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Functional interaction between Trace Amine Associated Receptor 1 (TAAR1) and dopamine D2 receptor

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TAAR1 and dopamine D2 receptor interaction

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Abstract

The ability of dopamine receptors to interact with other receptor subtypes may provide mechanisms for modulating dopamine-related functions and behaviors. In particular, there is evidence suggesting that the Trace Amine-Associated Receptor 1 (TAAR1) affects the dopaminergic system by regulating the firing rate of dopaminergic neurons or by altering dopamine D2 receptor (D2R) responsiveness to ligands. TAAR1 is a G α s protein-coupled receptor that is activated by biogenic amines, “trace amines” (TAs), such as β -phenylethylamine (β -PEA) and tyramine that are normally found at low concentrations in the mammalian brain. In the present study, we investigated the biochemical mechanism of interaction between TAAR1 and D2R and the role this interaction plays in D2R-related signaling and behaviors. Using a bioluminescence resonance energy transfer (BRET) biosensor for cAMP, we demonstrated that the D2R antagonists haloperidol, raclopride and amisulpride were able to enhance selectively a TAAR1-mediated β -PEA increase of cAMP. Moreover, TAAR1 and D2R were able to form heterodimers when co-expressed in HEK 293 cells and this direct interaction was disrupted in the presence of haloperidol. In addition, in mice lacking TAAR1, haloperidol-induced striatal c-Fos expression and catalepsy were significantly reduced. Taken together, these data suggest that TAAR1 and D2R have functional as well as physical interactions that could be critical for the modulation of the dopaminergic system by TAAR1 *in vivo*.

Introduction

The trace amines (TAs) β -phenylethylamine (β -PEA), p-tyramine, octopamine, tryptamine and synephrine are endogenous biogenic amines that are present in mammalian brain at very low concentrations compared to classical monoamines (Baldessarini, 1978; Grandy, 2007). For many years TAs were thought to have a minor role in neurotransmission and they were traditionally referred to as side products of the synthesis of classical monoamines. Their function as sympathomimetic compounds has been known since the last century and in humans TA activity is particularly evident in subjects treated with monoamine oxidase inhibitors or in individuals that consume food containing TAs in high concentrations (McCabe and Tsuang, 1982). The “amphetamine-like” effect of TAs is believed to occur at high, non-physiological concentrations and has been explained by their interaction with the plasma membrane monoamine transporters, particularly the dopamine transporter (DAT) (Berry, 2004). In normal mice, β -PEA administration induces hyperactivity and an increase in dopamine release, and these effects are disrupted in mice lacking the DAT (Sotnikova et al., 2004). The dysregulation of TAs has been linked to different neurological and psychiatric disorders, such as schizophrenia, depression, Parkinson’s disease (PD), attention deficit hyperactivity disorder (ADHD) and migraine (Grandy, 2007; Sotnikova et al., 2009). Altered levels of β -PEA have been found in patients with depression and psychotic episodes (Davis and Boulton, 1994; Sabelli and Mosnaim, 1974).

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The recent discovery of a class of G protein-coupled receptors (GPCR) that can be activated by TAs, trace amine-associated receptors (TAARs), has further increased interest in these amines and their roles in both physiology and pathology (Borowsky et al., 2001; Bunzow et al., 2001). Six TAAR genes and three TAAR pseudogenes exist in humans while rodents express an even greater number of TAAR genes. Only TAAR1 and TAAR4, however, possess any demonstrable TA responsiveness.

TAAR1 signals through the stimulatory G protein (Gas) and is localized in several brain areas including the limbic regions and in the nuclei containing monoaminergic cell bodies (Lindemann et al., 2008). This expression pattern makes TAAR1 a potential therapeutic target to modulate critical behaviors related to monoamine systems (Lindemann and Hoener, 2005; Sotnikova et al., 2009). In animal studies, TAAR1 deficient mice (TAAR1-KO mice) display an increased sensitivity to the neurochemical and locomotor effects of amphetamine (Lindemann et al., 2008; Wolinsky et al., 2007). While *in vitro* studies have suggested that TAAR1 may directly alter DAT function (Miller et al., 2005) there is substantial evidence that TAAR1 is able to modulate firing activity of dopaminergic neurons in the ventral tegmental area (VTA) (Lindemann et al., 2008) via potential interaction with dopamine D2 receptor (D2R) signaling. Particularly, the D2R agonist quinpirole appears to be more potent, and the D2R-mediated electrophysiological responses desensitize less well in TAAR1-KO mice (Bradaia et al., 2009).

The D2R serves as the main target of antipsychotic drugs like haloperidol (Strange, 2001) and we sought to determine if a functional interaction occurs between

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TAAR1 and D2R that modulates their signaling. First, we measured TAAR1 cAMP production in the presence of D2R/antagonist complexes using a cAMP-responsive bioluminescence resonance energy transfer (BRET) biosensor (Barak et al., 2008). We found that the D2R antagonists haloperidol, raclopride, and amisulpride enhanced β -PEA/TAAR1 mediated production of cAMP in a Gi-dependent and D2R selective manner. Furthermore, we discovered in cells that plasma membrane TAAR1 and D2R form constitutive heterodimers that can be disrupted in the presence of haloperidol, and in mice lacking TAAR1 that haloperidol-induced catalepsy and striatal c-Fos expression are reduced. These data suggest that the *in vivo* effects of TAAR1 ligands and antipsychotic drugs may depend on the mutual regulation of signaling that occurs between TAAR1 and D2R.

Materials and Methods

Animals and Reagents

TAAR1 knockout (TAAR1-KO) mice of mixed C57BL/6J x 129Sv/J background were generated as described (Sotnikova et al., 2010; Wolinsky et al., 2007). 3-6 months old mice of both genders were used in these experiments.

All cell culture reagents and buffers were from Invitrogen (Carlsbad, CA) and Sigma (St. Louis, MO), and FBS from JRH Biosciences (Lenexa, KS). Coelenterazine *h* was purchased from Promega (Madison, WI). Anti-HA antibody was from Roche Applied Sciences (Indianapolis, IN) and anti-FLAG from Sigma. Plasmids containing the cDNA for the human trace amine receptor were obtained from the cDNA resource center at the University of Missouri-Rolla and the American Type Culture Collection (Manassas, VA). All compounds used in this study were obtained from Sigma.

Construction of Expression Vectors

A modified version of human TAAR1 was used as described previously in order to enable plasma membrane expression of the mature receptor. This construct, described below, is hereafter referred to in the paper as TAAR1 (Barak et al., 2008). Briefly, full-length human TAAR1 cDNA without a stop codon was amplified by PCR with 5' and 3' in-frame restriction enzyme sites of EcoRI and KpnI, respectively and PCR product was cloned into a pcDNA3.1 vector with N-terminal triple HA tag. The cDNA corresponding to the first nine amino acids of the β 2-adrenergic receptor was inserted

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in-frame between the triple HA and TAAR1 to generate the triple HA- β 2N9-TAAR1-GFP. The Rluc version was generated amplifying by PCR using specific primers with 5' and 3' in-frame restriction enzyme sites of *Xho* I and *Kpn* I, respectively, and subcloned into a phRluc N3 vector (PerkinElmer).

Mouse D2R long dopamine receptor tagged on the C-terminus with Rluc or YFP was modified as described previously (Barak et al., 2008; Salahpour and Masri, 2007). Briefly, it was amplified by PCR using a 5' primer containing an Eco RV restriction site and a 3' primer containing a Not I restriction site. PCR product was cloned into a pcDNA3 vector downstream three hemagglutinin tags (HA) or FLAG tag which generated amino-terminally HA-tagged or FLAG tagged D2RLR. For D2R-YFP version the receptor was directly cloned into pEYFP N1 vector.

The BRET sensor for cAMP (Barak et al., 2008) was constructed by the modification of an existing FRET-based intramolecular biosensor EPAC (Ponsioen et al., 2004) in which residues 148–881 of EPAC1 were surrounded by eCFP upstream and citrine downstream (DiPilato et al., 2004; Violin et al., 2008). Using the restriction enzymes *Bam*H I and *Kpn* I, the eCFP was removed and replaced by a humanized *Renilla* luciferase gene that was PCR amplified from phRluc-C1 (PerkinElmer) and cloned using the same restriction sites to preserve the frame of translation (Barak et al., 2008).

Cell Culture and Transfection of Cell Lines

Human embryonic kidney 293 cells (HEK293T) were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% (vol/vol) of FBS, 2 mM L-glutamine

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and 0.05 mg/ml of gentamicin at 37°C in a humidified atmosphere at 95% air and 5% CO₂. Transient transfections were performed 24 h after cells seeding using calcium phosphate protocol. 5 µg of TAAR1 and 2 µg of D2R for each milliliter of transfection solution were used for the experiments. For the BRET experiments, 24 h after transfection, the cells were plated in poly-D-lysine coated 96-well microplates (well assay plate with clear bottom, Fisher Scientific) at a density of 80,000 cells per well in phenol red free Minimum Essential Medium containing 2% of FBS, 10 mM Hepes, 2 mM L-glutamine. The cells were then cultured for an additional 24 h.

