO₄, 10 mM MgSO₄, 90 mM (NH₄)₂SO₄, pH 9.1), 1 μL of 40 mM dNTPs, 10 pmol of each oligonucleotide primer, and 50–100 ng of gDNA template. The reaction conditions were 94 $^{\circ}\text{C}$, 1 min for one cycle; 94 $^{\circ}\text{C}$, 12 s then 54 $^{\circ}\text{C}$, 30 s then 72 $^{\circ}\text{C}$, 3 min for 35 cycles; and 72 $^{\circ}\text{C}$, 10 min for one cycle. PCR products were run on 1% agarose gels containing ethidium bromide. A single band was

excised from the gel on a DarkReader transilluminator (Clare Chemical Research, Denver, CO), and the DNA was purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). Isolated DNA was cloned into pcDNA3.1/V5/His-TOPO (Invitrogen) according to the manufacturer's protocol and transformed into bacteria for plasmid DNA preparation using either a Promega miniprep kit or QIAGEN maxiprep kit.

DNA Sequencing

Plasmid DNA prepared from selected clones was subjected to sequencing for determination of TAAR6 sequence using a CEQ8000 Genetic Analysis System and CEQ DTSC Quick Start Kit (Beckman Coulter, Fullerton, CA). The selected clone had 100% sequence identity with other clones derived from three different rhesus monkeys.

Protein Prediction

TAAR6 protein primary structure was determined from TAAR6 gene sequence using internet-based programs for DNA translation (<http://www.bioinformatics.org/sms/index.html>). TMpred Server (http://www.ch.embnet.org/software/TMPRED_form.html) was employed to determine transmembrane regions of TAAR6 protein. NetPhos 2.0 Server (<http://www.cbs.dtu.dk/services/NetPhos/>) was used to predict potential phosphorylation sites in TAAR6 protein. PhosphoBase Version 2.0 (<http://www.cbs.dtu.dk/databases/PhosphoBase>) was used to predict potential kinases that may phosphorylate the selected residues.

Cell Culture and Transfection

Cells were grown in DMEM supplemented with FBS (10%), penicillin (100 U/ml), streptomycin 100 µg/ml and non essential amino acids (0.1 mM) at 5% CO₂ and 37°C, and geneticin (G418) was used for selection and maintenance of stable cell lines. Calcium phosphate transfection was performed as described elsewhere (Xie et al., 2007a and 2007b) to introduce the necessary receptors and transporters into different cells for assays. For co-transfection, the ratio of constructs and amount of total DNA was held constant with pcDNA3.1.

Cell Compartmental Protein Extraction and Surface Protein Isolation

Cells were placed in the 145 mm plates, grown to 90 ~ 95% confluence, and collected through treatment with 0.05% trypsin/EDTA. Following rinse with ice-cold 1× phosphate buffered saline (PBS, pH 7.4), the cells were used to fractionally extract compartmental proteins using Qiagen Qproteome Cell Compartment Kit (Valencia, CA), according to the procedures provided by the manufacturer. Briefly, washed cells (1×10^7) were resuspended in 1 ml ice-cold Extraction Buffer CE1 to prepare cytosolic proteins, centrifuged and then resuspended in 1 ml Extraction Buffer CE2 to prepare membrane proteins, then in Extraction Buffer CE3 to obtain nuclear proteins, and finally in Extraction Buffer CE4 to get cytoskeletal proteins. All buffers were supplemented with protease inhibitor solution. Pierce Cell Surface Protein Isolation Kit (Rockford, IL) was used to isolate cell surface proteins according to the manufacturer's procedures. Briefly, washed cells (1×10^7) were incubated with 10 ml biotin solution for 30 min at 4°C on rocking platform, and then harvested by centrifugation for 3 min at 500g. The cell pellet was rinsed with ice-cold 1× PBS, mixed with 500 µl of the provided lysis buffer, and homogenized with a motor-driven pellet pestle for 30 up-and-down strokes. The homogenate was incubated on ice for 30 min, and then centrifuged at 10,000g for 2 min at 4°C to obtain the supernatant. The supernatant was incubated with Immobilized NeutrAvidin Gel which was loaded into the provided column for 60 min at room temperature. Following centrifugation (1 min, 1,000g) and rinse with

provided wash buffer which was supplemented with protease inhibitor solution, the gel-trapped proteins were eluted by incubation with 400 μ l DTT-containing SDS-PAGE sample buffer for 60 min at room temperature and centrifugation for 2 min at 1,000g, and then subjected to further analysis by Western blotting. The total proteins in the cells were isolated using Qiagen Mammalian Protein Preparation Kit according to the provided procedures. Briefly, washed cells (1×10^7) were incubated with 1 ml of the provided mammalian lysis buffer for 5 min at 4°C. The lysis buffer was complemented with nuclease and protease inhibitors before use. The lysate was subjected to centrifugation at 12,000g for 10 min at 4°C, and the supernatant was collected for analysis.

