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β-Phenylethylamine Alters Monoamine Transporter Function via Trace Amine-Associated Receptor 1: Implication for Modulatory Roles of Trace Amines in Brain

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Running Title: β-PEA Modulation of Transporters via TAAR1

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Abbreviations: $5HT_{1A}$ and $5HT_{1B}$, subtype A and B of serotonin receptor 1; α_{2A} and α_{2B}, subtype A and B of alpha adrenal receptor 2; CRE-Luc, cAMP response element-driven luciferase reporter; DAT, dopamine transporter; D2s, dopamine D2 receptor short isoform; DMEM, Dulbecco's modified Eagle's medium; NET, norepinephrine transporter; SERT, serotonin transporter; TAAR1, trace amine-associated receptor 1; SEM, standard error of the mean.

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Abstract

Brain monoamines include common biogenic amines (dopamine, norepinephrine and serotonin) and trace amines (β-phenylethylamine (β-PEA), tyramine, tryptamine and octopamine). Common biogenic amines are well established as neurotransmitters, but the roles and functional importance of trace amines remain elusive. Here, we re-evaluated the interaction of trace amines with trace amine-associated receptor 1 (TAAR1) and investigated effects of β-PEA on monoamine transporter function and influence of monoamine autoreceptors on TAAR1 signaling. We confirmed that TAAR1 was activated by trace amines and demonstrated that TAAR1 activation by β-PEA significantly inhibited uptake and induced efflux of [^{3}H]dopamine, [^{3}H]norepinephrine and [^{3}H]serotonin in transfected cells. In brain synaptosomes, β-PEA significantly inhibited uptake and induced efflux of [^{3}H]dopamine and [^{3}H]serotonin in striatal and [^{3}H]norepinephrine in thalamic synaptosomes of rhesus monkeys and wild type mice, but it lacked the same effects in synaptosomes of TAAR1 knockout mice. The effect of β-PEA on efflux was blocked by transporter inhibitors in either the transfected cells or wild type mouse synaptosomes. We also demonstrated that TAAR1 signaling was not affected by monoamine autoreceptors at exposure to trace amines that we show have poor binding affinity for the autoreceptors relative to common biogenic amines. These results reveal that β-PEA alters monoamine transporter function via interacting with TAAR1 but not monoamine autoreceptors. The functional profile of β-PEA may reveal a common mechanism by which trace amines exert modulatory effects on monoamine transporters in brain.

Introduction

Trace amines, including β-phenylethylamine (β-PEA), tyramine, tryptamine and octopamine, have been known to be heterogeneously distributed in mammalian brain tissues for decades, and their distribution spatially parallels the origins and terminal projection areas of the monoaminergic neurons (Durden et al., 1973; Philips et al., 1978). In monoaminergic neurotransmission, common biogenic amines, including dopamine, norepinephrine and serotonin, are well-established as neurotransmitters, which are synthesized from their precursor amino acids in neurons, stored in vesicles of neuron terminals and released into the synaptic clefts to interact with both presynaptic and postsynaptic receptors. Although trace amines share similarities with common biogenic amines in structure, metabolism and distribution, their roles and functional profiles in the brain are far from being as clear as for common biogenic amines.

With high turnover rates, trace amines are dynamically regulated by enzymes and are present in the brain at generally low levels (Durden and Philips 1980; Durden and Davis, 1993). No dedicated neurons to date have been found to use any of the trace amines exclusively, and therefore for a long period, the trace amines were thought of as by-products or false neurotransmitters (Parker and Cubeddu, 1988; Janssen et al., 1999). However, the rate in synthesis of trace amines is equivalent to that of dopamine and norepinephrine (Durden and Philips 1980; Paterson et al., 1990), and altered levels of the trace amines are associated with various neuropsychiatric disorders (Boulton, 1980; Davis and Boulton, 1994; McClung and Hirsh, 1999; D'Andrea et al. 2003) which are hallmarked by changes in monoaminergic activity. These properties and amphetamine-like effects of the trace amines (Parker and Cubeddu, 1988; Paterson, 1993; Barroso and Rodriguez, 1996) suggest that trace amines may serve as

neuromodulators in brain. Over the years, several studies have reported specific binding sites for [^{3}H]β-PEA, [^{3}H]tryptamine and [^{3}H]tyramine in brain (Hauger et al., 1982; Kellar and Cascio, 1982; Vaccari, 1986), which displayed a high correlation in their density with levels of endogenous trace amines (Karoum et al., 1981; Hauger et al., 1982).

In 2001, a family of related G protein-coupled receptors, now referred to as trace amine-associated receptors (TAARs), were identified and the first solid evidence of TAARs in mammalian brain was reported (Borowsky et al., 2001). Among TAARs, TAAR1 has been well established to be functionally related with trace amines (Borowsky et al., 2001; Bunzow et al., 2001), and TAAR1 signaling has been demonstrated to be associated with the cAMP signaling pathway (Borowsky et al., 2001; Bunzow et al., 2001; Miller et al., 2005; Xie et al., 2007b). We previously revealed that TAAR1 is widely expressed in rhesus monkey brain, notably in monoaminergic nuclei (Miller et al., 2005; Xie et al., 2007b), and that rhesus monkey TAAR1 responds to a wide spectrum of chemicals, including trace amines (Miller et al., 2005, Xie et al., 2007b). However, the functional interaction of TAAR1 with trace amines and the role that TAAR1 plays in the brain remain unclear. Our previous data showed that TAAR1 signaling is enhanced by monoamine transporters and that TAAR1 activation can modulate DAT kinetics *in vitro* (Miller et al., 2005; Xie et al., 2007b; Xie and Miller, 2007). Our data also showed co-localization of TAAR1 and DAT in a subset of dopamine neurons in the rhesus monkey and mouse substantia nigra (Xie et al., 2007b; Xie and Miller, 2007). We have also observed TAAR1 expression in NET-positive neurons in the locus coerulius of rhesus monkey (unpublished data). Lindemann et al. recently reported TAAR1 expression in serotonin neurons in the dorsal raphe nucleus (Lindemann et al., 2007). These findings suggest that TAAR1 may serve as a

presynaptic regulator in functional modulation of monoamine transporters. With respect to monoamine autoreceptors, it has been reported that their activation by monoamine neurotransmitters provides feedback regulation of monoamine neurotransmitter release (Hjorth et al., 2000; Garcia et al., 2004), but it is unknown whether trace amines exert any effects via monoamine autoreceptors.

In this study, we evaluated the interaction of trace amines with TAAR1 and monoamine autoreceptors, and investigated whether monoamine autoreceptors influence TAAR1 signaling and monoamine transporter function in response to trace amines. We used transfected HEK293 cells and brain synaptosomes derived from non-human primates, wild-type mice and TAAR1 knockout mice to investigate the effects of β-PEA activation of TAAR1 on the uptake and efflux function of DAT, norepinephrine transporter (NET) and serotonin transporter (SERT). The data confirm that all the trace amines tested recognize and excite TAAR1 and reveal that trace amines do not interact with monoamine autoreceptors to alter TAAR1 activity. The data also reveal that β-PEA alters monoamine transporter function via interacting with TAAR1 but not monoamine autoreceptors, which may suggest a common mechanism by which trace amines exert a modulatory role on monoamine transporters in brain.

Methods

Materials. β-phenylethylamine, indatraline, methylphenidate, desipramine, citalopram, raclopride, SKF86466, and methiothepin were purchased from Sigma Aldrich (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM), 100× non-essential amino acids, fetal bovine serum, 100× penicillin/streptomycin, geneticin and trypsin/EDTA were purchased from InVitrogen (Carlsbad, CA). Dual-Luciferase® Reporter Assay System kit, pGL4.73 and Passive Lysis Buffer (5×) were obtained from Promega (Madison, WI). [^{3}H]Dopamine (60 Ci/mmol), [^{3}H]norepinephrine (49.5 Ci/mmol) and [^{3}H]serotonin (20.3 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). ReadySafe™ scintillation cocktail was obtained from Beckman Coulter (Fullerton, CA). Rabbit anti-human trace amine receptor 1 antibody was obtained from ABR Affinity BioReagents (Golden, CO). Goat anti-rabbit IgG (H+L) was purchased from Chemicon International (Temecula, CA). SuperSignal West Pico Chemiluminescent Substrate was from Pierce Chemical (Rockford, IL). The expression constructs of human α_{2A}, α_{2B}, 5-HT_{1A} and 5-HT_{1B} were purchased from UMR cDNA Resource Center (Rolla, MO). Rhesus monkey dopamine D2 receptor short isoform (D2s) was constructed in pcDNA3.1 (+) in our laboratory.

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 or maintenance of selection of stable cell lines. Calcium phosphate transfection was performed as described elsewhere (Xie et al., 2007a, 2007b) to introduce the necessary receptor and transporter into different cells for assays.

For co-transfection, the ratio of constructs and the amount of total DNA was held constant with pcDNA3.1.

Generation of Cell Lines for Assays. All cell lines in this study are generated originally from HEK293 cells (American Type Culture Collection, Manassas, VA). *TAAR1*, HEK293 cells transiently transfected with rhesus monkey TAAR1, CRE-Luc, and pGL4.73; *HEK*, HEK293 cells transiently transfected with pcDNA 3.1, CRE-Luc, and pGL4.73; *D2s-TAAR1*, stable D2s cells transiently transfected with rhesus monkey TAAR1, CRE-Luc, and pGL4.73; *α_{2A}-TAAR1*, stable α_{2A} cells transiently transfected with rhesus monkey TAAR1, CRE-Luc, and pGL4.73; *α_{2B}-TAAR1*, stable α_{2B} cells transiently transfected with rhesus monkey TAAR1, CRE-Luc, and pGL4.73; *$5HT_{1A}$-TAAR1*, stable $5HT_{1A}$ cells transiently transfected with rhesus monkey TAAR1, CRE-Luc, and pGL4.73; *$5HT_{1B}$-TAAR1*, stable $5HT_{1B}$ cells transiently transfected with rhesus monkey TAAR1, CRE-Luc, and pGL4.73; *DAT*, HEK293 cells transiently transfected with human DAT; *NET*, HEK293 cells transiently transfected with human NET; *SERT*, HEK293 cells transiently transfected with human SERT; *TAAR1-DAT*, stable TAAR1 cells transiently transfected with human DAT; *TAAR1-NET*, stable TAAR1 cells transiently transfected with human NET; *TAAR1-SERT*, stable TAAR1 cells transiently transfected with human SERT; *D2s-DAT*, stable D2s cells transiently transfected with human DAT; *α_{2A}-NET*, stable α_{2A} cells transiently transfected with human NET; *$5HT_{1B}$-SERT*, stable $5HT_{1B}$ cells transiently transfected with human SERT.