Bioluminescence Resonance Energy Transfer Measurement

For BRET assays, at the day of the experiment the phenol red free medium was removed from HEK293T cells and replaced by Phosphate Buffer Saline (PBS) containing calcium and magnesium and 0.003% (wt/vol) of ascorbic acid. The assay was started by adding 10 µl of the cell-permeant substrate specific for *Renilla* luciferase, coelenterazine *h* to the well to yield a final concentration of 5 µM. The antagonist compounds were added 5 minutes before the agonist. For time course experiments the plate was read immediately after the addition of the agonist and for approximately 30 minutes. For dose response experiments the plate was read 10 minutes after agonist addition. All the experiments were conducted in presence of the phosphodiesterase inhibitor IBMX (Sigma) at 200 µM final concentration as previously described (Barak et al., 2008). BRET readings were collected using a Mithras LB940 instrument that allows the sequential integration of the signals detected in the 465 to 505nm and 515 to 555 nm

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windows using filters with the appropriate band pass and by using MicroWin 2000 software. For titration experiments, constant concentration of TAAR1-Rluc was used with increasing amount of D2R-YFP. The acceptor/donor ratio was calculated as previously described (Salahpour and Masri, 2007). Curve was fitted using a non linear regression and one site specific binding with GraphPad Prism 5. For evaluation of the basal cAMP level, data were expressed as percentage of vehicle-treated cells.

Cellular fractionation

Cells were lysed in cold hypotonic buffer (20 mM HEPES, pH 7.4, 2mM EDTA, 2mM EGTA, 6 mM MgCl₂ and protease inhibitor cocktail) using a Dounce homogenizer. Cellular debris were removed by centrifugation (1000 x *g* for 5 minutes at 4 °C). Sucrose was added to the supernatant to a final concentration of 0.2 M and then lysates were layered to a discontinuous sucrose gradient (0.5, 0.9, 1.2, 1.35, 1.5, 2.0 M). Samples were centrifuged at 28,000 rpm for 16 hours at 4°C using a Beckman rotor SW32Ti. Thirty-two fractions of 1 ml each were collected and BRET was measured from 100 µl aliquots of each fraction. As ER and PM markers anti-KDEL (Abcam) and anti-Na⁺/K⁺-ATPase (Millipore) antibodies were used.

Quantitative Measurement of Cell Surface Receptors

Cell surface expression of HA-GPCRs or FLAG-GPCRs constructs was determined by ELISA using monoclonal anti-HA or anti-FLAG antibody and the horseradish peroxidase-conjugated secondary anti-mouse antibody. The peroxidase

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activity was detected by specific reagent (SigmaFast, Sigma) and the colorimetric reaction was measured using a spectrophotometer (Beckman Coulter) using a 492 nm filter. Total receptor level was assessed in the same sample by measuring the *Rluc* activity as in the BRET experiments (Salahpour et al., 2004).

Immunofluorescence

1 hour after i.p. administration of haloperidol (0.5 mg/kg) or saline mice were rapidly anesthetized with ketamine-xylazine and perfused transcardially with 4%(w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were post-fixed overnight in the same solution and stored at 4°C. Thirty-micrometer thick sections were cut with a Cryostat (Leica) and stored at -20°C in a solution containing 30% (v/v) ethylene glycol, 30% (v/v) glycerol, and 0.1 M sodium phosphate buffer, until they were processed for immunofluorescence. Striatum was identified using a mouse brain atlas (Paxinos and Franklin, 2001) and sections were processed as follows.

Day 1. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5) three times for 10 min each. After a 20 min incubation in 0.2% Triton X-100 in TBS, sections were rinsed three times in TBS again. Finally, they were incubated overnight at 4°C with the c-Fos primary antibody (1:1000; Santa Cruz Biotechnology).

Day 2. Sections were rinsed three times for 10 min in TBS and incubated for 45 min with AlexaFluor 488 (Invitrogen). Sections were rinsed for 10 min twice in TBS and twice in Tris buffer (0.25 M Tris) before mounting in Vectashield (Vector Laboratories).

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Confocal microscopy and images from each region of interest (ROI) were obtained bilaterally using sequential laser-scanning confocal microscopy (SP2; Leica). Neuronal quantification was performed in 375x375 μm images by counting c-Fos positive nucleus.

Haloperidol-induced catalepsy

Wild type (WT) and TAAR1 heterozygous (HET) and TAAR1 knockout (KO) mice were treated with different doses of haloperidol or vehicle and catalepsy was measured 3 hours later in the bar test as described (Sotnikova et al., 2005). Briefly, the presence of catalepsy was determined and measured by placing the animal's forepaws on a horizontal wooden bar (0.7 cm in diameter), 4 cm above the tabletop. The time until the mouse removed both forepaws from the bar was recorded, with a maximum cut-off time of 180 seconds.

Statistical Analysis

Data were analyzed by two-tailed Student's t test, one-way ANOVA or two-way ANOVA with Bonferroni post-hoc test. Values in graphs were expressed as mean \pm SEM.

Results

Haloperidol enhances β -PEA induced stimulation of TAAR1

We measured the accumulation of cAMP induced by the activation of TAAR1 and evaluated the capacity of D2R to modulate this process. $G_{\alpha s}$ -coupled TAAR1 signals through cAMP (Borowsky et al., 2001; Bunzow et al., 2001) whereas agonists of D2R decrease adenylyl cyclase activity by coupling to an inhibitory $G_{\alpha i}$ protein (Masri et al., 2008). To measure cAMP we used an EPAC biosensor validated for TAAR1 and D2R (Barak et al., 2008; Masri et al., 2008; Violin et al., 2008). With TAAR1 expressed alone, β -PEA at $1\mu\text{M}$ induced a robust increase in cAMP levels as measured by a reduction in BRET signal (Fig. 1A). In these cells expressing TAAR1 alone, haloperidol at $1\mu\text{M}$ was not able to alter cAMP concentrations either under basal conditions or in β -PEA stimulated cells. When we co-transfected TAAR1 with D2R, we observed a reduced (by about 25%) β -PEA stimulation to cells expressing TAAR1 alone and in this case haloperidol enhanced the β -PEA induced cAMP production without affecting the basal level of cAMP (Fig. 1B). This enhancement lasted for the duration of the 30 minute experiment. It is also evident that the basal level of cAMP did not differ between TAAR1 and TAAR1-D2R expressing cells, indicating a lack of effect of D2R on basal cAMP levels (TAAR1 = 1.213 ± 0.005 vs TAAR1+D2R = 1.217 ± 0.002 , $p \geq 0.05$). However, β -PEA responses were significantly lower in cells co-expressing TAAR1 and D2R (β -PEA in TAAR1 cells: 1.124 ± 0.002 vs TAAR1+D2R 1.151 ± 0.002 , $p < 0.05$, $n = 7$).

D2R dopamine receptor mediates haloperidol effects on TAAR1 signaling

It has been reported that haloperidol does not interact with TAAR1 (Barak et al., 2008; Bunzow et al., 2001) and, accordingly, we have observed that haloperidol does not directly stimulate TAAR1 in our assay (Fig. 1A, B). To confirm that an enhancement in TAAR1 signaling by haloperidol is mediated through D2R we investigated two other D2R antagonists, raclopride and amisulpride. The first is a classical selective D2R antagonist and the second is an atypical antipsychotic with good D2R selectivity (Moller, 2003). In cells expressing TAAR1 and D2R, each of these compounds at 1 μ M was able to enhance β -PEA stimulation without affecting basal cAMP (Fig. 1C, D, E, and F). Since D2R receptor is coupled to G $_{\alpha i}$ protein, we verified whether this protein was the mediator of the observed enhancement. We therefore pretreated cells overnight with pertussis toxin (PTX) to prevent the coupling between G $_{\alpha i}$ protein and D2R receptor. In control experiments pretreatment with PTX prevented the inhibitory effect of dopamine on the isoproterenol stimulation of cAMP production mediated by β 2-AR (DA+iso = 1.10 \pm 0.004, PTX+DA+iso = 1.06 \pm 0.001, $p < 0.05$, Fig. 2A). Similarly, PTX treatment prevented the ability of haloperidol to potentiate TAAR1 stimulation of cAMP production (PTX+ β -PEA = 1.09 \pm 0.005, PTX+ β -PEA+halo = 1.09 \pm 0.006, $p \geq 0.05$ Fig. 2B). A loss of the stimulatory effects of raclopride and amisulpride on TAAR1-mediated cAMP signaling was also observed in PTX-treated cells (data not shown).

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D2R blockade selectively enhances TAAR1 signaling

The above data indicate that the D2R is able to modulate TAAR1-induced cAMP production. To validate the selectivity of this effect for TAAR1, we performed a similar experiment using 1 μ M isoproterenol activation of endogenous β 2-AR. Cells expressing only biosensor or biosensor and D2R showed robust response to isoproterenol and this response was not affected by haloperidol (1 μ M) treatment (data not shown). Similarly, isoproterenol produced a robust increase of cAMP in cells transfected with only TAAR1 or in cells co-expressing TAAR1 and D2R (Fig. 3A, B). Haloperidol at 1 μ M had no effect on basal cAMP concentration and did not augment isoproterenol cAMP increases thus confirming specificity of the TAAR1/D2R interaction for eliciting this phenomenon (iso = 1.025 ± 0.003 , iso+halo = 1.027 ± 0.005 , $p \geq 0.05$ in TAAR1 expressing cells; iso = 1.016 ± 0.003 , iso + halo = 1.012 ± 0.005 , $p \geq 0.05$ in TAAR1-D2R expressing cells). Isoproterenol tested at lower concentrations (10 and 100 nM) with haloperidol also showed no potentiation in cAMP production (data not shown).

