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Trace Amine Associated Receptor 1 Signaling in Activated Lymphocytes

Michael W. Panas¹, Zhihua Xie², Helen N. Panas², Marius C. Hoener³, Eric J. Vallender², and Gregory M. Miller^{2, #}

¹Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts USA 02115 ²New England Primate Research Center, Harvard Medical School, Southborough, Massachusetts USA ³Neuroscience Research, Pharmaceuticals Division, F. Hoffmann-La Roche Ltd., CH-4070 Basel, Switzerland

Abstract

Although most research to date on Trace Amine Associated Receptor 1 (TAAR1) has focused on its role in the brain, it has been recognized since its discovery in 2001 that TAAR1 mRNA is expressed in peripheral tissues as well, suggesting that this receptor may play a role in non-neurological pathways. This study reports TAAR1 expression, signaling and functionality in rhesus monkey lymphocytes. We detected a high level of TAAR1 protein in immortalized rhesus monkey B cell lines and a significant upregulation of TAAR1 protein expression in rhesus monkey lymphocytes following PHA treatment. Through screening a wide range of signaling pathways for their upregulation following TAAR1 activation by its potent agonist methamphetamine, we identified two transcription factors, CREB and NFAT, which are commonly associated with immune activation. Furthermore, we observed a TAAR1-dependent phosphorylation of PKA and PKC following treatment with methamphetamine in transfected HEK293 cells, immortalized rhesus monkey B cells and PHA-activated rhesus monkey lymphocytes. Accordingly, the high levels of TAAR1 that we observed on lymphocytes are inducible and fully functional, capable of transmitting a signal likely via PKA and PKC activation following ligand binding. More importantly, an increase in TAAR1 receptor expression is concomitant with lymphocyte immune activation, suggesting a possible role for TAAR1 in the generation or regulation of an immune response. TAAR1 is emerging as a potential therapeutic target, with regard to its ability to modulate brain monoamines. The current data raises the possibility that TAAR1-targeted drugs may also alter immune function.

Introduction

Trace Amine Associated Receptor 1 (TAAR1) is a G protein coupled receptor (GPCR) that responds to a wide spectrum of agonists, including endogenous trace amines, common biogenic amines and thyronamines, as well as exogenous psychostimulant drugs of the amphetamine class, including methamphetamine. Whereas endogenous common biogenic amines bind to a variety of receptors, trace amines and amphetamines show a greater specificity for TAAR1 and have served as useful probes for characterizing TAAR1 pharmacology and functionality. These TAAR1 agonists are also monoamine transporter substrates. Accordingly, much of the research on TAAR1 has focused on its role as a modulator of monoaminergic function and mediator of psychostimulant action in the brain.

[#]To whom correspondence should be addressed: Phone: 508-624-8023, gmiller@hms.harvard.edu.

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Stemming from this work is a conceptualization that TAAR1 may be a potential target for novel therapeutics aimed at treating drug addiction and other neuropsychiatric conditions which are hallmarked by aberrations in brain monoaminergic systems, but highly selective drugs that target TAAR1 have been slow in coming.

In addition to its expression in brain, TAAR1 is also expressed in a number of peripheral tissues, including liver, kidney, spleen, pancreas, heart and gastrointestinal tract tissues (Borowsky et al., 2001), but functionality of TAAR1 in non-neurological tissue has been less examined. Also, TAAR1 expression has been reported in cells of the immune system (Nelson et al., 2007). Our previous work has shown that methamphetamine produces a TAAR1-dependent increase in cyclic AMP (cAMP) activation, as indicated using a CRE-luciferase assay, as well as phosphorylation-dependent downstream effects on monoamine transporter kinetic function that are attenuated with PKA or PKC inhibitors, suggesting that both the PKA and PKC pathways are activated by methamphetamine binding to TAAR1 (Miller et al., 2005, Xie and Miller, 2007, 2009).

The present study was initiated to more formally investigate which signaling pathways are activated by TAAR1. We first determined which signal transduction pathways are activated by methamphetamine in the presence and absence of TAAR1 in transfected HEK293 cells. In doing so, we identified two pathways that are upregulated in a TAAR-1 dependent manner, CREB and NFAT, along with concurrent changes in the phosphorylation status of PKA and PKC. As both of these pathways are known to be induced traditionally following lymphocyte receptor-activation, these data led us to investigate the expression of TAAR1 by lymphocytes following lymphocyte immune activation. We next verified TAAR1 expression and then determined whether the TAAR1-mediated signal transduction pathways get activated by methamphetamine in rhesus monkey PHA-activated PBMC and immortalized B lymphocytes. We then used a newly-identified TAAR1 antagonist, N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoro-methyl-benzamide [EPPTB] (Bradaia et al., 2009), to selectively inhibit TAAR1 signal transduction. Finally, we used the newly established methamphetamine/EPPTB system as a tool to demonstrate the functional capability of TAAR1 that is upregulated on lymphocytes following immune activation to transduce a signal and activate downstream pathways.

