The selective antagonist EPPTB reveals TAAR1-mediated regulatory mechanisms in dopaminergic neurons of the mesolimbic system

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Edited by Shigetada Nakanishi, Osaka Bioscience Institute, Osaka, Japan, and approved September 29, 2009 (received for review June 11, 2009)

Trace amine-associated receptor 1 (TAAR1) is a G protein-coupled receptor (GPCR) that is nonselectively activated by endogenous metabolites of amino acids. TAAR1 is considered a promising drug target for the treatment of psychiatric and neurodegenerative disorders. However, no selective ligand to identify TAAR1-specific signaling mechanisms is available yet. Here we report a selective TAAR1 antagonist, EPPTB, and characterize its physiological effects at dopamine (DA) neurons of the ventral tegmental area (VTA). We show that EPPTB prevents the reduction of the firing frequency of DA neurons induced by p-tyramine (p-tyr), a nonselective TAAR1 agonist. When applied alone, EPPTB increases the firing frequency of DA neurons, suggesting that TAAR1 either exhibits constitutive activity or is tonically activated by ambient levels of endogenous agonist(s). We further show that EPPTB blocks the TAAR1-mediated activation of an inwardly rectifying K+ current. Importantly, these EPPTB effects were absent in Taar1 knockout mice, ruling out off-target effects. We additionally found that both the acute application of EPPTB and the constitutive genetic lack of TAAR1 increase the potency of DA at D2 receptors in DA neurons. In summary, our data support that TAAR1 tonically activates inwardly rectifying K+ channels, which reduces the basal firing frequency of DA neurons in the VTA. We hypothesize that the EPPTB-induced increase in the potency of DA at D2 receptors is part of a homeostatic feedback mechanism compensating for the lack of inhibitory TAAR1 tone.

Results

Identification of the TAAR1 Antagonist EPPTB. For high-throughput compound screening, we stably expressed human TAAR1 in HEK293 cells. To identify antagonists, we activated the receptor with β-phenylethylamine (1.5 μM, corresponding to the EC50 value) and measured the compound-mediated inhibition of cAMP accumulation. Compounds with an IC50 < 3 μM in the primary screen were optimized for their physicochemical, pharmacodynamic, and pharmacokinetic properties using medicinal chemistry. This resulted in the identification of the TAAR1 antagonist EPPTB (Fig. 1). EPPTB potently antagonized cAMP production induced by activating mouse TAAR1 with 1.5 μM β-phenylethylamine (IC50 = 27.5 ± 9.4 nM, Fig. S1). Schild plot analysis revealed a competitive mode of action of EPPTB (Fig. S2). Interestingly, EPPTB reduced TAAR1-stimulated cAMP production in stably transfected HEK293 cells below basal levels (−12.3 ± 4.7%). This suggests that TAAR1 exhibits agonist independent constitutive activity and that EPPTB is an inverse agonist. Consistent with this notion, cAMP levels were dose-dependently reduced by EPPTB in HEK293 cells in the absence of TAAR1 agonist (−10.2 ± 4.5%, IC50 = 19 ± 12 nM). EPPTB was significantly more potent in antagonizing cAMP production by mouse, as compared to rat (IC50 = 4539 ± 2051 nM)


The authors declare no conflict of interest.

This article contains supporting information online at www.pnas.org/cgi/content/full/0906522106/DCSupplemental.

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TAAR1 binding to TAAR1 engages Gα-type G proteins that activate adenylyl cyclases (1). However, because TAs not only activate TAAR1 but also influence the activity of TAAR4, DA transporters, adrenergic, as well as serotonin receptors it was difficult to assign specific physiological functions to TAAR1 (1, 6). With the availability of Taar1 knockout mice (7, 8) it became clear that TAAR1 can inhibit DA neurons in the VTA via a receptor-mediated pathway. The genetic absence of TAAR1 clearly increased the spontaneous firing rate of DA neurons but the underlying signaling mechanism remained unclear (7). Taar1 knockout mice also display behavioral and neurochemical signs of DA supersensitivity, a feature thought to relate to positive symptoms of schizophrenia (8). In addition, TAs were implicated in the etiology of depression, addiction, attention-deficit/hyperactivity disorder, and Parkinson’s disease (5, 9, 10). However, validation of therapeutic concepts was hampered by the lack of a ligand that specifically regulates TAAR1 activity in vivo. Here we report the identification of a selective TAAR1 antagonist, N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide [EPPTB, CAS Registry Number 1110781–88-8, (11)], which we used to study the signaling mechanisms of TAAR1 in DA neurons of the VTA. Surprisingly, we found that TAAR1 not only tonically activates inwardly rectifying K+ channels, but that acute blockade of TAAR1 also increases the affinity of DA at D2 receptors. We discuss the implications of our findings for DA neurotransmission and drug discovery.