D2R blockade increases maximal response but not potency of TAAR1 co-expressed with D2R

We examined a range of β -PEA concentrations from 10^{-11} to 10^{-4} M in cells expressing exclusively TAAR1 and in cells expressing both TAAR1 and D2R with and without haloperidol. As expected, haloperidol had no effect in cells expressing TAAR1 alone; in contrast, in cells expressing both TAAR1 and D2R, haloperidol doubled the maximum effect of β -PEA ($E_{max} = 209 \pm 13\%$, $p < 0.001$) with little change in the EC50

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(basal EC50=28 ± 69 nM, haloperidol treated EC50=125 ± 123 nM) (Fig. 4A, B).

Raclopride and amisulpride had similar effects, increasing the efficacy of β -PEA for TAAR1 stimulation (raclopride treated E_{max} = 195 ± 8%, $p<0.001$); (amisulpride treated E_{max} = 252 ± 24%, $p<0.05$) with minor changes in EC50s (basal EC50=48 ± 119 nM, raclopride treated EC50=151 ± 115 nM); (basal EC50=111 ± 213 nM, amisulpride treated EC50=215 ± 326 nM) (Fig. 4 C-D, E and F).

D2R co-expression modulates TAAR1 levels

Next, we analyzed the membrane expression of co and singularly expressed HA-TAAR1 and FLAG-D2R by ELISA in non-permeabilized cells (Salahpour et al., 2004). When TAAR1 and D2R were present in the same cells, TAAR1 membrane expression was reduced by about 50% compared to cells without D2R or in cells co-expressing D1R (Fig. 5A). D2R membrane expression was not influenced by TAAR1 presence (Fig 5C). We performed an additional experiment to exclude the possibility that this modulation could be a non specific effect by measuring membrane expression of D1R in presence of D2R. Co-expression of D2R did not affect D1R membrane immunoreactivity (Fig. 5B). Since we used a TAAR1 that was HA tagged on N-terminus and tagged with *Rluc* on C-terminus, we were able to simultaneously measure its membrane and total expression. The same cells that were used for ELISA experiments were detached from their dishes and *Rluc* counts measured as an indicator of total presence of TAAR1. The total amount of TAAR1 was reduced in the presence of D2R but not in the presence of D1R (Fig. 5D). Finally, we investigated if haloperidol could modulate TAAR1 membrane expression. Cells were treated with haloperidol at 1 μ M for

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15 minutes and, as shown in Fig. 5E, haloperidol did not modulate TAAR1 surface expression in cells co-expressing TAAR1 and D2R.

TAAR1 and D2R form a heterodimer in living cells

GPCR dimerization occurs for many receptors and this process has important functional consequences (Milligan, 2009). For instance, the dimerization of the two subunits of the metabotropic GABA_B receptor is a prerequisite for the correct cell-surface expression and activation of the receptor (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). BRET is a versatile and validated method to study protein-protein interactions in living cells (Bouvier, 2001), and we used this technique to study the dimerization of TAAR1 tagged with *Rluc* and D2R tagged with YFP. We performed a titration curve between the two receptors using a fixed TAAR1-*Rluc* (BRET donor) expression and an increasing amount of D2R-YFP (BRET acceptor). The hyperbolic nature of the curve indicates association between TAAR1 and D2R (Fig. 6) and heterodimer formation (Salahpour and Masri, 2007). Pretreatment with haloperidol at 1 μ M reduced the BRET signal and resulted in a linear titration curve suggesting the disassembling of the dimer. As a negative control, we co-transfected TAAR1-*Rluc* and increasing amount of D1-YFP and we observed no detectable BRET signal between these two receptors (Fig. 6A.). Moreover, by co-transfecting the cells with an excess of untagged D2R, we saw a significant reduction of BRET between TAAR1-*Rluc* and D2-YFP (-53.53% \pm 17.66, $p < 0.005$, Fig. 6B), indicating the specificity of the dimer formation. In addition, we also investigated possibility of co-internalization of the receptors. Since agonist pretreatment may induce a physiological desensitization of a

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receptor by its internalization, we verified if TAAR1 stimulation could trigger D2R internalization. Using the same ELISA approach described above we used HA-TAAR1 and FLAG-D2R to monitor receptors membrane expression. Quinpirole at 1 μ M was able to decrease D2R membrane expression (-23.97 % \pm 0.03, $p < 0.05$, Fig. 6C). Interestingly, TAAR1 stimulation by β -PEA at 1 μ M induced a weak but significant decrease in D2R membrane expression (-6.16 % \pm 0.01, $p < 0.05$). The observed co-internalization of the receptors following selective stimulation of only one of them further strengthens the evidence of TAAR1-D2R heterodimer formation.

TAAR1 and D2R heterodimer is formed mainly at the plasma membrane

We further investigated whether the heterodimer is formed at the level of the endoplasmic reticulum (ER) or at the plasma membrane (PM). In order to study dimer localization, we transfected the cells with TAAR1-Rluc and D2-YFP or with only TAAR1-Rluc and we separated ER and PM by centrifugation of the cell lysates using a discontinuous sucrose gradient (Salahpour et al., 2004). We obtained ER in fractions 2-6 and PM in 6-14, using Na⁺/K⁺ ATPase and KDEL as PM and ER markers respectively (data not shown). Under these conditions we could detect significant Rluc activity in the first 11 fractions, but as shown in Fig.7, TAAR1-D2R heterodimer was found mainly in the plasma membrane fractions.

Haloperidol induced c-Fos expression is reduced in TAAR1-KO mice

Haloperidol treatment can induce the expression of several marker proteins in the brain indicative of neuronal activity, and in particular c-Fos expression in the dorsal striatum of mice (Nguyen et al., 1992). We injected TAAR1-KO mice with saline or with haloperidol at a dose of 0.5 mg/kg i.p. and evaluated by immunofluorescence the c-Fos expression in dorsal striatum at one hour post injection. As shown in Fig. 8A, vehicle did not induce c-Fos expression in any of the genotypes, while haloperidol induced a marked fluorescence in neurons of both WT and TAAR1-KO mice, indicating expression of the c-Fos protein. Importantly, the number of neurons activated by haloperidol was significantly reduced in TAAR1-KO animals by about 30% ($p < 0.05$) (Fig. 8B).

Haloperidol-induced catalepsy is reduced in TAAR1-KO mice

To further evaluate the consequences of a TAAR1-D2R interaction *in vivo* we tested the ability of haloperidol to induce classical D2R mediated cataleptic behaviors in mice deficient in TAAR1. As shown in Fig. 8C, haloperidol caused a dose-dependent increase in cataleptic behaviors in WT, TAAR1-HET and TAAR1-KO mice, however the responses to haloperidol were significantly reduced in TAAR1-HET and TAAR1-KO mice (two-way ANOVA revealed significant effect of dose, $p < 0.001$; genotype, $p < 0.001$ and interaction dose x genotype, $p < 0.05$) These observations taken together with the c-Fos data demonstrate that the interaction between TAAR1 and D2R observed *in vitro* may have important physiological consequences *in vivo*.

Discussion

In this study, we demonstrate that TAAR1 is able to physically and functionally interact with the D2R both in *in vitro* and *in vivo*. D2R co-expression with TAAR1 results in formation of haloperidol-sensitive constitutive heterodimers in plasma membranes of cells. Additionally, the inhibition of D2R signaling by specific D2R antagonists enhances β -PEA - induced TAAR1 signaling. This D2R-dependent potentiation of β -PEA stimulation is $G_{\alpha i}$ protein-dependent and the stimulatory effect appears limited specifically to TAAR1. Furthermore, we observe *in vivo* in TAAR1-KO mice a role for TAAR1 in D2R-related signaling and behavior; a reduction in haloperidol induced c-Fos expression that parallels a reduction in haloperidol-induced catalepsy.

The clinical efficacies of antipsychotics are related to their abilities to antagonize dopamine action at D2Rs (Creese et al., 1976; Strange, 2001) and even the newer serotonin receptor active, atypical antipsychotics antagonize D2R signaling through G-protein and β -arrestin2-dependent pathways (Masri et al., 2008). TAAR1 is also well positioned to modulate brain dopaminergic activity. For example, TAAR1-KO mice show increased sensitivity to amphetamine (Lindemann et al., 2008; Wolinsky et al., 2007) and dopaminergic drugs (Bradaia et al., 2009; Sotnikova et al., 2008). These mutants demonstrate also a deficit in pre-pulse inhibition and have a larger proportion of striatal D2Rs in a high affinity state ($D2R^{high}$) (Wolinsky et al., 2007). Dopamine supersensitivity and increased activity of D2R have been observed in patients with schizophrenia (Breier et al., 1997), and consequently TAAR1-KO mice have been suggested as an animal model of this disorder (Wolinsky et al., 2007).