SDS-PAGE and Western Blotting

Detailed procedures are described elsewhere (Xie et al., 2005a; Xie et al., 2007a). Cells were grown to 90 ~ 95% confluence and collected through treatment with 0.05% trypsin/EDTA. The cells were disrupted through violent shaking in ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) at 10^7 cells/ml, and then incubated for 30 min at 4°C. The RIPA buffer was supplemented with 1 $\times$ protease inhibitor cocktail. The cell lysates were centrifuged at 12,000g for 10 min at 4°C. The supernatants were collected, mixed with Bio-Rad Laemmli sample buffer at 1:2 (v/v), heated at 95°C for 5 min, and centrifuged at 12,000g for 5 min at 4°C. The subsequent supernatants were subjected to SDS-PAGE (10% acrylamide separating gel, 4% acrylamide stacking gel), and the proteins were electrotranslocated onto a polyvinylidene difluoride (PVDF) membrane (0.45 μ m) presoaked in 100% methanol for 10 min. The membrane was then blocked with blocking buffer (10% nonfat milk, 10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) and incubated with the receptor antibody at 1:1,000 overnight at 4°C, and goat anti-rabbit IgG (H+L) at 1:5,000 for 2 h at room temperature, in blocking buffer. SuperSignal West Pico Chemiluminescent Substrate was used to visualize the blots under a luminescent image analyzer (LAS-1000; Fujifilm, Tokyo, Japan).

Immunocytochemical Staining

HEK293 cells stably expressing TAAR6 were placed in 8-well Lab-Tek II chamber slides coated with human fibronectin from BD Biosciences (Bedford, MA) at a density of 2,000 cells/well in 400 μ l growth medium, and incubated at 5% CO₂ and 37°C for 18 h. Following removal of the medium, the cells were washed once with 1 $\times$ PBS (pH 7.4) containing calcium and magnesium. The washed cells were fixed with 200 μ l of 10% neutral buffered formalin for 10 min, washed in 1 $\times$ calcium- and magnesium-free PBS (pH 7.4) for 5 min, and then permeabilized in 1 $\times$ calcium- and magnesium-free PBS (pH 7.4) containing 0.1% Triton X-100 for 5 min, at room temperature. The permeabilized cells were blocked in DAKO's (Carpinteria, CA) serum-free protein block complemented with 10% normal goat serum from Vector Laboratory (Burlingame, CA) for 30 min, and then incubated with TAAR6 antibody (1.1 mg/ml, at a 1:500 dilution) or DAKO's rabbit immunoglobulin fraction (15 mg/ml, at a 1: 6818 dilution as negative control) in DAKO's antibody diluent for 1 h at room temperature. The cells were washed in 0.1% TTBS (Tris buffered saline with tween 20) twice for 5 min, then incubated with Vector's biotinylated secondary antibody (goat anti-rabbit) at a 1:200 dilution for 30 min, and finally incubated with Vector's Elite ABC reagent for 30 min at room temperature. Cells were visualized with DAKO's liquid DAB⁺ (chromagen diaminobenzidine) and counterstained with Mayer's hematoxylin. Following counterstaining and dehydration through alcohols, the cells were cleared with xylene and mounted with Permount mounting media. For membrane staining, the cells growing in the chamber slides were quickly washed once with 1 $\times$ PBS (pH 7.4) containing calcium and magnesium, and then incubated with wheat germ agglutinin, Alexa Fluor® 555 conjugate (Invitrogen, Carlsbad, CA) at a concentration of 1 μ g/ml for 5 min. After a quick wash with

1× calcium-and magnesium-free PBS (pH 7.4), the cells were fixed for 10 min in neutral buffered formalin. Following fixation, the cells were washed and mounted with Vector's Vectashield hardset mounting media. Fluorescent membrane stain images were obtained using an Olympus IX51 fluorescent microscope and an Olympus DP70 camera (Tokyo, Japan). TAAR6 stain and negative control images were obtained with an Olympus BX45 microscope and an Olympus DP12 camera.