T amines (TAs) such as p-tyr, β-phenylethylamine, octopamine, and tryptamine are metabolites of amino acids that are found at low concentrations in the brain (1). Because of their structural similarity to classical biogenic amines, TAs were for a long time believed to modulate neurotransmission by displacing biogenic amines from vesicular stores or by acting on transporters in an amphetamine-like manner. It was not until TAs were found to bind to members of a family of GPCRs, the TA-associated receptors (TAARs), that receptor-mediated mechanisms were evoked (2–5). While several TAARs were identified, only TAAR1 and, to a lesser extent, TAAR4 respond to typical TAs (5). TAs such as p-tyr and β-phenylethylamine activate human, mouse, and rat TAAR1 with EC50 values of 0.2–1.7 μM. Other TAs (octopamine, tryptamine), classical biogenic amines, and amphetamine-related psychostimulants have much reduced potency and efficacy at TAAR1.

Desensitization | dopamine supersensitivity | Kir3 | trace amines | VTA
Blocking of TAAR1 Activity Increases the Firing Frequency of DA Neurons in the VTA. We first addressed whether EPPTB inhibits the p-tyr-mediated inhibition of the spontaneous firing frequency of DA neurons in slice preparations of the VTA (7, 12). Bath application of EPPTB (10 nM) under current-clamp conditions not only prevented the inhibition of the firing frequency of DA neurons induced by p-tyr (10 μM), but significantly increased the firing frequency over the basal level (Fig. 2A). As a control, p-tyr by itself or in combination with EPPTB failed to alter the firing frequency of DA neurons in Taar1−/− mice (Fig. 2B). However, as reported earlier (7), the basal firing rate in Taar1−/− mice was significantly increased compared to wild-type (WT) littermate mice, suggesting that TAAR1 in DA neurons of the VTA is normally constitutively active or tonically activated by ambient levels of endogenous agonist(s). Accordingly, we found that EPPTB by itself significantly and reversibly increased the basal firing rate of DA neurons in WT but not in Taar1−/− mice (Fig. 2C and D). Under current-clamp conditions, the p-tyr-induced decrease in the firing frequency of WT DA neurons was associated with a hyperpolarization of the membrane potential from −49.6 ± 0.4 to −57.6 ± 0.7 mV (n = 15), consistent with earlier findings (7, 12). Since in voltage-clamp recordings p-tyr application to DA neurons caused a drop in input resistance [see below and (12)], this hyperpolarization suggests the opening of K+ channels. Conversely, the application of EPPTB to WT DA neurons was accompanied with a depolarization of the membrane potential to −35.8 mV (n = 15), indicating that TAAR1 mediates an inhibitory tone.

DA neurons possess cation-permeable Ii channels that are activated by hyperpolarization and positively modulated by cAMP (13, 14). Therefore, a concomitant TAAR1-mediated hyperpolarization and increase in the intracellular cAMP concentration may support the activation of Ii currents. However, blocking Ii channels in WT mice with the inhibitor ZD7288 (20 μM) had no significant effect on the firing frequency in the presence or absence of p-tyr (control, 2.1 ± 0.8 Hz, n = 10; control + ZD7288, 2.2 ± 0.7 Hz, n = 5; p-tyr, 0.5 ± 0.1 Hz, n = 10; p-tyr + ZD7288, 0.4 ± 0.1 Hz, n = 5). This supports that Ii channels in DA neurons of the VTA are neither activated by TAAR1 nor involved in pacemaker frequency control, as already suggested by an earlier report (14).