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In *in vitro* model system, we observed that our reference compound and prototypical antipsychotic drug haloperidol significantly enhanced TAAR1-dependent β -PEA signaling in cells co-expressing TAAR1 and D2R, while it is known that haloperidol does not act on TAAR1 directly (Barak et al., 2008; Bunzow et al., 2001). The ability of other D2R antagonists, raclopride and amisulpride, to mimic the characteristic effects of haloperidol further indicated that those effects are due to modulation by D2R. Importantly, β -PEA stimulated, but not basal, cAMP levels were decreased in cells co-expressing TAAR1 and D2R, while D2R blockade with antagonists enhanced the efficacy of β -PEA TAAR1 signaling. An analogous response to D2R expression was not observed with isoproterenol acting on endogenously expressed $G_{\alpha s}$ – coupled β_2 -AR indicating that the result for TAAR1 is not simply due to cross talk between the agonist of $G_{\alpha s}$ – coupled TAAR1 and the antagonist of $G_{\alpha i}$ -coupled D2R. While several mechanisms may be responsible for the potentiation in TAAR1 signaling, including enhancement of TAAR1 G-protein coupling, it is certain that the D2R and $G_{\alpha i}$ subunits are critical, since overnight pretreatment of cells with PTX prevents the increase in TAAR1 signaling caused by D2R antagonist.

GPCRs may interact at multiple subcellular locations, such as those that occur during cell trafficking (Dong et al., 2007). We therefore studied receptor expression levels in cells co-expressing both receptors. Our results show that D2R co-expression decreases both the surface and total levels of TAAR1 by about 50%, whereas the co-expression of D1R had no effect on TAAR1 membrane levels. In addition D2R co-expression did not affect surface levels of D1R. Importantly, while haloperidol treatment

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did not affect the level of the surface expression of TAAR1, TAAR1-mediated signaling is markedly increased when D2R is blocked by the antagonist. Thus, the observed alterations in TAAR1 expression found in cells co-expressing D2R cannot be a basis of the increase in TAAR1 signaling caused by haloperidol under these conditions. Rather, it is possible that the pharmacological properties of TAAR1 are changed when it is co-expressed with D2R.

A simple hypothesis to explain our findings is receptor heterodimerization (Angers et al., 2002). GPCR homo- and hetero-dimerization has been demonstrated for many receptors (Dalrymple et al., 2008), and direct interaction between receptors could lead to a modulation of their function, such as differential trafficking and/or changes in their pharmacological profile (Milligan, 2009; Salahpour et al., 2004). We tested this hypothesis by using a BRET approach and performed a titration curve analysis of heterodimerization between TAAR1-Rluc and D2R-YFP. This method is commonly used to study homo and heterodimerization between different GPCRs (Pfleger and Eidne, 2006). Using this approach we showed that TAAR1 and D2R formed constitutive heterodimers and that haloperidol treatment abolished the BRET signal resulting from the formation of the heterodimer. We further demonstrated that heterodimer formation was specific to D2R, and that heterodimers formed mainly on the plasma membrane. While further detailed studies are necessary to understand the molecular mechanisms of altered pharmacological properties of these heterodimers, it is likely that the disruption of the heterodimer formation or conformational change in the complex structure caused by haloperidol contributes to the enhanced TAAR1 signaling.

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To directly investigate if this interaction of D2R and TAAR1 has physiological consequences at the *in vivo* level, we first studied the effect of haloperidol treatment on c-Fos expression. c-Fos is an immediate early gene that has been widely used as an indicator of neuronal activation. It has been demonstrated that haloperidol as well as many other antipsychotics can induce c-Fos expression in several brain regions and, particularly, typical antipsychotics can activate neurons located in dorsal striatum (Nguyen et al., 1992). We observed that c-Fos expression in dorso-lateral striatum, after haloperidol treatment, was decreased in TAAR1-KO mice, suggesting that D2R mediated signaling is affected. Haloperidol, as well as other D2R antagonists induce cataleptic behaviors in animals and these extrapyramidal side-effects are commonly used in pharmacological modeling of Parkinson's Disease (Sotnikova et al., 2005). Using a bar test to evaluate catalepsy, we have shown that haloperidol is less potent in inducing catalepsy in TAAR1 full and partial (heterozygous) knockout mice, indicating that TAAR1 modulates D2R-related physiology *in vivo*.

In conclusion, we report that antagonism of D2R enhances TAAR1 cAMP signaling. The enhancement appears unique to this pair of receptors and possibly is a result of the disruption of TAAR1-D2R complex. Moreover, TAAR1 modulates *in vivo* D2R antagonism-related signaling and behaviors. The observations that both TAAR1 antagonism and a constitutive genetic lack of TAAR1 result in increased dopamine potency at D2R in dopaminergic neurons (Bradaia et al., 2009), combined with our data showing enhancement of TAAR1 signaling under conditions of D2R blockade indicate that the TAAR1 and D2R can potently modulate each other's activity. Thus, the

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observed functional D2R/TAAR1 interaction may have important consequences for current and future therapeutic strategies based on application of dopaminergic drugs and TAAR1 ligands in the treatment of dopamine-related disorders (Revel et al., 2011).

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Authorship Contribution

Participated in research design: Espinoza, Salahpour, Masri, Sotnikova, Barak, Caron, and Gainetdinov.

Conducted experiments: Espinoza, Salahpour, Masri, Sotnikova, and Messa.

Contributed new reagents or analytical tools: Espinoza, Salahpour, Masri, Barak, Caron, and Gainetdinov.

Performed data analysis: Espinoza, Salahpour, Masri, Sotnikova, Messa, and Gainetdinov.

Wrote or contributed to the writing of the manuscript: Espinoza, Salahpour, Masri, Barak, Caron, and Gainetdinov.

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Footnotes

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Figure legends

Fig.1. D2R antagonists enhance TAAR1-mediated stimulation of cAMP by β -PEA in HEK-293 cells. A, time course effects of haloperidol and β -PEA in cells transiently transfected with EPAC and TAAR1. BRET ratio is measured as YFP/Rluc ratio and the reading are started right after β -PEA addition. Cells are exposed to 1 μ M of β -PEA or control medium in presence or absence of haloperidol 1 μ M. The decrease in BRET ratio indicates an increase in cAMP concentration. β -PEA induces a robust increase in cAMP level while haloperidol does not alter neither basal nor the stimulated response. B, the same time course experiment is performed in cells co-expressing TAAR1 and D2R. In this case 1 μ M of haloperidol enhances β -PEA stimulation without altering basal cAMP. C, D, an analogous time course experiments with raclopride, another D2R antagonist. In cells expressing only EPAC biosensor and TAAR1, raclopride at 1 μ M had no effect on β -PEA stimulation (C) while demonstrating the ability to increase this effect in cells co-expressing D2R (D). E, F, similar results are obtained with D2R antagonist amisulpride. At 1 μ M amisulpride induced long lasting enhancement in cAMP levels only in cells expressing D2R with TAAR1 (F), but in the cells expressing TAAR1 alone (E). These panels are representative of 3-4 independent experiments.

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Fig. 2. Pertussis toxin disrupts D2R-mediated effect of haloperidol on TAAR1 signaling. A, HEK-293 cells transfected with EPAC biosensor and D2R are treated with pertussis toxin (PTX) overnight to prevent coupling of G_i protein to D2R. These cells are exposed to isoproterenol $1\mu\text{M}$ to stimulate β_2 -AR endogenously expressed and compared to PTX untreated cells. Isoproterenol readily stimulates cAMP production and this effect is partly inhibited by dopamine at $0.1\mu\text{M}$, as a consequence of D2R activation. This inhibition is abolished in cells pretreated with PTX indicating the efficacy of this toxin to disrupt $G_{\alpha i}$ - mediated effect. B, time course experiment is conducted to evaluate PTX effect on haloperidol enhancement of β -PEA response. Cells transfected with EPAC, TAAR1 and D2R are pretreated with PTX and the day after the cells were stimulated with β -PEA that induce an increase in cAMP level. In PTX treated cells, haloperidol loses its modulatory action on β -PEA effect. These panels are representative of 3-4 independent experiments.

Fig. 3. Lack of effect of haloperidol on isoproterenol – induced stimulation of β_2 -AR. A, the selectivity of haloperidol ability to enhance TAAR1 response was tested by assessing the effects of haloperidol on β_2 -AR stimulation. Cells expressing TAAR1 and the EPAC biosensor were exposed to isoproterenol ($1\mu\text{M}$) or control medium. The activation of endogenously expressed β_2 -AR induced a robust increase in cAMP levels that are not modulated by haloperidol co-administration. B, when D2R is co-expressed with TAAR1 in the same cells haloperidol is still not able to increase cAMP production induced by isoproterenol. These panels are representative of 3-4 independent experiments.

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Fig. 4. Dose-response of β -PEA effect on TAAR1-dependent cAMP accumulation under conditions of D2R blockade. A, various concentrations of β -PEA were applied to cells expressing TAAR1 and cAMP levels were detected using the BRET cAMP biosensor. BRET signal was measured 10 minutes after the addition of β -PEA. Effects of 10^{-11} to 10^{-4} M of TAAR1 agonist β -PEA and $1\mu\text{M}$ of haloperidol were assessed. β -PEA induced an increase in cAMP level that was not modulated by an addition of haloperidol in cells expressing TAAR1 alone. B, the same experiment was conducted in cells co-expressing TAAR1 and D2R. In this case, haloperidol enhanced TAAR1 response with a two-fold increase in maximum effect ($p < 0.001$) and no change in EC50. C, D, modulatory effect of raclopride at $1\mu\text{M}$ on the ability of different β -PEA concentrations to stimulate cAMP via TAAR1. Like haloperidol, raclopride doubled the β -PEA maximum effect ($p < 0.001$) only in cells co-expressing D2R (D), with no change in EC50. E, F, analogous experiment with amisulpride at $1\mu\text{M}$ reveals an increase in maximum effect of β -PEA ($p < 0.05$) in cells co-expressing D2R without alteration in EC50. 3-4 independent experiments were performed for each compound and condition. Data were analyzed using a two-way ANOVA with repeated measures and Bonferroni post-hoc test.

