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Normal thermoregulatory responses to 3-iodothyronamine, trace amines and amphetamine-like psychostimulants in trace amine associated receptor 1 knockout mice

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Abstract

3-Iodothyronamine (T1AM) is a metabolite of thyroid hormone. It is an agonist at trace amine-associated receptor 1 (TAAR1), a recently identified receptor involved in monoaminergic regulation and a potential novel therapeutic target. Here, T1AM was studied using rhesus monkey TAAR1 and/or human dopamine transporter (DAT) co-transfected cells, and wild-type (WT) and TAAR1 knock-out (KO) mice. The IC₅₀ of T1AM competition for binding of the DAT-specific radio-ligand [³H]CFT was highly similar in DAT cells, WT striatal synaptosomes and KO striatal synaptosomes (0.72–0.81 μM). T1AM inhibition of 10 nM [³H]dopamine uptake (IC₅₀: WT, 1.4±0.5 μM; KO, 1.2±0.4 μM) or 50 nM [³H]serotonin uptake (IC₅₀: WT, 4.5±0.6 μM; KO, 4.7±1.1 μM) in WT and KO synaptosomes was also highly similar. Unlike other TAAR1 agonists that are DAT substrates, TAAR1 signaling in response to T1AM was not enhanced in the presence of DAT as determined by CRE-luciferase assay. In vivo, T1AM induced robust hypothermia in WT and KO mice equivalently and dose dependently (maximum change degrees Celsius: 50 mg/kg at 60 min: WT -6.0±0.4, KO -5.6±1.0; and 25 mg/kg at 30 min: WT -2.7±0.4, KO -3.0±0.2). Other TAAR1 agonists including beta-phenylethylamine (β-PEA), MDMA (3,4-methylenedioxymethamphetamine) and methamphetamine also induced significant, time-dependent thermoregulatory responses that were alike in WT and KO mice. Therefore, TAAR1 co-expression does not alter T1AM binding to DAT in vitro nor T1AM inhibition of [³H]monoamine uptake ex vivo, and TAAR1 agonist-induced thermoregulatory responses are TAAR1-independent. Accordingly, TAAR1-directed compounds will likely not affect thermoregulation nor are they likely to be cryogens.

Keywords

TAAR1; thyronamine; psychostimulant; thermoregulation; monoamine

Introduction

3-Iodothyronamine (T1AM) is an endogenous derivative of thyroid hormone and itself a possible chemical messenger (Scanlan et al., 2004). In various studies in which the biological response to T1AM has been measured, changes in temperature, heart rate and cardiac output have been observed. Most strikingly, T1AM produces a profound hypothermic response when administered in vivo (Scanlan et al., 2004). The robust hypothermic action of T1AM suggests that the thyronamine could serve as a neuroprotectant, and that its receptor could be a potential therapeutic target for cryogenic agents. In this regard, T1AM has been assessed for its potential neuroprotective action in a stroke injury model. Treatment with T1AM was found to reduce infarct volume by about one third when tested in a middle cerebral artery occlusion stroke model in mice (Doyle et al., 2007). The study by Doyle et al. was the first to use endogenous chemicals including T1AM to induce prophylactic hypothermia to treat stroke, and to suggest a therapeutic use of T1AM as a cryogen. The body's varied and rapid response to T1AM, as well as the potential use of thyronamine as a cryogenic agent has made determining the mechanism of action of T1AM as well as its biological role a relevant goal of recent experimental work.