EPPTB Inhibits a TAAR1-Induced G Protein-Dependent Outward Current in DA Neurons of the VTA. Voltage-clamp experiments revealed that at a physiological extracellular [K+], bath application of p-tyr to DA neurons in the VTA induced an outward current with an EC50 value of 305 ± 11 nM, which agrees with EC50 values obtained at recombinant mouse receptors in other assays (Table 1 and Fig. S3). The outward current induced by p-tyr (10 μM) was associated with a 21% decrease of the apparent input resistance and inhibited by EPPTB (10 nM) (Fig. 3A and B). In the presence of 1.5 nM EPPTB the p-tyr dose–response curve exhibited a parallel rightward shift with no significant reduction of the maximal response, consistent with the competitive mode of action of EPPTB (Fig. S3). It was proposed that trace amines cause an indirect activation of DA D2 receptors by enhancing the efflux of dopamine (12). However, the D2 antagonist sulpiride (10 μM) did not inhibit the p-tyr-induced current, showing that the current is not mediated by D2 receptors (Fig. 3B). Since TAAR1 is positively coupled to adenylyl cyclase, we analyzed whether cAMP-dependent protein kinase A (PKA) modulates the p-tyr-induced outward current. In the presence of the PKA inhibitor H8 (10 μM), the amplitude of the outward current remained unchanged (Fig. 3B). Consistent with constitutive activity or tonic TAAR1 activation the holding current was reduced below baseline following EPPTB application. Neither p-tyr nor EPPTB had any effects on the holding current in Taar1−/− mice. In further support of constitutive or tonic TAAR1 activity, application of EPPTB in the absence of p-tyr elicited an apparent inward current in WT but not in Taar1−/− mice (Fig. 3C and D). The EPPTB-induced inward current in WT neurons was associated with a 16% increase in input resistance, supporting the closure of K+ channels. In WT mice, the EPPTB-induced inward current was significantly increased.

Table 1. Comparison of binding affinities and EC50 values of p-tyr and EPPTB at human and rodent TAAR1

<table>
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<tr>
<th>Compound</th>
<th>Parameter, assay, preparation</th>
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<th>Rat</th>
<th>Human</th>
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<td>p-tyr</td>
<td>K&lt;sub&gt;i&lt;/sub&gt;, binding, HEK293*</td>
<td>404 ± 129</td>
<td>70 ± 12</td>
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;, CAMP, HEK293†</td>
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<td>125 ± 36</td>
<td>1664 ± 135</td>
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;, GIRK, Xenopus oocytes‡</td>
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<td>nd</td>
<td>nd</td>
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;, patch-clamp, VTA slices§</td>
<td>305 ± 11</td>
<td>nd</td>
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<td></td>
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<tr>
<td>EPPTB</td>
<td>K&lt;sub&gt;i&lt;/sub&gt;, binding, HEK293*</td>
<td>0.9 ± 0.1</td>
<td>942 ± 133</td>
<td>nd</td>
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</table>

*Radioligand [3H]-rac-2-(1,2,3,4-tetrahydro-1-naphthyl)-2-imidazolinedione (12).
†Biotin-Enzyme Immunoassay for CAMP.
‡Current mediated by Kir3.1 and Kir3.2 co-expressed with TAAR1.
§Current at −50 mV holding potential; nd, not determined. Values are given as nM (mean ± SEM). The results were obtained from at least four independent experiments.

Fig. 1. Chemical structure of the selective TAAR1 antagonist N-(3-Ethoxyphenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide (EPPTB).
Following preincubation of the slices with 100 μM pargyline (Fig. 3D), a nonspecific monoamine oxidase inhibitor increasing the extracellular TA concentration (15), TAAR1 may, similar to other GPCRs, gate Kir3-type K⁺ channels via the G₂₅₄, subunits of the activated G protein (16). Consistent with this proposal, dialysis of DA neurons with GDPβS (2 mM for 40 min), a blocker of G protein activation, significantly reduced the p-tyr-mediated outward current (Fig. 3E).