SDS-PAGE and Western Blotting. Cells were grown to more than 90% confluence and collected through treatment with ice-cold 0.05% trypsin/EDTA and centrifugation at 1,000g for 5 min at 4°C. The cell pellets were rinsed once with ice-cold phosphate-buffered saline and

centrifuged again. Cell lysates were prepared by addition of ice-cold radioimmunoprecipitation assay buffer (150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) in a ratio of 10^7 cells/ml to break cells for 30 min at 4°C, which was supplemented with 1× protease inhibitor cocktail. The cell lysates were centrifuged at 12,000g for 10 min at 4°C, and the supernatants were analyzed for protein concentration using Quick Start™ Bradford Protein Assay (Bio-Rad, Hercules, CA). The supernatant was mixed with Bio-Rad Laemmli sample buffer at 1:2 (v/v), heated for 5 min at 95°C, and centrifuged at 12,000g for 5 min at 4°C. Proteins (10 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide running gel and 4% acrylamide stacking gel) and electrotranslocated onto polyvinylidene difluoride membrane (0.45 μm). The membrane was rinsed three times with TTBS (25 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) and blocked for 12 h at 4°C in TTBS with 5% nonfat dry milk, and then incubated with rabbit anti-human trace amine receptor 1 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).

Animals and Synaptosome Preparation. Monkey brains were obtained from juvenile rhesus (*Macaca mulatta*) and tamarin (*Sanguinus Oedipus*) monkeys that were sacrificed for other purposes or euthanized for necropsy, and the striatum (only putamen) and thalamus were collected. TAAR1 knockout and wild-type mouse colonies were established in New England Primate Research Center (Southborough, MA) from six pairs of heterozygous mice given to us as a gift by Lundbeck Research USA, Inc. (Paramus, NJ). The mice were sacrificed at 8-10 weeks old to collect the striatum and thalamus. The operations were conducted in accordance

with the Animal Experimentation Protocol approved by the Harvard Medical Area Standing Committee on Animals. Fresh tissues were homogenized in 1.5 ml Eppendorf's centrifuge tubes with 10 volume of ice-cold unbuffered 0.32 M sucrose solution (pH 7.0), using a motor-driven pellet pestle purchased from Sigma Aldrich (St. Louis, MO) for 30 up-and-down strokes. The homogenate was centrifuged (1,000g, 10 min at 4°C) to yield crude nuclear pellet and low speed supernatant. The low speed supernatant fraction was carefully transferred into another fresh tube and centrifuged at 10,000g and 4°C for 20 min to yield synaptosome-containing pellet. The pellet was resuspended in an appropriate volume of ice-cold uptake buffer (a modified Kreb's 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) for further assays.

Dual Luciferase Reporter Assay. Cells were placed in wells of 48-well plates (75,000 cells/well in 0.5 ml DMEM). At 60-70% cell confluence, a luciferase reporter construct CRE-Luc (cAMP sensitive) and a reporter control construct pGL4.73 (cAMP irresponsive) were introduced into the cells along with rhesus monkey TAAR1. Following 12 h incubation under transfection condition, the cells were exposed to vehicle or different concentrations (10^{-9}-10^{-4} M) or 1 μM of trace amines in serum-free DMEM for 18 h. Passive lysis buffer (PLB) and luciferase assay substrate reagents were prepared according to manufacturer's protocol. 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 RLUs for 12 sec on a Wallac 1420 multilabel counter, Victor 3V (PerkinElmer, Shelton, CT).

Binding Assays. The binding assays were performed according to procedures described elsewhere (Xie et al., 2005). Briefly, stable D2s, α_{2A}, α_{2B}, $5HT_{1A}$ and $5HT_{1B}$ cells were placed in 145 mm cell culture dishes (1.5×10^5 cells/ml) and grown to approximately 90% confluence, dispersed with ice-cold Trypsin/EDTA, and collected via centrifugation at 1000g for 5 min. The cell pellets were rinsed once with ice-cold phosphate-buffered saline (PBS, pH 7.4) and resuspended in serum-free DMEM at a density of 5×10^6 cells/ml. The cell suspension (100 μl) was added into binding buffer (25 mM HEPES, 120 mM NaCl, 1.5 mM $CaCl_2$, 5 mM KCl, and 1.5 mM $MgCl_2$, pH 7.4) to perform competition binding assay in which whole cells were incubated at 25°C for 1 h with [^{3}H]dopamine (20 nM), [^{3}H]norepinephrine (10 nM) or [^{3}H]serotonin (10 nM), along with different concentrations (10^{-10}~10^{-4} M) of each of the trace amines or common biogenic amines in a final volume of 500 μl. Nonspecific binding was determined in the presence of 10 μM of raclopride (D2s), SKF86466 (α_{2A} and α_{2B}) or methiothepin (5-HT_{1A} and 5-HT_{1B}). The bound radiant was separated from the free by rapid filtration through a Brandel 24-well harvester using Whatman GF/C glass fiber filter paper, presoaked in a 0.25% solution of polyethyleneimine in water. The filter patches with trapped cells were rinsed three times with 25 mM HEPES buffer (pH 7.4, pre-cooled to 4°C), and placed in scintillation vials with 4.0 ml of Beckman ReadySafe scintillation cocktail and counted on Beckman LS6000IC scintillation spectrophotometer (Fullerton, CA) for 1 min/sample.

[^{3}H]Monoamine Uptake Assays. Cells were placed in 145 mm cell culture dishes (1.5×10^5 cells/ml) and grown up to 60-70% confluence for transfection. Following transfection (incubation under transfection condition for 24 h and then in fresh growth medium for 12 h), the cells were harvested and prepared into suspension at a density of 5×10^6 cells/ml. Brain

synaptosomes were prepared from fresh striatal and thalamic tissues of monkey and mouse brains. 50 μl of the cell suspension or synaptosome preparation was added into 1.5 ml Eppendorf's centrifuge tubes and pretreated with vehicle or 1 μM of β-PEA in serum-free DMEM (for cells) or in uptake buffer (for synaptosomes) at 25°C for 10 min. Following the drug pretreatment, the samples were centrifuged at 1,000g (for cells) or at 10,000g (for synaptosomes) at 4°C for 3 min and washed twice with 1 ml of ice-cold serum-free DMEM (for cells) or ice-cold uptake buffer (for synaptosomes). [^{3}H]dopamine (10 nM), [^{3}H]norepinephrine (20 nM) or [^{3}H]serotonin (20 nM) diluted with serum-free DMEM or uptake buffer were then used to load the cells or synaptosomes at 25°C for 5 min. The samples pretreated with serum-free DMEM (for cells) or uptake buffer (for synaptosomes) only were taken as baseline (100% uptake). For time-course uptake assays, synaptosome samples were exposed to [^{3}H]dopamine (10 nM), [^{3}H]norepinephrine (20 nM) or [^{3}H]serotonin (20 nM) only or their mixture with 100 nM β-PEA at 25°C in uptake buffer for 1, 2, 3, 4, 5, 10, 20, 30 min. The uptake of the synaptosomes at 30 min in [^{3}H]dopamine, [^{3}H]norepinephrine or [^{3}H]serotonin only was taken as maximal uptake (100%). Non-specific uptake was defined in the presence of 10 μM indatraline for cells, or 10 μM of methylphenidate, desipramine or citalopram for synaptosomes. Uptake reactions were terminated by addition of 1 ml of ice-cold serum-free DMEM (for cells) or uptake buffer (for synaptosomes) into the tubes and immediate centrifugation at 1,000g (for cells) or 10,000g (for synaptosomes) at 4°C for 3 min. The resulted pellets were rinsed twice with 1 ml of ice-cold serum-free DMEM (for cells) or uptake buffer (for synaptosomes) through centrifugation as above. The washed pellets were incubated in 1 × PLB buffer for 30 min on a shaking platform at 200 rpm, and then transferred into scintillation vials containing 4 ml of Beckman ReadySafe

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scintillation cocktail and counted on Beckman LS6000IC scintillation spectrophotometer (Fullerton, CA) for 1 min/sample.

[^{3}H]Monoamine Efflux Assays. Cells were placed in wells of 48-well plates (75,000 cells/well in 0.5 ml medium) and grown up to 60-70% confluence for transfection (incubation under transfection condition for 24 h and then in fresh growth medium for 12 h). Brain synaptosomes were prepared from fresh striatal and thalamic tissues of monkey and mouse brains. The cells and synaptosomes were loaded with [^{3}H]dopamine (10 nM), [^{3}H]norepinephrine (20 nM) or [^{3}H]serotonin (20 nM) at 25°C for 20 min in serum-free DMEM (for cells) or uptake buffer (for synaptosomes), and the assays were conducted in plate wells for cells and in 1.5 ml Eppendorf's tubes for synaptosomes. [^{3}H]Monoamine uploading is terminated by addition of 1 ml of serum-free DMEM into the wells or uptake buffer into the tubes and immediate aspiration of the DMEM or centrifugation at 10,000g at 4°C for 3 min (for synaptosomes). Non-specific loading was defined in the presence of 10 μM indatraline for cells, or 10 μM of methylphenidate, desipramine or citalopram for synaptosomes. Following uploading, the samples were washed once and exposed to vehicle, 1 μM β-PEA or 1 μM β-PEA plus 10 μM of methylphenidate, desipramine or citalopram in serum-free DMEM (for cells) or efflux buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 μM pargyline, 2 mg/ml glucose, 0.2 mg/ml ascorbic acid, pH 7.5) (for synaptosomes) at 25°C for 30 min. Efflux process was terminated by addition of 1 ml of ice-cold serum-free DMEM into the wells or efflux buffer into the tubes. Following aspiration of the DMEM (for cells) or centrifugation at 10,000g at 4°C for 3 min (for synaptosome), the samples were then washed twice and incubated in 1 × PLB buffer for 30 min on a shaking platform at 200 rpm. The lysates were transferred into scintillation vials containing

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4 ml of Beckman ReadySafe™ scintillation cocktail and counted on Beckman LS6000IC scintillation spectrophotometer (Fullerton, CA) for 1 min/sample. The samples treated with efflux buffer after [^{3}H]monoamine uploading were taken as baseline (100% retention).

Data Analysis. 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 cAMP accumulation in response to the drug challenge. Specific [^{3}H]monoamine uptake and retention were compared to the baseline or the maximal level and expressed as percentage values in each set of assays. Results were finalized as mean ± SEM of the indicated number of observations. Student's T test was used to judge the difference between two samples, and the results were considered significantly different at $p < 0.05$.