Materials and Methods

Chemicals, Reagents and Antibodies

(+)-Methamphetamine hydrochloride, 8-br-cAMP (8-bromoadenosine 3',5'-cyclic monophosphate), β -PMA (phorbol 12-myristate 13-acetate), H89 dihydrochloride hydrate, Ro32-0432 hydrochloride and the T cell mitogen PHA (Phytohemagglutinin, PHA-M, L8902) were purchased from Sigma Aldrich (St. Louis, MO). All reagents for cell culture were purchased from Invitrogen (Carlsbad, CA). Cell Surface Protein Isolation Kit, SuperSignal West Pico Chemiluminescent Substrate and Goat anti-rabbit IgG (H+L) (31460) were purchased from Pierce (Rockford, IL). Cignal™ Finder Reporter Arrays, Cignal™ Lenti CRE Reporter kit and Cignal™ Lenti NFAT Reporter kit, SureEFCT and SureENTRY transfection reagents were obtained from SABiosciences (Frederick, MD). Phospho-PKA antibody (ab5815) and phospho-PKC antibody (ab23513) were purchased from abcam (Cambridge, MA). The TAAR1 antibody (IMG-71855) targeted to the 3rd cytoplasmic domain of human Trace Amine Associated Receptor 1 was purchased from Imgenex (San Diego CA). Dual luciferase assay reagents were purchased from Promega (Madison, WI). The TAAR1 antagonist, N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoro-methyl-benzamide [EPPTB] was supplied by F. Hoffmann-La Roche Ltd, Pharma Research (CH-4070 Basel, Switzerland). TAAR1 expression constructs were generated in our laboratory using pcDNA3.1 from Invitrogen (Carlsbad, CA). A CRE-luciferase reporter

construct, CRE-Luc, was made by Dr. Graeme Bilbe, Novartis, Basel, Switzerland and was obtained from Dr. Walter Borne, University of Zurich, Zurich, Switzerland). pGL4.7 Renilla luciferase control vector was purchased from Promega (Madison, WI).

Cell Culture and Transfection/Transduction

HEK293 cells were transfected or transduced to introduce DNA encoding target proteins for analysis using calcium phosphate, SureFECT or SureENTRY transfection reagents. Geneticin (G418) and/or puromycin were used for selection and maintenance of selection of stable cell lines. HEK293 cells were grown in DMEM supplemented with FBS (10%, heat-inactivated for 30 min at 56 °C), penicillin (100 u/ml), streptomycin (100 µg/ml) and non essential amino acids (0.1 mM) and then grown at 5% CO₂ and 37 °C.

Generation of PBMC

PBMCs were generated from rhesus monkey blood by layering fresh blood over Lymphocyte Separation Medium (MP Biomedicals, LLC) followed by centrifugation. The lymphocyte layer that was formed was transferred to a separate tube and Phosphate Buffered Saline (PBS) was added to rinse the layer. The tube was centrifuged and the remaining pellet was incubated in the presence of ACK Lysing Buffer to remove any remaining red blood cells. After centrifugation, the pellet was rinsed with PBS by resuspending in PBS, centrifuging and aspirating the PBS. This was followed by a similar pellet rinse in RPMI 1640 Media plus 1% FBS. PBMCs were then counted and resuspended in R20 media (RPMI 1640 Media plus 20% FBS, 25 mM HEPES, 100 units/ml Penicillin, 100 µg/ml Streptomycin and 2 mM L-Glutamine) at one million PBMCs/ml with or without 1 µg/ml PHA for 48 hours.

Generation of immortalized B cells

PBMCs were resuspended in R20 media and Cyclosporin A was added at 2 µg/ml followed by the addition of an equal volume of filtered S594 supernatant containing Herpesvirus papio (supplied by Dr. W. Johnson, Harvard Medical School, Southborough, MA). The cells were cultured until transformation occurred as evidenced by cell clumping. AZT was then included in the media at a concentration of 4 µM and the cells were cultured until a sufficient number of B cells were generated.

Pharmacological characterization of methamphetamine and EPPTB using the CRE-luciferase reporter assay

A dual luciferase assay was performed to characterize CRE-luciferase responses to methamphetamine and EPPTB. HEK293 cells stably transfected with mouse TAAR1 and untransfected HEK293 cells were subjected to transient co-transfection with luciferase reporter vectors (CRE-Luc along with pGL4.73) using a Calcium phosphate procedure (Xie et al., 2007). The total amount of DNA was held constant with pcDNA3.1. The cells were exposed to the transfection media for 24 hours and then incubated in serum-free DMEM and subjected to drug challenges for 20 hours. Cells were then lysed with 1 × PLB buffer (25 µl/well) by incubation on a shaking platform for 30 min at 25 °C. The lysates (20 µl) were transferred to wells of opaque 96-well microplates for measurement of luciferase expression on a Wallac 1420 multilabel counter, Victor 3V (PerkinElmer, Shelton, CT). Luciferase substrate reagents (50 µl) were injected into each well, and after a 2 sec delay, luciferase levels were measured as Relative Light Units (RLUs) for 12 sec.

Signal™ Finder 45-Pathway Reporter Array

Signal™ 45-Pathway Reporter Arrays were used to simultaneously assess 45 different signaling pathways in HEK293 and derived cell lines. Cells were seeded into wells (50,000

cells/well) of the Cignal™ Finder 96-well plates (SABiosciences, Frederick, MD) for introducing pathway reporters into cells via reverse transfection according to manufacturer's protocol. Briefly, reporter DNA constructs resident in each plate well were resuspended with 50 μ l Opti-MEM and then mixed with 50 μ l diluted SureFECT transfection reagent. Cells were suspended in Opti-MEM supplemented with 10% of fetal bovine serum and 0.1 mM NEAA at a density of 6×10^5 cells/ml, and then 50 μ l of the cell suspension was added into each plate well and mixed with DNA resident in the plate and added transfection reagent. The cells were incubated for 48 h at 5% CO₂ and 37 °C. Following transfection, the cells were treated with vehicle (DMEM) or methamphetamine (1 μ M) for 18 hours in fresh Opti-MEM. Cells were then lysed and luciferase expression was determined as described above.