Dual Luciferase Reporter Assay

The detailed procedures are described elsewhere (Xie and Miller, 2007; Xie et al., 2007b). Briefly, cells were placed in 48-well plates (75,000 cells/well in 0.5 ml DMEM). At 60–70% cell confluence, the luciferase reporter construct CRE-Luc (cAMP sensitive) or pTL-ELK-1 (an ERK specific trans-activation domain expression vector) and pTL-Luc (a phosphorylation-dependent luciferase expression vector), plus a reporter control construct pGL4.73 (cAMP insensitive) were introduced into the cells along with rhesus monkey TAAR1 or TAAR6. Following 12 h incubation under transfection condition, the cells were exposed to vehicle and the indicated chemicals in serum-free DMEM for 18 h. Passive lysis buffer (PLB) and luciferase assay substrate reagents were prepared according to manufacturer's protocol (Promega, Madison WI). Cell lysates were prepared by adding 100 µl of 1× PLB into each well to break the cells and shaking the plate on a platform at 25°C for 30 min. The lysate (20 µl) from each well was transferred into wells of opaque 96-well microplates (PerkinElmer, Shelton, CT). Luciferase substrate reagents (50 µl) were injected into each well, and after a 2 sec delay, luciferase levels were measured as RLU for 12 sec on a Wallac 1420 multilabel counter, Victor 3V (PerkinElmer, Shelton, CT). Relative Light Units (RLUs) represent the luciferase level (firefly and renilla). The average ratio of firefly luciferase RLU to renilla luciferase RLU was calculated for each triplicate and then divided by the value at baseline (vehicle treatment), and the results were converted into a percentage value. The RLU increase (%), which is the percentage value above baseline, represents the level of cAMP accumulation ERK/MAKase phosphorylation in response to the drug challenge.

RT-CES Assay

The RT-CES (real-time cell electronic sensing) system can monitor functional activation of GPCRs without addition of labels and sample collection. The detailed experimental procedures are described elsewhere (Solly et al., 2004; Yu et al., 2006). Briefly, 50 µL of media was added to 16-well E-plates to obtain background readings prior to addition of 100 µL of cell suspension (10,000 ~ 15,000 cells). The cells placed in the E-plates were incubated at room temperature for 15–20 minutes, and then placed in the device station in the incubator and incubated overnight (12 h) to allow for cell attachment before continuous recording of impedance as reflected by cell index. Baseline recording was collected every two minutes for 10–20 min before drug challenge. Chemicals were prepared as 40× stock solutions and 5 µl of the stock was added to the cells in the wells and recording was continuously obtained for a period. Forskolin was added to the cells 10 min after addition of monoamines. The data were expressed as normalized cell index, which are derived from the ratio of cell index before and after drug addition.

Real-Time Polymerase Chain Reaction

Dissected brain regions from three rhesus monkey brains obtained at necropsy from animals being euthanized for other purposes were collected and placed in RNAlater and stored at 4°C for less than 1 week. The tissue was homogenized with a pellet pestle (Sigma-Aldrich), and total RNA was extracted from tissue homogenate using a Versagene RNA purification system (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol. In addition to DNase treatment during processing with the Versagene kit, each 1 µg RNA

sample was further treated with 2 U of RQ1 RNase-free DNase for 1 h and then reverse transcribed using Superscript III reverse transcriptase, according to the manufacturer's protocols. TAAR6 and TAAR1 were amplified from rhesus monkey genomic DNA as a positive control, and an exon 2-flanking sequence of tryptophan hydroxylase 2 (TPH2) was amplified using intronic primers from rhesus monkey genomic DNA as a positive control and from each cDNA sample for the detection of trace genomic DNA. cDNA was also prepared from TAAR6 stable cells and assessed for TAAR6 expression. β -actin was amplified from each cDNA sample as positive control when the amplification for the target gene expression was negative. Assays were designed using the Roche Universal ProbeLibrary Assay Design Center for the Human ProbeLibrary (www.roche-applied-science.com). Human ProbeLibrary probes were used in assays designed with rhesus monkey sequence data. Probe 7 and a set of primers (5'-ccatggagagctgctgtat-3' and 5'-catcacagcaggtgtggaa-3') were used in assays for TAAR6, probe 50 and a set of primers (5'-gtccctgctgttttgcatt-3' and 5'-tcttcagcgccttgaagtt-3') were used in assays for TAAR1; probe 38 and a set of intronic primers (5'-tggaaccctaactacgtttcg-3' and 5'-caggtttgtaaccagcaca-3') that flank TPH2 exon 2 (Chen et al., 2006) were used in control assays for TPH2 to monitor genomic DNA contamination; and probe 64 and a set of primers (5'-ccaaccgcgagaagatag-3' and 5'-ccagaggcgtacaggatag-3') were used in assays for β -actin. For each reaction, a LightCycler Taqman Master kit (Roche Diagnostics) was used with 10 μ M of each primer, 0.2 μ L of each probe, and either 2 ng of genomic DNA or 50 ng of cDNA in a total volume of 20 μ L. All amplifications were carried out in glass capillary tubes with rapid cycling polymerase chain reaction in a LightCycler 2.0 instrument under the following conditions: preheat for 1 cycle at 95°C for 15 min; amplification for 45 cycles: 95°C for 10 s, 58°C for 30 s, 72°C for 3 s; and final cooling to 40°C.