Results

TAAR1 is Activated by Trace Amines. We and others have previously reported that TAAR1 is expressed in brain monoaminergic nuclei and activated by trace amines *in vitro*. To assess the influence of their interaction on monoamine transporter function, we herein re-evaluated TAAR1 activation by trace amines to confirm that TAAR1 is recognized and excited by trace amines, using dual luciferase assays. Rhesus monkey TAAR1 was transiently transfected into HEK293 cells along with CRE-Luc and pGL4.73, and CRE-Luc expression was determined as a measurement of cAMP accumulation to assess TAAR1 activity in the cells following exposure to trace amines. The data were normalized to vehicle treatment and presented as RLU increase (%). β-PEA increased CRE-Luc expression in a dose-dependent manner in the presence of TAAR1, but the CRE-Luc expression did not vary with the dose increase of β-PEA in the absence of TAAR1. Similarly, tyramine, tryptamine and octopamine also increased CRE-Luc expression in a TAAR1-dependent manner (Fig. 1). These results confirm our previous findings that trace amine are agonists at rhesus monkey TAAR1 (Miller et al., 2005; Xie et al., 2007b) and give further support for interaction of trace amines with TAAR1 through cAMP signaling pathway.

TAAR1 Activation by β-PEA Modulates Monoamine Transporter Function in Transfected Cells. To determine whether trace amines modulate monoamine transporter function via activating TAAR1, we heroin investigated the effect of TAAR1 signaling in response to β-PEA on DAT, NET and SERT function. Human DAT, NET or SERT were transiently transfected into HEK293, stable TAAR1 and stable D2s, α_{2A} and $5HT_{1B}$ cells to generate different cell lines, DAT, NET, SERT, TAAR1-DAT, TAAR1-NET, TAAR1-SERT, D2s-DAT, α_{2A}-NET and $5HT_{1B}$-SERT, respectively. DAT, NET and SERT cells were used as control.

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We first evaluated the influence of TAAR1 activation by β-PEA on the uptake function of monoamine transporters in the cells. The cells were pretreated with vehicle or 1 μM β-PEA for 10 min, and then washed twice and uploaded with 10 nM [^{3}H]dopamine, 20 nM [^{3}H]norepinephrine or 20 nM [^{3}H]serotonin for 5 min. The uptake values were reported as % of baseline (uptake in cells pretreated with DMEM for 10 min). β-PEA reduced [^{3}H]dopamine uptake in TAAR1-DAT cells by 29.4 ± 5.2%, relative to vehicle treatment ($p < 0.01$). However, β-PEA had no such effect on [^{3}H]dopamine uptake in DAT and D2s-DAT cells (Fig. 2A). Similarly, β-PEA reduced [^{3}H]norepinephrine uptake in TAAR1-NET cells by 29.6 ± 4.3%, relative to vehicle treatment ($p < 0.01$). However, β-PEA had no such effect on [^{3}H]norepinephrine uptake in NET and α_{2A}-NET cells (Fig. 2B). Likewise, β-PEA reduced [^{3}H]serotonin uptake in TAAR1-SERT cells by 34.6 ± 6.6%, relative to vehicle treatment ($p < 0.01$). However, β-PEA had no such effect on [^{3}H]serotonin uptake in SERT and $5HT_{1B}$-SERT cells (Fig. 2C).

Next, we evaluated the influence of TAAR1 activation by β-PEA on the efflux function of monoamine transporters in the cells. The cells were uploaded with 10 nM [^{3}H]dopamine, 20 nM [^{3}H]norepinephrine or 20 nM [^{3}H]serotonin for 20 min, and then washed twice and treated with vehicle, 1 μM β-PEA or 1 μM β-PEA plus 10 μM indatraline for 30 min. The [^{3}H]monoamine retention values were reported as % of baseline (retention in cells treated with DMEM for 30 min following [^{3}H]monoamine uploading). β-PEA reduced [^{3}H]dopamine retention by 22.5 ± 3.6% in TAAR1-DAT cells, in comparison to vehicle treatment ($p < 0.01$), but it had no such effect in DAT cells. Indatraline attenuated the effect of β-PEA in TAAR1-DAT cells (Fig. 3A). Similarly,

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β-PEA reduced [^{3}H]norepinephrine retention by 20.6 ± 4.4% in TAAR1-NET cells, in comparison to vehicle treatment ($p < 0.01$), but it had no such effect in NET cells. Indatraline attenuated the effect of β-PEA in TAAR1-NET cells (Fig. 3B). Likewise, β-PEA reduced [^{3}H]serotonin retention by 22.2 ± 4.8% in TAAR1-SERT cells, in comparison to vehicle treatment ($p < 0.01$), but it had no such effect in SERT cells. Indatraline attenuated the effect of β-PEA in TAAR1-SERT cells (Fig. 3C).

TAAR1 Activation by β-PEA Modulates DAT Function in Brain Striatal Synaptosomes. To investigate whether TAAR1 activation by β-PEA can modulate DAT function in brain, we prepared synaptosomes from monkey and mouse striatal tissues to evaluate DAT modulation by β-PEA, as there is a high density of DAT expressed in brain striatum (Ciliax et al., 1995; Miller et al., 2001). To distinguish effects at DAT from other monoamine transporters also expressed in the striatum, we used the DAT-selective inhibitor methylphenidate for determination of non specific uptake of [^{3}H]dopamine. We first evaluated the influence of β-PEA pretreatment on uptake of [^{3}H]dopamine. The synaptosomes were pretreated with vehicle or 1 μM β-PEA for 10 min, and then washed twice and uploaded with 10 nM [^{3}H]dopamine for 5 min. The uptake values were reported as % of baseline (uptake in synaptosomes pretreated with uptake buffer for 10 min). β-PEA reduced [^{3}H]dopamine uptake in the synaptosomes of wild type mice by 17.4 ± 3.2%, relative to vehicle treatment ($p < 0.01$), but it had no such effect on [^{3}H]dopamine uptake in the synaptosomes of TAAR1 knockout mice (Fig. 4A). We further performed time course studies to observe the effect of β-PEA on [^{3}H]dopamine uptake. The synaptosomes were exposed to 10 nM [^{3}H]dopamine or 10 nM [^{3}H]dopamine plus 100 nM β-PEA for different times. The

uptake values were reported as % of the maximal uptake (uptake of 10 nM [^{3}H]dopamine alone at 30 min). Under this condition, [^{3}H]dopamine uptake was apparently inhibited – the uptake curves shifted right and down. Notably, [^{3}H]dopamine uptake temporarily halted after 3 min in synaptosomes of rhesus monkeys and wild type mice, but no such uptake halt occurred in the synaptosomes of TAAR1 knockout mice (Fig. 4B). Owing to such a halt in the uptake, the reduction in the percentage value of [^{3}H]dopamine uptake in striatal synaptosomes of rhesus monkeys (42.8 ± 7.1% at 10 min) and wild type mice (45.6 ± 6.8% at 10 min) were significantly greater than the reduction in striatal synaptosomes of TAAR1 knockout mice (26.5 ± 4.2% at 10 min) ($p < 0.01$). We then investigated the influence of β-PEA on efflux of [^{3}H]dopamine. The synaptosomes were uploaded with 10 nM [^{3}H]dopamine for 20 min, and then washed twice and exposed to vehicle, 1 μM β-PEA or 1 μM β-PEA plus 10 μM methylphenidate for 30 min. The [^{3}H]dopamine retention was reported as % of baseline (retention in synaptosomes treated with efflux buffer for 30 min following [^{3}H]dopamine uploading), and the loss of the [^{3}H]dopamine retention was taken as the efflux values. β-PEA promoted [^{3}H]dopamine efflux (retention is reduced) in the synaptosomes of rhesus monkeys and wild type mice by 20.1 ± 3.5% and 27.6 ± 4.2%, respectively, but it had no such effect on [^{3}H]dopamine efflux in the synaptosomes of TAAR1 knockout mice. Methylphenidate blocked the β-PEA effect in the synaptosomes of wild type mice (Fig. 4C).

TAAR1 Activation by β-PEA Modulates NET Function in Brain Thalamic Synaptosomes. NET has been found to be expressed in brain thalamus at a relatively high level (Ding et al., 2003; Madras et al., 2006). We therefore collected monkey and mouse thalamic tissues to prepare synaptosomes for evaluation of NET modulation by β-PEA. We used the NET-selective

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inhibitor desipramine for determination of non specific uptake of [^{3}H]norepinephrine. We first evaluated the influence of β-PEA pretreatment on uptake of [^{3}H]norepinephrine. The synaptosomes were pretreated with vehicle or 1 μM β-PEA for 10 min, and then washed twice and uploaded with 20 nM [^{3}H]norepinephrine for 5 min. The uptake values were reported as % of baseline (uptake in synaptosomes pretreated with uptake buffer for 10 min). β-PEA reduced [^{3}H]norepinephrine uptake in the synaptosomes of wild type mice by 20.0 ± 3.9%, relative to vehicle treatment ($p < 0.01$), but it had no such effect on [^{3}H]norepinephrine uptake in the synaptosomes of TAAR1 knockout mice (Fig. 5A). We further performed time course studies to observe the effect of β-PEA on [^{3}H]norepinephrine uptake. The synaptosomes were exposed to 20 nM [^{3}H]norepinephrine, or 20 nM [^{3}H]norepinephrine plus 100 nM β-PEA for different times. The uptake values were reported as % of the maximal uptake (uptake of 20 nM [^{3}H]norepinephrine alone at 30 min). Under this condition, [^{3}H]norepinephrine uptake was apparently inhibited – the uptake curves shifted right and down. [^{3}H]Norepinephrine uptake temporarily halted after 3 min in synaptosomes of rhesus monkeys and wild type mice, but no such uptake halt occurred in the synaptosomes of TAAR1 knockout mice (Fig. 5B). Owing to such a halt in the uptake, the reduction in the percentage value of [^{3}H]norepinephrine uptake in thalamic synaptosomes of rhesus monkeys (41.0 ± 5.6% at 10 min) and wild type mice (39.3 ± 4.5% at 10 min) were significantly greater than the reduction in thalamic synaptosomes of TAAR1 knockout mice (26.6 ± 4.9% at 10 min) ($p < 0.01$). We then investigated the influence of β-PEA on the efflux of [^{3}H]norepinephrine. The synaptosomes were uploaded with 20 nM [^{3}H]norepinephrine for 20 min, and then washed twice and exposed to vehicle, 1 μM β-PEA or 1

μM β-PEA plus 10 μM desipramine for 30 min. The [^{3}H]norepinephrine retention was reported as % of baseline (retention in synaptosomes treated with efflux buffer for 30 min following [^{3}H]norepinephrine uploading), and the loss of the [^{3}H]norepinephrine retention was taken as the efflux values. β-PEA promoted [^{3}H]norepinephrine efflux (retention is reduced) in the synaptosomes of rhesus monkeys and wild type mice by 27.2 ± 5.5% and 26.8 ± 5.1%, respectively, but it had no such effect on [^{3}H]norepinephrine efflux in the synaptosomes of TAAR1 knockout mice. Desipramine blocked β-PEA effect in the synaptosomes of wild type mice (Fig. 5C).