Central to recent studies of T1AM is the speculation of the involvement of trace amine associated receptor 1 (TAAR1) in its mechanism of action. TAAR1 is a G protein-coupled receptor that is activated by a wide spectrum of compounds, including common biogenic amines, trace amines, and amphetamine-like psychostimulant drugs, and plays an important role in modulating brain monoamine systems (reviewed in Xie et al., 2009). T1AM has been found to be a potent agonist of rat and mouse TAAR1 (EC₅₀: 14 nM and 112 nM, respectively) and to stimulate cAMP production in rat and mouse TAAR1-expressing cells in a dose dependent manner (Scanlan et al., 2004). It is also recognized that T1AM is a monoamine transporter inhibitor, making this TAAR1 agonist different from other TAAR1 agonists such as endogenous monoamines (e.g., trace amines and common biogenic amines) and exogenous amphetamine-like psychostimulants (e.g., amphetamine and methamphetamine) that are monoamine transporter substrates (Snead et al., 2007; Xie et al., 2007). Accordingly, the demonstrated effects of T1AM could be mediated by TAAR1 or could be a result of altered monoamine concentration via blockade of monoamine transporters, or some other mechanism. Also, TAAR1 is under current investigation as a potential target for novel therapeutics which may regulate monoaminergic function, and so determining whether TAAR1 mediates thermoregulatory responses is important with regard to medications development.

Accordingly, we took advantage of a TAAR1 knockout mouse model to directly address the question of whether TAAR1 mediates the thermoregulatory response of T1AM in vivo, and compared responses of T1AM to two trace amines, tyramine and beta-phenylethylamine (β -PEA), and two amphetamine-like psychostimulants, methamphetamine and MDMA, which also affect thermoregulation. We verified the interaction of T1AM with TAAR1 and monoamine transporters in transfected cells and striatal synaptosomes prepared from wild-type (WT) and TAAR1 knockout mice (KO). We measured cAMP production using a CRE-Luciferase (CRE-Luc) reporter assay in TAAR1 and TAAR1/DAT cells after exposure to T1AM and assessed whether DAT could potentiate TAAR1 signaling in response to T1AM, as is the case for other TAAR1 agonists that are DAT substrates (Miller et al., 2005; Xie et al., 2007). In striatal synaptosomes, we measured IC₅₀ values for T1AM binding to DAT as well as for [³H]dopamine and [³H]serotonin uptake by the DAT and SERT, respectively. A comparison of mouse synaptosomal [³H]serotonin uptake blockade, using both WT and KO mice, was also performed.

Materials and Methods

Animals

Animals were approximately 3 months old at the time of the studies and only males were tested. TAAR1 KO and WT mouse colonies were established at the New England Primate Research Center (Southborough, MA) from six pairs of heterozygous mice received as a gift from Lundbeck Research USA, Inc. (Paramus, NJ). All animals were maintained on a 12 hour light/dark schedule at a room temperature of 22 ± 1 °C with free access to food and water. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and all procedures were conducted in accordance with the Animal Experimentation Protocol approved by the Harvard Medical Area Standing Committee on Animals.

Synaptosome Preparation

Synaptosomes were prepared from adult male TAAR1 KO and WT mice as described previously (Xie and Miller, 2008). Briefly, freshly dissected striatal tissue was homogenized in cold 0.32 M sucrose ($10 \times$ volume) using a Kontes Pellet Pestle with 15 up and down strokes. The mixture was centrifuged at low speed ($1,000 \times g$, 10 minutes, 4 °C) and the supernatant carefully removed and centrifuged at high speed ($10,000 \times g$, 20 minutes, 4 °C) to yield the pelleted synaptosomes. The pellet was resuspended in ice-cold uptake buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 1 μM pargyline, 2 mg/ml glucose, 0.2 mg/mL ascorbic acid, pH 7.5) and used immediately in assays. All reagents and the pestles used in the preparation of synaptosomes were purchased from Sigma-Aldrich, St. Louis, MO.

Cell Culture

Cell lines were generated as described previously (Xie et al., 2008). Cell lines were cultured in untreated tissue culture dishes (Greiner America, Inc., Lake Mary, FL) in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 0.1 mM nonessential amino acids. Cell transfections were performed using calcium phosphate (250 mM calcium chloride and $2\times$ HBS buffer [50 mM HEPES, 1.5 mM Na_2HPO_4 (Fisher Scientific, Pittsburgh, PA), 280 mM NaCl, 10 mM KCl, 12 mM glucose, pH 7.05]). With the exception of reagents noted above, all reagents were obtained from Invitrogen Corporation, Carlsbad, CA.