Lenti Reporter Assays

A Cignal™ Lenti CRE Reporter kit and a Cignal™ Lenti NFAT Reporter kit (SABiosciences) were used to transduce lenti reporters into cells for evaluation of CREB and NFAT pathway activation according to the manufacturer's protocols. Briefly, stable rhesus monkey TAAR1-expressing HEK293 cells were seeded into wells (50,000 cells/well) of 96-well plates and were grown at 37 °C for 24 hours in a humidified 5% CO₂ incubator. Then, the cells were transduced with Lenti CRE Reporter or Lenti NFAT Reporter (40 μ l/well of lentiviral particles and 60 μ l growth medium without antibiotics containing 14 μ g/ml SureEntry transduction reagent) for 24 hours and then incubated in fresh growth medium for another 24 h at 37 °C and 5% CO₂. Thereafter, the cells were treated with 5.0 μ g/ml puromycin in growth medium for selection of transduced cells. The puromycin-resistant cell colonies were expanded in 100 mm cell culture dishes and then harvested and seeded in 96-well plates for assays. Upon 60% confluence, the cells were exposed to test drugs in serum-free DMEM for 20 hours and then were lysed with 1 $\times$ PLB buffer (25 μ l/well) by incubation on a shaking platform for 30 min at 25 °C. The lysates (20 μ l) were transferred to wells of opaque 96-well microplates for measurement of luciferase expression as described above.

Drug treatments, Protein isolation and immunoblotting

We used immunoblotting to detect TAAR1 expression and to measure levels of phosphorylated PKA and phosphorylated PKC in HEK293 and rhesus monkey TAAR1-transfected cells, immortalized rhesus monkey B cells and rhesus monkey PBMC with various drug treatments in the following dosages and combinations: vehicle, 8-Br-cAMP (100 μ M), β -PMA (1 μ M), methamphetamine (1 μ M) in the absence or presence of the PKA inhibitor H89 (10 μ M) or the PKC inhibitor Ro32-0432 (10 μ M), EPPTB (10 μ M), or methamphetamine (1 μ M) alone or in combination with EPPTB (10 μ M) for 10 min. The density of the phosphor-PKA and phosphor-PKC were normalized to β -actin in the same amount of the cells to obtain percentage values for comparing the effects of vehicle and methamphetamine treatment relative to the untreated case. The experimental procedures were modified from previous studies (Xie et al., 2007, Xie et al., 2008). Briefly, cells were lysed in an appropriate volume of ice-cold RIPA buffer (150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) at 10^7 cells/ml or 1:5 (w/v) supplemented with 1 $\times$ protease inhibitor cocktail for 30 min at 4 °C. The lysates were centrifuged at $12,000 \times g$ for 10 min at 4 °C, and the supernatants were collected and mixed with BioRad Laemmli sample buffer at 1:1 (v/v), heated at 95 °C for 5 min and centrifuged at $12,000 \times g$ for 5 min at 4 °C. The prepared samples were subjected to SDS-PAGE (10% acrylamide separating gel, 4% acrylamide stacking gel), and the proteins were electrotranslocated onto a PVDF membrane (0.45 μ m) that had been activated by presoaking in 100% methanol for 10 min. The membrane was then blocked with blocking buffer (10% non-fat milk, 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.5) and then incubated with primary antibodies against TAAR1, phosphorylated PKA and phosphorylated PKC at 1:1,000 overnight at 4 °C. Thereafter, the membrane was washed

and then incubated with secondary antibody (goat anti-rabbit IgG, (H+L)) at 1:5,000 for 2 hours at room temperature in blocking buffer. SuperSignal West Pico Chemiluminescent Substrate was employed to visualize the blots under a luminescent image analyzer (LAS-1000; Fujifilm, Tokyo, Japan).

Statistics

In all cases, the One-Way ANOVA/Turkey post-hoc test is applied.

Results

Although most research has focused on TAAR1 in brain, it has been recognized since its discovery in 2001 that TAAR1 mRNA is also expressed in peripheral tissues, suggesting that TAAR1 may play a role in non-neurological pathways (Borowsky et al., 2001). To investigate the intracellular signaling pathways triggered by TAAR1 ligand binding, we screened a wide range of signaling pathways for their upregulation following TAAR1 activation by the potent agonist methamphetamine. We have previously shown that methamphetamine induces strong CRE-luciferase activation in a TAAR1-dependent manner in TAAR1-transfected HEK293 cells as well as in brain synaptosome preparations (Xie and Miller, 2009). HEK293 cells were chosen because they do not, by themselves, express a functional TAAR1 receptor (Xie and Miller, 2007, Xie et al., 2007). We first performed a dose-response study using methamphetamine and EPPTB (Fig. 1), using a CRE-luciferase construct that we had previously used to characterize rhesus monkey TAAR1 pharmacological responsivity (Miller et al., 2005, Xie and Miller, 2008). This was done to assist us in selecting appropriate doses of methamphetamine and EPPTB for further study and also to verify that untransfected HEK293 cells do not respond to either drug with regard to CRE reporter signaling.