Characterization of the p-Tyr-Mediated Current in DA Neurons of the VTA. To determine whether the p-tyr-mediated current is carried by K⁺ ions, we determined its current–voltage (I–V) relationship. We applied brief voltage ramps ranging from −20 to −140 mV in the absence and presence of p-tyr. The I–V relationship of the current induced by p-tyr was then calculated by subtracting the I–V curve in the absence of p-tyr from the I–V curve in the presence of p-tyr. In physiological extracellular [K⁺] the current induced by p-tyr showed inward rectification and reversed near the calculated K⁺ equilibrium potential (2.5 mM [K⁺]ₑ, Vₑᵥₑᵣ = −101 mV) (Fig. 4A). This supports that p-tyr selectively activates a K⁺ current, further ruling out a contribution of a mixed Na⁺/K⁺ current from L⁴₉ channels (see above). In the presence of EPPTB a p-tyr-induced response was mostly lacking, similar to what is observed in Taar¹⁻/⁻ mice (Fig. 4D). The reversal potential of the p-tyr-induced current is shifted by rising the extracellular [K⁺] (12.5 mM [K⁺]ₑ, Vₑᵥₑᵣ = −60 mV), confirming that the gating of K⁺ channels underlies the p-tyr-induced current (Fig. 4B). The nonselective K⁺ channel blocker Ba²⁺ (300 μM) and the Kir3 channel blocker tertiapin (10 μM) (17) occluded the p-tyr-induced current, suggesting that the activation of Kir3 channels underlies the K⁺ current (Fig. S4).
TAAR1 Couples to Kir3 Channels in Xenopus Oocytes. To confirm that TAAR1 can activate Kir3 channels we coexpressed human Kir3.1 and Kir3.2 subunits together with mouse TAAR1 in Xenopus oocytes. At a holding potential of −50 mV and in the presence of elevated K⁺ (45 mM) oocytes expressing Kir3 produced a tonic inward current, consistent with the reported constitutive activation of K⁺ channels by excess free Gαq (18, 19). Application of p-tyr evoked an additional inward current (Fig. 5A). The E_{SC50} of 167 nM for the p-tyr-induced current is comparable to the E_{SC50} values measured in other assays (Table 1). The K⁺-current in response to p-tyr (10 μM) was robustly reduced by EPPTB (3 μM) (Fig. 5A and B). Intriguingly, EPPTB inhibited receptors heterologously expressed in Xenopus oocytes or HEK293 cells (cAMP assay) less potently than neuronal receptors. This may be attributable to differences in posttranslational receptor modifications or to interacting proteins allosterically influencing the antagonist binding-site. The p-tyr-induced current in Xenopus oocytes was not sensitive to pertussis toxin (PTX), supporting that the activation of Kir3 channels is mediated by Gαq and not by Gαo-type G proteins (Fig. 5C). Accordingly, coexpression of the G protein subunit αq increased the p-tyr-induced current (Fig. 5D), similarly as previously described for the β2-adrenergic receptor (19). No p-tyr-evoked current was seen in control oocytes injected with TAAR1 mRNA or Kir3.1/3.2 mRNA alone.

Increased Potency of DA at D2 Receptors in the Absence of TAAR1 Activity. Taar1⁻/⁻ mice exhibit an increase in the high-affinity states of striatal D2 receptors (8), which is indicative of DA supersensitivity (20, 21). We therefore investigated whether acute blockade of TAAR1 activity modulates D2 receptors in DA neurons of the VTA. Bath application of the D2/D3 DA receptor agonist quinpirole (30 μM) induced an outward current that decreased during prolonged application (Fig. 6A). Preincubation of slices with EPPTB prevented this current desensitization. Likewise, DA neurons in Taar1⁻/⁻ mice exhibited non-desensitizing, quinpirole-mediated currents, which were not altered in the presence of EPPTB (Fig. 6B). In the presence of the D2 receptor antagonist sulpiride (10 μM) the holding currents in WT slices preincubated with EPPTB were reduced below baseline (Fig. 6A). This suggests that D2 receptors become constitutively activated or tonically active in the absence of TAAR1 activity. Lack of TAAR1 activity not only correlates with a reduced desensitization rate and tonic or constitutive D2 receptor activity, but also with a simultaneous approximately 4-fold increase in agonist potency at D2 receptors (Fig. 6C). Of note, the potency of quinpirole at DA neurons in the presence of EPPTB was comparable to the potency of quinpirole at Taar1⁻/⁻ DA neurons. In the presence of 40 nM p-tyr a parallel rightward shift of the quinpirole dose-response curve is observed, in support of TAAR1 activity reducing agonist potency at D2 receptors (Fig. 6C).

