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Molecular mechanisms of serotonergic action of the HIV-1 antiretroviral efavirenz

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Abstract

Efavirenz is highly effective at suppressing HIV-1, and the WHO guidelines list it as a component of the first-line antiretroviral (ARV) therapies for treatment-naïve patients. Though the pharmacological basis is unclear, efavirenz is commonly associated with a risk for neuropsychiatric adverse events (NPAEs) when taken at the prescribed dose. In many patients these NPAEs appear to subside after several weeks of treatment, though long-term studies show that in some patients the NPAEs persist. In a recent study focusing on the abuse potential of efavirenz, its receptor psychopharmacology was reported to include interactions with a number of established molecular targets for known drugs of abuse, and it displayed a prevailing behavioral profile in rodents resembling an LSD-like activity. In this report, we discovered interactions with additional serotonergic targets that may be associated with efavirenz-induced NPAEs. The most robust interactions were with 5-HT_{3A} and 5-HT_6 receptors, with more modest interactions noted for the 5-HT_{2B} receptor and monoamine oxidase A. From a molecular mechanistic perspective,

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Author Contribution:

Dhwanil A. Dalwadi – designed, performed experiments, and helped with data interpretation and writing of the manuscript
Seongcheol Kim – performed experiments for early phases of this work
Shahnawaz M. Amdani – performed experiments for early phases of this work
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Authors declare no competing financial interest.

efavirenz acts as a 5-HT_6 receptor inverse agonist of G_s-signaling, 5-HT_{2A} and 5-HT_{2C} antagonist of G_q-signaling, and a blocker of the 5-HT_{3A} receptor currents. Efavirenz also completely or partially blocks agonist stimulation of the M_1 and M_3 muscarinic receptors, respectively. Schild analysis suggests that efavirenz competes for the same site on the 5-HT_{2A} receptor as two known hallucinogenic partial agonists (±)-DOI and LSD. Prolonged exposure to efavirenz reduces 5-HT_{2A} receptor density and responsiveness to 5-HT. Other ARVs such as zidovudine, nevirapine and emtricitabine did not share the same complex pharmacological profile as efavirenz, though some of them weakly interact with the 5-HT_6 receptor or modestly block $GABA_A$ currents.

Keywords

Efavirenz; 5-HT_{2A}; LSD; DOI; 5-HT_{2C}; 5-HT_6

1. Introduction

Efavirenz [(4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one] is a potent non-nucleoside reverse transcriptase inhibitor (NNRTI) and one of the preferred components of highly active antiretroviral therapy (HAART) [1, 2]. Though very effective at suppressing replication of the virus that causes AIDS, a standard dose of efavirenz is known to carry a significant risk for CNS-mediated NPAEs [3–8]. However, little is known from a mechanistic perspective as to why these NPAEs occur and which CNS off-targets might be involved. The findings of a previous report [9] led us to examine the molecular mechanisms of efavirenz across a broader range of serotonergic targets.

Though efavirenz is considered to have good CNS penetration [10] consistent with its physicochemical properties, only low levels are detected in cerebral spinal fluid (CSF) with a mean concentration across studies of approximately 44 nM that corresponds to CSF levels 0.52% of plasma concentrations [11–13]. Efavirenz has a very high tendency to bind proteins (e.g, approximately 99.8% is bound to plasma proteins) [14, 15], and healthy CSF contains very little protein compared to that in blood plasma (< 1%). Since the concentration of free efavirenz in the aqueous fraction is expected to be low and it binds to protein-rich brain tissue [16], one might expect that 44 nM in CSF, if it accounted for approximately 0.2% (100%−99.8%) of the efavirenz dose, would correspond to an estimated 22 μM (44 nM ÷ 0.002) concentration of efavirenz in brain tissue. A study in rats suggests that efavirenz readily accumulates in the brain to levels that exceed 4.6 times the plasma levels within 1 hr of an *i.p.* dose of 15 mg/kg [16]. Physiological effects have been reported in rodent studies at efavirenz doses in the same range: 10–30 mg/kg depending upon the behavioral measure examined [9, 17]. High plasma levels of efavirenz (1 – 4 μg/mL) are needed to keep the HIV-1 virus suppressed to clinically meaningful levels [18], but efavirenz also has a narrow therapeutic window [3]: plasma levels less than 1 μg/mL result in virologic failure and while those greater than 2.74 μg/mL result in NPAE [3, 18]. Assuming a similar level of brain accumulation occurs in humans as in rats, then efavirenz plasma levels >2.74 μg/mL would correspond to brain concentrations >40 μM (2.74×10^{-3} g/L • 1/315.7 g/mole • 4.6). Based upon these types of assumptions and calculations, it would appear that efavirenz can rapidly

accumulate in the brain to concentrations in the range of tens of micromolars. Rapid accumulation of relatively high concentrations of efavirenz in the brain and evidence of CNS behavioral effects in animals and humans suggest that this reverse transcriptase inhibitor has CNS off-targets.

It has been almost two decades since efavirenz was approved by the FDA and to date, there is only one study that has sought to investigate the receptor neuropharmacology underpinning efavirenz's CNS targets and this was in the context of its reported recreational use [9]. In that study, a rationalized mechanistic approach was utilized in that receptor targets known to interact with drugs of abuse were selected to narrow the receptor profiling effort leading to a number of CNS receptors being identified as possible targets for efavirenz. At the receptor level, efavirenz was shown to interact with the 5-HT_{2A}, 5-HT_{2C} and $GABA_A$ receptors, as well as DAT, SERT, and $VMAT_2$ transporters [9]. Mechanistically, efavirenz potentiated GABA-mediated chloride currents at the $GABA_A$ receptor, and functioned as a DAT and SERT blocker [9]. However, *in vivo*, efavirenz failed to strongly substitute in tests of discrimination for drugs known to have these same functional properties on DAT, SERT, $VMAT_2$ transporters, or the $GABA_A$ receptor [9]. In contrast, when efavirenz was used as the discriminative stimulus, LSD partially substituted for efavirenz. Moreover, in rats trained to discriminate LSD from saline, efavirenz partially substituted for LSD and this substitution was blocked by pretreatment with the 5-HT_{2A} receptor selective antagonist MDL100,907 [9]. In a rodent head-twitch assay, efavirenz produced head-twitch responses in *wild type* but not 5-HT_{2A}-KO mice; though, the response was far weaker than for LSD and initiated much more rapidly [9]. There is a strong positive correlation between compounds that are hallucinogens in humans and those that induce a head-twitch response in rodents [19–21]. Efavirenz also dose-dependently depressed novel open field locomotor activity, similar to LSD in the same strain of mice [9]. For these reasons, it was concluded that efavirenz's predominate behavioral profile in rodents is most consistent with an LSD-like effect mediated by the 5-HT_{2A} receptor [9].

In this study we report for the first time that efavirenz interacts with the 5-HT_3, 5-HT_6 receptors and the enzyme MAO-A, and elucidate its molecular mechanisms of action at these targets as well as at the 5-HT_{2A} and 5-HT_{2C} receptors. Within a similar concentration range, efavirenz also blocks agonist responses at the M_1 and M_3 muscarinic receptors. Schild shift analysis suggests efavirenz acts at the same binding site on the 5-HT_{2A} receptor as the partial agonists LSD and (±)-DOI, but it is unique in that it does not activate the G_q-signaling pathway. Chronic exposure of the 5-HT_{2A} receptor to efavirenz reduces receptor density and responsiveness to 5-HT. We also demonstrate here that efavirenz's mechanism of action across a range of CNS targets is distinct from other prominent HIV-1 medications like emtricitabine (FTC), nevirapine (NVP) and zidovudine (ZDV).

2. Materials and Methods

2.1. Chemicals

Radiochemicals were from Perkin Elmer (Saint Louis, MO): 4-(2″-Methoxy)-phenyl-1-[2″-(N-2-pyridinyl)-p-fluorobenzamido]ethyl-piperazine ([3H]MPPF, NET-1109, 80 Ci/mmol); [3H]methylspiperone ([3H]MSP, NET-856, 84 Ci/mmol); [3H]Lysergic acid diethylamide

([3H]LSD, NET638, 70 Ci/mmol); [3H]Mesulergine (NET1148, 80 Ci/mmol); [3H]BRL-43694 (granisetron) (NET1030, 65 Ci/mmol); [3H]GR113808 (NET1152, 85 Ci/mmol). Efavirenz, [(4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one], and the other antiretroviral drugs (emtricitabine (FTC), zidovudine (ZDV) and nevirapine (NVP)) were purchased from Sequoia Research Products Limited (Pangbourne, UK). MDL100,907 was synthesized and kindly provided by Dr. Kenner C. Rice (NIDA/NIAAA). Unless otherwise noted, all other drugs and reagents were purchased from Tocris Biosciences (via R&D Systems, Inc. in Minneapolis, MN) or Sigma-Aldrich (St. Louis, MO). All ARV drugs were solubilized in DMSO at concentrations ranging from 10–100 mM and diluted at least 1:1000 v/v in the final assay solution.

2.2. Profiling Serotonin Receptors by Radioligand Binding

The interaction of efavirenz (10 μM) with serotonin receptors was measured by its ability to displace specifically bound radioligands from the metabotropic 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_4, 5-HT_{5A}, 5-HT_6 and 5-HT_7 receptors, and an ionotropic 5-HT_{3A} receptor using the conditions outlined in Table 1. With the exception of the 5-HT_4 receptor, which was sourced from Duncan Hartley guinea pig striatal tissue, all other serotonin receptors were cloned receptors heterologously expressed in mammalian cell lines lacking the receptor subtypes of interest. Radioligand, purified membranes expressing individual serotonin receptors and a fixed concentration of 10 μM efavirenz in a total volume of 1 mL binding buffer were allowed to equilibrate. Receptors were then isolated by rapid filtration through glass fiber filters pretreated for 10 min with 0.5% polyethyleneimine (Sigma-Aldrich) and three rapid washes with 3 mL of ice-cold wash buffer (50 mM Tris, pH 7.4 at 0–2°C). GF/C filters were used for membranes derived from cell cultures and GF/B filters for membranes derived from brain tissue (Brandel, Gaithersburg, MD). Dried filters were cut into individual scintillation vials, filled with 3.5 ml of scintillation fluid, mixed, and the radioactivity bound to the filters was quantified via scintillation spectroscopy. Membrane protein concentrations varied from 0.02–0.06 mg/mL. All data for each experiment was measured in triplicate. Each experiment was then repeated two or three times and the mean values of these experiments were reported with their associated SEM. A one-way ANOVA followed by a Bonferroni post-hoc analysis set to high stringency ($P < 0.001$) was used to determine significant displacement of the radioligand.