We utilized a commercially available Cignal™ 45-Pathway Reporter Array (SABiosciences, Frederick, MD) to detect upregulated signaling pathways following receptor activation (Fig. 2). The array is capable of simultaneously analyzing 45 different signaling pathways in order to pinpoint particular pathways for further analysis. Activated pathways are identified by the comparison of two reporter constructs, one that is a pathway-focused transcription factor-responsive Firefly luciferase reporter, and one that is a constitutively expressing Renilla luciferase construct. This dual luciferase technology allows for quantification of the degree of activation of each particular signaling pathway in a 96 well format. This array was used to assess activated signaling pathways in untransfected HEK293 following vehicle treatment (Fig. 2a), and this baseline signaling profile was compared to that of untransfected HEK293 cells treated with 1 μ M methamphetamine (Fig. 2b) and to HEK293 cells stably transfected with rhesus monkey TAAR1 and treated with 1 μ M methamphetamine (Fig. 2c). A comparison of these arrays along with additional data generated from arrays performed with another TAAR1 agonist (β -phenylethylamine) and with HEK293 cells that stably express monoamine transporters with and without TAAR1 co-expression (*data not shown*) verified the identification of two pathways that specifically and consistently were upregulated by TAAR1 activation: NFAT and CREB. Based on these screening data, we then sought to validate the findings. These two pathways were further investigated using ready-to-transduce Cignal™ Lenti NFAT and CREB Reporters as lentiviral particles, which we used to transduce stable rhesus monkey TAAR1-expressing HEK293 cells.

In stable rhesus monkey TAAR1 cells, the transduced CREB reporter construct indicated signal pathway activation as the ratio of Firefly:Renilla luciferase increased over four-fold following methamphetamine treatment (Fig. 3). This CREB-mediated luciferase expression is indicative of an increase in cAMP levels following TAAR1 activation. In parallel experiments, the transduced NFAT reporter construct indicated an increase in NFAT

signaling, as the ratio of Firefly:Renilla luciferase increased three-fold following methamphetamine treatment (Fig. 3). This increase in the NFAT signaling pathway is commonly associated with the release of intracellular calcium stores, suggesting that any member of the entire calcium dependent signal transduction pathway, including a number of conventional Protein Kinase C (cPKC) factors, may be activated following TAAR1 signaling. These data are consistent with our current array data and with our previous reports of downstream TAAR1-dependent effects on monoamine transporter kinetic function that are PKA- and PKC-dependent (Xie and Miller, 2007, 2008, 2009). The addition of EPPTB, a specific antagonist of TAAR1, was used to demonstrate the specificity of the methamphetamine-TAAR1 interaction on downstream CREB and NFAT signaling. The transduced cell lines treated with both methamphetamine and EPPTB failed to produce levels above baseline with either reporter construct. Therefore, the CREB activation associated with increased cAMP and the NFAT activation associated with PKA and PKC/ Ca^{++} activation were dependent on TAAR1 expression and signaling. Further, these data demonstrate that TAAR1-dependent methamphetamine-induced phosphorylation of PKA and PKC occurs coincidentally with the CREB and NFAT reporter activation.

Next, we further characterized the involvement of the PKA and PKC/ Ca^{++} pathways in the methamphetamine-TAAR1 interaction. We observed robust phosphorylation of both PKA and PKC following TAAR1 stimulation with methamphetamine in stably transfected rhesus monkey TAAR1-expressing HEK293 cells, but not in control HEK293 cells (Fig 4). The PKA inhibitor H89 blocked methamphetamine-induced PKA phosphorylation selectively, whereas the PKC inhibitor R032-0432 blocked methamphetamine-induced PKC phosphorylation selectively in the TAAR1-transfected cells, but had no significant effect in HEK293 cells. However, the cyclic AMP analog 8-Br-cAMP evoked PKA phosphorylation and the phorbol ester β -PMA evoked PKC phosphorylation comparably in TAAR1 cells as well as HEK293 cells.

We next assessed TAAR1 expression by Western blot in immortalized rhesus monkey B cell lines (Fig. 5a). We generated four immortalized B cell lines from different rhesus monkeys and tested cell lysates for the presence of TAAR1 protein. TAAR1 protein was observed in all B cell lineages tested. Slight variations between the different B cell lines were apparent, with lines B-248.05 and B-125.02 producing high levels of TAAR1 comparable to TAAR1-transfected HEK293, while lines B-165.01 and B-28.01 produced somewhat lower levels of TAAR1.

The ability of endogenously expressed TAAR1 on B cells to transmit a functional signal following agonist binding was then tested. Western blot analysis of whole cell lysate from the immortalized B cell line B-248.05 was performed using antibodies capable of only recognizing phosphorylated PKC and phosphorylated PKA molecules. Figure 5b shows robust phosphorylation of both PKA and PKC following TAAR1 stimulation with methamphetamine. The addition of EPPTB was used to demonstrate the specificity of the methamphetamine-TAAR1 interaction on the phosphorylation of PKA and PKC. Data from two independent experiments demonstrates that treatment of immortalized B cells with both methamphetamine and EPPTB show dramatically attenuated phosphorylation of PKA and PKC relative to treatment with methamphetamine alone (Fig. 5c). Therefore, phosphorylation of both PKA and PKC following TAAR1 stimulation with methamphetamine is dependent on TAAR1 expression and signaling in immortalized B cells.