Fig. 5. Surface and total expression of TAAR1 is modulated by D2R co-expression. A, the analysis of surface expression of TAAR1 alone or when co-expressed with D2R or D1R was performed in HEK-293 cells. ELISA assay was performed in non permeabilized cells by using HA tagged TAAR1 and FLAG tagged D2R and D1R and specific antibodies for these two tags. Using an appropriate substrate for the peroxidase

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(see *Materials and Method*) linked to secondary antibody the absorbance of the supernatant was correlated with the presence of the receptors in the membrane. When TAAR1 is co-expressed with D2R, its presence on the membrane is significantly reduced by about 50% ($p < 0.001$) compared to cells in which this receptor expressed alone. The reduction in TAAR1 levels was not observed in cells expressing TAAR1 and D1R. B, D2R co-expression does not affect membrane levels of D1R By using a HA-D1R we measured the effect of D2R on D1R expression. D2R co-expression did not cause significant changes in D1R membrane levels. C, D2R membrane expression is not modulated by co-expression with TAAR1. In cells expressing both TAAR1 and D2R receptors, D2R surface immunoreactivity is not changed compared to cells only expressing D2R. D, D2R co-expression, but not D1R, significantly reduces TAAR1 total expression. TAAR1 tagged with *Renilla* luciferase in the C-terminus was used to monitor the total expression of the receptor by using the cell permeable *Rluc* substrate coelenterazine *h*. The cells were detached from their dishes and approximately 90000 cells were placed for each well in a 96-well plate. Coelenterazine *h* was added at a final concentration of 5 μM and 10 minutes later the luminescence was measured. Similarly to surface expression, total amount of TAAR1 was also reduced in presence of D2R by about 60% ($p < 0.01$). All values are expressed as means \pm SEM ($n=3-5$ independent experiments for each experimental condition).

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Fig. 6. BRET titration curve of physical interaction between TAAR1-Rluc and D2R-YFP.

A, fixed amount of TAAR1-Rluc (donor) and increasing amount of D2R-YFP (acceptor) were co-expressed in the same cells. BRET was measured 10 minutes after the addition of the substrate coelenterazine h in presence or absence of 1 μ M of haloperidol. To test specificity of BRET signal between TAAR1 and D2R, BRET was also measured between TAAR1-Rluc and increasing amount of D1R-YFP. The hyperbolic shape of the curve indicates that TAAR1-Rluc and D2R-YFP form a constitutive heterodimer when co-expressed in the same cells. Haloperidol (1 μ M), on the contrary, abolished the BRET signal between the two receptors, suggesting the disassembling of the dimer. A linear increase in the BRET signal is observed between TAAR1-Rluc and D1-YFP indicating a non-specific, bystander BRET between these receptors. B, fixed amount of TAAR1-Rluc and D2-YFP were transfected and BRET was measured. To evaluate the specificity of the complex an untagged D2 was also transfected. The cotransfection on the untagged-D2 reduced the complex formation between TAAR1-Rluc and D2-YFP as measured by BRET. C, Co-internalization of TAAR1 and D2 was measured by an ELISA approach (see *Materials and Method*). HA-TAAR1 and D2-FLAG were expressed in cells. Upon stimulation by quinpirole, D2-FLAG surface expression decreased. Similarly, also HA-TAAR1 membrane expression was reduced. All values are expressed as means \pm SEM All values are expressed as means \pm SEM (n=3-5 independent experiments for each experimental condition).

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Fig. 7. Subcellular distribution of TAAR1-D2R heterodimer. HEK-293T cells expressing TAAR1-Rluc and D2-YFP or with only TAAR1-Rluc were lysed and ER and PM were fractionated on a discontinuous sucrose gradients as described in *Materials and Methods*. TAAR1-D2R heterodimer was found mainly in the PM fractions and was determined by measuring BRET in every fraction.

Fig. 8. Disrupted effects of haloperidol in TAAR1 deficient mice. A, haloperidol induced c-Fos expression is reduced in the striatum of TAAR1-KO mice. WT and TAAR1-KO mice were treated with haloperidol 0.5 mg/kg i.p or saline for 1 hour. c-Fos expression was evaluated by immunofluorescence staining with specific antibody. Confocal microscopy and images from each region of interest were obtained bilaterally using sequential laser-scanning confocal microscopy. B, quantification of haloperidol-induced c-Fos positive neurons in WT and TAAR1-KO mice. Neuronal quantification was performed in 375x375 μm images by counting c-Fos positive nucleus. Please note that no c-Fos positive neurons were noted in vehicle-treated WT or TAAR1-KO mice. Positive neurons in striatal slices from haloperidol-treated TAAR1-KO mice were significantly reduced compared to WT control ($P < 0.05$). C. haloperidol-induced catalepsy in WT, TAAR1-HET and TAAR1-KO mice measured 3 hours after the treatment. Two-way ANOVA with Bonferroni post-hoc test revealed significant differences in all doses of haloperidol tested in both TAAR1-HET and TAAR1-KO mice in comparison to WT mice (**- $p < 0.01$; ***- $p < 0.001$).