TAAR1 Activation by β-PEA Modulates SERT Function in Brain Striatal Synaptosomes. SERT has been also found to be expressed in brain striatum at a high level (Sur et al., 1996; Kish et al., 2005). We therefore collected monkey and mouse striatal tissues to prepare synaptosomes for evaluation of SERT modulation by β-PEA. We used the SERT-selective inhibitor citalopram for determination of non specific uptake of [^{3}H]serotonin. We first evaluated the influence of β-PEA pretreatment on uptake of [^{3}H]serotonin. The synaptosomes were pretreated with vehicle or 1 μM β-PEA for 10 min, and then washed twice and uploaded with 20 nM [^{3}H]serotonin for 5 min. The uptake values were reported as % of baseline (uptake in synaptosomes pretreated with uptake buffer for 10 min). β-PEA reduced [^{3}H]serotonin uptake in the synaptosomes of wild type mice by 22.0 ± 5.1%, relative to vehicle treatment ($p < 0.01$), but it had no such effect on [^{3}H]serotonin uptake in the synaptosomes of TAAR1 knockout mice (Fig. 6A). We further performed time course studies to observe the effect of β-PEA on [^{3}H]serotonin uptake. The synaptosomes were exposed to 20 nM [^{3}H]serotonin or 20 nM [^{3}H]serotonin plus 100 nM β-PEA

for different times. The uptake values were reported as % of the maximal uptake (uptake of 20 nM [^{3}H]serotonin alone at 30 min). Under this condition, [^{3}H]serotonin uptake was apparently inhibited – the uptake curves shifted right and down. [^{3}H]Serotonin uptake temporarily halted after 4 min in synaptosomes of rhesus monkeys and wild type mice, but no such uptake halt occurred in the synaptosomes of TAAR1 knockout mice (Fig. 6B). Owing to such a halt in the uptake, the reduction in the percentage value of [^{3}H]serotonin uptake in striatal synaptosomes of rhesus monkeys (45.8 ± 6.6% at 10 min) and wild type mice (44.1 ± 5.8% at 10 min) were significantly greater than the reduction in striatal synaptosomes of TAAR1 knockout mice (30.0 ± 5.1% at 10 min) ($p < 0.01$). We then investigated the influence of β-PEA on efflux of [^{3}H]serotonin. The synaptosomes were uploaded with 20 nM [^{3}H]serotonin for 20 min, and then washed twice and exposed to vehicle, 1 μM β-PEA or 1 μM β-PEA plus 10 μM citalopram for 30 min. The [^{3}H]serotonin retention was reported as % of baseline (retention in cells treated with DMEM or synaptosomes treated with efflux buffer for 30 min following [^{3}H]serotonin uploading), and the loss of the [^{3}H]serotonin retention was taken as the efflux values. β-PEA promoted [^{3}H]serotonin efflux (retention is reduced) in the synaptosomes of rhesus monkeys and wild type mice by 20.0 ± 3.6% and 16.8 ± 3.3%, respectively, but it had no such effect on [^{3}H]serotonin efflux in the synaptosomes of TAAR1 knockout mice. Citalopram blocked the β-PEA effect in the synaptosomes of wild type mice (Fig. 6C).

TAAR1 Signaling is not Altered by Monoamine Autoreceptors at Exposure to Trace Amines. Monoamine autoreceptors, including dopamine D2, adrenergic α_2 and serotonin $5HT_1$ receptors, are presynaptic feedback regulators for the respective monoamine neurotransmitter release. To determine whether the trace amines interact with these receptors to alter TAAR1

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signaling, we assessed the influence of autoreceptors, D2s, α_{2A}, α_{2B}, $5HT_{1A}$ and $5HT_{1B}$ co-expressed with TAAR1, respectively, on TAAR1 activation in the cells at exposure to trace amines. We transiently transfected rhesus monkey TAAR1 along with CRE-Luc and pGL4.73 into HEK293 and stable D2s, α_{2A}, α_{2B}, $5HT_{1A}$ and $5HT_{1B}$ cells to generate different cell lines, TAAR1, D2s-TAAR1, α_{2A}-TAAR1, α_{2B}-TAAR1, $5HT_{1A}$-TAAR1 and $5HT_{1B}$-TAAR1, and then determined CRE-Luc expression as a measurement of cAMP accumulation to assess TAAR1 activity. The data were normalized to vehicle treatment and presented as RLU increase (%).

We first evaluated TAAR1 activation by β-PEA in the cells. When the cells were exposed to 1 μM β-PEA, the monoamine autoreceptors D2s, α_{2A}, α_{2B}, $5HT_{1A}$ or $5HT_{1B}$ had no significant effect on TAAR1 signaling - TAAR1 activation in the presence of each type autoreceptor was similar in amplitude to that in the absence of the autoreceptor (Fig. 7A). In contrast, parallel to β-PEA, we also tested dopamine, norepinephrine and serotonin, and we found that TAAR1 signaling was largely attenuated by D2s at exposure to 1 μM dopamine, by α_{2A} or α_{2B} at exposure to 1 μM norepinephrine, or by $5HT_{1A}$ or $5HT_{1B}$ at exposure to 1 μM serotonin (data not shown), suggesting that the autoreceptors were expressed well and indicating that β-PEA differs from dopamine, norepinephrine or serotonin in that β-PEA 1 μM at did not interact with the monoamine autoreceptors. Then, we further evaluated TAAR1 activation by other trace amines in the cells. Similarly, TAAR1 signaling in response to 1 μM tyramine (Fig. 7B), 1 μM tryptamine (Fig. 7C) or 1 μM octopamine (Fig. 7D) was not affected by any of the monoamine autoreceptors tested. To clarify whether there is a significant difference in TAAR1 expression in

the cells tested, we performed Western blotting to determine TAAR1 level. We found that D2s, α_{2A}, α_{2B}, $5HT_{1A}$ or $5HT_{1B}$ did not significantly alter TAAR1 expression (Fig. 7E).

Trace Amines Have Extremely Low Binding Affinity for Monoamine Autoreceptors. To further determine whether trace amines interact with monoamine autoreceptors, we assessed the binding affinity of trace amines to the monoamine autoreceptors in comparison to dopamine (for D2s), norepinephrine (for α_{2A} and α_{2B}) and serotonin (for $5HT_{1A}$ and $5HT_{1B}$). Stable D2s, α_{2A}, $\alpha_{2B,}$ $5HT_{1A}$ and $5HT_{1B}$ cells were used to perform competition binding assays to determine IC_{50} values of the trace amines. We found that β-PEA, octopamine, tryptamine and tyramine had poor binding affinity for D2s relative to dopamine (Fig. 8A), for α_{2A} and α_{2B} relative to norepinephrine (Fig. 8B), and for $5HT_{1A}$ and $5HT_{1B}$ relative to serotonin (Fig. 8C). The IC_{50} of dopamine for D2s was 0.19 ± 0.06 μM, the IC_{50} of norepinephrine for α_{2A} and α_{2B} was 0.025 ± 0.008 and 0.058 ± 0.018 μM, respectively, and the IC_{50} of serotonin for $5HT_{1A}$ and $5HT_{1B}$ was 0.048 ± 0.013 and 0.032 ± 0.011 μM, respectively. However, in each case, the IC_{50} of β-PEA, octopamine, tryptamine or tyramine was greater than 100 μM.

Discussion

The data presented in this study confirm that trace amines are potent agonists at TAAR1 and demonstrate that TAAR1 signaling in response to trace amines is not affected by monoamine autoreceptors co-expressed with TAAR1 in transfected cells. Trace amines lack interaction with monoamine autoreceptors while they recognize and excite TAAR1. β-PEA activation of TAAR1 modulates monoamine transporter function in the transfected cells and in brain striatal and thalamic synaptosomes, which suggests a modulatory role for trace amines in the functional regulation of monoamine transporters in brain.

β-PEA has been thought of as an endogenous amphetamine due to its similarity to amphetamine in structure and effects. Numerous studies have reported the influence of β-PEA in the central nervous system, especially on catecholaminergic activity in the mammalian brain. It has been reported that β-PEA increases the release of catecholamines and inhibits catecholamine uptake (Paterson et al., 1990). The present study provides evidence that β-PEA alters the uptake and efflux functions of DAT, NET and SERT via interaction with TAAR1. In time course assays in which brain synaptosomes were exposed to [^{3}H]monoamines in combination with β-PEA, we observed that β-PEA induced a halting in uptake of [^{3}H]monoamines after 3~4 min in monkey and wild type mouse synaptosomes. Because such uptake halting did not occur in TAAR1 knockout mouse synaptosomes, it can be deduced that TAAR1 is mediating this effect. In our pretreatment assays, in which cells and synaptosomes were exposed to β-PEA and then washed and exposed to [^{3}H]monoamine, β-PEA did not alter [^{3}H]monoamine uptake in transfected cells lacking TAAR1 or in the TAAR1 knockout mouse synaptosomes, but did significantly inhibit [^{3}H]monoamine uptake in cells expressing TAAR1 and in monkey and wild type mouse

synaptosomes, providing further evidence that TAAR1 is a mediator of β-PEA effects on the monoamine transporters. In efflux assays, we also observed that β-PEA promoted [^{3}H]monoamine efflux in a TAAR1-dependent manner. Although TAAR1 is not specific for β-PEA, TAAR1 may be essential for β-PEA exerting effects on monoamine transporters in brain.

In addition to β-PEA, brain trace amines also include tyramine, tryptamine and octopamine. These trace amines are spatially and closely associated with the dopamine, norepinephrine and serotonin neurotransmitter systems (Philips et al., 1974; Axelrod and Saavedra, 1977; Durden and Philips 1980; Paterson et al.1990), and their specific binding sites in brain have been known for decades (Hauger et al., 1982; Kellar and Cascio, 1982; Vaccari, 1986). In this study, we confirmed that the trace amines are all agonists at TAAR1. We focused on β-PEA for functional analysis, as β-PEA has been independently reported to be associated with various neuropsychiatric disorders/diseases. It is likely that the functional profile demonstrated for β-PEA in modulation of monoamine transporters is indicative of a common mechanism for trace amines. Although trace amines are generally present in brain at a very low level (Durden and Philips 1980; Durden and Davis, 1993), this does not exclude their interaction with TAAR1 in brain. It is possible that trace amines reach levels suitable for TAAR1 activation at very discrete locations in brain, particularly under certain pathological or pharmacological conditions (Bergman et al., 2001). Notably, aberrant trace amine levels occur in depression and other neuropsychiatric disorders (Boulton, 1980; Davis and Boulton, 1994; McClung and Hirsh, 1999; D'Andrea et al. 2003). Accordingly, our finding that β-PEA acts via TAAR1 to modulate monoamine transporter function may reveal a principal mechanism for trace amines exerting their effects on brain monoaminergic systems.