Discussion
To our knowledge, no other selective TAAR1 antagonist has been identified. EPPTB is more potent at mouse TAAR1 than at rat and human TAAR1. We characterized the pharmacological and physiological effects of EPPTB in the mouse brain, using Taar1⁻/⁻ mice as a control. Trace amines such as p-tyr are known to reduce the firing frequency of DA neurons (7, 12, 22, 23),
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PHARMACOLOGY

November 24, 2009

vol. 106

no. 47

20085

Here, we show that in slices of the VTA, EPPTB antagonizes the p-tetramer mediated inhibition of DA neuron firing. In fact, incubation of VTA slices with EPPTB increases the spontaneous firing rate of DA neurons above the basal level, consistent with an increased firing rate of DA neurons observed in Taar1 knockout mice (7). This corroborates the idea that TAAR1 normally exerts an inhibitory tone on DA neurons. We further show that activation of TAAR1 induces a G protein-dependent inwardly rectifying K⁺ current that is inhibited by Ba²⁺ and tertiapin, suggesting that TAAR1 reduces the firing rate of DA neurons by activating Kir3 channels. It was proposed that trace amines cause an increased efflux of newly synthesized dopamine (12) that could activate a K⁺ current via D2 receptors. However, pharmacological inhibition of D2 receptors with sulpiride does not inhibit the TAAR1-induced K⁺ current, showing that the K⁺ current is not triggered by D2 receptors. Instead, we found that in Xenopus oocytes TAAR1 directly activates Kir3 channels via PTX-insensitive G-proteins, most likely Gₛ proteins. This challenges an earlier report failing to demonstrate TAAR1 coupling to Kir3 channels (24). Our data reinforce that GPCRs coupled to G proteins other than Gₛ/o can gate Kir3 channels (25–29). Federici and colleagues showed that the application of p-tetra to DA neurons at concentrations higher than those used in our study (at least 30 µM) attenuates GABAₐ₅-mediated K⁺ currents through a G protein-dependent process (30). While they did not study direct effects of TAAR1 on Kir3 channels, they suggested that a reduced coupling between GABA receptors and Kir3 channels underlies this K⁺ current attenuation. Possibly, low-affinity p-tetra receptors distinct from TAAR1 are involved in this uncoupling. However, since Kir3 current responses in DA neurons desensitize (31) it is also conceivable that a TAAR1-mediated K⁺ current desensitization contributes to the attenuation of the GABA response.

We observed a functional link between TAAR1 and D2 receptors in DA neurons. Specifically, we found that the TAAR1 antagonist EPPTB increased agonist potency at D2 receptors while at the same time reducing their desensitization rate. A simultaneous increase in agonist potency and lack of desensitization makes D2 receptors ideally suited to sense ambient levels of DA. Presumably, this increases D2-mediated inhibition of DA neurons and balances the loss of TAAR1-mediated inhibition. The data support that TAAR1 is part of a homeostatic regulatory mechanism that prevents excess activity of DA neurons. It is currently unknown by which molecular events TAAR1 influences D2 receptors. It is conceivable that TAAR1 regulates the phosphorylation state of D2 receptors, which may explain the rapid effects on D2 receptor pharmacology and kinetics after acute TAAR1 blockade with EPPTB.

It was proposed that TAAR1 inhibits locomotor activity via a downmodulation of DA neurotransmission (7). This is supported by our study demonstrating a TAAR1-mediated activation of K⁺ channels in DA neurons. Because TAAR1 activates K⁺ channels in DA tonically, the overruling effect of blocking TAAR1 is a net increase in the firing rate of DA neurons. Antagonizing TAAR1 with compounds like EPPTB may therefore potentially provide an approach to enhance the action of L-dopa in Parkinson’s disease. Activating TAAR1, on the other hand, may be of therapeutic benefit in the treatment of schizophrenia, addiction, or attention deficit/hyperactivity disorder.