RESULTS

Rhesus Monkey TAAR6 Sequence

Borowsky et al identified four human trace amine-associated receptor subtype genes TA1 (TAAR1), TA3 (TAAR9), TA4 (TAAR6), and TA5 (TAAR8) that are intronless and occur in tandem in the human chromosomal region of 6q23.2 (Borowsky et al., 2001). We previously cloned and characterized TAAR1 from rhesus monkey genomic DNA (Miller et al., 2005; Xie et al., 2007b). Prior to publication of the rhesus genome, we designed primer sets based on the human DNA sequence on chromosome 6 (clone RP11-295F4, AL513524) that flank the TAAR6 coding region. From this we were able to obtain the full-length TAAR6 (1,038bp) coding region from rhesus monkey genomic DNA using a PCR-based strategy. A blast analysis indicated that rhesus monkey TAAR6 was 96.1% (997/1038) identical to human TAAR6 in the coding sequence. The translated amino acid sequence of rhesus monkey TAAR6 putatively contains seven transmembrane (TM) domains, two glycosylation sites within the N terminal, two casein kinase II (CKII) sites at S236 and S240, and a protein kinase C site at S244 (Fig. 1 A). Sequence comparison indicated that rhesus monkey TAAR6 bears a 94.5% similarity to human TAAR6 on the amino acid level overall and high identity to human TAAR6 in each putative domain (Fig 1B).

TAAR6 did not Alter cAMP Level at Exposure to Monoamines or Methamphetamine

Our previous studies have established that rhesus monkey TAAR1 interacts with a spectrum of monoamines and stimulates cAMP signaling (Miller et al., 2005; Xie et al., 2007b) using CRE-Luc expression assays. Here we evaluated the effect of TAAR6 on cAMP-driven CRE-Luc expression at exposure to monoamines. Rhesus monkey TAAR6 or pcDNA 3.1 was transiently transfected into HEK293 cells along with CRE-Luc and pGL4.73. The cells were then exposed to vehicle and each monoamine or methamphetamine, and CRE-Luc

expression was determined as a measure of cAMP accumulation in the cells and normalized to vehicle treatment and presented as RLU increase (%). In parallel, TAAR1 was assayed as a positive control. Unlike TAAR1, TAAR6 did not alter cAMP levels at exposure to 10 μ M of common biogenic amines, including dopamine, norepinephrine and serotonin, 10 μ M of trace amines, including β -PEA, octopamine, tryptamine and tyramine, or 10 μ M methamphetamine (Fig. 2A). Given the intracellular localization of TAAR1 expressed in HEK293 cells and the fact that monoamine transporters enhance TAAR1 activation by monoamines (Xie and Miller, 2007; Xie et al., 2007), we decided to test whether a TAAR6 response to monoamines is dependent on co-expression of the dopamine transporter (DAT). Rhesus monkey TAAR6 along with CRE-Luc and pGL4.73 was transiently transfected into HEK293 and stable DAT cells to generate cell lines, TAAR6 and DAT-TAAR6. The transfected cells were treated with 10 μ M β -PEA for 18 h. TAAR1 was assayed as control. DAT co-expression did not alter the TAAR6 response to β -PEA in induction of CRE-Luc expression but dramatically enhanced TAAR1 activation by β -PEA (Fig. 2B). Western blotting showed that TAAR6 expression in the stable TAAR6 cells used in this assay was positive (Fig. 2C). These results demonstrate that rhesus monkey TAAR6 is unresponsive to monoamines with respect to cAMP signaling in vitro.

TAAR6 did not Alter Cell Impedance at Exposure to Monoamines

The RT-CES system can monitor in real time the functional activation of GPCRs without the addition of labels and later sample collection and processing. We employed this system to assess the influence of TAAR6 on cell impedance at exposure to monoamines. Stable TAAR6 cells were seeded in 16-well E-plates, incubated overnight (12 h), and then subjected to treatment with each monoamine. Cell impedance was measured and normalized to the baseline (before drug treatment). When the cells were exposed to 20 μ M of dopamine, serotonin, β -PEA, octopamine, tryptamine or tyramine, the cell impedance remained unaltered during the observation period. However, 20 μ M forskolin induced a time-dependent increase in normalized cell index (Fig. 3A), suggesting that TAAR6 is unresponsive to all drugs tested. We also assessed whether the drugs could inhibit the forskolin response via action on TAAR6. The cells were exposed to 20 μ M of the monoamines 10 min prior to the addition of 20 μ M forskolin. Dopamine, serotonin, β -PEA, octopamine, tryptamine or tyramine did not affect the forskolin effect on cell impedance (Fig. 3B), providing further evidence that TAAR6 is unresponsive to all drugs tested. Western blotting showed that TAAR6 expression in stable TAAR6 cells was positive (Fig. 3C).