2.3. Measuring the affinity of efavirenz for selected serotonin receptor subtypes using competition radioligand binding

Selected receptors were examined further in competition-type concentration-response curves to measure their affinity for efavirenz. The binding conditions and procedure were the same as those described above and in Table 1 except that increasing concentrations, instead of a fixed concentration, of efavirenz was used in order to generate concentration-response curves and extract IC_{50} values. Equilibrium inhibition constants (K_i), representing binding affinities, were calculated from the IC_{50} values using the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [\text{ligand}]/K_D)$. Concentration-response curves were fitted with a four parameter logistic equation that included a variable slope using a 95% confidence interval for all curve-fits using Graphpad Prism version 4.0.

2.4. Intracellular Calcium and IP-One Assay: measurement of G_q-coupled response

Individual cloned human 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2c} receptors were stably expressed in HEK293 cells. Since an endogenous human M_3 muscarinic receptor [22–24] is expressed in HEK293 cells, untransfected HEK293 cells served as the source of the M_3 receptor. The cloned human M_1 muscarinic receptor was stably expressed in CHO cells. HEK293 cells were grown in Dulbecco's modified eagles medium (DMEM) complete which is DMEM supplemented with 50 units/mL streptomycin, 50 μg/mL penicillin, 1 mM sodium pyruvate, and 10% fetal bovine serum. The media of transfected cells additionally contained 0.1 mg/mL G418 to maintain selection. In the case of the 5-HT_{2c} receptor, dialyzed serum (Innovative Research Inc., Novi, MI) was employed as no signal could be obtained when using undialyzed FBS (ThermoFisher Scientific, Waltham, MA); however, undialyzed FBS was used in the case of the 5-HT_{2A} receptor since virtually identical responses were observed regardless of the serum type. CHO cells stably expressing the cloned human M_1 muscarinic receptor were grown in Ham's F12 supplemented with 50 units/mL streptomycin, 50 μg/mL penicillin, 1 mM sodium pyruvate, and 10% fetal bovine serum, and 0.1 mg/mL G418 to maintain selection.

Cells expressing the receptor of interest were seeded into Poly-L-Lysine coated, black-walled 96 well plates at a density of 120,000 cells per well. The following day, media was removed and cells were loaded for three hours in the dark with the FLIPR Calcium-6 QF dye (Molecular Devices, Sunnyvale CA) dissolved in Hank's buffered saline supplemented with 20 mM HEPES pH = 7.4. In cases where drug pretreatments were needed (as in the case of antagonist blocking an agonist response), the pretreatment drug or vehicle was added to the loading dye solution. Following dye loading and any pretreatments, changes in intracellular calcium were detected as rapid changes in fluorescent signal measured at 485 nm excitation, 525 nm emission with a cutoff filter set at 515 nm using a Flexstation 3 (Molecular Devices, Sunnyvale, CA). The baseline calcium signal was measured for 120 seconds followed by injection of the test drug and reading of any change in the calcium signal for an additional 780 seconds. At the termination of the experiment all signals were baseline subtracted and the change in intracellular calcium was quantified as the area under the curve, measured by integration (Prism version 4.0). All data were normalized to maximal functional response defined as the asymptote of the sigmoidal curve.

IP-One levels were measured in HEK293 cells stably expressing the 5-HT_{2A} receptor. Cells were seeded into Poly-L-Lysine coated, black walled 96 well plates at a density of 20,000 cells per well. IP-One levels were measured using the IP-One HTRF® assay kit (cisbio Assays, Bedford, MA) per manufacturer's protocol.

2.5. Effect of prolonged exposure to efavirenz on receptor density and G_q-coupled activation of the 5-HT_{2A} receptor

HEK293 cells stably expressing the 5-HT_{2A} receptor were split into four T-25 flasks to 20% confluency and were allowed 24 hrs to attach. Media was then removed by aspiration and replaced with either vehicle (1:1000 v/v DMSO), 30 μM 5-HT, or 30 μM efavirenz. Two flasks were designated for the vehicle treatment as one of them would be used later for a very short (15 min) exposure to efavirenz. For three days, the media was replaced with fresh

media containing the same treatments every 12 hrs. At the end of the third day, one of the vehicle treated flasks was also treated with 30 μM efavirenz for only 15 min. This group served as a control to ensure all efavirenz could be removed with our washing procedure prior to measuring the change in intracellular Ca^{2+} or receptor density (B_{max}). For the washing procedure, media was removed from all four flasks and cells were washed twice by incubating for 30 min in 10 mL DMEM complete. Following the second wash, cells were harvested for either the calcium assay as described above with no more than 8 hrs total between replating cells and performing the assay, or for radioligand binding to estimate receptor density (B_{max}). For the radioligand binding assay, membranes were prepared as described above for each of the four treated groups. The 5-HT_{2A} receptor density was then estimated using 0.5 nM [^{3}H]MSP followed by the calculation of B_{max} at saturation using a rectangular hyperbola model $B_{max} = (Y \bullet (K_D + X))/X$, where Y is [specific binding] and X = [radioligand concentration]. The K_D for [^{3}H]MSP at the cloned human 5-HT_{2A} receptor had been previously determined to be 246 pM [25]. The protein concentration for each sample was determined using the BCA assays as described in section 2.8.

2.6. Cyclic adenosine monophosphate (cAMP) Assay: measurement of G_s-coupled responses

Changes in intracellular cAMP levels were measured in HeLa cells stably expressing the rat 5-HT_6 receptor using a luminescent cAMP-Glo kit (Promega, Madison, WI) terminal assay as per the manufacturer's protocol with the following modifications. A total of 10,000 cells were seeded per well in a 96 well plate and grown in DMEM complete (see above). The following day, media was removed and cells were pretreated for 30 min in DMEM supplemented with 500 μM of the phosphodiesterase inhibitors 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, St. Louis, MO) and 100 μM Ro 20-1724 (Santa Cruz Biotechnology, Inc., Dallas, Texas). Next, cells were treated either alone or with a combination of vehicle, efavirenz or 5-HT for 30 min and then lysed in 20 μL lysis buffer for an additional 30 min with vigorous shaking (600 rpm on an IKA MTS 2/4 digital microplate shaker). A total of 10 μL of this lysate was transferred to a half-area plate containing 20 μL cAMP-Glo™ detection solution and the plate was mixed for 1 minute at 600 rpm and incubated at room temperature for 20 min. Finally, 40 μL of kinase glo® reagent was added to each well, mixed for 1 minute at 600 rpm and incubated at room temperature for 10 min. Luminescence measurements were performed using the Flexstation 3 set to luminescence mode with 100 millisecond integration time. All data were normalized to maximal stimulatory concentration of forskolin (10 μM).

2.7. Inhibition of monoamine oxidase A (MAO-A) activity

Recombinant human MAO-A was overexpressed in insect Hi5 cells and lysates of these cells containing approximately 4 μg/mL of enzyme were washed in 100 mM potassium phosphate, pH = 7.4, then pretreated for 15 min at 37°C with vehicle or 10 μM efavirenz, followed by treatment with 50 μM of the MAO-A substrate kynuramine for 60 min at 37°C. The reaction was terminated at by the addition of 6 N NaOH and the fluorescent 4-hydroxyquinoline cleavage product (Ex_{325}/Em_{465}) served as a measure of MAO-A activity.

2.8. Protein Assay

Membrane protein concentrations were measured by bicinchoninic acid assay (Pierce, IL) according to the manufacturer's instructions. Protein standard curves were constructed using purified bovine serum albumin.

2.9. Measurement of 5-HT3$_A$ and GABA$_A$ receptor currents using electrophysiology

Currents were obtained in the whole cell configuration from HEK293 cells stably expressing the rat or human $GABA_A$ receptors ($\alpha_1\beta_2\gamma_2$ (short isoform of the γ_2 subunit) [26], or the mouse 5HT3A receptors transiently transfected into HEK293 cells using a modified polyethylenimine method described previously [27]. Cell were voltage-clamped at −60 mV and the recordings were made at room temperature in the whole cell configuration using borosilicate pipettes with a tip resistance of 1–2.5 MΩ. The pipette solution contained 140 mM CsCl, 10 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, pH = 7.2. Coverslips containing cultured cells were placed in a 1.5 mL chamber on the stage of an inverted light microscope and superfused continuously (5–8 mL/min) with an extracellular solution containing 125 mM NaCl, 5.5 mM KCl, 0.8 mM $MgCl_2$, $CaCl_2$ (1.2 mM in 5-HT_{3A} studies and 3 mM in $GABA_A$ studies), 10 mM HEPES, 10 mM D-glucose pH = 7.3. GABA or 5-HT-induced currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder, and stored for subsequent analysis. A 60–80% series resistance compensation was applied at the amplifier. Any change in access resistance observed during the recording period resulted in the patch being aborted and that data was excluded from the analysis.

5-HT or GABA, with or without antiretroviral drugs, was dissolved in the extracellular solution and then applied to cells for 10–20 sec from independent reservoirs by gravity flow using a Y-shaped tube positioned within 100 μm of the cells. Using this system, the 10–90% rise time of the junction potential at the open tip is 12–51 milliseconds [28]. Receptors were activated with approximately an EC_{30} concentration of the appropriate neurotransmitter (0.6 μM for 5-HT_{3A} receptors, 5 μM for rat $\alpha_1\beta_2\gamma_2$ and 10 μM for human $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors), because this level of occupancy elicits minimal desensitization. Once a positive (baseline) control GABA or 5-HT response was established, the effect of ARV drug was examined by co-application with GABA or 5-HT. 5-HT or GABA responses were again monitored after each ARV drug application to ensure adequate washout and to ensure no loss of signal and integrity of the patch. Using this strategy, multiple concentrations of ARV drug could generally be tested for each cell studied. ARV drug stocks were dissolved in DMSO and diluted in saline so that the final DMSO concentration (v/v) was ≤ 0.2%. The concentration-response curve for efavirenz's modulatory effect on 5-HT_{3A} receptors was fitted to the following equation: $I/I_{max} = 1/(1+(IC_{50}/[efavirenz])^n)$, where I is the peak 5-HT current normalized to control at a given efavirenz concentration and I_{max} represents the normalized 5-HT-induced current, IC_{50} is the half-maximal blocking concentration, and n is the Hill coefficient. All data are presented as mean values ± SEM of a minimum of six individual experiments. A student's paired t-test was used to determine statistical significance ($p < 0.05$) for experiments comparing responses in the presence or absence of a single concentration ARV drug.