Materials and Methods

Generation of Cell Lines Stably Expressing TAAR1 and CAMP Measurements. Full-length genomic DNA was used to construct pREsne2 expression vectors encoding human, rat, and mouse TAAR1 (4). TAAR1 expressing cell lines were generated by transfecting HEK293 cells (ATCC) with expression plasmids using Lipofectamine 2000 (Invitrogen). Cells were cultured for 10 days in the presence of 1 mg/mL G418 (Sigma). Individual colonies were expanded and tested for responsiveness to TAs (Sigma) using the cAMP Biotrak Enzyme Immunoassay (EIA).

Membrane Preparation and Radioligand Binding. Cells were rinsed with ice-cold PBS (without Ca²⁺ and Mg²⁺) containing 10 mM EDTA and pelleted at 1,000 rpm for 5 min at 4 °C. The pellet was washed twice with ice-cold PBS, frozen in liquid nitrogen, and stored at −80 °C. Frozen pellets were suspended in 20 mL Hepes-NaOH (20 mM, pH 7.4) containing 10 mM EDTA and homogenized with a Polytron (PT 3000, Kinematica) at 10,000 rpm for 10 s. The homogenate was centrifuged at 48,000 × g for 30 min at 4 °C and the pellet suspended in 20 mL Hepes-NaOH containing 0.1 mM EDTA (buffer A). The procedure was repeated twice, the final pellet resuspended at 200 µg protein per mL in Hepes-NaOH (20 mM, pH 7.0) containing MgCl₂ (10 mM) and CaCl₂ (2 mM) (buffer B) and homogenized with a Polytron at 10,000 rpm for 10 s. Binding assays were performed for 30 min at 4 °C in a final volume of 1 mL. The TAAR1 radioligand [³²P]-rac-2-(1,2,3,4-tetrahydroy-1-naphthyl)-2-imidazoline (11) was used at a concentration equal to the calculated Kᵢ value of 60 nM, which resulted into approximately 0.1% of the radioligand and a specific binding representing approximately 70–80% of the total binding. Nonspecific binding was defined as the amount of [³²P]-rac-2-(1,2,3,4-tetrahydroy-1-naphthyl)-2-imidazoline bound in the presence of 10 µM unla-
belled ligand. All compounds were tested at a broad range of concentrations (10 pM to 10 μM) in duplicate. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company) and glass filters GF/C presoaked for at least 2 h in polyethyleneimine 0.3%. Tubes and filters were washed three times with 1 mL of cold buffer B. Wet filters were soaked in Ultima gold (45 μL/well) and the radioactivity counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company).

Electrophysiology in Brain Slices. Horizontal slices (250-μm) of the midbrain were made from adult mice (3–6 months of age) in cooled artificial cerebrospinal fluid containing (in mM): NaCl 119, KCl 2.5, MgCl2 1.3, CaCl2 2.5, NaH2PO4 1.0, NaHCO3 26.2, and glucose 11, bubbled with 95% O2 and 5% CO2. After 1 h slices were transferred to the recording chamber and superfused with 1.5 mL min⁻¹ artificial cerebrospinal fluid. Visual whole-cell voltage-clamp or current-clamp recording techniques were used to measure response of D2 DA neurons at −50 mV or to record spontaneous spiking activity, respectively. DA cells were identified by their large Ih current and an outward current in response to the D2 receptor agonist quinpirole (10 μM). The internal solution contained (in mM): potassium gluconate 130, MgCl2 4, EGTA 1.1, HEPES 5, NaATP 3.4, sodium creatine-phosphate 10, and NaGTP 0.1. Signals were amplified, filtered at 1 kHz (Axopatch 200B), digitized at 5 kHz (Digidata 1322A card) and saved on hard disk (pClamp 9, Axon Instruments). Current-voltage (I–V) relationships were determined by ramp-command protocols (from −20 to −140 mV, 250 ms duration) in the presence of TEA and 4-AP in the external solution (32). Data are presented as mean ± SEM. For statistical comparison of two groups the nonparametric Mann-Whitney test was used (GraphPad InStat 3.0). For statistical comparison of multiple groups two-way ANOVA was used. For statistical comparison of frequencies the Kolmogorov-Smirnov test was used. Quinpirole, sulpiride, ZD7288, and ptyr were from Sigma.