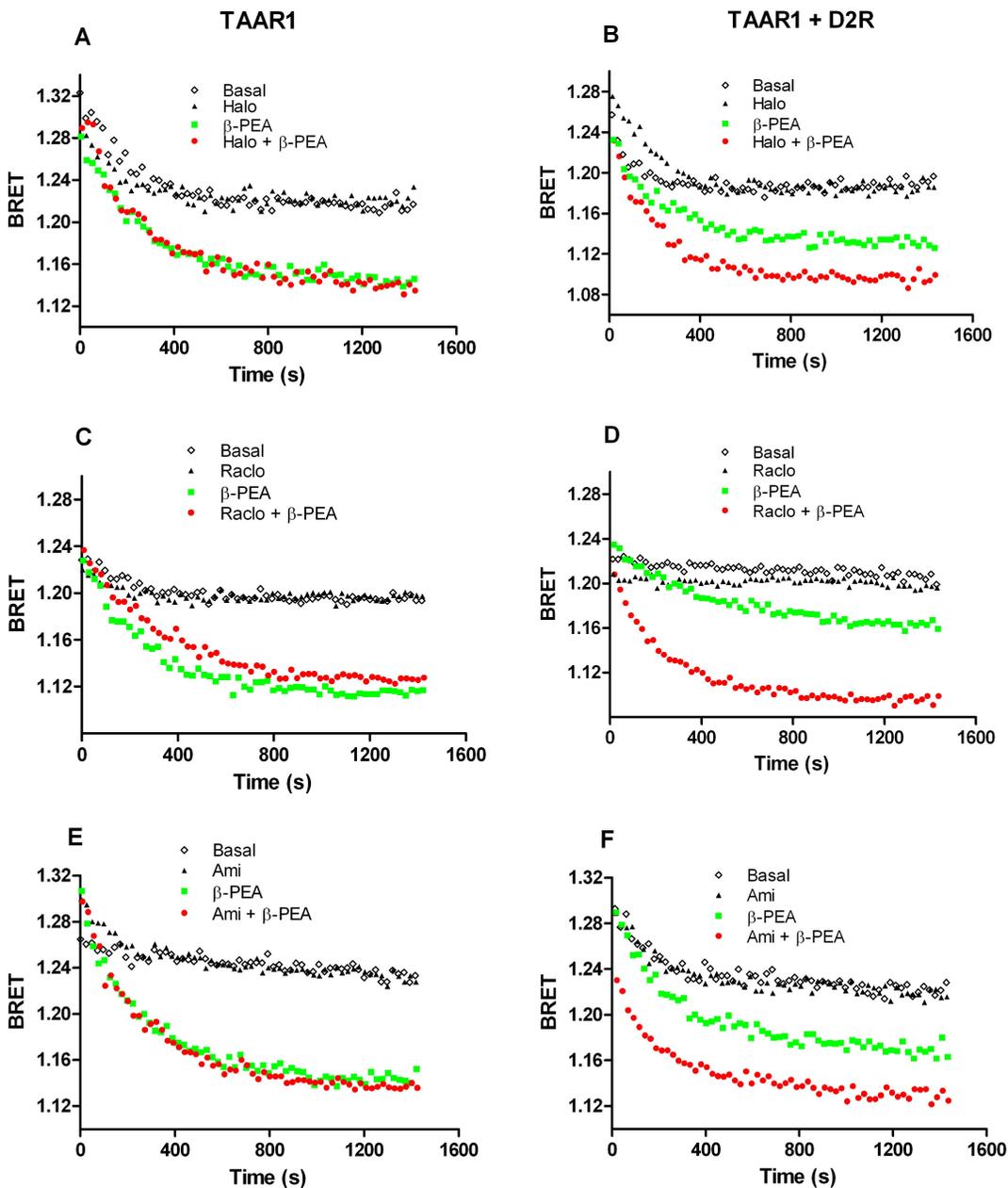


Figure 1

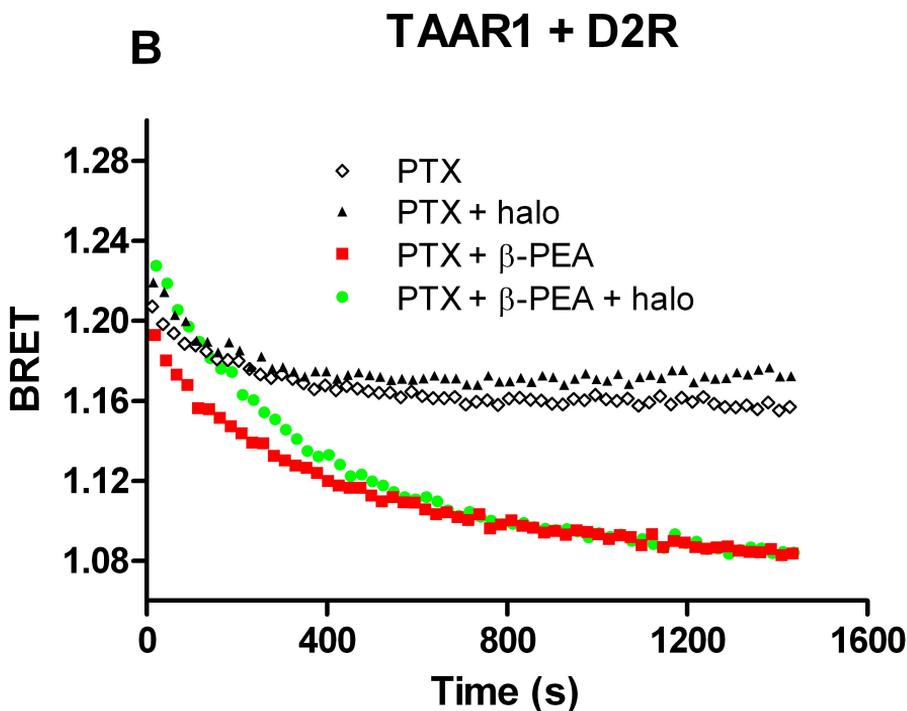
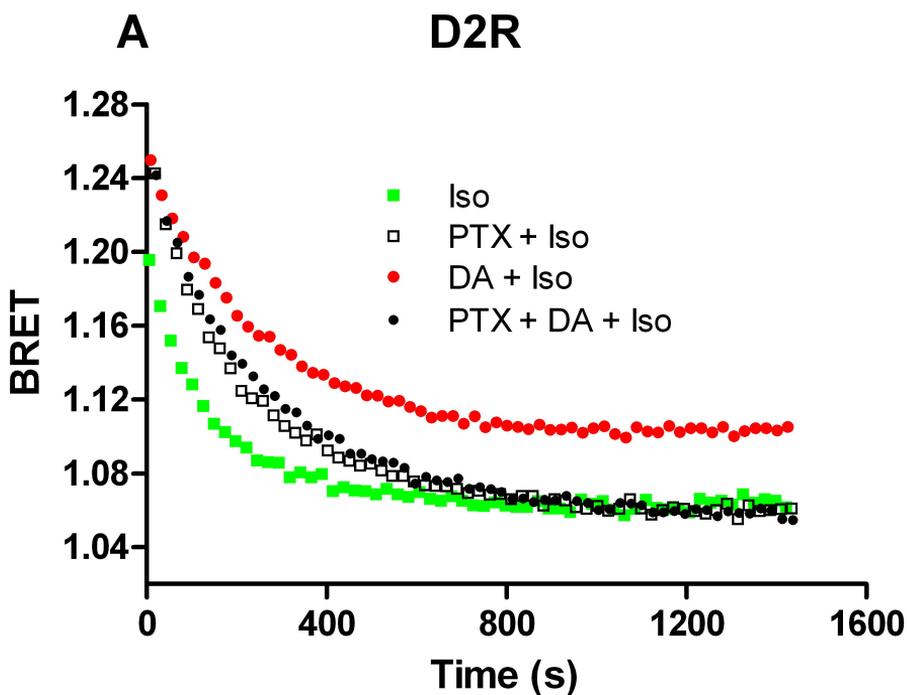


Figure 2

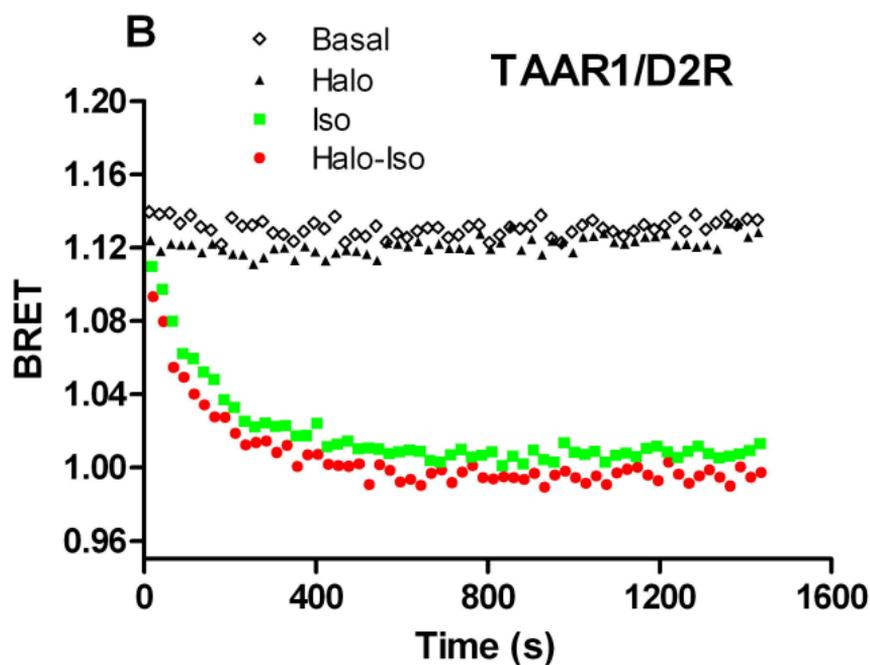
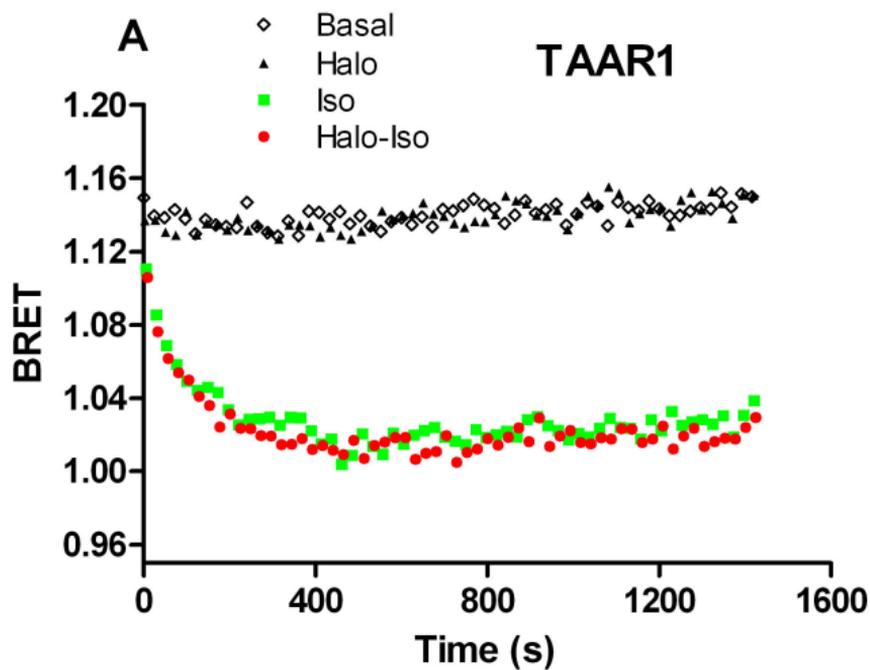