3. Results

In a previous report [9], CNS targets for efavirenz were identified that may contribute to its attractiveness as a drug of abuse. In this report, the molecular mechanisms of action for efavirenz were examined across a broader range of serotonergic targets in an effort to enhance our understanding of some of the observed NPAEs in patients taking it as prescribed. This included a comparison of efavirenz's mode of action with those of the other HIV-1 medications FTC, NVP and ZDV.

3.1. Efavirenz interacts with a number of receptors in the 5-HT family

Potential interactions of efavirenz with serotonin receptor subtypes from each of the seven subfamilies, 5-HT_1 through 5-HT_7, were probed initially by measuring the ability of a fixed concentration of efavirenz (10 μM) to displace radioligands specifically bound to individual receptor subtypes (Figure 1). The concentration selected was based upon our investigation of numerous receptor systems, including the 5-HT_{2A} and 5HT_{2C} receptors, and the estimated levels of efavirenz's brain exposure (> 40 μM) in those at risk for experiencing NPAEs following a standard oral dose (see **Introduction**). Using this assay format, we showed in our previous report that efavirenz interacted with the 5-HT_{2A} and 5-HT_{2C} receptors, and in this report we show that efavirenz additionally had interactions with the 5-HT_{2B}, and 5-HT_6 receptors, which reached statistical significance with a stringent cutoff ($P < 0.001$, ANOVA followed by a Bonferroni post-hoc) (Figure 1). Of these interactions, the most robust were with the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_6 receptors as efavirenz displaced significantly more radioligand from these three 5-HT receptor subtypes than from the 5-HT_{2B} receptor ($P < 0.05$, ANOVA followed by a Bonferroni post-hoc).

Those receptors experiencing ≥ 75% displacement of radioligand in the presence of 10 μM efavirenz (i.e., 5-HT_{2A}, 5-HT_{2C} and 5-HT_6 receptors) were examined further in competition-type concentration-response curves to measure their affinity for efavirenz (Figure 2). The calculated affinities (K_i) for efavirenz were similar in magnitude ranging from 1.7 to 4.4 μM. The highest affinity was for the 5-HT_{2C} receptor and the lowest for the 5-HT_{2A}, which had significantly lower affinity than for the other two receptors ($P < 0.05$ ANOVA with a Bonferroni post-hoc). While a variable slope model was not the statistically preferred model over a model with the pseudo Hill slope fixed to unity (e.g. −1.0), in order to evaluate the pseudo Hill slope parameter all the slopes were treated as a variable (instead of a fixed value) for the purpose of curve fitting. Using this approach, in all cases the pseudo Hill slopes were moderately greater than unity (−1.4 to −1.2) but the variance was such that none of them deviated significantly from unity when strict criteria were applied ($P < 0.001$, t-test), and further none of them differed significantly from one another ($P < 0.05$ ANOVA with a Bonferroni post-hoc). Slopes not different from unity are consistent with efavirenz binding to a single site on each of the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_6 receptors. As a reference, the affinities for serotonin at the 5-HT_{2A}, 5-HT_{2C} and 5-HT_6 receptors were measured as well, yielding $K_i \pm$ SEM values equal to 360 ± 29 nM, 16 ± 3.5 nM and 105 ± 8 nM, respectively, which are consistent with previous reports [29⁻31].

3.2. Efavirenz functions as a 5-HT_{2A} and 5-HT_{2C} receptor antagonist, a 5-HT_6 receptor inverse agonist, a 5-HT_3 receptor pore blocker, and an MAO-A inhibitor

The individual functional properties of the stably expressed cloned G_q-coupled 5-HT_{2A} and 5-HT_{2C}, and the G_s-coupled 5-HT_6 receptors were assessed starting with their ability to be stimulated by the endogenous full agonist serotonin (Figure 3). Serotonin activated the 5-HT_{2A} receptor with a potency that was unaffected by the serum condition in the culture media: 13.3 ± 2.5 nM in absence of serum, 13.9 ± 4.1 nM in presence of dialyzed FBS, and 17.2 ± 7.5 nM in presence of FBS. In stark contrast, the potency of 5-HT at the 5-HT_{2C} receptor spanned from the sub-nanomolar to the low nanomolar range: 0.039 ± 0.0039 nM in absence of serum, 1.5 ± 0.18 nM in presence of dialyzed FBS, and undetectable in the presence of FBS. Most notable was that no 5-HT response above baseline was observed when the 5-HT_{2C} receptor was maintained in FBS prior to measuring the Ca^{2+} response. Serotonin had potency in the lower nanomolar range, 12.7 nM, for the 5-HT_6 receptor. The 5-HT_{2C} receptor had a significantly higher potency for serotonin under serum free conditions compared with the 5-HT_{2A} receptor ($P < 0.05$ Student's t-test) or the 5-HT_6 receptor ($P < 0.05$ Student's t-test). Large differences in pseudo Hill slope values were measured as well ranging from 0.55 to 1.5, with the 5HT_{2A} receptor having the shallowest slope and the 5HT_{2C} receptor having the steepest slope. However, due to the variance in the data, these pseudo Hill slopes were not significantly different between receptors ($P > 0.05$ ANOVA with a Bonferroni post-hoc) and did not differ significantly from unity ($P > 0.05$, Student's t-test). More importantly, these values set a baseline for a full agonist response under our assays conditions and were useful for comparing and contrasting the ARV drug responses mediated by these receptors. Also, the mean EC_{50} values reported here for the 5-HT_{2A}, 5-HT_{2C} (for each of the serum conditions tested) and the 5-HT_6 receptors are in agreement with previously published values [32–34].

In a previous collaborative report, efavirenz was determined to have LSD-like properties mediated by the 5-HT_{2A} receptor based primarily upon its behavioral properties in rodents [9]. In this report, we employed detection of intracellular calcium or IP_1 accumulation as robust measures of 5-HT_{2A}–mediated activation of the G_q-PLC-IP_3-$[Ca^{2+}]_i$ signaling pathway. When a change in intracellular Ca^{2+} was employed as the measure of G_q-signaling, efavirenz did not stimulate the 5-HT_{2A} receptor at the maximum concentration (10 μM efavirenz) that could be tested as an agonist under the assay conditions (Figure 4A). To completely rule out the possibility that efavirenz might be a weak partial agonist, a higher concentration needed to be tested; due to efavirenz's solubility restriction in the Ca^{2+} assay, the IP-One assay was employed. Using the maximum concentration that could be tested, 100 μM efavirenz did not activate the G_q-pathway when IP-One was used as the readout of G_q activity (Figure 4B). This prompted us to test whether efavirenz might have other actions given that the radioligand binding data indicated that it interacts with the 5-HT_{2A} receptor. Accordingly, efavirenz was tested for its ability to modify functional responses of different classes of the 5-HT_{2A} receptor agonists: 5-HT, TCB-2 and α-methyl-5-hydroxytryptamine (α-Me-5HT). Like the selective 5-HT_{2A} receptor antagonist MDL100,907, efavirenz was able to antagonize the effects of all three agonists (Figure 4A). Given these results, efavirenz was also tested for its ability to modify the *in vitro* functional response of the well-known hallucinogens (±)-DOI and LSD by measuring changes in intracellular calcium. Both (±)-

DOI and LSD behaved as partial agonists (Figure 5B & 5E), which is consistent with previously published reports [35–37]. In the presence of increasing concentrations of efavirenz, both (±)-DOI and LSD functional responses were progressively shifted to the right without a significant decrease in the maximal response (Figure 5A, $P > 0.05$ ANOVA followed by Bonferroni's post-hoc for maximal responses) suggestive of either a negative heterotropic allosteric modulation with high cooperativity or a purely competitive interaction at a single site. Schild plot transformation of the dose-ratio shift data yielded slopes equal to 1.06 ± 0.054 and 1.20 ± 0.16 for (±)-DOI and LSD, respectively, that were not significantly different from unity ($P > 0.05$ t-test) suggesting that efavirenz competes with (±)-DOI and LSD for binding to a single site on the 5-HT_{2A} receptor (Figure 5C, 5E). From the Schild plots, the pA_2 value (also known as the antagonist potency (K_B)) for efavirenz was calculated to be 1.3 ± 0.2 μM and 0.31 ± 0.18 μM using either (±)-DOI or LSD, respectively, as the agonist in competition with efavirenz. However, these efavirenz antagonist potency values were not statistically different from one another or from the measured affinity (K_i = 4.37 ± 0.43 μM, Figure 2A) of efavirenz for the 5-HT_{2A} receptor ($P > 0.01$ ANOVA with a Bonferroni post-hoc). Collectively, the results suggest that efavirenz competitively interacts with a single site on the 5-HT_{2A} receptor that is indistinguishable from the site where LSD and (±)-DOI bind.

While efavirenz produces NPAEs in the majority of patients, the initial clinical finding was that in many of those affected, the NPAEs appear to lessen or disappear after several weeks [7]. Thus, we attempted to model a chronic exposure scenario *in vitro* and evaluate the possibility that efavirenz might induce a pharmacodynamic tachyphylaxis type response, meaning that its ability to antagonize a 5-HT_{2A} receptor response would diminish upon repeated exposure. HEK293 cells stably expressing the cloned human 5-HT_{2A} receptor were exposed to vehicle, 30 μM 5-HT, or 30 μM efavirenz for 3 days. At the end of the 3 day treatment, cells were thoroughly washed and suppression of 5-HT-induced Ca^{2+} response by efavirenz was assessed. In all the treatment groups, 10 μM efavirenz treatment resulted in a 48–49% suppression of the calcium response induced by a submaximal concentration of 5-HT (100 nM). This suppression was statistically significant in the two vehicle and the serotonin pre-treatment groups (Figure 6A, $P < 0.05$ ANOVA followed by Bonferroni post-hoc), but was not significant in the 3 day 30 μM efavirenz pre-treatment group (Figure 6A, $P > 0.05$ ANOVA followed by Bonferroni post-hoc) even though a 49% suppression is evident. Unexpectedly, the overwhelming effect of chronic treatment with efavirenz was a large (81%) and significant reduction in the 5-HT_{2A} receptor's responsiveness to 5-HT (Figure 6A, $P < 0.05$ ANOVA followed by Bonferroni post-hoc). This drastic reduction was not caused by the inability of efavirenz to dissociate from the receptor, because the responses in cells treated with 30 μM efavirenz for 15 min after 3 day treatment with DMSO were not significantly different from the responses in cells that underwent the 3 day treatment with DMSO only. To assess if the reduction in the 5-HT response in the 3 day efavirenz treatment group was due to the reduction in receptor density, B_{max} was estimated following the same series of 3 day treatments. Three day exposure to efavirenz resulted in a statistically significant 52% reduction in B_{max} (Figure 6B, $P < 0.05$ ANOVA followed by Bonferroni post-hoc), which is consistent with the observed reduction in the functional response to agonist following 3 days chronic treatment with efavirenz.