Monoamine autoreceptors co-localize with monoamine transporters on presynaptic membranes of monoaminergic neurons and provide feedback regulation of monoamine neurotransmitter release. It has been previously recognized that D2 autoreceptor activation can modulate DAT function and expression (Mayfield and Zahniser, 2001; Bolan et al., 2007). Recently, Wolinsky et al reported that the knockout of TAAR1 elicited a large increase of striatal high-affinity D2 receptors (Wolinsky et al., 2006), suggesting that TAAR1 function may be related to monoamine autoreceptors. In this regard, we evaluated whether trace amines interact with monoamine autoreceptors, including dopamine D2s, adrenergic α_{2A} and α_{2B}, and serotonin $5HT_{1A}$ and $5HT_{1B}$ receptors, to alter TAAR1 signaling. Our data show that these monoamine autoreceptors are unresponsive to trace amines. TAAR1 signaling in response to trace amines is not affected by these autoreceptors concurrently exposed to trace amines. We previously reported that dopamine, norepinephrine and serotonin, which are agonists for the respective autoreceptors, are also rhesus monkey TAAR1 agonists *in vitro* (Xie et al., 2007b). Indeed, we have also demonstrated that TAAR1 signaling was largely attenuated by D2s at exposure to 1 μM dopamine, by α_{2A} or α_{2B} at exposure to 1 μM norepinephrine, or by $5HT_{1A}$ or $5HT_{1B}$ at exposure to 1 μM serotonin (unpublished data). Accordingly, trace amines may differ from common biogenic amines with regard to modulation of monoamine transporters.

Monoamine transporters are important regulators of brain monoaminergic neurotransmission in clearance of released monoamine neurotransmitters from synaptic clefts and termination of neural signaling (Uhl and Johnson, 1994; Giros et al., 1996; Masson et al., 1999), though diffusion and enzymatic degradation also contribute to reducing the synaptic concentration of the neurotransmitters. Trace amines are substrates for DAT, NET and SERT, and therefore they can

compete with dopamine, norepinephrine and serotonin for transport though the respective monoamine transporters. Indeed, in time course uptake assays, we observed an uptake inhibition in TAAR1 knockout mouse synaptosomes - the uptake curves in the presence of β-PEA shifted right and down, which also occurred in monkey and wild type mouse synaptosomes. Such conserved inhibition is most likely due to substrate competition. However, the extracellular concentration of the trace amines in brain is much lower than for common biogenic amines (Durden and Philips, 1980; Durden and Davis, 1993), suggesting that the competition effects of the trace amines may not significantly affect the transport of the common biogenic amines *in vivo*. In contrast, TAAR1-mediated effects of the trace amines may play a key role.

That β-PEA activation of TAAR1 modulates monoamine transporter function, demonstrated in this study, provides a novel insight into understating the modulatory roles of brain trace amines. Full elucidation of the influence of TAAR1 interaction with trace amines in the brain is directly dependent on clarification of the cellular distribution of the trace amines and trace amine-associated receptors. Unlike many other G protein-coupled receptors, TAAR1 apparently remains largely intracellularly sequestered in transfected cells (Bunzow et al., 2001; Miller et al., 2005; Xie et al., 2007b). It is predictable that the localization of TAAR1 in the neurons will play an important role determining its signaling in response to trace amines and other activators, for which further studies are needed. In addition, although the present study provides initial evidence that TAAR1 may serve as a presynaptic regulator of monoamine transporters, we lack direct evidence for TAAR1 distribution on presynaptic membranes. It is likely that TAAR1 is located on/in neurons both presynaptically and postsynaptically, and plays a wider role in monoaminergic regulation than we present here. Nevertheless, this study demonstrates that

TAAR1 is a modulator of monoamine transporter function in brain and reveals a mechanism by which β-PEA, and possibly other trace amines, can affect monoamine transporter function.

Monoamine transporter regulators are among the most important drugs used to treat a spectrum of neuropsychiatric disorders and most of the drugs, such as the selective serotonin reuptake inhibitors, directly interact with monoamine transporters. However, these drugs display a dramatically delayed onset in their clinical effects, and have potentially serious adverse effects particularly in children and adolescents with depression, which stimulates efforts for new drug development. Our data reveal a receptor-mediated mechanism in the functional regulation of monoamine transporters and suggest that novel drugs which activate TAAR1 but not monoamine autoreceptors could be able to improve the medication of neuropsychiatric disorders/diseases. Accordingly, the present study may provide an impetus for drug development. Furthermore, our findings also suggest that the clinical efficacy of monoamine autoreceptor inhibitors may in part result from changes in receptor signaling in which the TAAR1 response to endogenous monoamine neurotransmitters is reinforced due to deprivation of autoreceptor activation. Owing to the similarity of β-PEA to amphetamine, our data also suggest that amphetamines may regulate monoamine transporter function similarly to β-PEA, and in this regard, drugs which can antagonize TAAR1 may have clinical efficacy as therapeutics for drug abuse and addiction of amphetamines.

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Footnotes

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Legends for Figures

Fig. 1. TAAR1 activation by trace amines *in vitro*. The rhesus monkey TAAR1 expression construct along with CRE-Luc and pGL4.73 was transiently transfected into HEK293 cells to generate TAAR1 cells, and the pcDNA 3.1 along with CRE-Luc and pGL4.73 was transiently transfected into HEK293 cells to generate HEK cells as control. The cells were incubated in transfection medium for 12 h and then exposed to vehicle and a range of concentrations (10^{-9}-10^{-4} M) of β-PEA, tyramine (TYR), tryptamine (TRY) or octopamine (OCT) 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 (%). Filled circles (●) indicate CRE-Luc expression in TAAR1 cells following exposure to the trace amines; open circles (o) indicate CRE-Luc expression in HEK cells following exposure to the trace amines. Data are presented as mean ± SEM for three independent experiments performed in triplicate.

Fig. 2. Influence of TAAR1 activation by β-PEA on the uptake function of the monoamine transporters in the cells. HEK293, stable TAAR1 cells and stable D2s, α_{2A} and $5HT_{1B}$ cells were transiently transfected with human DAT, NET or SERT to generate different cell lines: DAT, NET, SERT, TAAR1-DAT, TAAR1-NET, TAAR1-SERT, D2s-DAT, α_{2A}-NET and $5HT_{1B}$-SERT, respectively. The cells were pretreated with vehicle (VEH) or 1 μM β-PEA for 10 min, and then washed twice and uploaded with [^{3}H]dopamine (10 nM), [^{3}H]norepinephrine (20 nM) or [^{3}H]serotonin (20 nM) for 5 min. The uptake values are % of baseline (uptake in cells pretreated with DMEM for 10 min). A, Influence of β-PEA on [^{3}H]dopamine uptake in TAAR1-DAT, DAT and D2s-DAT cells. B, Influence of β-PEA on [^{3}H]norepinephrine uptake in

TAAR1-NET, NET and α_{2A}-NET cells. C, Influence of β-PEA on [^{3}H]serotonin uptake in TAAR1-SERT, SERT and $5HT_{1B}$-SERT cells. Data are presented as mean ± SEM for three independent experiments performed in triplicate. ** $p < 0.01$. [^{3}H]DA, [^{3}H]dopamine; [^{3}H]NE, [^{3}H]norepinephrine; [^{3}H]5-HT, [^{3}H]serotonin.

Fig. 3. Influence of TAAR1 activation by β-PEA on the efflux function of the monoamine transporters in the cells. HEK293 and stable TAAR1 cells were transiently transfected with human DAT, NET or SERT to generate different cell lines, DAT, NET, SERT, TAAR1-DAT, TAAR1-NET and TAAR1-SERT, respectively. The cells were uploaded with [^{3}H]dopamine (10 nM), [^{3}H]norepinephrine (20 nM) or [^{3}H]serotonin (20 nM) for 20 min, and then washed twice and exposed to vehicle (VEH) or 1 μM β-PEA for 30 min. 10 μM indatraline was added to the cells during the exposure to β-PEA following [^{3}H]monoamine uploading. The retention values are % of baseline (retention in cells treated with DMEM for 30 min following [^{3}H]monoamine uploading). A, Effect of β-PEA on [^{3}H]dopamine efflux in TAAR1-DAT, DAT cells, and the influence of indatraline on β-PEA effect in TAAR1-DAT cells. B, Effect of β-PEA on [^{3}H]norepinephrine efflux in TAAR1-NET and NET cells, and the influence of indatraline on β-PEA effect in TAAR1-NET cells. C, Effect of β-PEA on [^{3}H]serotonin efflux in TAAR1-SERT and SERT cells, and the influence of indatraline on β-PEA effect in TAAR1-SERT cells. Data are presented as mean ± SEM for three independent experiments performed in triplicate. ** $p < 0.01$. [^{3}H]DA, [^{3}H]dopamine; [^{3}H]NE, [^{3}H]norepinephrine; [^{3}H]5-HT, [^{3}H]serotonin; IND, indatraline.

Fig. 4. DAT modulation by β-PEA in brain striatal synaptosomes. Synaptosomes were prepared from fresh brain striatal tissues, and the experiments were performed in triplicate for 3 rhesus monkeys, and 3 pairs of wild type and TAAR1 knockout mice. A, Effect of β-PEA on [^{3}H]dopamine uptake in wild type and TAAR1 knockout mouse synaptosomes. The synaptosomes were pretreated with vehicle (VEH) or 1 μM β-PEA for 10 min, and then washed twice and uploaded with 10 nM [^{3}H]dopamine for 5 min. The uptake values are % of baseline (uptake in synaptosomes pretreated with uptake buffer for 10 min). B, The synaptosomes were exposed to 10 nM [^{3}H]dopamine alone or 10 nM [^{3}H]dopamine plus 100 nM β-PEA for the indicated times. The uptake values are % of the maximal uptake (uptake of 10 nM [^{3}H]dopamine alone at 30 min). Left: [^{3}H]Dopamine uptake in rhesus monkey synaptosomes. Middle: [^{3}H]Dopamine uptake in wild type mouse synaptosomes. Right: [^{3}H]Dopamine uptake in TAAR1 knockout mouse synaptosomes. C, The synaptosomes were uploaded with [^{3}H]dopamine (10 nM) for 20 min, and then washed twice and exposed to vehicle (VEH) or 1 μM β-PEA for 30 min. For blockade of DAT, 10 μM methylphenidate (MPH) was mixed with 1 μM β-PEA. The [^{3}H]dopamine retention values are % of the baseline (retention in the synaptosomes treated with efflux buffer for 30 min following [^{3}H]dopamine uploading). Left: Effect of β-PEA on [^{3}H]dopamine efflux in the synaptosomes of rhesus monkeys, wild type and TAAR1 knockout mice. Right: Influence of methylphenidate on β-PEA effect in wild type mouse synaptosomes. Data are presented as mean ± SEM.. ** $p < 0.01$. [^{3}H]DA, [^{3}H]dopamine; WT, wild type mouse; TAAR1-KO, TAAR1 knockout mouse.