Dual Luciferase Reporter Assays

Cells were plated into 48-well plates (75,000 cells/well in 0.5 mL DMEM) 24 hours prior to transfection. On the following day the cells were transfected with CRE-Luc, a reporter control construct pGL4.73 (cAMP insensitive) and either a rhesus TAAR1 expression construct or pcDNA3.1 control vector added to keep the amount of DNA used equivalent between the cells (Miller et al., 2005; Xie et al., 2007). After 24 hours, the transfection media was replaced with 0.5 mL of serum-free DMEM immediately followed by treatment with T1AM (3-iodothyronamine; synthesized according to the procedure described in Hart et al., 2006) or β -PEA (Sigma-Aldrich, St. Louis, MO) and incubated for 18 hours. Alternatively, cells were immediately exposed to various DAT blockers. The DAT blockers were CFT (WIN 35428; Organix, Inc., Woburn, MA), GBR 12909 (Sigma-Aldrich, St. Louis, MO), bupropion HCl (Burroughs-Wellcome Co., Research Park, NC), cocaine (NIDA, Bethesda, MD), indatraline (Sigma-Aldrich, St. Louis, MO) and methylphenidate HCl (Sigma-Aldrich, St. Louis, MO). All DAT blockers, T1AM and β -PEA were tested at 10 μM . A 10 mM stock solution was made fresh daily for each compound, and diluted 1:10 into DMEM for a 1mM solution. 5 μL of this solution was added into 495 μL of DMEM in

each well for a final concentration of 10 μ M. GBR 12909 is believed to be less stable in solutions so it was made and used immediately. All 10 mM stock solutions were made with water with the exception of T1AM (0.1% DMSO at final dilution) and CFT (0.1% ethanol at final dilution). All blockers were incubated with the cells for 15 min with the exception of CFT, which was incubated for 1 hour. Cells were then treated with T1AM or β -PEA for 18 hours. The passive lysis buffer (PLB) and luciferase assay substrate reagents were prepared following the manufacturer's protocol (Promega Corporation, Madison, WI). Following drug treatments, media was removed from the cells and the cells were lysed by the addition of 100 μ L of 1 $\times$ PLB into each well and shaking the plate for 1 hour at room temperature. An aliquot of the lysate (20 μ L) from each well was transferred into wells of an opaque 96-well microplate (PerkinElmer, Shelton, CT). Luciferase substrate reagents (25 μ L) were injected into each well and luciferase levels were measured as relative light units (RLUs) for 12 seconds on a Wallac 1420 multilabel counter, Victor 3V (PerkinElmer, Shelton, CT). To analyze the data, a ratio of the Firefly RLU reading and the Renilla RLU reading for each well was divided by the similar ratio for the baseline wells and changed to a percentage. In experiments utilizing DAT blockers, the baseline was derived from cells treated with the DAT blocker alone. An increase in the value above baseline indicates cAMP accumulation in response to the drug exposure.

Binding and uptake assays

Cell lines used in binding assays were grown to 80% confluence in cell media, rinsed with PBS and removed from the plate by repeated PBS wash. The cells collected were centrifuged at 1000 $\times$ g for 5 minutes and the supernatant removed. The cells were resuspended and brought to a concentration of 1.25×10^6 cells/mL in buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 1 μ M pargyline, 2 mg/mL glucose, 0.2 mg/mL ascorbic acid, pH 7.5) and used immediately in assays. In radio-receptor binding assays, equal volumes of serially diluted T1AM and 1 nM [^3H]CFT (PerkinElmer Life and Analytical Sciences, Boston, MA; 85.9 Ci/mMol) were mixed, followed by the addition of the cells in solution. This mixture was incubated for 2 hours at room temperature with the reaction terminated by centrifugation (12,500 $\times$ g, 15 minutes) and the supernatant aspirated. The remaining pellet was solubilized in 1% SDS and the radioactivity counted. The experimental points were done in triplicate. Non-specific binding was determined using cocaine at a final concentration of 30 μ M.