As the B cells lines are immortalized and may be in a constant state of immune activation, we wanted to test the expression and functionality of TAAR1 on lymphocytes from peripheral blood, a more physiologically relevant system. A low level of TAAR1 was

present on unactivated PBMC (Fig 6a, vehicle). Following activation with the T cell mitogen PHA (1 μ g/ml for 48 hours), a significant upregulation in TAAR1 protein was detected (Fig. 6a, PBMC (PHA)). We then sought to test the functionality of the increased TAAR1. Using Western blot analysis of whole cell lysates with antibodies capable of only recognizing phosphorylated PKC and phosphorylated PKA molecules, we detected robust phosphorylation of both PKA and PKC following TAAR1 stimulation with methamphetamine in PHA-treated PBMC (Fig. 6b, 1 μ g/ml PHA). A low but detectable level of PKC activation and no PKA activation was present 48 hours after lymphocytes were treated with PHA in the absence of TAAR1 stimulation. TAAR1 stimulation led to robust detection of phosphorylated PKA and PKC. Here too, the addition of EPPTB along with methamphetamine attenuated the phosphorylation of PKA and PKC. The activation of PKA and PKC was not observed in PBMC treated with methamphetamine that were not first activated using PHA (Fig. 6b, Non-PHA), indicating that the observed PKC and PKA activity first requires T cell activation to raise the levels of TAAR1 expression. Data from two independent experiments with PBMC generated from two different rhesus monkeys are shown in Fig. 6c.

We conclude that TAAR1 protein expression is upregulated following lymphocyte immune activation. Furthermore, the high levels of TAAR1 expressed in activated lymphocytes are capable of transducing CREB and NFAT signaling, and do so by the activation of PKA and PKC following TAAR1 activation.

Discussion

TAAR1 has a demonstrated function in neurological signaling, including downstream effects on monoamine transporter function (Xie et al., 2008, Miller, 2011), but it may also play a role in peripheral tissue. It is possible that the expression, function, and signaling of TAAR1 in the periphery is different than in the brain, and that these signals play a separate and distinct role mediated by different ligands. To begin to address the role of TAAR1 in the periphery, we first investigated the cellular signaling pathways that get activated by TAAR1 by screening TAAR-dependent signal upregulation. In doing so, we identified pathways that are known to be induced traditionally following lymphocyte receptor activation. These data led us to investigate the expression of TAAR1 in lymphocytes and the capacity of TAAR1 in lymphocytes to transmit a functional signal. We demonstrate that TAAR1 expression is significantly increased following lymphocyte immune activation, and that the increased TAAR1 expression on activated lymphocytes is capable of mediating activation of the CREB and NFAT signaling pathways.

Our screening of signal pathways was done in a manner to identify pathways activated by TAAR1 ligation with various ligands. Two such pathways, NFAT and CREB, were consistently and invariably induced in a TAAR1-dependent manner. Several other pathways (NF κ B, AP-1, both transcription factors for T cell IL-2 production and proliferation) were upregulated in an inconsistent manner, suggesting that they may be transiently activated by the methamphetamine-TAAR1 interaction, or that other unexamined factors dominate the activation of these signal pathways and the methamphetamine-TAAR1 signal plays a minor role in modulating these pathways. Alternatively, the kinetics of the TAAR1 modulation of other pathways, such as NF κ B and AP-1, may be more or less rapid making our assessments with using the CignalTM array and prolonged drug treatments (18 hours) appear inconsistent.

CREB and NFAT are known to play key roles in B cell and T cell activation following B cell receptor (BCR) and T cell receptor (TCR) activation, respectively. NFAT activation is triggered by the release of calcium following both TCR and BCR activation, and is associated with IL-2 promoter binding as well. CREB activation via phosphorylation,

leading to survival and proliferation, is observed following BCR activation (Blois et al., 2004). The identification of these two pathways commonly associated with lymphocyte receptor activation, in combination with a recent report of TAAR1 mRNA observed in lymphocytes (Nelson et al., 2007), led us to investigate the protein expression and functionality of TAAR1 in lymphocytes.

We observed constitutive high level expression of TAAR1 in all four immortalized rhesus monkey B cell lines that we produced and investigated, but inducible TAAR1 expression in PBMC. A low level of TAAR1 expression was detected by Western blot at steady state in PBMC, but the activation of PBMC by the T cell mitogen PHA induced high levels of TAAR1. In fact, the B cell lineages used in this investigation are immortalized using Herpesvirus papio, and such a treatment most likely results in a chronic state of activation (Samanta et al., 2006, Martin et al., 2007). Therefore, our observation of the high levels of TAAR1 on B cells may be related to B cell activation.

A previous report investigating the mRNA profile of TAAR1 in immune cells found similar results, specifically constitutive TAAR1 mRNA expression in B cells and NK cells but undetectable mRNA in T cells until activation (Nelson et al., 2007). Because of the many layers of regulation separating gene expression from final functional protein expression, we felt it was important to demonstrate the presence of both protein and receptor functionality.