Fig. 5. NET modulation by β-PEA in brain thalamic synaptosomes. Synaptosomes were prepared from fresh brain thalamic tissues, and the experiments were performed in triplicate for 3 rhesus monkeys, and 3 pairs of wild type and TAAR1 knockout mice. A, Effect of β-PEA on [^{3}H]norepinephrine uptake in wild type and TAAR1 knockout mouse synaptosomes. The synaptosomes were pretreated with vehicle or 1 μM β-PEA for 10 min, and then washed twice and uploaded with 20 nM [^{3}H] norepinephrine for 5 min. The uptake values are % of baseline (uptake in synaptosomes pretreated with uptake buffer for 10 min. B, The synaptosomes were exposed to 20 nM [^{3}H]norepinephrine alone or 20 nM [^{3}H] norepinephrine plus 100 nM β-PEA for the indicated times. The uptake values are % of the maximal uptake (uptake of 20 nM [^{3}H]norepinephrine alone at 30 min). Left: [^{3}H]Norepinephrine uptake in rhesus monkey synaptosomes. Middle: [^{3}H]Norepinephrine uptake in wild type mouse synaptosomes. Right: [^{3}H]Norepinephrine uptake in TAAR1 knockout mouse synaptosomes. C, The synaptosomes were uploaded with 20 nM [^{3}H]norepinephrine for 20 min, and then washed twice and exposed to vehicle (VEH) or 1 μM β-PEA for 30 min. For blockade of NET, 10 μM desipramine (DMI) was mixed with 1 μM β-PEA. The [^{3}H]norepinephrine retention values are % of the baseline (retention in the synaptosomes treated with efflux buffer for 30 min following [^{3}H]norepinephrine uploading). Left: Effect of β-PEA on [^{3}H]norepinephrine efflux in the synaptosomes of rhesus monkeys, wild type and TAAR1 knockout mice. Right: Influence of desipramine on β-PEA effect in wild type mouse synaptosomes. Data are presented as mean ± SEM. ** $p < 0.01$. [^{3}H]NE, [^{3}H]norepinephrine; WT, wild type mouse; TAAR1-KO, TAAR1 knockout mouse.

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Fig. 6. SERT modulation by β-PEA in brain striatal synaptosomes. Synaptosomes were prepared from fresh brain striatal tissues, and the experiments were performed in triplicate for 3 rhesus monkeys, and 3 pairs of wild type and TAAR1 knockout mice. A, Effect of β-PEA on [^{3}H]serotonin uptake in wild type and TAAR1 knockout mouse synaptosomes. The synaptosomes were pretreated with vehicle (VEH) or 1 μM β-PEA for 10 min, and then washed twice and uploaded with 20 nM [^{3}H]serotonin for 5 min. The uptake values are % of baseline (uptake in synaptosomes pretreated with uptake buffer for 10 min. B, The synaptosomes were exposed to 20 nM [^{3}H]serotonin alone or 20 nM [^{3}H]serotonin plus 100 nM β-PEA for the indicated times. The uptake values are % of the maximal uptake (uptake of 20 nM [^{3}H]serotonin alone at 30 min). Left: [^{3}H]Serotonin uptake in rhesus monkey synaptosomes. Middle: [^{3}H]Serotonin uptake in wild type mouse synaptosomes. Right: [^{3}H]Serotonin uptake in TAAR1 knockout mouse synaptosomes. C, The synaptosomes were uploaded with 20 nM [^{3}H]serotonin for 20 min, and then washed twice and exposed to vehicle (VEH) or 1 μM β-PEA for 30 min. For blockade of SERT, 10 μM citalopram (CIT) was mixed with 1 μM β-PEA. The [^{3}H]serotonin retention values are % of the baseline (retention in the synaptosomes treated with efflux buffer for 30 min following [^{3}H]serotonin uploading). Left: Effect of β-PEA on [^{3}H]serotonin efflux in the synaptosomes of rhesus monkeys, wild type and TAAR1 knockout mice. Right: Influence of citalopram on β-PEA effect in wild type mouse synaptosomes. Data are presented as mean ± SEM. ** $p < 0.01$. [^{3}H]5-HT, [^{3}H]serotonin; WT, wild type mouse; TAAR1-KO, TAAR1 knockout mouse.

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Fig. 7. Effects of monoamine autoreceptors on TAAR1 activation by trace amines and TAAR1 expression in the cells. HEK293 and stable D2s, α_{2A}, α_{2B}, $5HT_{1A}$ and $5HT_{1B}$ cells were transiently transfected with rhesus monkey TAAR1, CRE-Luc and pGL4.73 to generate different cell lines: TAAR1, D2s-TAAR1, α_{2A}-TAAR1, α_{2B}-TAAR1, $5HT_{1A}$-TAAR1 and $5HT_{1B}$-TAAR1. The cells were incubated in transfection medium for 12 h and then exposed to vehicle and 1 μM of β-PEA, tyramine (TYR), tryptamine (TRY) or octopamine (OCT) 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 (%). The same amount (10 μg) of the proteins prepared from the cells was subjected to SDS-PAGE/Western blotting, and grey values of the blots were normalized to TAAR1 cells and presented as % of TAAR1. Shown are the influences of the monoamine autoreceptors on TAAR1 activation by β-PEA (A), tyramine (B), tryptamine (C) and octopamine (D), and the influence of the monoamine autoreceptors on TAAR1 expression in the cells (E). Data are presented as mean ± SEM for three independent experiments performed in triplicate.

Fig. 8. Binding of trace amines to monoamine autoreceptors. Stable D2s, α_{2A}, α_{2B}, $5HT_{1A}$ and $5HT_{1B}$ cells were prepared to conduct competition binding assays and IC50 values were calculated. [^{3}H]dopamine (20 nM), [^{3}H]norepinephrine (10 nM) and [^{3}H]serotonin (10 nM) were used as tracers. A, Binding of the trace amines to the D2s receptor. B, Binding of the trace amines to the α_{2A} and α_{2B} receptors. C, Binding of the trace amines to the $5HT_{1A}$ and $5HT_{1B}$ receptors. Data are presented as mean ± SEM for three independent experiments performed in triplicate. DA, dopamine; NE, norepinephrine; 5-HT, serotonin; TYR, tyramine; TRY,

tryptamine; OCT, octopamine; [^{3}H]DA, [^{3}H]dopamine; [^{3}H]NE, [^{3}H]norepinephrine; [^{3}H]5-HT, [^{3}H]serotonin.

Figure 1

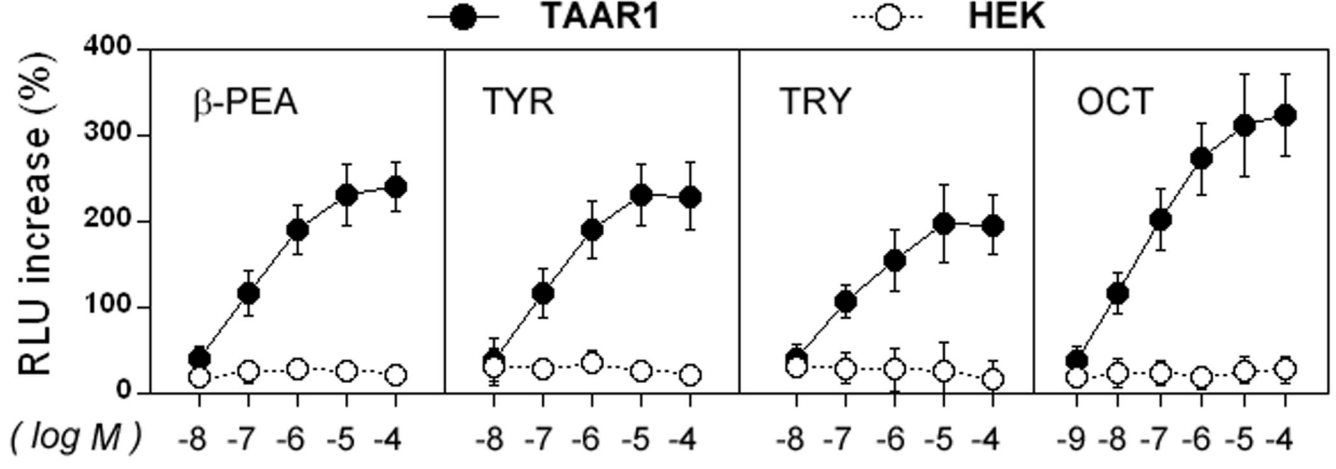

TAAR1
HEK
RLU increase (%)
400
300
200
100
0
β-PEA
TYR
TRY
OCT
(log M)
-8 -7 -6 -5 -4
-8 -7 -6 -5 -4
-8 -7 -6 -5 -4
-9 -8 -7 -6 -5 -4

Figure 2

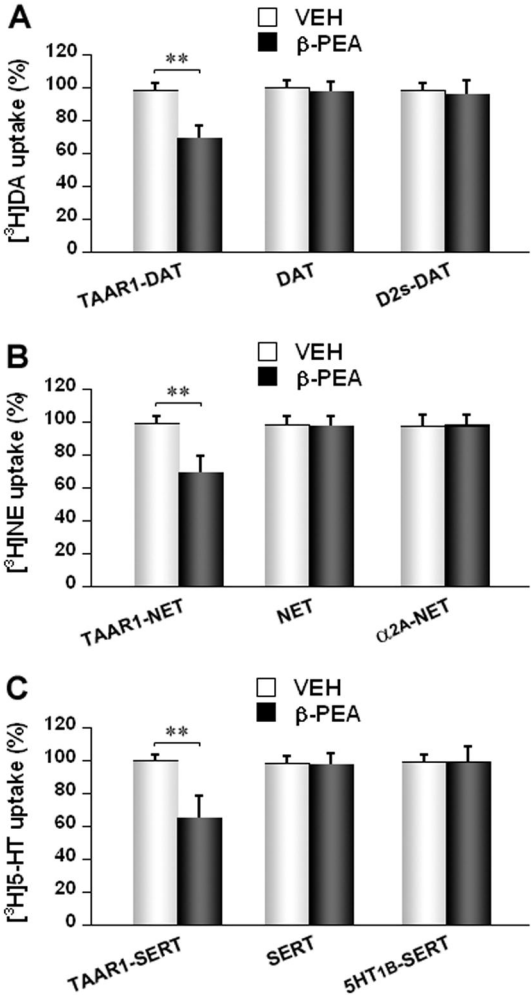

A
VEH
β-PEA
[3H]DA uptake (%)
120
100
80
60
40
20
0
**
TAAR1-DAT
DAT
D2s-DAT
B
VEH
β-PEA
[3H]NE uptake (%)
120
100
80
60
40
20
0
**
TAAR1-NET
NET
α2A-NET
C
VEH
β-PEA
[3H]5-HT uptake (%)
120
100
80
60
40
20
0
**
TAAR1-SERT
SERT
5HT1B-SERT