For the synaptosomal binding experiments, equal volumes of serially diluted T1AM and 1 nM [^3H]CFT were mixed, followed by the addition of the synaptosomes in solution. This mixture was incubated for 2 hours at room temperature and the reaction was then terminated by centrifugation (12,500 $\times$ g for 15 minutes) and aspiration of the supernatant. The remaining pellet was solubilized in 1% SDS and the radioactivity counted. The experimental points were done in triplicate. The baseline drug was cocaine at a final concentration of 30 μ M.

When synaptosomes were used for [^3H]dopamine uptake experiments, serially diluted T1AM was added to the synaptosomes and allowed to incubate for 15 minutes at room temperature. 10 nM [^3H]dopamine (PerkinElmer Life and Analytical Sciences, Boston, MA; 48.1 Ci/mMol) was added to the mixture and incubated for 15 minutes at room temperature. The uptake was terminated by plunging the vials into an ice water bath. After chilling for at least 2 minutes, the vials were centrifuged as described above. The baseline drug was indatraline at a final concentration of 10 μ M.

For the synaptosomal [^3H]serotonin uptake experiments, serially diluted T1AM was added to the synaptosomes and allowed to incubate for 30 minutes. 50 nM [^3H]serotonin (PerkinElmer Life and Analytical Sciences, Boston, MA; 28.1 Ci/mMol) was added and the

mixture was incubated for 15 minutes at room temperature. The uptake was terminated by plunging the vials into an ice water bath. After chilling for at least 2 minutes, the vials were centrifuged as described above. The baseline drug was fluoxetine (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 μ M.

Body temperature measurement

All animals were relocated from the colony into a behavioral study holding room and habituated to the new environment for at least 72 hours prior to testing. During this time, all animals were weighed, marked for identification, and had their lower backs shaved (Oster Turbo A5 Clippers with an Elite CryogenX-AgION size 50 blade, McMinnville, TN). On the testing day, each animal was individually housed in a new, bedding free, open clean cage and relocated to a testing area for 1 h prior to receiving a single intraperitoneal (i.p.) injection of 50 mg/kg T1AM, 25 mg/kg T1AM, 50 mg/kg β -PEA, 50mg/kg tyramine (Sigma-Aldrich, St. Louis, MO), 25 mg/kg (+/-)MDMA (NIDA, Bethesda, MD), 3 mg/kg (+)methamphetamine (Sigma-Aldrich, St. Louis, MO) or saline. Body temperature readings were captured using a wireless infra-red thermometer (La Crosse Technology Ltd., La Crescent, MN) over the shaved back area of the mouse at 5 minutes prior to the drug injection and at post injection times: 15, 30, 45, 60, 75, 90, 120, 150, 180, 240, and 320 minutes. Room temperature of 22 ± 1 °C was continuously monitored throughout the experiment. The research assistant obtaining the temperature readings was blind to the treatment conditions of the animals.

Data analysis

A one-way ANOVA was used to test for significant differences between doses of T1AM or β -PEA in HEK cells transfected with CRE-Luc and pGL4.73 only. A t-test was used to compare the effect of 10 μ M T1AM or 10 μ M β -PEA on TAAR1 transfected HEK cells either with or without a DAT co-transfection. One-way ANOVA with Dunnett's correction for multiple testing was used to compare the effect of DAT blockers on TAAR1-transfected cells in the presence or absence of DAT. Binding and uptake assays in cells and synaptosomes vary in radiation counting between cells and synaptosome preparations from different sources, and accordingly, data are normalized to the maximum level or the baseline and expressed as percentage values in each set of assays. Data for the drug effects on thermoregulation were normalized to the saline controls prior to statistical analyses. A two-way ANOVA with repeated measures was employed to determine if there was a significant difference in thermoregulatory responses over all time points between WT and KO mice and across different drug treatments. For each drug treatment in each set of mice, a one way ANOVA followed by Bonferroni post-hoc analysis was also used to determine significance of each time point versus the baseline temperature reading taken at the -5 time point. All results were finalized as mean $\pm$ SEM of the indicated number of observations.