Electrophysiology in Xenopus Oocytes. Plasmids encoding mouse TAAAR1 and human Kir3.1/3.2 were linearized, mRNA synthesized (mMESSAGE mMA- CHINE Ultra, Ambion) and mRNA purified (MEGAgold kit, Ambion). Capped and polyA tailed mRNA was microinjected into Xenopus laevis oocytes (Ecotye Biotech). Each oocyte was injected with 50 nL of an aqueous solution containing 20 pg/nL mouse TAAAR1 mRNA and 0.5 pg/nL Kir3.1 and Kir3.2 mRNA. The Roboocyte instrument (Multi Channel System) was used for oocyte injection and voltage-clamp experiments. Experiments were performed 4–6 days after mRNA injection. During electrical recording the oocytes were constantly superfused with a solution containing (in mM): NaCl 46, KCl 45, MgCl2 1, CaCl2 1, HEPES 5, pH adjusted to 7.4 by addition of NaOH. In some experiments PTX (−5 ng/oocyte, Sigma) was injected into the oocytes 2 days before recording. Quantitative data are presented as mean ± SEM. The t-test was used for statistical comparison.

ACKNOWLEDGMENTS. We thank S. Chaboz, V. Metzler, P. Biry, P. Pifilmin, and V. Graf for technical assistance and R. Seder and R. Turecek for comments on the manuscript. This work was supported by F. Hoffmann-La Roche Ltd, Swiss Science Foundation Grant 3100A0-117816 (to B.B.) and European Community’s Seventh Framework Program Grant FP7/2007-2013 under Grant Agreement 201714.

**Fig. S1.** Inhibition of the TAAR1-mediated increase in cAMP levels by EPPTB in transfected HEK293 cells. Open circles, cAMP accumulation in response to increasing concentrations of p-tyr (EC\(_{50}\) = 545 ± 179 nM). Data were normalized to the cAMP level obtained with 10 μM β-phenylethylamine. Filled squares, inhibition of β-phenylethylamine (1.5 μM)-mediated cAMP accumulation by increasing concentrations of EPPTB (IC\(_{50}\) = 27.5 ± 9.4 nM). Bars show mean values ± SEM of four experiments. Data analysis and curve fitting was performed using nonlinear regression (GraphPad Prism Version 5.01).
**Fig. S2.** Schild plot analysis of EPPTB in transfected HEK293. (A) Concentration-response curves for the β-phenylethylamine-induced accumulation of cAMP under control conditions and in the presence of increasing concentrations of EPPTB (48–768 nM, 2-fold increases). A representative experiment is shown. (B) Schild plot derived from the concentration-response curves of two independent experiments. The concentration ratio r is the EC50 for β-phenylethylamine in the presence of EPPTB divided by the EC50 for β-phenylethylamine under control conditions. The slope of the line fitted to the concentration ratios is 1.065, which suggests that EPPTB is a competitive antagonist.
Fig. S3. The dose-response curve of the p-tyr-induced K⁺ current amplitude at DA neurons is shifted to the right in the presence of 1.5 nM EPPTB, with no significant reduction in the maximally p-tyr-induced current amplitude (EC₅₀ values: p-tyr, 305 ± 11 nM; p-tyr + EPPTB, 650 ± 11 nM). This is consistent with a competitive mode of action of EPPTB at the p-tyr binding-site.
Fig. S4. The p-tyr induced K⁺ current in DA neurons of the VTA is sensitive to Ba²⁺ and tertiapin. The p-tyr induced currents were obtained by calculating the difference between the I–V curves before and after addition of p-tyr (10 μM), both in the absence (black traces) and presence (gray traces) of the nonselective K⁺ channel blocker Ba²⁺ (300 μM) and the Kir3 channel blocker tertiapin (10 μM), respectively. This suggests that the activation of Kir3 channels underlies the p-tyr induced K⁺ current in DA neurons of the VTA.
Table S1. Percent inhibition of control specific binding (mean ± SEM) is shown for EPPTB (10 μM) on several receptors and transporters

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<tr>
<th>Receptors/Transporters</th>
<th>Assay</th>
<th>% Inhibition of Control Specific Binding</th>
<th>SEM</th>
<th>% Control</th>
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The results were obtained from three independent experiments.