Figure 3

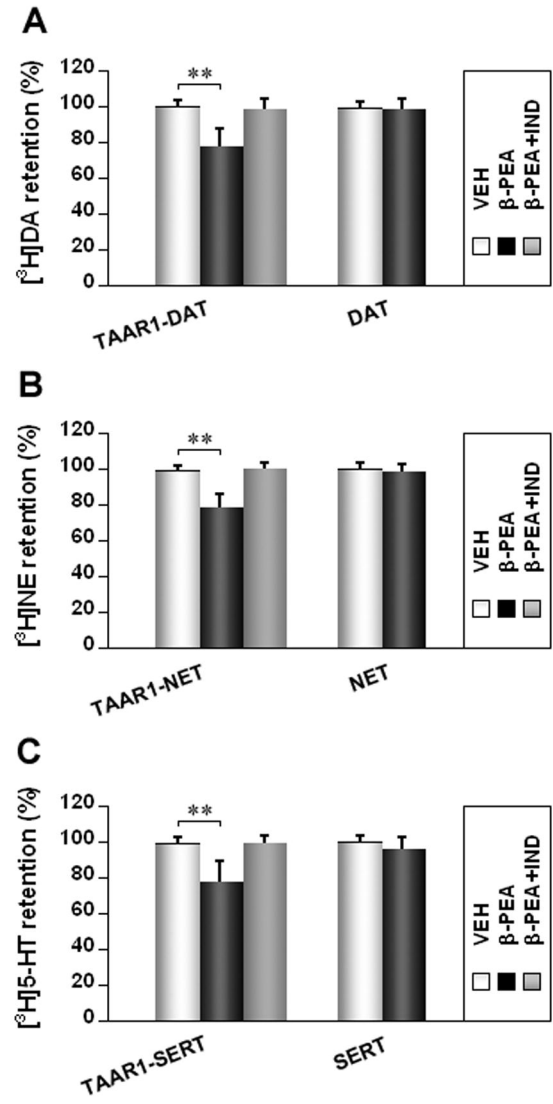

A
[3H]DA retention (%)
120
100
80
60
40
20
0
**
TAAR1-DAT
DAT
VEH
β-PEA
β-PEA+IND
B
[3H]NE retention (%)
120
100
80
60
40
20
0
**
TAAR1-NET
NET
VEH
β-PEA
β-PEA+IND
C
[3H]5-HT retention (%)
120
100
80
60
40
20
0
**
TAAR1-SERT
SERT
VEH
β-PEA
β-PEA+IND

Figure 4

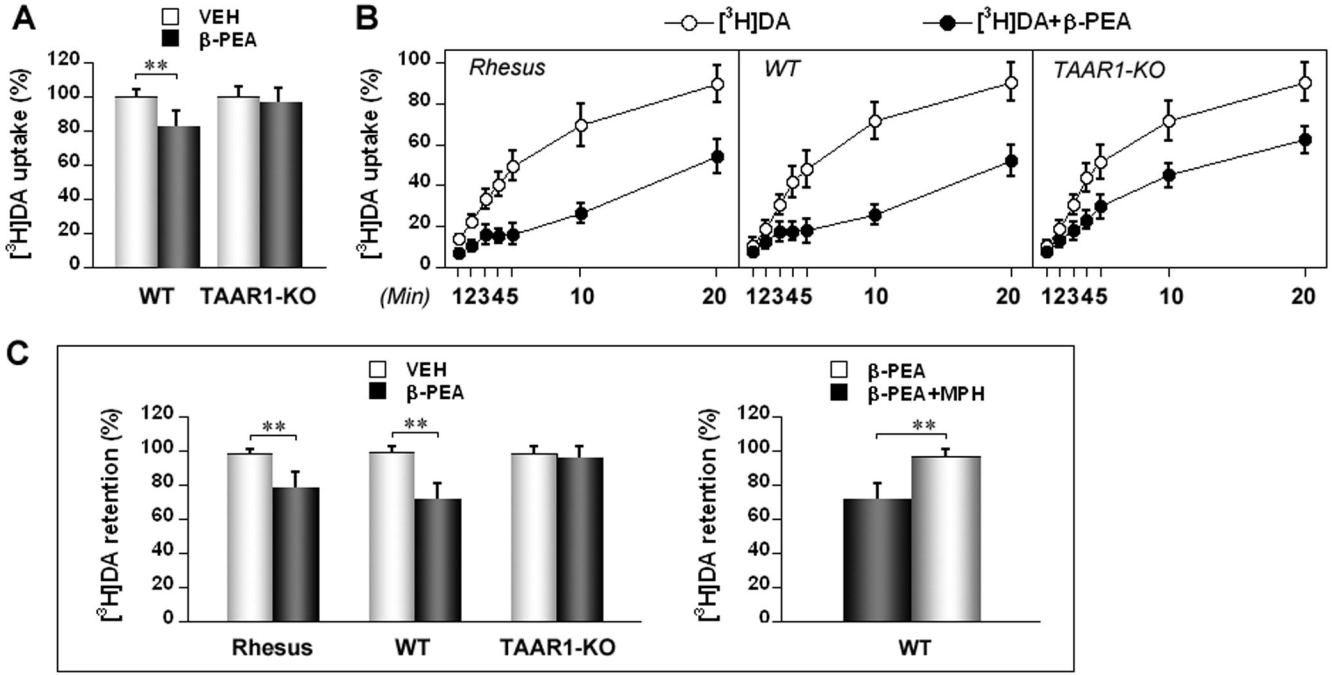

A
VEH
β-PEA
[3H]DA uptake (%)
120
100
80
60
40
20
0
**
WT
TAAR1-KO
B
[3H]DA
[3H]DA+β-PEA
Rhesus
WT
TAAR1-KO
[3H]DA uptake (%)
100
80
60
40
20
0
(Min)
12345
10
20
C
VEH
β-PEA
[3H]DA retention (%)
120
100
80
60
40
20
0
**
Rhesus
WT
TAAR1-KO
β-PEA
β-PEA+MPH
[3H]DA retention (%)
**
WT

Figure 5

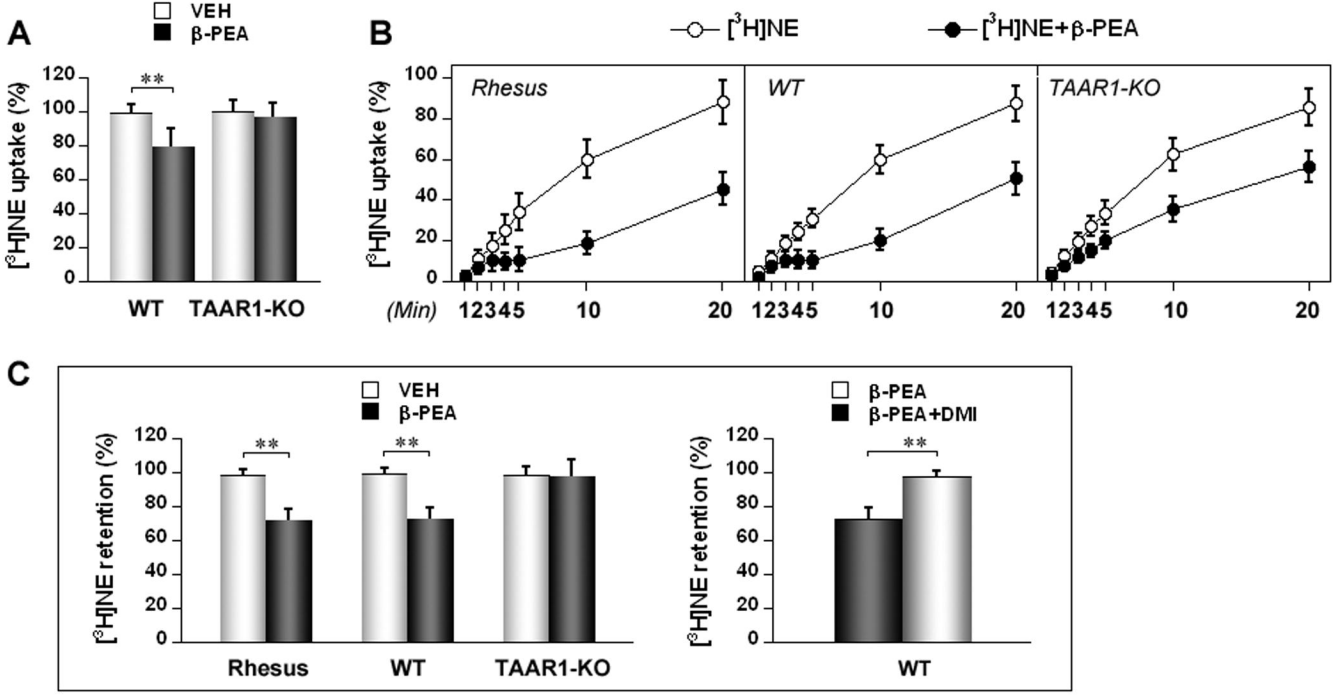

A
VEH
β-PEA
[3H]NE uptake (%)
120
100
80
60
40
20
0
**
WT
TAAR1-KO
B
[3H]NE
[3H]NE+β-PEA
Rhesus
WT
TAAR1-KO
[3H]NE uptake (%)
100
80
60
40
20
0
(Min)
1 2 3 4 5
10
20
C
VEH
β-PEA
[3H]NE retention (%)
120
100
80
60
40
20
0
**
Rhesus
WT
TAAR1-KO
β-PEA
β-PEA+DMI
[3H]NE retention (%)
120
100
80
60
40
20
0
**
WT