Results

T1AM activation of TAAR1 and comparison to β -PEA

Neither T1AM nor β -PEA significantly stimulated CRE-Luc expression in HEK cells transfected with CRE-Luc and pGL-4.73 across concentrations tested (100 nM, 1 μ M and 10 μ M; data not shown). T1AM (10 μ M) treatment resulted in a 680% increase in RLU in TAAR1 cells relative to vehicle treatment, demonstrating that T1AM acts as an agonist at rhesus monkey TAAR1 (Figure 1A). Previous work in our lab has demonstrated a robust enhancement of TAAR1 signaling when DAT is also present, presumably because the TAAR1 agonists previously tested (e.g., β -PEA, tyramine, dopamine, methamphetamine) are also substrates for the DAT, and the TAAR1 receptor is largely intracellularly sequestered and associated with an intracellular membrane fraction (Miller et al., 2005; Xie

et al., 2008b). Accordingly, we assessed whether the presence of DAT would enhance TAAR1 signaling in response to T1AM. T1AM (10 μ M) exposure resulted in a 760% increase in TAAR1-DAT cells relative to vehicle treatment, which did not significantly differ from the response of T1AM in the TAAR1 cells. In contrast and similar to our previous findings (Xie and Miller, 2008), β -PEA (10 μ M) resulted in a 531% increase in RLU in TAAR1 cells versus a 1750% increase in RLU in TAAR1-DAT cells ($p < 0.001$; Figure 1A). The data demonstrate a significant enhancement by DAT of TAAR1 signaling in response to β -PEA but not T1AM.

Next, we assessed whether a series of DAT blockers could alter TAAR1 signaling in response to T1AM or β -PEA in TAAR1-DAT cells. None of the DAT blockers used are agonists at TAAR1 nor cause any elevation in CRE-Luc expression in TAAR1 cells (data not shown). TAAR1 and TAAR1-DAT cells were exposed to the various DAT blockers at a concentration of 10 μ M or vehicle (no DAT blocker) and were then treated with 10 μ M T1AM or 10 μ M β -PEA for 18 hours. Pretreatment with DAT blockers had no significant effect on the response to 10 μ M T1AM in TAAR1-DAT cells (Figure 1B), whereas all DAT blockers significantly attenuated the enhanced response to 10 μ M β -PEA in the TAAR1-DAT cells, to the same level as in TAAR1 cells without DAT ($p < 0.01$, Figure 1C).

T1AM binding to DAT and blockade of DAT and SERT in striatal synaptosomes

T1AM binding to DAT was measured by competitive binding versus [3 H]CFT in stable human DAT-transfected cells and in WT and KO striatal synaptosomes. The IC_{50} was determined to be $8.0 \pm 1.2 \times 10^{-7}$ M ($n = 2$; each point done in triplicate; Figure 2A), 7.2×10^{-7} M in WT synaptosomes and 8.1×10^{-7} M in KO synaptosomes ($n = 1$; each point done in triplicate; Figure S1, supplemental data). The IC_{50} of T1AM inhibition of [3 H]dopamine or [3 H]serotonin uptake versus at 15 minutes was also measured in WT and KO striatal synaptosomes. The IC_{50} of T1AM inhibition of [3 H]dopamine was $1.4 \pm 0.5 \times 10^{-6}$ M in WT synaptosomes and $1.2 \pm 0.4 \times 10^{-6}$ M in KO synaptosomes ($n = 2$; each point done in triplicate; Figure 2B). The IC_{50} of T1AM inhibition of [3 H]serotonin was $4.5 \pm 0.6 \times 10^{-6}$ M in WT synaptosomes and $4.6 \pm 1.1 \times 10^{-6}$ M in KO synaptosomes ($n = 2$; each point done in triplicate; Figure 2C).