TAAR6 did not Alter ERK Phosphorylation at Exposure to Monoamines or Methamphetamine

To investigate whether TAAR6 interacts with monoamines via another signaling pathway, we evaluated the influence of TAAR6 on ERK phosphorylation-driven luciferase expression at exposure to monoamines. Rhesus monkey TAAR6, human KOR (κ opioid receptor) or pcDNA 3.1 along with pTL-ELK-1, pTL-Luc and pGL4.73 was transiently transfected into HEK293 cells to generate cell lines, TAAR6, KOR and HEK, respectively. The KOR cell line was used as positive control, and the HEK cell line was used as negative control. The cells were then exposed to the monoamines or U69593 (a κ opioid receptor agonist) for 18 h. ERK phosphorylation-driven luciferase expression was determined and normalized to vehicle treatment and presented as RLU increase (%). In contrast to KOR which significantly increased the luciferase expression at exposure to 1 μ M U69593 ($p < 0.01$), TAAR6 did not alter the luciferase expression at exposure to 10 μ M of each monoamine (dopamine, norepinephrine, serotonin, β -PEA, octopamine, tryptamine or tyramine) or methamphetamine (Fig. 4A).

To test whether a TAAR6 response to the monoamines is dependent on co-expression of DAT, we assessed the influence of TAAR6 on the ERK phosphorylation-driven luciferase expression at exposure to β -PEA in the presence and absence of DAT. Rhesus monkey TAAR6 along with pTL-ELK-1, pTL-Luc and pGL4.73 was transiently transfected into HEK293 cells and stable DAT cells to generate cell lines TAAR6 and DAT-TAAR6. The transfected cells were treated with 10 μ M β -PEA for 18 h and luciferase expression was determined. DAT did not alter the β -PEA effect on the luciferase expression in the cells (Fig. 4B). Again, western blotting showed that TAAR6 expression in TAAR6 cells was positive (Fig. 4C). These results demonstrate that rhesus monkey TAAR6 is unresponsive to monoamines with regard to the stimulation of MAPK/ERK phosphorylation in vitro.

TAAR6 mRNA expression and cellular localization in transfected cells

We used real-time RT-PCR to verify TAAR6 mRNA expression in transfected cells (Fig. 5A). To determine the distribution of TAAR6 protein in the cells and whether TAAR6 is located on the cell surface plasma membrane, we first extracted and analyzed the compartmental proteins from the cells using SDS-PAGE and western blotting. TAAR6 cells were analyzed for comparison. TAAR6 associates with the total membrane fraction, and TAAR1 also associates with the total membrane fraction but is apparently detected in the compartments of cytosol, nucleus and cytoskeleton as well (Fig. 5B), which is similar to the signal we have previously observed using this TAAR1 antibody in total protein derived from untransfected HEK293 cells (Xie et al., 2007b). Accordingly, this faint band may be non-specific or reflect TAAR1 localization. The receptor localization on the cell surface plasma membrane was assessed using biotinylation assays and Western blotting. TAAR6 was clearly detected on the cell surface, whereas TAAR1 was not (Fig. 5C). These data suggest that both TAAR1 and TAAR6 are associated with cellular membranes and that TAAR6 is expressed on the cell surface. Immunocytochemical staining of stable TAAR6 cells using a TAAR6 antibody showed apparent TAAR6 staining near the cell surface, in comparison to the negative control and the membrane staining with a membrane-staining dye (Fig. 5D).

TAAR6 mRNA is Undetectable in Selected Brain Regions

We employed a real-time RT-PCR strategy to detect expression of TAAR6 mRNA derived from a set of rhesus monkey brain regions. TAAR6 was successfully amplified from rhesus monkey genomic DNA, and β -actin was positively amplified from cDNAs of the brain regions tested, but TAAR6 was completely undetectable in these brain regions (Fig. 6A). Similar results were obtained from cDNAs derived from two other monkeys. In comparison, TAAR1 expression was detected in all brain regions assayed (Fig. 6B). The cDNA samples were negative for genomic DNA contamination as indicated by the lack of signal for an intron-flanked sequence of TPH2 exon 2, which was readily amplified from rhesus monkey genomic DNA included as a positive control (Fig. 6B). Experiments were repeated at least two times. These data indicated that, unlike TAAR1, TAAR6 mRNA is not expressed in the brain regions tested.