Despite the high levels of TAAR1 protein on PHA-activated lymphocytes and immortalized B cells, TAAR1 is apparently not signaling at steady state in these T and B cells. This is consistent with our previous observations of a very faint band on TAAR1 Western blots in HEK293 cells using the same TAAR1 antibody, coincident with a complete lack of detectable signaling. Accordingly, the faint band in the non-PHA-treated PBMC may be an antibody artifact. Nonetheless, we observed a significant increase in the phosphorylation of the downstream signaling molecules PKA and PKC following methamphetamine-TAAR1 interaction in the PHA-treated PBMC. These data do not preclude the fact that *in vivo*, TAAR1 may signal through endogenous ligands at different times and/or via other pathways, such as at the time of immune activation or at another time point in the natural course of an immune response.

Calcium release is a hallmark of TCR ligation and mitogen stimulation (Majumdar et al., 1990, O'Donovan et al., 1995). We initially had concerns that PHA mitogen-induced calcium flux would complicate TAAR1-dependent PKC activity and analysis. However, calcium flux resulting from PHA or TCR activation is usually reduced to levels approaching baseline 1-2 hours following release (Donnadieu et al., 1992, Verheugen et al., 1997), and so we chose 48 hours after initial PHA activation to examine calcium signaling in response to TAAR1 activation. We observed only minimal PKC activity at 48 hours post PHA stimulation, and a large NFAT reporter signal, indicative of a large calcium flux, in response to TAAR1 activation.

In this paper, we used the newly identified TAAR1 antagonist EPPTB in combination with the TAAR1 agonist methamphetamine as pharmacological tools to demonstrate the functional signaling capability of TAAR1. This combination allowed for the identification of pathways activated in a TAAR1-dependent manner, and the strength of the methamphetamine-TAAR1 binding is potent enough to induce a robust downstream signal for molecular analysis. Indeed, given our identification of TAAR1 expression on lymphocytes, it is quite possible that the wide range of immunosuppressive effects attributed to methamphetamine may be mediated, in part, through TAAR1. The potent methamphetamine-TAAR1 signaling on lymphocytes may well be causing reduced lymphocyte function, including but not limited to: suppression of antibody production (Wey

et al., 2008), an inability of T cells to produce IL-2 and proliferate (Potula et al., 2010), and an increase in host susceptibility to pathogens (Martinez et al., 2009). In our analysis, we have used methamphetamine solely for its ability to stimulate a potent signal from TAAR1. Within the host, however, it is possible that the signal propagated from TAAR1 in response to its ligation by different endogenous substrates, including common biogenic amines such as dopamine, trace amines and thyronamines, could give rise to different signaling patterns than the ones identified here.

Importantly, TAAR1 is emerging as a potential therapeutic target, with regard to its ability to modulate dopamine and perhaps other monoamines in the brain. The current data raises the possibility that TAAR1-targeted drugs may alter both brain and immune function. Given the emergence of expression of TAAR1 following activation, TAAR1 may play a role in the generation of an immune response. Our data demonstrating that TAAR1 activates signal transduction pathways that are shared with the TCR signal that initiates the adaptive immune response supports a hypothesis that TAAR1's natural physiological function is to augment an immune response. Rapid upregulation of TAAR1 following lymphocyte activation, which in turn increases CREB and NFAT transcription factor signaling, may allow for longer, stronger, more durable transcription factor activation, leading to greater lymphocyte proliferation and cytokine responses. An alternative hypothesis can be drawn from the role of TAAR1 in the brain, wherein TAAR1 limits the signaling of other receptors. Studies in transfected cells and in mouse and rhesus monkey brain synaptosomes demonstrate that the dopamine D2 autoreceptor, as well as other monoamine autoreceptor signaling (i.e., 5-HT_{1A}, 5-HT_{1B}, α_{2A} and α_{2B}), is highly attenuated when TAAR1 is present and signaling (Xie et al., 2008). To draw a parallel to the immune system, it is possible that TAAR1 signaling decreases the signaling of other GPCRs, in particular chemokine receptors and receptors for lipid mediators of inflammation, which share inhibitory signaling properties (e.g., inhibition of cAMP) with monoamine autoreceptors. Ultimately, such TAAR1 signaling to endogenous ligands may limit the cell's responsiveness to chemokines or lipid mediators of inflammation, acting as a break to prevent run-away immune system activation that can give rise to autoimmunity, an abundance of non-specifically activated T cells, or whole body systemic inflammation.

In conclusion, the high levels of TAAR1 that we observed on lymphocytes are inducible and fully functional, capable of transmitting a signal via PKA and PKC activation following ligand binding. More importantly, an increase in TAAR1 receptor expression is concomitant with lymphocyte immune activation, suggesting a possible role for TAAR1 in the generation or regulation of an immune response.