Thermoregulatory responses of T1AM, β -PEA, tyramine, MDMA and methamphetamine in WT and KO mice

WT and KO mice were exposed to a single intraperitoneal injection of drug or saline and body temperature was measured over time. WT and KO mice did not significantly differ in body temperature at the baseline temperature measurement collected at the -5 time point (WT: 36.3 ± 0.7 °C, $n = 71$; KO: 36.2 ± 0.7 °C, $n = 70$). Both WT and KO mice showed a dose-dependent hypothermic response to T1AM, without a significant difference in the magnitude or duration of this response (Figure 3A). Similarly, the thermoregulatory response characteristics for other tested drugs were not significantly different between the WT and KO mice (Figure 3 B-E). Further analyses by one way ANOVA were performed on the normalized data for each drug treatment to determine whether the drug treatments caused significant thermoregulatory responses relative to the baseline temperature measurement collected at the -5 time point. All drug treatments except for tyramine showed significant effects on thermoregulation (summarized in supplemental Table S1). Whereas WT mice demonstrated a rapid and short-lived hyperthermic response to 50 mg/kg β -PEA, the KO mice displayed an additional hypothermic rebound effect that was not observed in the WT mice (60, 75 and 90 min time points; Figure 3B), though this did not reach statistical significance. Also, the hyperthermic response to both MDMA and methamphetamine treatments reached significance at earlier time points in the KO mice (15 min and 30 min, respectively) compared with the WT mice (30 min and 75 min, respectively). Saline-treated

mice showed a gradual decrease in body temperature over time and maintained this lower temperature for the entire duration of the experiment (Figure 3F). This was likely due to the absence of bedding in the test cage, and appears to be a typical finding in mice (Fantegrossi et al., 2008). Accordingly, we chose to present data for the drug effects (Figure 3 A-E) as normalized to the saline controls (Figure 3F) rather than the actual temperature change recorded in each mouse.

Discussion

The data presented here demonstrate that T1AM is an agonist at the rhesus monkey TAAR1 receptor and, unlike other TAAR1 agonists that we have tested to date using the CRE-luciferase assay, reporter expression indicative of cAMP accumulation is unaffected by the presence of DAT. We find that T1AM binds to the human DAT in transfected cells, and to the mouse DAT in WT synaptosomes and KO synaptosomes with similar potency. We also demonstrate that T1AM blocks [³H]dopamine uptake and [³H]serotonin uptake in WT and KO striatal synaptosomes with similar potency. And, we show that the time-dependent thermoregulatory responses to the TAAR1 agonists T1AM, β -PEA, tyramine, MDMA and methamphetamine are highly similar and do not significantly differ in WT and KO mice, indicating that TAAR1 is not involved in mediation of thermoregulation and that the KO mice do not have a compensatory phenotype with regard to pharmacologically-induced thermoregulatory responses. Accordingly, the robust cryogenic action of T1AM is not mediated via TAAR1, nor is the hyperthermic response to psychostimulants.

TAAR1 mediation in the physiological effects of T1AM was initially suggested by the finding that the rank order of potency of T1AM and related analogs at TAAR1 matched their potency to decrease body temperature in vivo (Scanlan et al., 2004; Hart et al., 2006). Also, TAAR1 mRNA is expressed in the mouse arcuate nucleus (ARC) and has been detected in human and rhesus monkey hypothalamus (Borowsky et al., 2001; Branchek and Blackburn, 2003; Lindemann et al., 2008), brain areas important in the regulation of food intake and energy balance. Dhillo et al (2009) have recently investigated the putative role of T1AM in appetite regulation and energy expenditure and found that following intracerebroventricular administration of T1AM into the ARC in rats, a three-fold increase in food consumption was observed, along with a significant increase in c-fos expression, indicating the ARC neurons were activated by T1AM to release cAMP, consistent with the possibility of mediation via TAAR1 receptor activation. Additionally, recent work has shown a connection between TAAR1, T1AM and hyperglycemia, suggesting a possible control of metabolism (Klieverik et al., 2009) and insulin secretion (Regard et al., 2007). These findings warranted our assessment of the role of TAAR1 in T1AM-induced thermoregulatory responses in the TAAR1 knockout mice.