Like at the 5-HT_{2A} receptor, efavirenz functioned as an antagonist at the 5-HT_{2C} receptor. Given that serum had a drastic effect on 5-HT's potency at the 5-HT_{2C} receptor (Figure 3B), we tested efavirenz's ability to modify the *in vitro* functional response to 5-HT in the absence of serum. Using an EC_{50} concentration of 5-HT (0.03 nM) to stimulate the 5-HT_{2C} receptor, up to 3 μM efavirenz had no significant effect on 5-HT responses; however, at concentrations ≥ 10 μM efavirenz inhibited the 5-HT agonist effect, and at a concentration ≥ 60 μM efavirenz completely suppressed it (Figure 7A, $P < 0.001$ ANOVA followed by Dunnett's multiple comparison). To verify the specificity of the results, we tested efavirenz's ability to modulate the activity of an endogenous G_q-coupled M_3 muscarinic receptors known to be expressed in HEK293 cells [22⁻24]. While efavirenz alone had no effect on intracellular Ca^{2+} levels in untransfected HEK293 cells (Figure 8A), at concentrations ≥ 10 μM it was able to partially suppress the G_q response mediated by an EC_{50} concentration of acetylcholine (ACh) (Figure 8B, $P < 0.05$ ANOVA followed by Dunnett's multiple comparison). Since it was previously reported based upon binding studies that efavirenz does not interact with muscarinic receptors [9], and our data indicated that it inhibits the agonist responses at the human M_3 receptor, we investigated if efavirenz interacts with a different G_q-coupled muscarinic receptor. The M_1 receptor was selected because it is the predominant muscarinic receptor in the brain, and because it plays an important role in cognition and learning and memory [38⁻41]. CHO cells stably expressing the human M_1 receptor were used, to test for possible interactions of efavirenz with the M_1 receptor. While efavirez alone failed to stimulate the M_1 receptor, at concentrations ≥ 10 μM it suppressed the G_q response mediated by an EC_{50} concentration of carbachol, and concentrations ≥ 30 μM resulted in complete inhibition (Figure 8C–D, $P < 0.05$ ANOVA followed by Dunnett's multiple comparison). No G_q-mediated response was elicited by efavirenz, acetycholine or carbachol in untransfected CHO cells (data not shown).

In HeLa cells stably expressing the G_s-coupled rat 5-HT_6 receptor, efavirenz significantly reduced the basal level of cAMP activity (Figure 9A). At a lower concentration (3 μM), efavirenz first diminished 5-HT's agonist effect, and at a higher concentration (30 μM), not only completely overcame the agonist effect of 5-HT but also reduced activity to below basal levels (Figure 9A). Clozapine is an atypical antipsychotic drug that has been reported to interact with the 5-HT_6 receptor [42] acting as an inverse agonist [34]. Like efavirenz, a high concentration of clozapine (30 μM) significantly reduced basal levels of cAMP, and not only completely overcame the agonist effects of 5-HT but also reduced cAMP levels to below basal (Figure 9A). However, in untransfected HeLa cells, which lack the 5-HT_6 receptor, neither efavirenz (30 μM), clozapine (30 μM), nor 5-HT (1 μM) produced significant functional effects compared with the vehicle control (Figure 9B). This demonstrates that the functional responses we observed for efavirenz, clozapine, and serotonin were mediated via the 5-HT_6 receptor. HeLa cells have been reported to express an endogenous G_s-coupled β-adrenergic receptor [43]. Thus, as a further control, we invoked a cAMP functional response in untransfected HeLa cells by stimulating an endogenous G_s-coupled β-adrenergic receptor with (−)-epinephrine, and this effect was blocked by the β-adrenergic receptor antagonist timolol (Figure 9B). In this case though, efavirenz failed to significantly modulate (−)-epinephrine's stimulation of the β-adrenergic receptor response (Figure 9B). Thus, efavirenz exerts no cAMP-mediated functional effect in untransfected

HeLa cells and it additionally failed to modulate functional responses produced by stimulation of the endogenous β-adrenergic receptors.

Although efavirenz did not significantly displace the binding of the 5-HT_3 receptor antagonist [^{3}H]-granisetron from the 5-HT_{3A} receptor (Figure 1), we reasoned that since this receptor subtype is a ligand-gated ion channel efavirenz might act as a pore blocker or a direct channel activator, and thus a follow-up functional assay was warranted. Consistent with the idea that it might act as a pore blocker, efavirenz (10 μM) alone had no effect (n = 5, data not shown), but when co-applied with an EC_{30} concentration of 5-HT, which will open the channel pore, efavirenz inhibited the 5-HT_{3A} receptor currents in a concentration-dependent manner (0.1–30 μM, Figure 10B). At higher concentrations, efavirenz had a greater blocking effect on the steady state current than peak current (Figure 10A), with 30 μM producing a complete inhibition of the steady-state currents. The efavirenz-induced inhibition of 5-HT activated current was completely reversible when treated with concentrations of efavirenz up to 10 μM and partially recovered at 30 μM after 2–3 min washout (data not shown).

We previously demonstrated [9] that efavirenz (10 μM) blocks reuptake of serotonin via the serotonin transporter (SERT). Here we demonstrate that the same concentration of efavirenz also significantly, though modestly, inhibits (36%) monoamine oxidase A, an enzyme responsible for metabolizing 5-HT (Figure 11).

3.3. Other ARVs (FTC, NVP, ZDV) have a different receptor profile than efavirenz

In light of the findings that efavirenz has the most pronounced binding interactions with the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_6 receptors, we sampled other ARV drugs to see if they also had notable interactions with these receptor subtypes. Although no significant interactions for ZDV, FTC or NVP were observed for any of these receptors at the original profiling concentration of 10 μM (data not shown), we tested these other ARVs to their limit of solubility for the particular assay (60 μM). At these highest concentrations, only NVP displayed a significant, albeit comparatively weak interaction, with the 5-HT_6 receptor (Figure 12).

Given that efavirenz was shown previously to potentiate $GABA_A$ receptor currents [9], we also investigated the effects of these same three ARV drugs on the $GABA_A$ receptor. Both ZDV and FTC inhibited the GABA-activated currents in $\alpha_1\beta_2\gamma_2GABA_A$ receptors with reductions of 36–50% of control at 30 μM (Figure 13). The inhibitory effect had a very rapid onset (msec), and was reversible upon removal of the drugs (Figure 13A). In contrast, co-application of NVP (30 μM) with GABA, caused only a small, though significant, 8% potentiation of GABA-activated currents that was also rapid and reversible (Figure 13B).

4. Discussion

In a previous study focusing on its recreational use [9], efavirenz was reported to interact with a number of known molecular targets for drugs of abuse (e.g., $GABA_A$ and 5-HT_{2A} receptors, and DAT, SERT and $VMAT_2$ transporters), and to have a prevailing behavioral effect in rodents most similar to an LSD-like activity mediated by the 5-HT_{2A} receptor. Here

our attention was primarily on discovering molecular mechanisms potentially relevant to the reported CNS toxicity of efavirenz in those taking a prescribed dose for the treatment of HIV-1. In this context, we additionally refined our focus on serotonergic systems as it was suspected that other components of this signaling family were likely involved. Indeed, efavirenz was found to interact with the entire 5-HT_2 subfamily of receptors, the ionotropic 5-HT_{3A} receptor, the 5-HT_6 receptor and MAO-A. The most prominent of the newly discovered interactions were followed up here with detailed mechanistic studies.

While efavirenz was reported to interact with the 5-HT_{2C} receptor [9], its activity at that receptor was never elucidated. We report here for the first time that efavirenz acts as an antagonist of the 5-HT_{2C} receptor as it has no agonist activity alone but it concentration-dependently blocked the agonist response by serotonin. Given that ligands which interact with both the 5-HT_{2C} and 5-HT_{2A} receptors typically display similar functional properties, we re-evaluated the activity of efavirenz on the 5-HT_{2A} receptors. Using cloned 5-HT_{2A} receptors and either changes in intracellular calcium or IP_1 accumulation as measures of activation of the G_q-PLC-IP_3-$[Ca^{2+}]_i$ signaling pathway, efavirenz had no detectable agonist activity, but instead blocked multiple different agonists (i.e., 5-HT, α-methyl-5-HT, TCB-2, (±)-DOI, and LSD) from activating the 5-HT_{2A} receptor. Though it is unclear what the exact reason might be for the discrepancy in efavirenz's activity reported here and a previous collaborative report employing different methodology and conditions [9], it cannot be due to a greater sensitivity of activation as measured by radiolabeled inositol phosphates because measurements of IP_3-mediated changes in intracellular calcium are much more sensitive than measures of changes in inositol phosphates (e.g., IP_3) [44]. Since radioligand binding assays clearly demonstrate efavirenz interacts with the 5-HT_{2A} receptor and in the present report efavirenz acts as an antagonist of G_q-mediated agonist responses, Schild analysis was performed to determine its antagonist potency (K_B). No significant difference in efavirenz's antagonist potency (K_B) was observed using either (±)-DOI or LSD as the agonist, indicating an absence of allosteric probe dependence [45, 46]. Regardless of whether the agonist employed was (±)-DOI or LSD, the slopes of the Schild plots for efavirenz were not different from unity. The implication is that efavirenz competes for the same binding site on the 5-HT_{2A} receptor as either of these known hallucinogens. Our finding that efavirenz behaves as an antagonist of the 5-HT_{2A} receptor-mediated G_q signaling is consistent with efavirenz affecting human sleep patterns in a manner similar to the 5-HT_{2A} receptor antagonist MK-0454 [47].