Figure 3

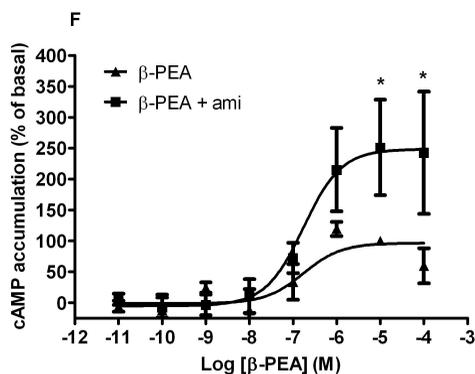
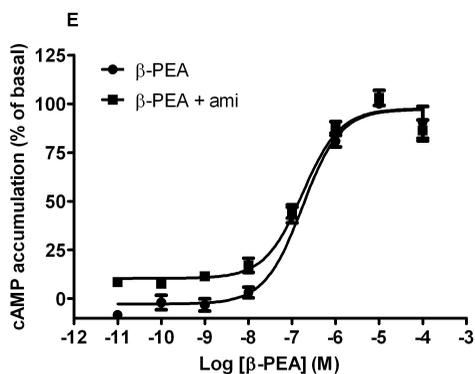
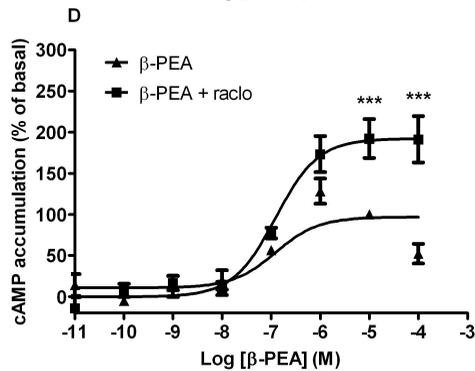
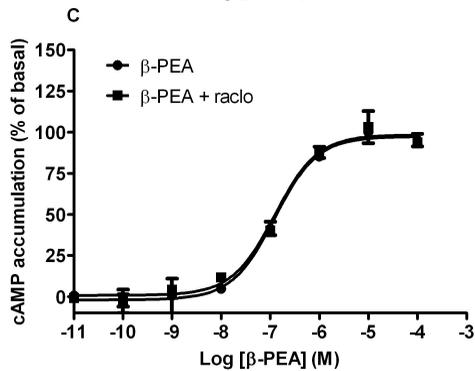
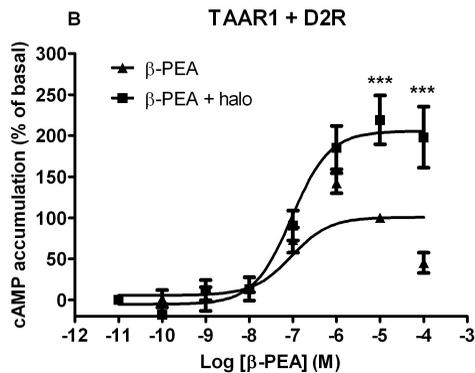
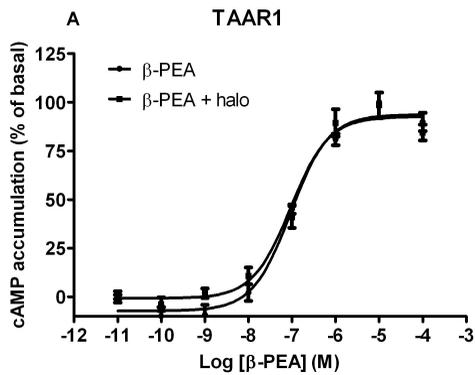


Figure 4

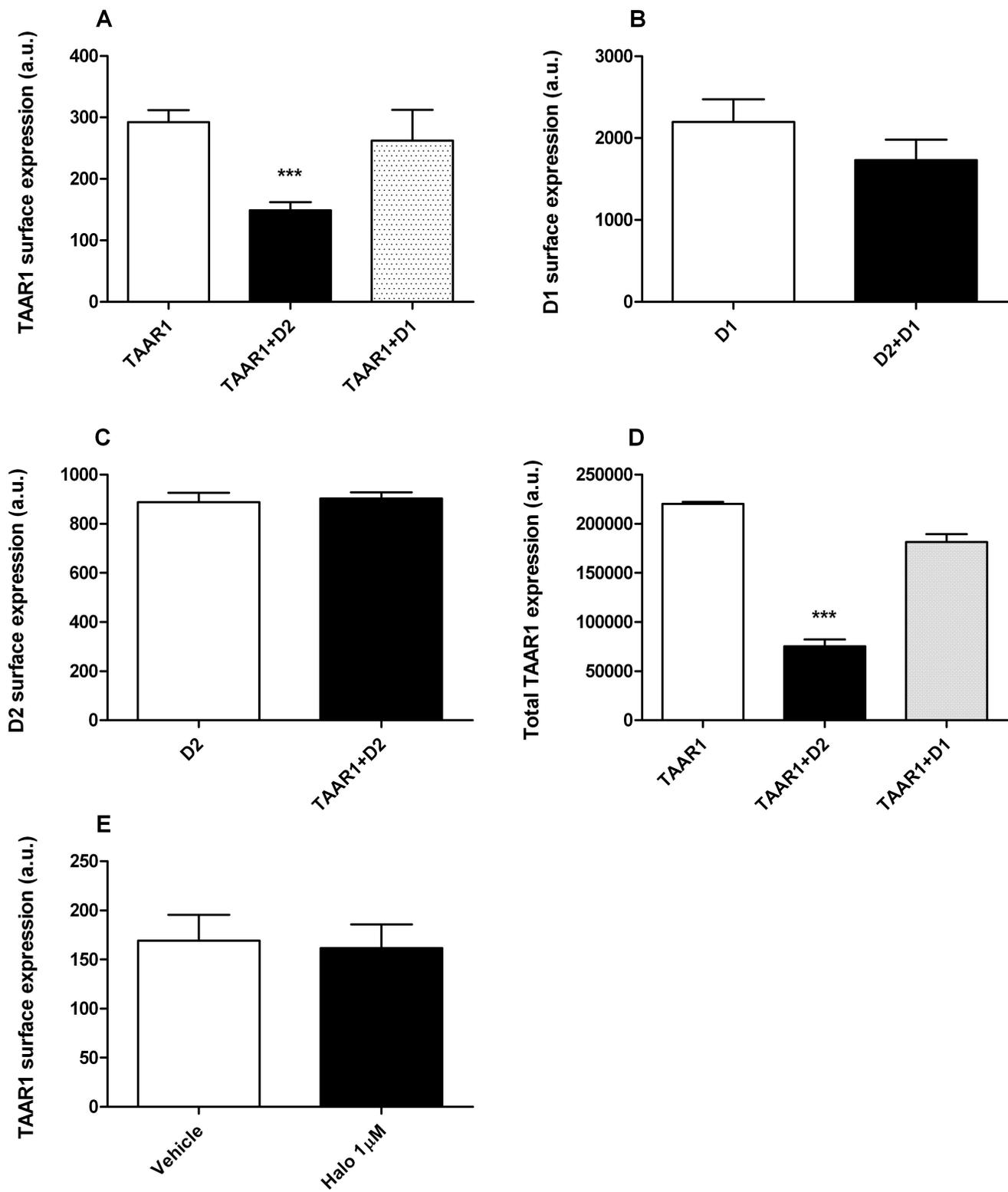


Figure 5

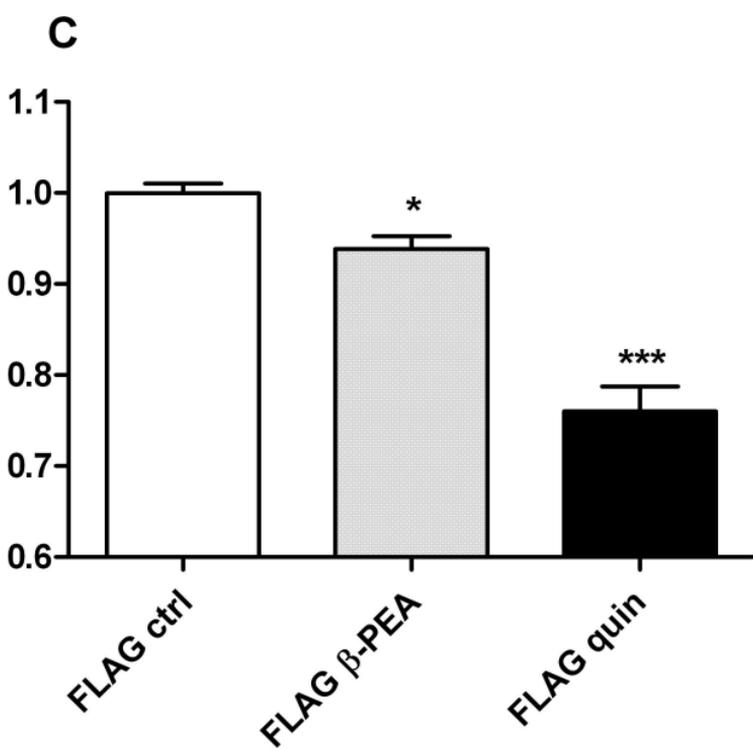
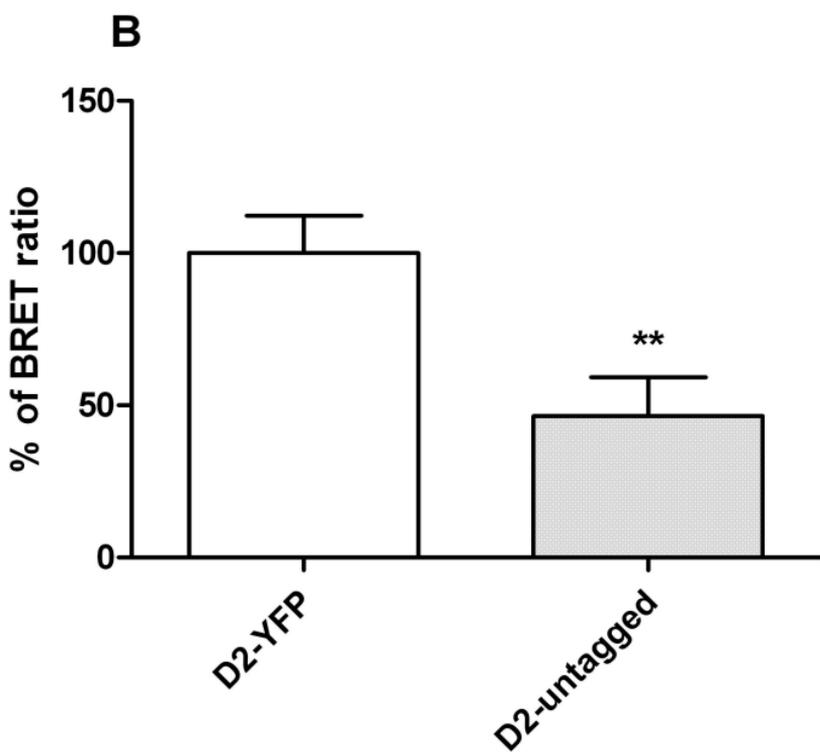
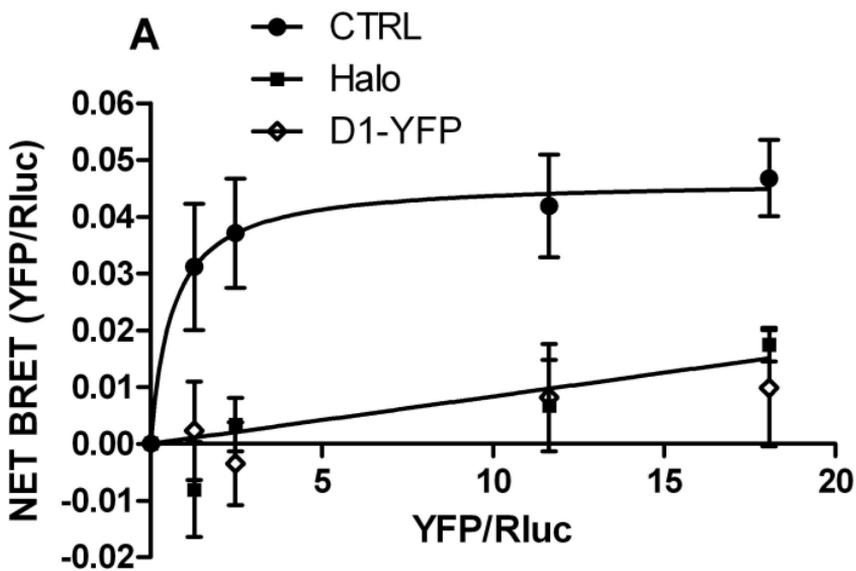