In agreement with our current findings, Frascarelli et al (2008) found that the rank order of potency for negative inotropic effects on isolated rat hearts as measured by cardiac output did not match that from cAMP production of either TAAR1 or TAAR4 in cells, and suggested that the effect on heart function is mediated by a different TAAR subtype than TAAR1 or TAAR4. It was suggested that a possible target of T1AM in the rat heart is TAAR8a, where the mRNA content of TAAR8a is 58 times that of TAAR1 (Chiellini et al., 2007). The TAAR family of receptors is rather large, and many are still orphan receptors, so it is possible that other TAAR family members are sensitive to T1AM and drive the thyronamine-mediated effects on hypothermia as well. Other results demonstrate that a primary target of T1AM is the central nervous system. Small doses of T1AM administered intracerebroventricularly can mirror larger systemic administration (Klieverik et al., 2009). In studies using the Djungarian hamster *Phodopus sungorus*, T1AM administration brought about a rapid decrease in metabolic rate, body temperature, respiratory quotient, ketonuria

and loss of body fat indicating a change in fuel utilization from carbohydrate to lipid (Braulke et al., 2007). These authors suggested that the hypothermia is a result of reduced metabolic rate and that T1AM results in depressed metabolism by decreasing carbohydrate utilization and increasing lipid metabolism. Another possible target of T1AM might be α_{2A} adrenergic receptors. The hyperglycemia induced in WT mice by T1AM is lost in α_{2A} adrenergic null mice and blocked in WT mice that are treated with the α_{2A} adrenergic antagonist yohimbine, although the concentration of T1AM needed to activate the receptor is in the micromolar range, a level high above that measured in tissue (Regard et al., 2007).

Nevertheless, our data demonstrate that, similar to rodent, T1AM is an agonist at rhesus monkey TAAR1. We also confirm that T1AM is a blocker at the human and mouse DAT as has been reported previously (Scanlan et al., 2004; Snead et al., 2007). It had been shown that T1AM (10 μ M) significantly inhibited [3 H]dopamine, [3 H]norepinephrine and [3 H]serotonin transport by 77.6%, 72.1% and 42.2% respectively, in rat brain synaptosomal preparations, whereas T1AM inhibited transport at the human dopamine transporter (DAT) and the human norepinephrine (NET) but failed to do so at the human serotonin transporter (SERT) in experiments utilizing monoamine transporter-transfected HeLa cells (Snead et al., 2007). We also demonstrated that T1AM can block the SERT in WT and KO mice, clarifying its role as a monoamine transporter blocker. Further, the current data suggest that T1AM may differ from other TAAR1 agonists in its ability to access the receptor in that T1AM activation of TAAR1 is not enhanced in the presence of DAT (Xie et al., 2007; 2008a). Particularly, our recent work has focused on methamphetamine, which produces very large enhancements in TAAR1 signaling in the presence of DAT. We have speculated that this enhanced signaling could contribute to adaptive cellular responses that are associated with addiction (Xie and Miller, 2009b). Accordingly, cellular actions of thyronamine that are mediated via TAAR1 could be predicted to be similar to other TAAR1 agonists in cells devoid of monoamine transporters, but different at monoaminergic cells where it will block monoamine transporters and also lack the enhanced signaling properties of the substrates agonists. Its ability to block monoamine transporters suggests that it would permit elevations in extracellular monoamine concentrations which in turn could produce downstream effects different from those of other TAAR1 agonists that are monoamine transporter substrates.

It has been reported that an i.p. injection of β -PEA in mice produces an initial hyperthermia followed by a prolonged hypothermia (Mantegazza and Riva, 1963; Jackson, 1975), although the hypothermic rebound effect at 50 mg/kg is quite subtle (Jackson, 1975). Our data reveals some indication of this effect in the KO but not the WT mice, though a more systematic investigation that directly addresses this question is required to reveal whether there are any significant differences between how WT and KO mice respond to β -PEA with regard to thermoregulation. We also observed that the hyperthermic response to both MDMA and methamphetamine treatments reached significance at earlier time points in the KO mice compared with the WT mice, perhaps indicative of a greater sensitivity to amphetamines in the KO mice. Lastly, we observed that tyramine did not cause significant thermoregulatory responses in either WT or KO mice, exemplifying that not all TAAR1 agonists cause significant changes in thermoregulation.