Figure 6

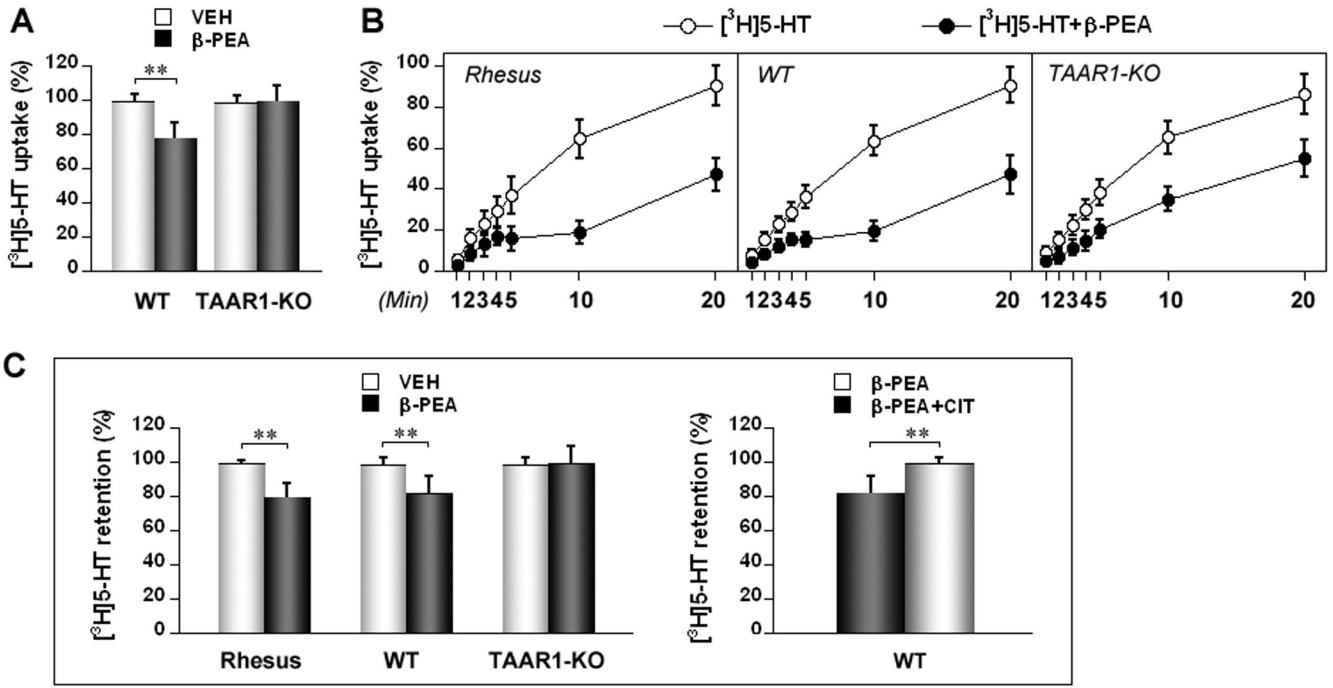

A
VEH
β-PEA
[3H]5-HT uptake (%)
120
100
80
60
40
20
0
**
WT
TAAR1-KO
B
[3H]5-HT
[3H]5-HT+β-PEA
[3H]5-HT uptake (%)
Rhesus
WT
TAAR1-KO
(Min)
1 2 3 4 5
10
20
C
VEH
β-PEA
[3H]5-HT retention (%)
Rhesus
WT
TAAR1-KO
β-PEA
β-PEA+CIT
WT

Figure 7

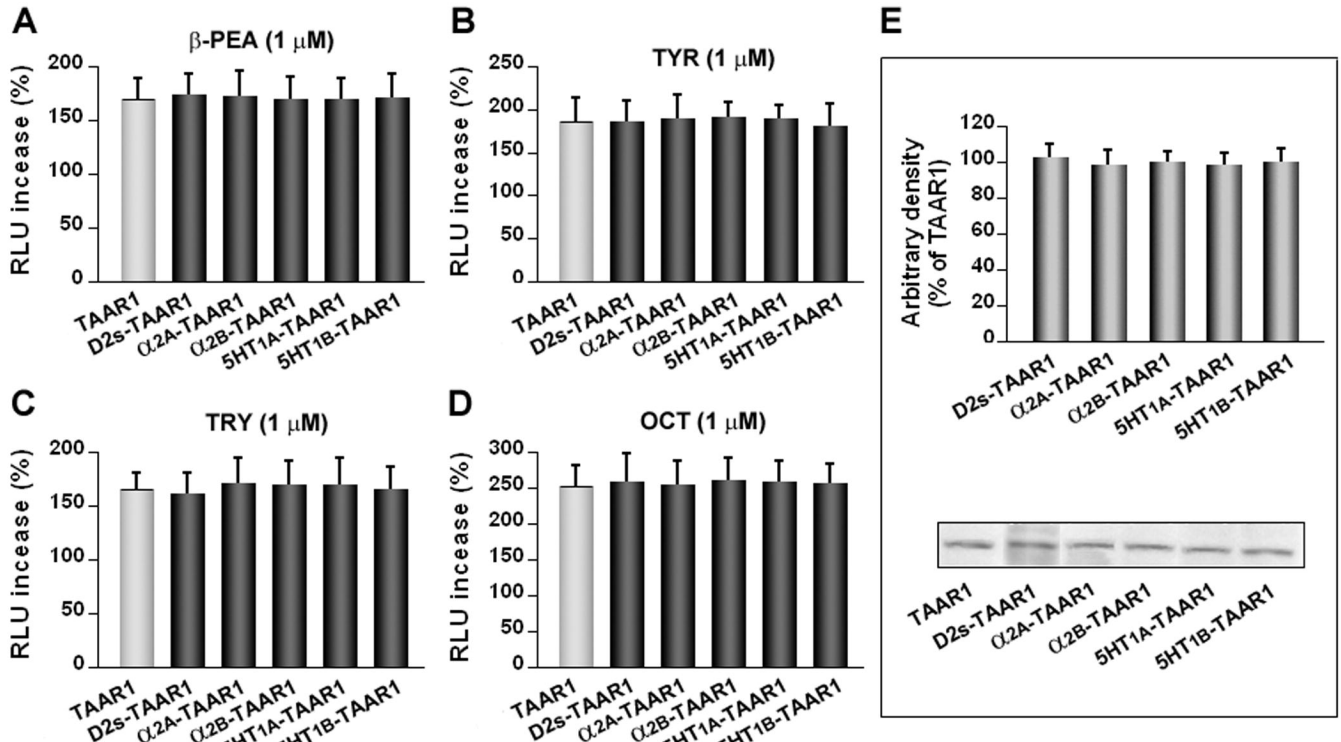
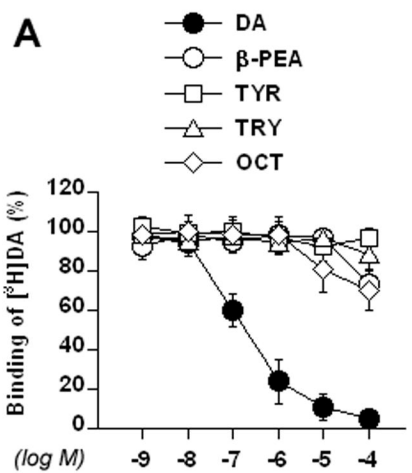
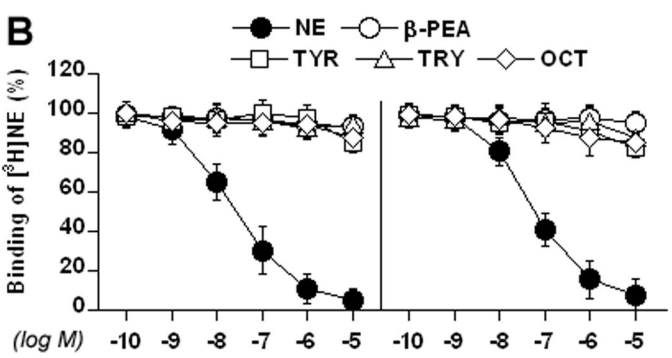
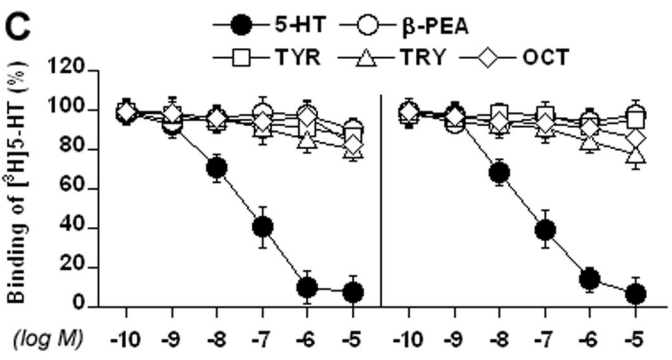

A
β-PEA (1 μM)
RLU incease (%)
200
150
100
50
0
TAAR1
D2s-TAAR1
α2A-TAAR1
α2B-TAAR1
5HT1A-TAAR1
5HT1B-TAAR1
B
TYR (1 μM)
RLU incease (%)
250
200
150
100
50
0
TAAR1
D2s-TAAR1
α2A-TAAR1
α2B-TAAR1
5HT1A-TAAR1
5HT1B-TAAR1
C
TRY (1 μM)
RLU incease (%)
200
150
100
50
0
TAAR1
D2s-TAAR1
α2A-TAAR1
α2B-TAAR1
5HT1A-TAAR1
5HT1B-TAAR1
D
OCT (1 μM)
RLU incease (%)
300
250
200
150
100
50
0
TAAR1
D2s-TAAR1
α2A-TAAR1
α2B-TAAR1
5HT1A-TAAR1
5HT1B-TAAR1
E
Arbitrary density
(% of TAAR1)
120
100
80
60
40
20
0
D2s-TAAR1
α2A-TAAR1
α2B-TAAR1
5HT1A-TAAR1
5HT1B-TAAR1
TAAR1
D2s-TAAR1
α2A-TAAR1
α2B-TAAR1
5HT1A-TAAR1
5HT1B-TAAR1

Figure 8


A
DA
β-PEA
TYR
TRY
OCT
Binding of [³H]DA (%)
120
100
80
60
40
20
0
(log M)
-9
-8
-7
-6
-5
-4


B
NE
β-PEA
TYR
TRY
OCT
Binding of [³H]NE (%)
120
100
80
60
40
20
0
(log M)
-10
-9
-8
-7
-6
-5
-10
-9
-8
-7
-6
-5


C
5-HT
β-PEA
TYR
TRY
OCT
Binding of [³H]5-HT (%)
120
100
80
60
40
20
0
(log M)
-10
-9
-8
-7
-6
-5
-10
-9
-8
-7
-6
-5