In large clinical studies (1008 patients), hallucinations have been reported as one of the NPAEs associated with efavirenz-containing treatment regimes, but the incidents were relatively low (1.2%) compared to other NPAEs [7]. Further, the frequency and severity of all NPAEs are highest during the first couple weeks of treatment and tend to disappear with continued treatment in some, but not all patients [5, 48]. An even higher frequently (6–31%) of hallucinations associated with efavirenz treatment have been reported in three other clinical studies, each involving between 114–197 patients [49–51]. An HIV-negative individual with a history of psychedelic drug experience reported, upon first time experimental ingestion of efavirenz (initially 300 mg stepped up to a full 600 mg dose p.o.), that it has a classical psychedelic quality with acute effects somewhat comparable to the hallucinogen mescaline [52]. Thus, it seems clear that efavirenz, at the prescribed dose is

capable of inducing hallucinations in humans in some instances. Given our finding that efavirenz competitively antagonizes the 5-HT_{2A} receptor-mediated G_q signaling responses produced by the hallucinogenic 5-HT_{2A} receptor partial agonists (±)-DOI and LSD, the question becomes whether efavirenz mediates hallucinogenic responses via the 5-HT_{2A} receptor, as is the case for classical hallucinogenic drugs like LSD and DOI, or via some other receptor mechanism.

The 5-HT_{2A} receptor is an established site for hallucinogenic drug action. The hallucinogenic indoleamine psilocybin increases cerebral metabolic rate in the frontal cortex and anterior cingulate cortex [53], and pretreatment with the 5-HT_{2A} antagonist ketanserin dose-dependently prevents hallucinations caused by psilocybin in humans [54]. The frontal cortex-mediated headtwitch response [55, 56], a hallucinogenic behavioral proxy in rodents, is absent in 5-HT_{2A} knockout mice [57]. In *wild type* mice the head-twitch response is blocked by the 5-HT_{2A} antagonists, MDL100,907 and ketanserin, as is the increase in synaptic activity in the frontal cortex neurons induced by application of the hallucinogenic 5-HT_{2A} agonists DOI and α-methyl-5-HT [37, 58]. To our knowledge, all known hallucinogens which signal via the 5-HT_{2A} receptor happen to also act as G_q signaling agonists; however, we show here that efavirenz functions as an antagonist of G_q signaling. In G_q homozygous knockout mice, DOI is still capable of producing a significant, albeit blunted, head-twitch response, which suggests that the G_q signaling pathway is not the sole mediator of the 5-HT_{2A} receptor dependent behavioral effects of hallucinogens [59]. In a previous report, efavirenz was shown to invoke a head-twitch response of remarkably reduced duration and intensity compared to other hallucinogens like LSD and DOI in *wild type* mice, but failed to induce this response in 5-HT_{2A} knockout mice [9]. One possibility is that the comparatively diminished behavioral response for efavirenz, relative to other hallucinogens, might be related to efavirenz's lack of G_q-mediated agonist signaling. Efavirenz also has other behavioral properties similar to LSD, including in tests of discrimination where efavirenz partially substituted for LSD and vice versa [9]. Taken together, it seems clear that efavirenz interacts with the 5-HT_{2A} receptor and has psychoactive properties, though it also has properties distinct from classical hallucinogens including that it fails to stimulate G_q signaling via the 5-HT_{2A} receptor.

An alternative explanation is that the hallucinogenic properties of efavirenz are not exerted via its interactions with the 5-HT_{2A} receptor, but instead might be attributed to its interactions with a different receptor system. A previous study using individual cloned receptors capable of mediating hallucinations revealed that efavirenz (10 μM) did not have significant interactions with dopamine receptors or NMDA receptors [9]. That same study also reported that efavirenz (10 μM) did not have high affinity interactions with mixed populations of muscarinic receptors present in brain tissue [9]; however, this mixed receptor population approach utilizing a radiolabeled antagonist is not well suited to reveal moderate interactions with a single subtype nor would it reveal allosteric interactions with agonists. Though efavirenz alone had no effect on intracellular calcium levels in untransfected HEK293 cells, during the course of evaluating the possible modulation of endogenous G_q-coupled receptor-mediated responses in untransfected HEK293 cells, we discovered that efavirenz is able to partially antagonize agonist responses produced by endogenous M_3 muscarinic receptors. The plateauing of the inhibitory response for efavirenz at the

muscarinic M_3 receptor and the lack of any 5-HT response in untransfected cells, clearly demonstrates efavirenz has a different mechanistic profile at the endogenous M_3 receptor than at the exogenously expressed 5-HT_{2A} and 5-HT_{2C} receptors. Given that efavirenz exerted a modest and partial attenuation of agonist actions on the M_3 receptor, we investigated whether efavirenz might also affect the cloned human M_1 receptor. In contrast to its effect on the M_3 receptor, efavirenz was able to completely block agonist activation of the M_1 receptor, like the muscarinic receptor antagonists scopolamine and atropine. In this context it is worth noting that scopolamine [60] has psychoactive properties that include hallucinations, vivid dreams, and memory impairment [61–65]. Similarly, atropine, another muscarinic receptor antagonist [66], is also known to cause hallucinations [67–69]. Hence, partial and complete blockade of the M_3 and M_1 receptors, respectively, by efavirenz might account for or contribute to its hallucinogenic properties. The M_1 receptor is the most abundant muscarinic subtype in the brain and is known to play a role in cognition, learning and memory, and vigilance. M_1 knockout mice exhibit cognitive deficits [38, 39], hence blockade of the M_1 receptor by efavirenz may contribute to the cognitive impairment observed in patients taking efavirenz [7, 70].

Although efavirenz reduces the 5-HT_{3A} receptor currents, like a number of 5-HT_3 receptor antagonists, it did not displace [^{3}H]granisetron (also known as [^{3}H]BRL-43694) binding and thus is not a competitive antagonist for the 5-HT ligand binding site like granisetron is [71]. However, efavirenz does block 5-HT_3 receptor mediated currents in the presence of 5-HT, but in contrast to ondansetron, a competitive antagonist of the 5-HT site that reduces peak currents more strongly than steady-state currents [72], efavirenz reduces steady-state currents (as measured by end-application of current) more strongly than peak currents. This latter inhibition profile by efavirenz suggests it might act as an open-channel blocker of the 5-HT_{3A} receptor similar to picrotoxin [73], though the precise mechanism remains to be elucidated and we cannot rule out that efavirenz behaves as some sort of complex allosteric modulator.

In contrast to its actions on the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{3A} receptors, at the 5-HT_6 receptor efavirenz functions as an inverse agonist as evidenced by its ability to significantly reduce basal levels of cAMP in the absence of 5-HT. Also consistent with an inverse agonist, the 5-HT responses were first diminished then completely overcome by increasing concentrations of efavirenz even to the point where basal levels of activity were reduced. Clozapine, an accepted 5-HT_6 receptor inverse agonist [34], produced the same functional effect as efavirenz and served as our positive control for inverse agonism. These effects were observed using a rat receptor stably expressed in HeLa cells and did not require special measures to observe the inverse agonist activity as has been reported when the rat 5HT_6 receptor is expressed in other cell lines [74]. The functional responses we observed for efavirenz, clozapine, and serotonin were all mediated by the cloned 5-HT_6 receptor, since these compounds produced no significant changes in cAMP levels in untransfected HeLa cells which lack any detectable level of 5-HT_6 expression. Further, the agonist-induced increase in cAMP that is mediated by endogenous β-adrenergic receptors was unaffected by efavirenz and suggests that efavirenz is not exerting a non-specific effect in these cells.

4.1 CNS targets of other ARVs

Given that efavirenz has multiple off-target activities and some of these appear to correlate with certain NPAEs observed in humans, we investigated other ARV drugs for possible interactions with some of these off-target receptors. ZDV and NVP have been occasionally associated with reports of CNS toxicity, while FTC has not. For example, ZDV use has been associated with malaise, fatigue and insomnia [75, 76]. Further, a number of case reports have been published describing the onset of dose-related NPAEs associated with ZDV for the treatment of HIV-1, including depressive and manic syndromes primarily accompanied by auditory hallucinations and paranoid delusions [77, 78]. Case reports of NPAEs for NVP include hallucinations, vivid dreaming or psychosis in conjunction with depression and concomitant delusions soon after initiating NVP in patients with no previous history of psychiatric illness or substance use [79, 80]. These NPAEs subsided after withdrawal of NVP. We report here that, in general, ZDV, NVP and FTC did not share the same complex profile as efavirenz with respect to interactions with 5-HT_{2A}, 5-HT_{2C} and 5-HT_6 receptors. In fact, only in the case of the 5-HT_6 receptor and NVP, and only at a much higher concentration, was an interaction even detectable (estimated affinity would correspond to ~65 μM or approximately 30-fold lower than for efavirenz). Since we had found previously that efavirenz acted as an allosteric potentiator of $GABA_A$ receptors [9], the actions of ZDV, NVP and FTC on this receptor were examined as well. The most pronounced modulatory effect was observed for ZDV and FTC, and, in contrast to efavirenz, these ARV drugs inhibited, rather than potentiated the GABA response. While the investigation of other ARV drugs was limited in scope, it seems clear that the complex multiple off-target profile for efavirenz is unique amongst the ARV drugs examined. Though no rodent behavior clearly related to the $GABA_A$ receptor potentiating effects of efavirenz has been elucidated, the finding that efavirenz potentiates and ZDV and FTC inhibit GABA currents might still have relevance for HAART therapy given that the standard of HIV-1 care is combined drug treatments.

4.2 Speculation concerning efavirenz's CNS targets possibly contributing to NPAEs

Given the molecular mechanistic insights gained from our studies, we became curious about which one(s) of the prominent NPAEs might be most likely to be mediated by 5-HT_{3A} and 5-HT_6 receptors. The following is educated speculation based upon inference drawn from the *in vitro* receptor pharmacology. Dizziness and headache are amongst the most frequently reported adverse events for 5-HT_3 receptor antagonists [81–83]. Since efavirenz blocks 5-HT_3 receptor currents, it seems reasonable that 5-HT_3 receptor antagonism may contribute in part to efavirenz-induced dizziness and headache [4, 5, 7, 84]. HIV-1 patients are advised to take efavirenz before going to bed and sleep disturbances are commonly reported NPAEs including both insomnia and somnolence [4, 7, 85–87]. Several serotoninergic targets for efavirenz have been identified in this and a previous report (5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_6, 5-HT_{2B}, MAO-A, and SERT), and given the complex role that serotonin plays in the regulation of sleep and wakefulness and the known involvement of each of these serotonergic targets [88–93], it seems reasonable to infer that one or more of these targets would be implicated in efavirenz's sleep-related NPAEs. Consistent with this idea, and as noted above, efavirenz alters certain aspects of human sleep patterns in a manner similar to that of a 5-HT_{2A} receptor antagonist [47].