Accordingly, data presented here demonstrates that TAAR1 does not mediate the thermoregulatory responses of T1AM nor those of β -PEA or amphetamine-like psychostimulants. The very robust cryogenic actions of T1AM suggest that it may be a therapeutically-relevant cryogen, and its other physiological effects warrant further investigation into its biological role and mechanism of action. Importantly, compounds under development currently that target TAAR1 will likely not be limited in potential therapeutic efficacy by thermoregulatory side effects, nor are they likely to be cryogens

useful as neuroprotective agents in stroke. Nevertheless, T1AM produces robust thermoregulatory responses and is an agonist at TAAR1, warranting further investigation of its endogenous functions as well as its usefulness as a therapeutic agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Activation of TAAR1 by T1AM and comparison to β-PEA

T1AM and β-PEA activation of TAAR1 and TAAR1-DAT cells was determined by a CRE-Luc reporter assay and are reported as percent change in relative light units (RLU). A. T1AM (10 μM) resulted in a 680% increase in RLU in TAAR1 cells versus a 760% RLU increase in TAAR1-DAT cells, whereas β-PEA (10 μM) resulted in a 531% increase in RLU in TAAR1 cells and 1750% increase in RLU response in TAAR1-DAT cells (n=3). B and C. TAAR1-DAT cells were exposed to various DAT blockers at (10 μM) or to vehicle (no DAT blocker), and were then treated with T1AM (10 μM) or β-PEA (10 μM). Pretreatment with DAT blockers had no significant effect on the response to 10 μM T1AM in TAAR1-DAT cells, whereas all DAT blockers significantly attenuated the enhanced response to 10 μM β-PEA in the TAAR1-DAT cells (n=3). All data shown are values of mean ± SEM. **: p < 0.01; ***: p < 0.001.

Figure 2. T1AM binding and uptake in cells and synaptosomes

A. T1AM binding to the dopamine transporter was measured by competitive binding versus [3H]CFT in DAT/HEK cells. The IC_{50} was $8.0 \pm 1.2 \times 10^{-7}$ M (n=2). B. The IC_{50} of T1AM versus [3H]dopamine was $1.4 \pm 0.5 \times 10^{-6}$ M for WT and $1.2 \pm 0.4 \times 10^{-6}$ M for KO striatal synaptosomes (n=2). C. The IC_{50} of T1AM versus [3H]serotonin was $4.5 \pm 0.6 \times 10^{-6}$ M for WT and $4.6 \pm 1.1 \times 10^{-6}$ M for KO striatal synaptosomes (n=3). All data shown are values of mean $\pm$ SEM.

Figure 3. Thermoregulatory responses of T1AM, β -PEA, tyramine, MDMA, methamphetamine and saline in WT and KO mice

Wild type (WT) and TAAR1 knockout (KO) male mice were treated with 25 mg/kg or 50 mg/kg T1AM (A), 50 mg/kg β -PEA (B), 50 mg/kg tyramine (C), 25 mg/kg MDMA (D), 3 mg/kg methamphetamine (E) or saline (F). The number of mice in each group is indicated. Body temperature readings were captured using a wireless infra-red thermometer held over the shaved back area of each mouse at 5 minutes prior to the drug injection (-5) and at 15, 30, 45, 60, 75, 90, 120, 150, 180, 240, and 320 minutes post injection. Data was normalized to the saline controls (shown in F). A two-way ANOVA with repeated measures was used to determine if there was a significant difference in the thermoregulatory responses over all time points between WT and KO mice and across different drug treatments. There were no significant main or interactive effects. For each drug treatment, a one way ANOVA was

used to determine statistical significance of individual time points versus the baseline (-5) temperature (detailed in supplemental Table S1). All data shown are values of mean $\pm$ SEM. *, $p < 0.05$ or lower versus the -5 time point for both WT and KO analyses except as indicated: in A, *ns* refers to KO data; in D and E, *ns* refers to WT data. See supplemental Table S1 for more detail.