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References

- Blois JT, Mataraza JM, Mecklenbrauker I, Tarakhovsky A, Chiles TC. B cell receptor-induced cAMP-response element-binding protein activation in B lymphocytes requires novel protein kinase Cdelta. *J Biol Chem.* 2004; 279:30123–30132. [PubMed: 15138267]
- Borowsky B, Adham N, Jones KA, Raddatz R, Artymyshyn R, Ogozalek KL, Durkin MM, Lakhiani PP, Bonini JA, Pathirana S, Boyle N, Pu X, Kouranova E, Lichtblau H, Ochoa FY, Branchek TA, Gerald C. Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proc Natl Acad Sci U S A.* 2001; 98:8966–8971. [PubMed: 11459929]

- Bradaia A, Trube G, Stalder H, Norcross RD, Ozmen L, Wettstein JG, Pinard A, Buchy D, Gassmann M, Hoener MC, Bettler B. The selective antagonist EPPTB reveals TAAR1-mediated regulatory mechanisms in dopaminergic neurons of the mesolimbic system. *Proc Natl Acad Sci U S A*. 2009; 106:20081–20086. [PubMed: 19892733]
- Donnadieu E, Cefai D, Tan YP, Paresys G, Bismuth G, Trautmann A. Imaging early steps of human T cell activation by antigen-presenting cells. *J Immunol*. 1992; 148:2643–2653. [PubMed: 1349319]
- Majumdar G, Beachey EH, Tomai M, Kotb M. Differential signal requirements in T-cell activation by mitogen and superantigen. *Cell Signal*. 1990; 2:521–530. [PubMed: 2081094]
- Martin HJ, Lee JM, Walls D, Hayward SD. Manipulation of the toll-like receptor 7 signaling pathway by Epstein-Barr virus. *J Virol*. 2007; 81:9748–9758. [PubMed: 17609264]
- Martinez LR, Mihu MR, Gacser A, Santambrogio L, Nosanchuk JD. Methamphetamine enhances histoplasmosis by immunosuppression of the host. *J Infect Dis*. 2009; 200:131–141. [PubMed: 19473099]
- Miller GM. The emerging role of trace amine-associated receptor 1 in the functional regulation of monoamine transporters and dopaminergic activity. *J Neurochem*. 2011; 116:164–176. [PubMed: 21073468]
- Miller GM, Verrico CD, Jassen A, Konar M, Yang H, Panas H, Bahn M, Johnson R, Madras BK. Primate trace amine receptor 1 modulation by the dopamine transporter. *J Pharmacol Exp Ther*. 2005; 313:983–994. [PubMed: 15764732]
- Nelson DA, Tolbert MD, Singh SJ, Bost KL. Expression of neuronal trace amine-associated receptor (Taar) mRNAs in leukocytes. *J Neuroimmunol*. 2007; 192:21–30. [PubMed: 17900709]
- O'Donovan MR, Johns S, Wilcox P. The effect of PHA stimulation on lymphocyte subpopulations in whole-blood cultures. *Mutagenesis*. 1995; 10:371–374. [PubMed: 7476275]
- Potula R, Hawkins BJ, Cenna JM, Fan S, Dykstra H, Ramirez SH, Morsey B, Brodie MR, Persidsky Y. Methamphetamine causes mitochondrial oxidative damage in human T lymphocytes leading to functional impairment. *J Immunol*. 2010; 185:2867–2876. [PubMed: 20668216]
- Samanta M, Iwakiri D, Kanda T, Imaizumi T, Takada K. EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. *EMBO J*. 2006; 25:4207–4214. [PubMed: 16946700]
- Verheugen JA, Le Deist F, Devignot V, Korn H. Enhancement of calcium signaling and proliferation responses in activated human T lymphocytes. Inhibitory effects of K⁺ channel block by charybdotoxin depend on the T cell activation state. *Cell Calcium*. 1997; 21:1–17. [PubMed: 9056073]
- Wey SP, Wu HY, Chang FC, Jan TR. Methamphetamine and diazepam suppress antigen-specific cytokine expression and antibody production in ovalbumin-sensitized BALB/c mice. *Toxicol Lett*. 2008; 181:157–162. [PubMed: 18706494]
- Xie Z, Miller GM. Trace amine-associated receptor 1 is a modulator of the dopamine transporter. *J Pharmacol Exp Ther*. 2007; 321:128–136. [PubMed: 17234899]
- Xie Z, Miller GM. Beta-phenylethylamine alters monoamine transporter function via trace amine-associated receptor 1: implication for modulatory roles of trace amines in brain. *J Pharmacol Exp Ther*. 2008; 325:617–628. [PubMed: 18182557]
- Xie Z, Miller GM. A receptor mechanism for methamphetamine action in dopamine transporter regulation in brain. *J Pharmacol Exp Ther*. 2009; 330:316–325. [PubMed: 19364908]
- Xie Z, Westmoreland SV, Bahn ME, Chen GL, Yang H, Vallender EJ, Yao WD, Madras BK, Miller GM. Rhesus monkey trace amine-associated receptor 1 signaling: enhancement by monoamine transporters and attenuation by the D2 autoreceptor in vitro. *J Pharmacol Exp Ther*. 2007; 321:116–127. [PubMed: 17234900]
- Xie Z, Westmoreland SV, Miller GM. Modulation of monoamine transporters by common biogenic amines via trace amine-associated receptor 1 and monoamine autoreceptors in human embryonic kidney 293 cells and brain synaptosomes. *J Pharmacol Exp Ther*. 2008; 325:629–640. [PubMed: 18310473]

Figure 1. EPPTB blocks TAAR1 activation by methamphetamine in TAAR1-transfected HEK293 cells

A dual luciferase assay was performed to observe the response of mouse TAAR1 to EPPTB *in vitro*. HEK293-derived cells that stably express mouse TAAR1 (**top**) or HEK293 cells (**bottom**) were transiently transfected with a reporter vector CRE-Luc (cAMP sensitive) and a control vector pGL4.73 for 24 h and then treated with the indicated drugs for 20 h. (**Top left**) TAAR1 response to methamphetamine and EPPTB. (**Top right**) Blockade effect of EPPTB (10 μ M) on the response of TAAR1 to methamphetamine. (**Bottom left**) Effect of methamphetamine on cells in the absence of TAAR1. (**Bottom right**) Effect of EPPTB on cells in the absence of TAAR1.