DISCUSSION

In the present study, we demonstrate that rhesus monkey TAAR6 is unresponsive to brain monoamines or the psychostimulant drug methamphetamine in its ability to trigger cAMP signaling and MAP/ERK phosphorylation in vitro. We provide evidence that TAAR6 is associated with cellular membranes and is detectable on the cell surface plasma membrane in the transfected cells, and that TAAR6 lacks signaling linkage to Gs or Gi protein at exposure to monoamines. We show that TAAR6 mRNA is undetectable in selected rhesus monkey brain regions, including the prominent monoaminergic nuclei.

Several recent studies have reported that the TAAR6 gene is associated with susceptibility to schizophrenia and bipolar affective disorder. After an initial linkage study found a signal associated with schizophrenia in the 6q13-q26 region (Cao et al., 1997), follow up association studies in Americans of European and African ancestry were able to specifically find an association with SNPs in TAAR6 (Duan et al., 2004). These findings have since been replicated in Irish (Vladimirov et al., 2007) and Korean (Pae et al., 2008) populations. However, studies with other populations, including Japanese (Ikeda et al., 2005), Han Chinese (Duan et al., 2004), and Arab Israeli (Amann et al., 2006) have failed to replicate these results. A similar story is found with bipolar disorder. The 6q23.2 region in which TAAR6 is located (along with all other member of the human trace amine associated receptor family) was initially linked to bipolar disease (Farley et al., 1978; Ewald et al., 2002). Focusing on the trace amine associated receptors, an association between TAAR6 and bipolar affective disorder was found in a German population (Abou Jamra et al., 2005) as well as the European and African-American population in which the association with schizophrenia was found (Duan et al., 2004). The specific SNPs within TAAR6 that were found to be associated with the disease were different between the two studies, and an additional study of American families, largely of European descent but also including African and Asian ancestry, failed to find any association with TAAR6 despite a previous linkage signal in the region (Liu et al., 2007).

Recently, Reiners et al. used a constitutively active TAAR6 mutant (TA4_{CAM}) as a tool to investigate effects of a V265I polymorphism in human TAAR6 implicated in bipolar affective disorder. They found no functional difference in signaling between the two alleles tested (perhaps not surprising in a constitutively active mutant), but did demonstrate that TAAR6 signals through CREB (Reiners et al., 2007), suggesting that TAAR6 is linked to the cAMP signaling pathway. In this study, we used CRE-Luc assays to determine CRE-Luc expression as a measurement of cAMP accumulation to evaluate TAAR6 response to brain monoamines and methamphetamine. Given the previous reports that TAAR1 is activated by a spectrum of chemicals including brain monoamines and psychostimulant amphetamines (Borowsky et al., 2001; Bunzow et al., 2001; Miller et al., 2005; Hart et al., 2006; Grandy, 2007; Reese et al., 2007; Wainscott et al., 2007; Xie and Miller, 2007; Xie et al., 2007b; Xie and Miller, 2008; Xie et al., 2008), we herein tested TAAR1 in parallel to compare the receptor response. We indeed found that TAAR1 was positive but TAAR6 was negative in induction of CRE-Luc expression at exposure to monoamines. Our further study also demonstrated that TAAR6 is unresponsive to monoamines in its ability to trigger MAPK/ERK phosphorylation using luciferase assays.

The RT-CES system is capable of live measuring of minute changes in cell morphology utilizing microelectronic sensor arrays integrated into the bottom of specialized microtiter plates (E-plates) to monitor functional activation of G protein-coupled receptors linked to Gs or Gi without labeling and sample collection (Solly et al., 2004; Yu et al., 2006). In this study, we used this system to monitor variation of the cell impedance for determination of the TAAR6 response to monoamines. The data indicated that rhesus monkey TAAR6 at exposure to monoamines did not alter cell impedance and failed to influence forskolin-induced change of cell impedance, suggesting that TAAR6 is not linked to either Gs or Gi for signal transduction in response to monoamines to change cell status. Taken as a whole, these findings lead us to the conclusion that rhesus monkey TAAR6 is not a target for brain monoamines.