Figure 6

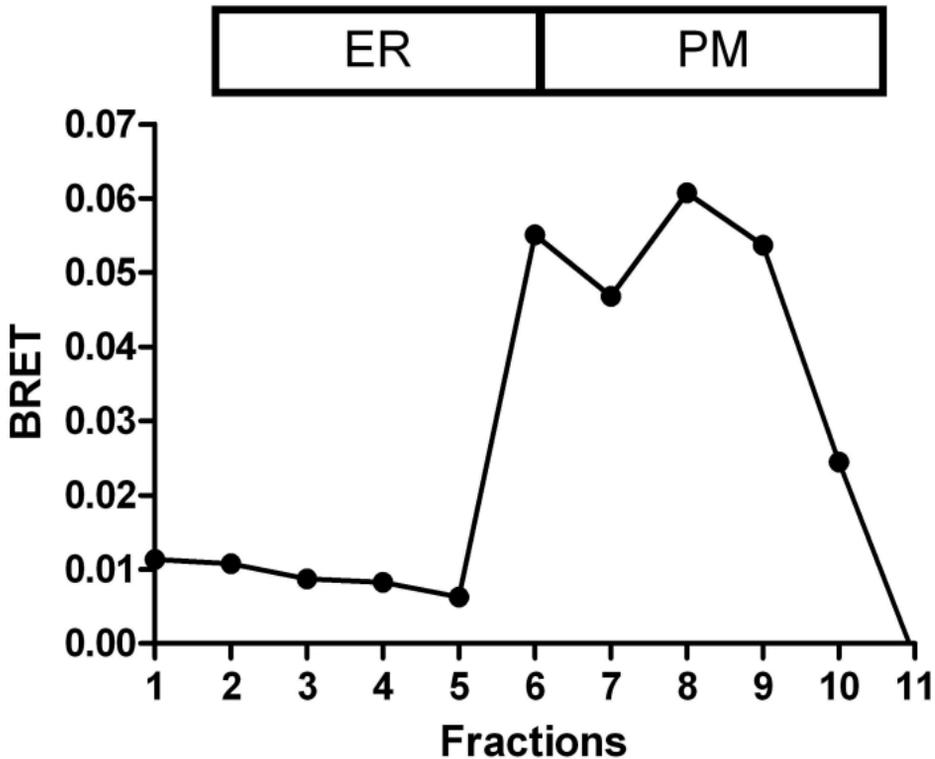
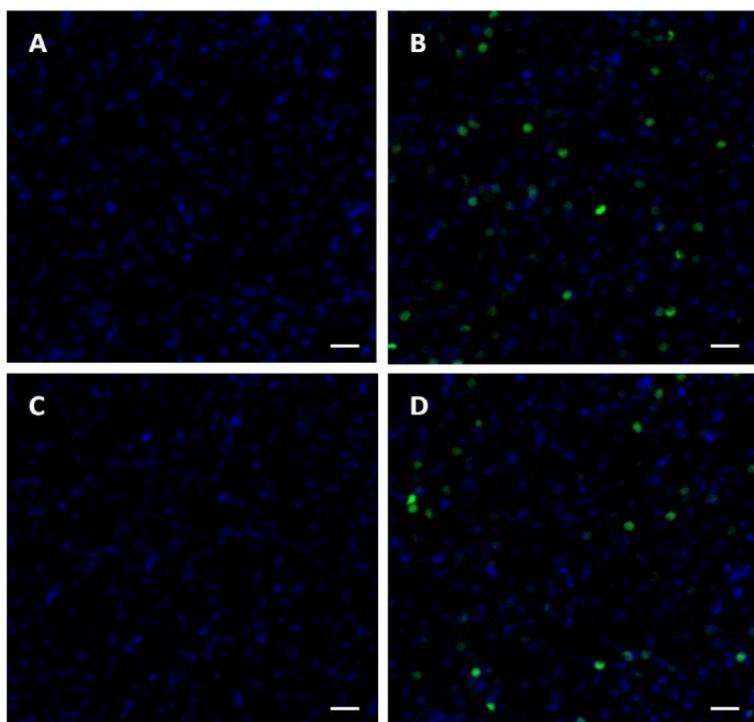
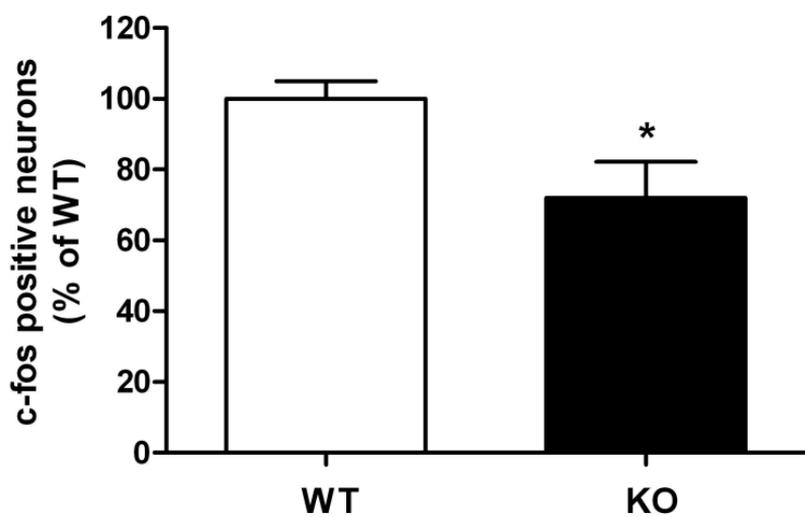
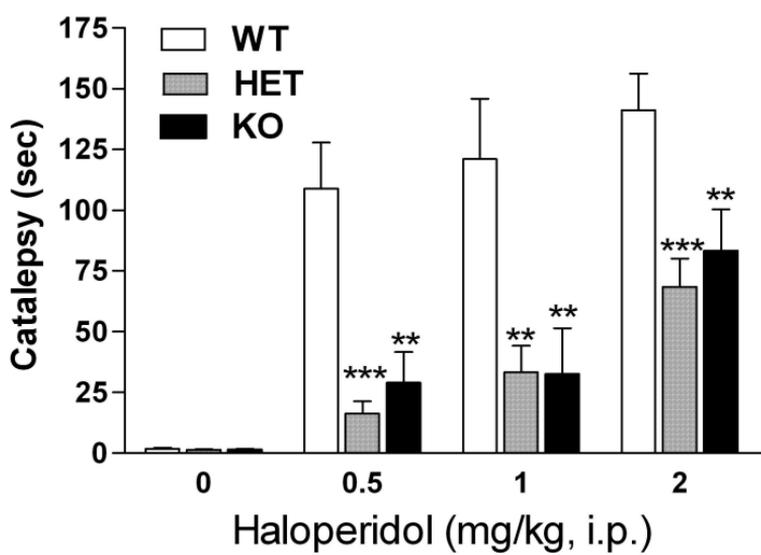


Figure 7

A**B****C****Figure 8**