Other reported NPAEs for efavirenz include depression, anxiety and feelings of stress [4, 6, 7, 85, 94]. Homozygous 5-HT_{2A} knockout mice exhibit an anxiodepressive-like phenotype compared to wild type mice, possibly due to diminished 5-HT_{2A} receptor mediated transmission [95]. Though we demonstrated that efavirenz acts as an antagonist of 5-$HT_{2A/2C}$–mediated G_q signaling, we also discovered that chronic application of efavirenz reduces 5-HT_{2A} receptor density and the responsiveness of this receptor to serotonin. The inference here would be that efavirenz could also be reducing 5-HT_{2A} mediated neurotransmission thus precipitating an anxiodepressive-like phenotype.

5. Conclusion

In this report, we detail the molecular mechanisms of action of efavirenz across a family of serotonergic targets. Our radioligand binding studies revealed efavirenz's interactions with the 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} and 5-HT_6 receptors. Functional studies showed that efavirenz acts as an antagonist of G_q-coupling at the 5-HT_{2A} and 5-HT_{2C} receptors, an inverse agonist at the G_s-coupled 5-HT_6 receptor, a 5-HT_{3A} receptor pore blocker, and a modest inhibitor of MAO-A. Within a similar concentration range, efavirenz also completely or partially blocks agonist responses at the M_1 and M_3 muscarinic receptors, respectively. Schild shift analysis suggests that efavirenz competes with LSD and (±)-DOI for binding to the same site on the 5-HT_{2A} receptor. 5-HT_{2A} receptor density and responsiveness to 5-HT are reduced upon prolonged treatment with efavirenz. Other ARVs like NVP, FTC, and ZDV did not share the same complex pharmacology as efavirenz; only NVP interacted with the 5-HT_6 receptor and potentiated $GABA_A$ receptors currents, like efavirenz, albeit with much smaller effect sizes. FTC and ZDV inhibited, instead of potentiating, $GABA_A$ receptor currents. Overall, the data suggests that efavirenz has complex receptor pharmacology with varied functional responses that is unique among ARV drugs tested in this report.

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Abbreviations

NPAEs	Neuropsychiatric adverse events
ARV	Antiretroviral
HAART	Highly active antiretroviral therapy
ZDV	Zidovudine
FTC	Emtricitabine
NVP	Nevirapine

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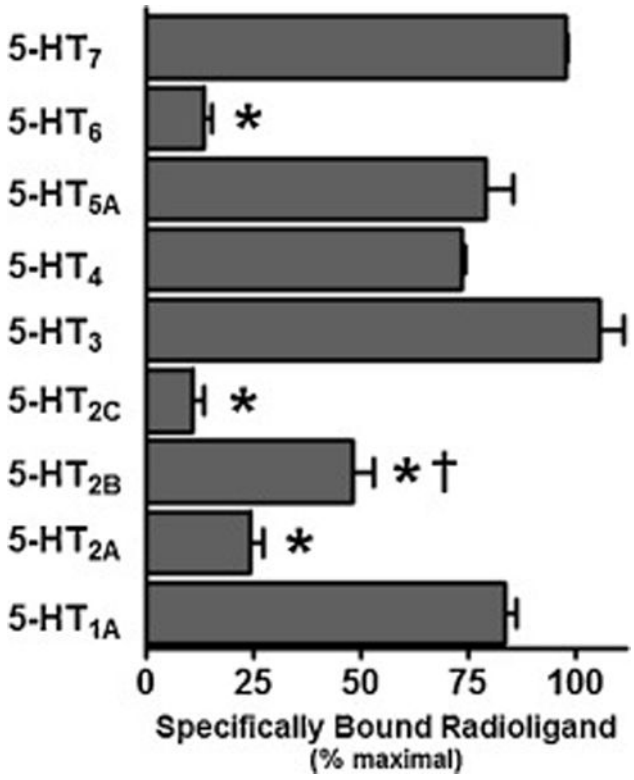

Figure 1. Efavirenz has significant interactions with the cloned metabotropic 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$ and 5-HT$_{6}$ receptors

At a concentration of 10 μM, efavirenz displaced greater than 50% of specifically bound radioligand from the 5-HT$_6$ receptor and 5-HT$_2$ subfamily of receptors, while having a less pronounced or no detectable interaction with the other serotonin receptor subtypes: 5-HT$_{1A}$, 5-HT$_{3A}$, 5-HT$_4$, 5-HT$_5$, 5-HT$_7$. A one-way ANOVA followed by a Bonferroni post-hoc analysis set to high stringency ($P < 0.001$) indicated significant displacement for those groups having an asterisk. When considered as a group, efavirenz has similar robust interactions with 5-HT$_{2A}$, 5-HT$_{2C}$ and 5-HT$_6$ receptors, but significantly weaker interactions with the 5-HT$_{2B}$ receptor as determined by a one-way ANOVA followed by a Bonferroni post-hoc analysis ($P < 0.05$) and indicated by the dagger. All data are presented as averaged values from multiple experiments ± SEM. Displacement data for the 5-HT$_{1A}$, 5-HT$_{2A}$ and 5HT$_{2C}$ receptors were adapted from Gatch et al., 2013 [9] and shown here for completeness.

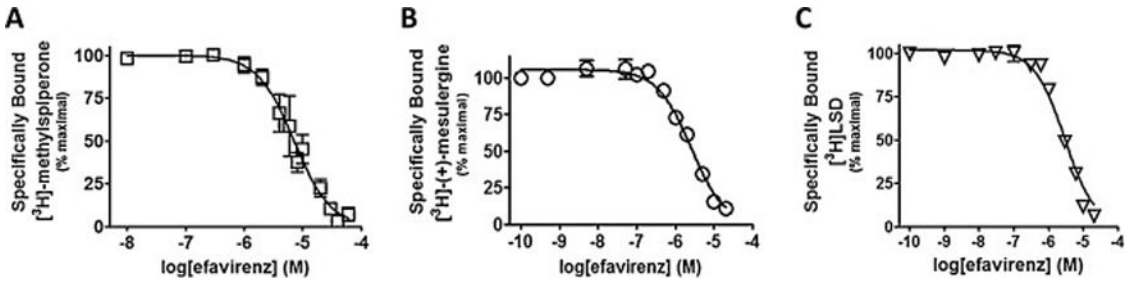

Figure 2. Efavirenz has low micromolar affinity for cloned metabotropic 5-HT$_{2A}$, 5-HT$_{2C}$ and 5-HT$_6$ receptors

The affinity of efavirenz for each receptor was determined at equilibrium by concentration-dependent competition binding of radiolabeled receptors using rapid filtration. All data are presented as averaged values from multiple experiments ± SEM. **A**, The 5-HT$_{2A}$ receptor binds to efavirenz with an affinity (K_i) = 4.4 ± 0.43 μM and a pseudo Hill slope = −1.39 ± −0.18. **B**, The 5-HT$_{2C}$ receptor binds to efavirenz with an affinity (K_i) = 1.7 ± 0.28 μM and a pseudo Hill slope = −1.22 ± −0.18. **C**, The 5-HT$_6$ receptor binds to efavirenz with an affinity (K_i) = 2.2 ± 0.27 μM and a pseudo Hill slope = −1.36 ± −0.14.

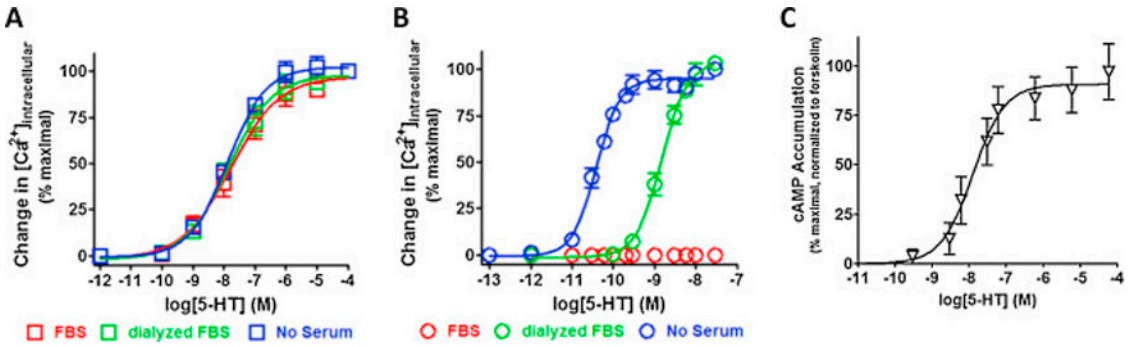

Figure 3. The cloned metabotropic 5-HT$_{2A}$, 5-HT$_{2C}$ and 5-HT$_6$ receptors are potently activated by serotonin

Functional dose-responses to serotonin for the G_q-coupled 5-HT$_2$ receptors were measured as an increase in intracellular calcium using the calcium sensitive fluorescent dye Calcium-6 QF (Ex_{485}/Em_{525}), while the functional dose-response for the G_s-coupled 5-HT$_6$ receptor was measured as an increase in cAMP detected using the bioluminescent cAMPGlo assay. All data are presented as averaged values from multiple experiments ± SEM. **A**, Serotonin activates the 5-HT$_{2A}$ receptor with an average potency (EC_{50}) range from 13–17 nM and a pseudo Hill slope range from 0.55–0.70 that was insensitive the serum condition used for culturing ($P > 0.05$ ANOVA with with a Bonferroni post-hoc). **B**, Serotonin activates the 5-HT$_{2C}$ receptor with a potency (EC_{50}) = 1.5 ± 0.18 nM and a pseudo Hill slope = 1.4 ± 0.19 when cells were cultured in dialyzed FBS and a potency (EC_{50}) = 0.039 ± 0.0039 nM and a pseudo Hill slope = 1.5 ± 0.17 when cells were cultured in absence of serum. No response was observed when cells were culture in FBS. **C**, Serotonin activates the 5-HT$_6$ receptor with a potency (EC_{50}) = 12.7 ± 6.2 nM and a pseudo Hill slope = 1.02 ± 0.35.