Figure 2. Selective upregulation of NFAT and CREB by methamphetamine in TAAR1-transfected HEK29 cells

Comparison of 45 different signal transduction pathways following vehicle or methamphetamine (1 μ M) treatment in untransfected and rhesus monkey TAAR1-transfected HEK293 cells. Signal transduction pathways that are active in untransfected HEK293 cells treated with vehicle (a) were compared with those detected in untransfected HEK293 cells treated with methamphetamine (b) or rhesus monkey TAAR1-transfected HEK293 cells treated with methamphetamine (c). Black bars highlight TAAR1-dependent activation of the cAMP/PKA and PKC/Ca⁺⁺ pathways in response to methamphetamine.

Figure 3. Methamphetamine-induced Lenti-CRE or Lenti-NFAT reporter expression is TAAR1-dependent

HEK293 cells stably transfected with rhesus monkey TAAR1 were transformed with a Lenti-CRE Reporter or with a Lenti-NFAT Reporter. Transformed cell lines were treated with vehicle, EPPTB (10 μ M), methamphetamine (1 μ M), or methamphetamine in combination with EPPTB. Levels of Firefly luciferase expressed by the reporter constructs were determined in cell lysates, and relative light units (RLU) are displayed as a percent of the baseline RLU values. **: $p < 0.0001$ for indicated comparisons.

Figure 4. TAAR1 signaling promotes phosphorylation of both PKA and PKC
HEK293 cells and HEK293 cells stably expressing rhesus monkey TAAR1 were left untreated (1) or were treated with vehicle (2), 1 μ M methamphetamine (3), 1 μ M methamphetamine and 10 μ M H89 (4), 1 μ M methamphetamine and 10 μ M R032-0432 (5), 100 μ M 8-Br-cAMP (6), or 1 μ M μ -PMA and were then collected and lysed to detect phosphorylated PKA and phosphorylated PKC by Western blot. Methamphetamine significantly increased phosphorylation of both PKA and PKC in TAAR1 cells but not in HEK293 cells. The PKA inhibitor H89 blocked methamphetamine-induced PKA phosphorylation selectively, whereas the PKC inhibitor R032-0432 blocked methamphetamine-induced PKC phosphorylation selectively in TAAR1 cells, but had no significant effect in HEK293 cells. The cyclic AMP analog 8-Br-cAMP evoked PKA phosphorylation and the phorbol ester β -PMA evoked PKC phosphorylation comparably in TAAR1 cells as well as HEK293 cells. ** (1) $p < 0.01$ by one-way ANOVA/Turkey post-hoc test, compared to the vehicle treatment; ** (2) $p < 0.01$ by one-way ANOVA/Turkey post-hoc test, compared to the methamphetamine treatment.

Figure 5. Phosphorylation of PKA and PKC by methamphetamine is TAAR1-dependent in rhesus monkey immortalized B lymphocytes

(a) TAAR1 expression in four immortalized rhesus monkey B cell lines compared to TAAR1 expression in rhesus monkey TAAR1-transfected HEK293 cells assessed by Western blot using an anti-TAAR1 antibody. (b) Phosphorylation status of PKA and PKC in untreated B cells and following vehicle, EPPTB (10 μ M), methamphetamine (1 μ M), and EPPTB plus methamphetamine treatments were assessed by Western blot using anti-phospho-PKA and anti-phospho-PKC antibodies. Data shown are representative of two independent experiments. (c) Quantification and comparison of PKA and PKC phosphorylation was done by densitometry using IMAGEJ software. Data are averaged from two different immortalized B cell lines assessed independently. **: $p < 0.0001$ for indicated comparisons.

Figure 6. TAAR1 expression upregulation following immune activation and TAAR1-dependent phosphorylation of PKA and PKC by methamphetamine in rhesus monkey PBMC

(a) TAAR1 expression was upregulated in PBMC by stimulation with PHA (1 μg/ml for 48 h) and is compared to TAAR1 expression in rhesus monkey TAAR1-transfected HEK293 cells, as assessed by Western blot in cells obtained from two different rhesus monkeys (top and bottom). (b) Phosphorylation status of PKA and PKC in untreated PBMC and following vehicle, EPPTB (10 μM), methamphetamine (1 μM), and EPPTB plus methamphetamine treatments were assessed by Western blot using anti-phospho-PKA and anti-phospho-PKC antibodies. Data shown are derived from one rhesus monkey and is representative of two independent experiments using PBMC generated from two different rhesus monkeys. (c) Quantification and comparison of PKA and PKC phosphorylation in PHA-treated PBMC was done by densitometry using IMAGEJ software. Data are averaged from the two independent experiments. **: $p < 0.0001$ for indicated comparisons.