Further studies demonstrated that TAAR6 protein expressed in the transfected cells is membrane-associated and localizes to the cell surface cytoplasmic membrane, and revealed an apparent difference between TAAR1 and TAAR6 with regard to cell surface localization. Notably, we previously reported that TAAR1 is largely intracellularly sequestered when

expressed in HEK293 cells, and that monoamine transporters, including dopamine transporter, norepinephrine transporter and serotonin transporter can significantly enhance TAAR1 activation by common biogenic amines and trace amines (Miller et al., 2005; Xie et al., 2007b). Since we cannot exclude the possibility that TAAR6 is partially intracellular, we also tested TAAR6 responsivity to drugs in the presence of DAT in both CRE-Luc and ERK expression assays. DAT did not alter the TAAR6 response to β -PEA in induction of either CRE-Luc or ERK expression, but dramatically enhanced TAAR1 activation by β -PEA with respect to the induction of CRE-Luc expression. These results further suggest that, unlike TAAR1, TAAR6 is not a target of the monoamines.

Real time RT-PCR studies revealed that TAAR6 mRNA is not detectable in selected rhesus monkey brain regions, including monoaminergic nuclei. A previous study has also reported a failure to detect TAAR6 mRNA in rodent brain (Liberles and Buck, 2006), suggesting that TAAR6 may not be directly expressed in brain monoaminergic systems. Our previous study showed that TAAR6 protein was detectable by Western blotting in the selected rhesus monkey brain areas that we assayed for mRNA detection (Xie et al., 2005b). Failing to observe TAAR6 mRNA expression in the rhesus monkey brain regions, we question the antibody specificity for TAAR6 in the proteins derived from brain tissues, though the antibody appears to specifically recognize TAAR6 in our TAAR6-transfected cells.

The cloned rhesus monkey TAAR6 gene is 96.1% identical to human TAAR6, and the translated protein shares a common structure of seven transmembrane domains for G protein-coupled receptors and a 94.5% similarity to the human ortholog. The high identity of rhesus monkey TAAR6 to human TAAR6, particularly in extracellular regions likely to be involved in ligand binding, suggests that TAAR6 may have similar functional relevance in non-human primates and humans. Notably, however, rhesus monkey TAAR6 has only 41.2% identity to rhesus monkey TAAR1 in the translated protein sequence and 32.2% identity in extracellular regions (40.9% and 31.1% for human TAAR6 to TAAR1 overall and in extracellular regions respectively), which may account for the discrepancy in their response to monoamines. We previously reported that TAAR1 is widely expressed in rhesus monkey brain and co-localizes with the dopamine transporter (DAT) in a subset of dopamine neurons in rhesus monkey substantia nigra (Miller et al., 2005; Xie et al., 2007b), and that TAAR1 activation modulates monoamine transporter function *in vitro* and in brain synaptosomes (Xie and Miller, 2007; Xie and Miller, 2008; Xie et al., 2008). These findings along with the present data suggest that TAAR1 and TAAR6 differentiate in their expression and their functional roles.

Methamphetamine is a widely abused, highly addictive psychostimulant and neurotoxic drug that interferes with cognition (memory and attention) and emotion (euphoria, surge in productivity, increase in self-esteem and impulsion) (Hart et al., 2001; Cretzmeyer et al., 2003). We previously demonstrated that methamphetamine interacts with TAAR1 and consequently modulates dopamine transporter function (Xie et al., 2007b). This study reveals that, unlike TAAR1, rhesus monkey TAAR6 is unresponsive to methamphetamine in its ability to stimulate cAMP accumulation and trigger MAPK/ERK phosphorylation, suggesting that TAAR6 is not associated with the psychostimulant effects of methamphetamine. This provides further indirect evidence that TAAR6 is not involved in monoamine neuronal function.

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Fig. 1. Sequence of the rhesus monkey TAAR6 protein and its similarity to the human analogue. A. The translated amino acid sequence of rhesus monkey TAAR6 with seven transmembrane (TM) domains, indicated by the lines and the numbers; two N-glycosylation sites (Gly) at the N-terminal; two casein kinase II (CKII) sites at S236 and S240; and a protein kinase C site at S244. B. The identity between rhesus monkey and human TAAR6, both overall as well as within various domains. TM: transmembrane domain, IL: intracellular loop, EL: extracellular loop.

Fig. 2.

Effect of TAAR6 on cAMP-driven CRE-Luc expression following exposure to monoamines and methamphetamine. Cells were incubated under transfection conditions for 12 h and then exposed to vehicle and 10 μ M of each chemical for 18 h. CRE-Luc expression was determined as a measurement of cAMP accumulation in the cells and normalized to vehicle treatment and presented as RLU increase (%). Data are presented as mean $\pm$ SEM for three independent experiments performed in triplicate. ** $p < 0.01$. A. Rhesus monkey TAAR1, TAAR6 or pcDNA3.1 along with CRE-Luc and pGL4.73 with the TAAR1 cells used as a positive control and the HEK cells used as a negative control. Unlike TAAR1, TAAR6 did not alter cAMP level at exposure to the indicated monoamines. B. Rhesus monkey TAAR1 or TAAR6 along with CRE-Luc and pGL4.73 was transiently transfected into HEK293 and stable DAT cells to generate cell lines, TAAR1 and DAT-TAAR1, or TAAR6 and DAT-TAAR6. Dopamine transporter (DAT) did not alter the effect of β -PEA effect on CRE-Luc

expression in TAAR6 cells but dramatically enhanced its effect in TAAR1 cells. C. TAAR6 expression detected by Western blotting in the transiently-transfected TAAR6 cells.