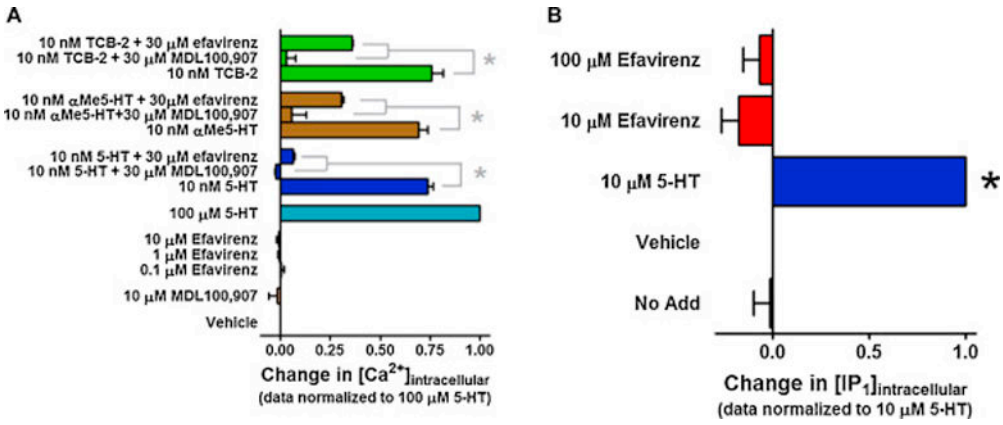

Figure 4. Efavirenz is a 5-HT$_{2A}$ receptor antagonist of G$_q$ signaling

All data are presented as averaged values from multiple experiments ± SEM. **A**, Efavirenz behaves as an antagonist at the cloned 5-HT$_{2A}$ receptor when a change in intracellular calcium is used as a measure of G$_q$-signaling and the 5-HT$_{2A}$ receptor is stimulated with different classes of agonists: 5-HT, TCB-2 and α-Me-5HT. MDL100,907, a potent and selective 5-HT$_{2A}$ receptor antagonist was used as a positive control for antagonist activity. A one-way ANOVA followed by a Bonferroni post-hoc analysis set to high stringency (*$P < 0.001$) indicated significant inhibition of agonist responses by both 30 μM efavirenz and 30 μM MDL100,907. **B**, To rule out the possibility that weak partial agonist properties of efavirenz might only be observed at a very high concentration, a higher maximal concentration of efavirenz was tested using IP-One assay conditions than was possible under the Ca^{2+} assay conditions. Nevertheless, at the highest concentration that could be tested (100 μM) in the IP-One assay, efavirenz did not display any agonist activity. A one-way ANOVA followed by Dunnett's multiple comparison test set to high stringency ($P < 0.001$) indicated by an asterisk revealed only the response by 5-HT to be significantly different from vehicle.

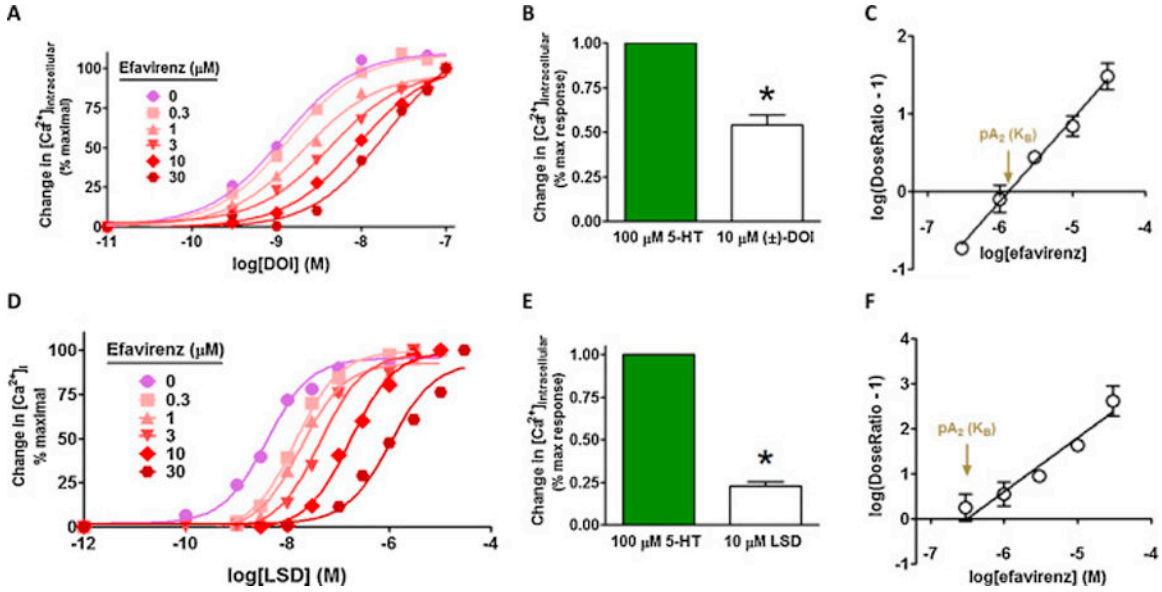

Figure 5. Schild shift analysis of efavirenz at the cloned 5-HT$_{2A}$ receptor suggests that it competes for the same binding site as (±)-DOI and LSD

A & D, G_q-signaling was measured as a change in intracellular calcium. Data are presented as averaged values from multiple experiments ± SEM. For clarity error bars are not shown and the Schild data are normalized to 100% response, even though both (±)-DOI and LSD are partial agonists of the 5-HT$_{2A}$ receptor, $P < 0.01$, two-tailed t-test as shown in **B** and **E**. (±)-DOI and LSD concentration-response curves of the G_q-mediated functional responses in the presence of increasing fixed concentrations of efavirenz induce a progressive shift to the right with no reduction in efficacy. **C**, Schild plot transformation of the data shown in panel **A** suggests that efavirenz's interaction with (±)-DOI is purely competitive given a slope equal to 1.06 ± 0.054. The pA_2 or antagonist potency (K_B) value for efavirenz competition of (±)-DOI is 1.3 ± 0.20 μM. Panel **F** suggests that efavirenz's interaction with LSD is also purely competitive with a slope of 1.2 ± 0.16. The pA_2 value for efavirenz competition of LSD is 0.31 ± 0.18 μM. In both panels **C** and **F**, the slopes of the Schild plot lines are not significantly different from unity ($P > 0.05$) and a two-site model is not significantly better than a one site model ($P > 0.05$).

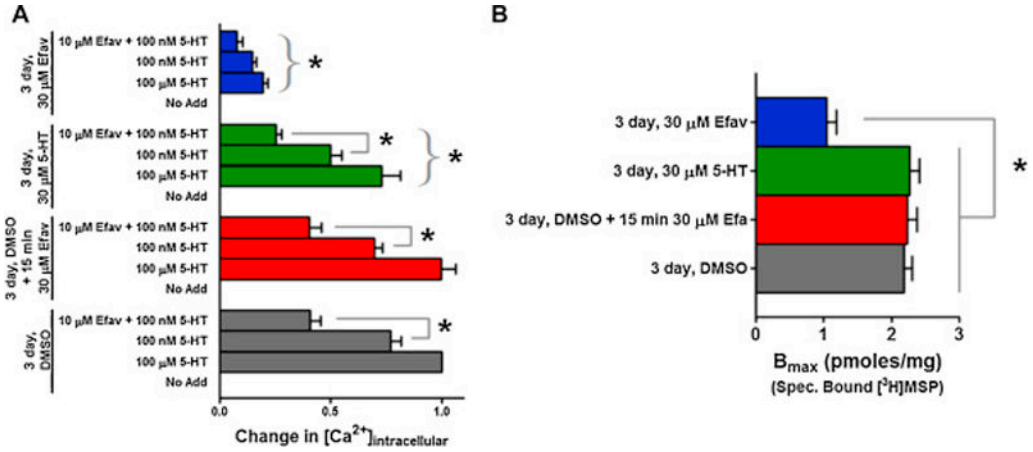

Figure 6. Chronic exposure to efavirenz reduces the 5-HT$_{2A}$ receptor density and 5-HT mediated G$_q$ activation at the 5-HT$_{2A}$ receptor

Cells were exposed (pretreated) for 3 days to DMSO vehicle [gray bars], serotonin (30 μM) [green bars] or efavirenz (30 μM) [blue bars], or to DMSO vehicle for 3 days and efavirenz for only 15 min on the third day [red bars]. The latter category was to ensure that our wash procedure was able to effectively remove all traces of efavirenz. After extensive washes, the cells from all groups were tested for their ability to mount functional responses to serotonin in the absence and presence of efavirenz (10 μM). All data were normalized to a maximal response generated by 100 μM 5-HT following the 3-day DMSO pretreatment and are presented as averaged values from multiple experiments ± SEM. **A**, Cells exposed to DMSO vehicle for 3 days elicited a robust calcium response when stimulated with a submaximal concentration of 5-HT (100 nM), and 10 μM efavirenz was able to antagonize this response by 48%. The same result was obtained when cells were exposed to efavirenz for only 15 min following 3 day exposure to DMSO, demonstrating that efavirenz could be easily removed by washing. Compared to the 3 day vehicle treated group, three day exposure to 30 μM 5-HT reduced the 100 μM maximal 5-HT response by 27%, and 10 μM efavirenz antagonized the 100 nM 5-HT submaximal response by 49%. In contrast to the other groups, 3 day exposure to 30 μM efavirenz drastically reduced the maximal 5-HT response by 81%, but the magnitude of efavirenz's antagonist effect on the 100 nM 5-HT submaximal response was similar to all the other groups (i.e, a 49% reduction). The comparisons being made between specified groups within a category are indicated by interconnecting gray lines and show a significant inhibition by efavirenz. A bracket with asterisk indicates a significant difference between the 3 day DMSO category and the remaining categories for comparisons between matched groups. A two-way ANOVA followed by Bonferroni multiple comparisons test was performed to identify significant differences within and between the various pretreatment categories at $P < 0.05$. **B**, Three day exposure to 30 μM efavirenz resulted in a significant 52% decrease in receptor density (B_{max}). However, none of the other exposure conditions had a significant effect on B_{max}. A one way ANOVA followed by Bonferroni's multiple comparisons test was performed to identify significant differences between various pretreatment categories. * $P < 0.05$ between the 3 day efavirenz pretreatment category and all the other pretreatment categories.