Fig. 3.

Effect of TAAR6 on cell impedance at exposure to monoamines. A, Stable TAAR6 cells were seeded in wells of 16-well E-plates and incubated overnight (12 h), and then subjected to treatment with the indicated chemicals. The RT-CES system was used to measure changes of cell impedance which was recorded as cell index. Data are presented as mean $\pm$ SD ($n = 4$). Upper: The cells were exposed to 20 μ M forskolin or 20 μ M of each monoamine. In contrast to forskolin, the monoamines did not alter the cell impedance. Bottom: The cells were exposed to 20 μ M of the monoamines 10 min prior to the addition of forskolin. Monoamines did not affect forskolin effect on the cell impedance. B, TAAR6 expression detected by Western blotting in stable TAAR6 cells.

Fig. 4.

Effect of TAAR6 on ERK phosphorylation-driven luciferase expression at exposure to monoamines and methamphetamine. The cells were exposed to the indicated chemicals for 18 h and then the ERK phosphorylation-driven luciferase expression was determined and normalized to vehicle treatment and presented as RLU increase (%). Data are presented as mean $\pm$ SEM for three independent experiments performed in triplicate. ** $p < 0.01$ (Student's t-test). A, Rhesus monkey TAAR6, human KOR (κ opioid receptor) or pcDNA 3.1 along with pTL-ELK-1, pTL-Luc and pGL4.73 was transiently transfected into HEK293 cells to generate the cell lines, TAAR6, KOR and HEK. The KOR cell was used as positive control, and the HEK cell was used as negative control. In contrast to KOR which significantly increased the luciferase expression at exposure to 1 μ M U69593, TAAR6 did not alter the luciferase expression at exposure to 1 μ M of each monoamine or methamphetamine. B, Rhesus monkey TAAR6 along with pTL-ELK-1, pTL-Luc and pGL4.73 was transiently transfected into HEK293 cells and stable DAT cells to generate cell lines TAAR6 and DAT-TAAR6. Dopamine transporter (DAT) did not alter β -PEA

effect on the luciferase expression in the cells. C, TAAR6 expression detected by Western blotting in TAAR6 cells.

Fig. 5. TAAR6 expression, distribution and localization in transfected cells. Cells stably transfected with TAAR6 were grown in 145 mm plates and harvested at 90–95% confluence to prepare cDNA and isolate compartmental or cell surface proteins, or in 8-well chamber slides for immunocytochemical staining. The prepared cDNA was subjected to real time PCR, and the isolated proteins were analyzed by SDS-PAGE and western blotting. A. TAAR6 mRNA expression in the transfected cells demonstrated by real time PCR. TPH2 intron was amplified as a control. B. TAAR6 distribution in the transfected cells demonstrated by Western blotting, with TAAR1 as control. C. Cell surface localization of TAAR6 in the transfected cells demonstrated by biotinylation assay and Western blotting, with TAAR1 as control. D. Immunochemical staining of TAAR6 in the transfected cells. TAAR6 cells were stained using a TAAR6 antibody (left) or rabbit immunoglobulin fraction as a negative control (middle) and the cell membrane was visualized with a membrane staining dye (right).

Fig. 6. TAAR6 mRNA in rhesus monkey brain. Real-time RT-PCR amplification for cDNAs derived from rhesus monkey brain regions (caudate dorsal, caudate ventral, putamen, globus pallidus, substantia nigra, thalamus, medial frontal cortex, nucleus accumbens, hippocampus, hypothalamus, cerebellum, pons, pituitary and locus coeruleus). A. TAAR6 mRNA was not detectable in any brain region. The amplification of TAAR6 from rhesus monkey genomic DNA and β -actin from the brain cDNAs was used as positive control. B. TAAR1 was amplified from the same rhesus monkey brain regions. The amplification of TAAR1 from rhesus monkey genomic DNA was used as positive control, and the amplification of an intron of the gene for tryptophan hydroxylase 2 (TPH2) from rhesus monkey cDNA was used as a control against genomic DNA contamination in the cDNA samples, and with genomic DNA as positive control.