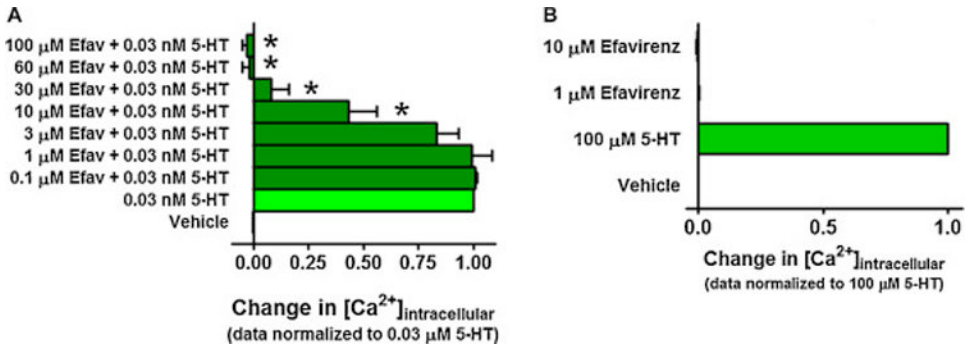

Figure 7. Efavirenz is an antagonist of the cloned 5-HT$_{2C}$ receptor

G_q-signaling was measured as a change in intracellular calcium. All data are presented as averaged values from multiple experiments ± SEM. **A**, Efavirenz dose-dependently antagonizes agonist responses produced by an EC_{50} concentration of 5-HT (EC_{50} data normalized to 100%). For these experiments cells were maintained in serum free media for at least 12 hours, pretreated with efavirenz, then stimulated with 0.03 nM serotonin, which is the EC_{50} for 5-HT for this receptor under serum free conditions (see Figure 3B). Values significantly different from 0.03 nM 5-HT, (Dunnett's multiple comparison test set to high stringency, $P < 0.001$) are denoted by asterisks. **B**, Efavirenz did not have any agonist activity at the 5-HT$_{2C}$ receptor under similar conditions.

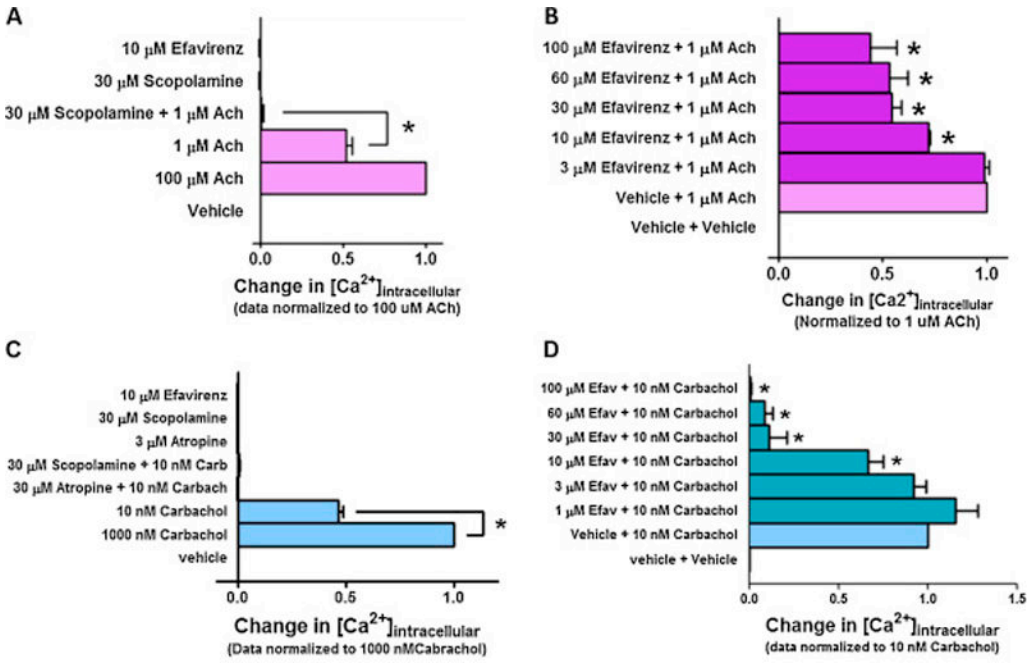

Figure 8. Efavirenz partially inhibits the activation of the M_3 muscarinic receptor, and completely inhibits the activation of the M_1 muscarinic receptor

G_q-signaling was measured as a change in intracellular calcium. All data are presented as averaged values from multiple experiments ± SEM. **A**, In untransfected HEK293 cells, efavirenz does not stimulate G_q-coupling as measured by changes in intracellular Ca^{2+}. However, these cells express an endogenous G_q-coupled M_3 muscarinic receptor [22–24] that was activated by acetylcholine, and this response was blocked by the muscarinic receptor antagonist scopolamine. **B**, Efavirenz partially suppresses ACh mediated M_3 receptor activation at concentrations ≥ 10 μM but was unable to produce a complete block at the highest concentration that could be tested under these assay conditions. **C**, Efavirenz fails to stimulate G_q-coupling in CHO cells stably expressing the human M_1 receptor, but this receptor can be stimulated with the muscarinic receptor agonist carbachol and the muscarinic receptor antagonists scopolamine and atropine completely block carbachol's effect. **D**, Efavirenz antagonizes carbachol-mediated M_1 receptor activation in a concentration dependent manner. At 10 μM there is a significant inhibition of the response produced by an EC_{50} concentration of carbachol (normalized to 100%) and a complete inhibition at concentrations ≥ 30 μM. A one-way ANOVA followed by Dunnett's multiple comparison test was performed to identify significant differences between the agonist response in the absence of efavirenz compared to the agonist response in the presence of different concentrations of efavirenz. * $P < 0.05$ for those responses in the presence of efavirenz that are significantly different from the agonist response in the absence of efavirenz.

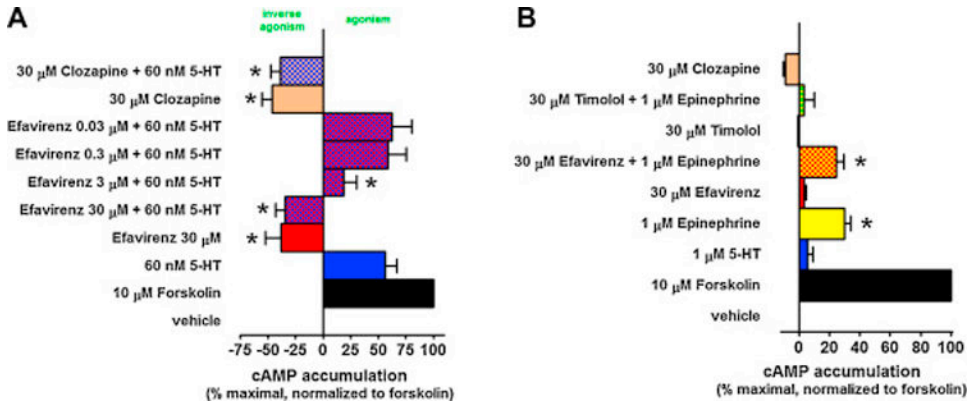

Figure 9. Efavirenz behaves as an inverse agonist of the cloned 5-HT$_6$ receptor

G_s-signaling was measured as a change in intracellular cyclic AMP (cAMP) levels. All data are presented as averaged values from multiple experiments ± SEM. **A**, Efavirenz (30 μM) acts as an inverse agonist of the cloned 5-HT$_6$ receptor stably expressed in HeLa cells as it lowers the basal levels of cAMP. Only at the higher concentrations (3–30 μM) can efavirenz overcome the stimulatory effect of 60 nM serotonin. The atypical antipsychotic clozapine (30 μM), a known inverse agonist, was used as a positive control for the 5-HT$_6$ inverse agonist response. One-way ANOVA followed by a Dunnett's post-hoc analysis comparing the 60 nM 5-HT group to groups containing increasing concentrations of efavirenz indicated that cAMP levels were significantly lower in experimental groups containing ≥ 3 μM efavirenz ($P < 0.05$). **B**, There is no stimulatory effect of 5-HT or inverse agonism by efavirenz or clozapine in untransfected HeLa cells. HeLa cells express endogenous β-adrenergic receptors whose stimulation by (−)-epinephrine can be blocked by the β-adrenergic receptor antagonist timolol but not by efavirenz.

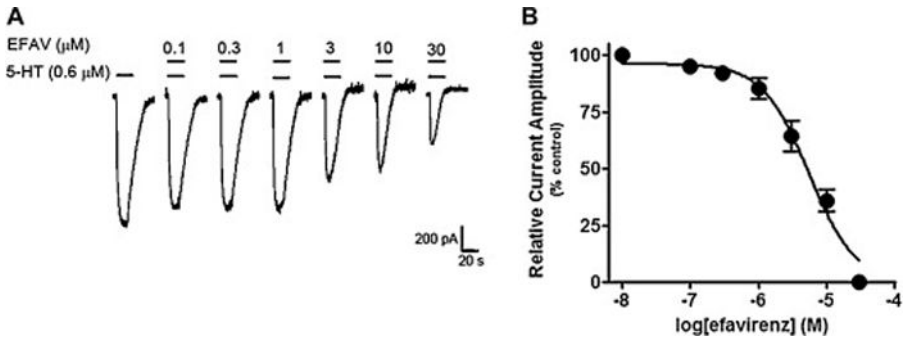

Figure 10. Efavirenz inhibits cloned 5-HT$_{3A}$ receptor currents

All recording were performed on HEK293 cells transiently expressing mouse 5-HT$_{3A}$ receptors. **A**, Representative traces showing whole-cell currents activated by an EC_{30} concentration 0.6 μM 5-HT in the absence and presence of co-applied (20 sec) increasing concentrations (0.1–30 μM) of efavirenz. Note that efavirenz produces a greater inhibition on the steady-state current amplitude as the concentration increases. **B**, Summary data for effect of efavirenz on 5-HT-induced currents. The currents at the end of ligand application were normalized to the current amplitude at the same time point as in the control (5-HT alone). Efavirenz's inhibitory concentration (IC_{50}) is 4.9 ± 0.7 μM with a Hill coefficient of 1.2 ± 0.19 (n = 10). All data are presented as means ± SEM.

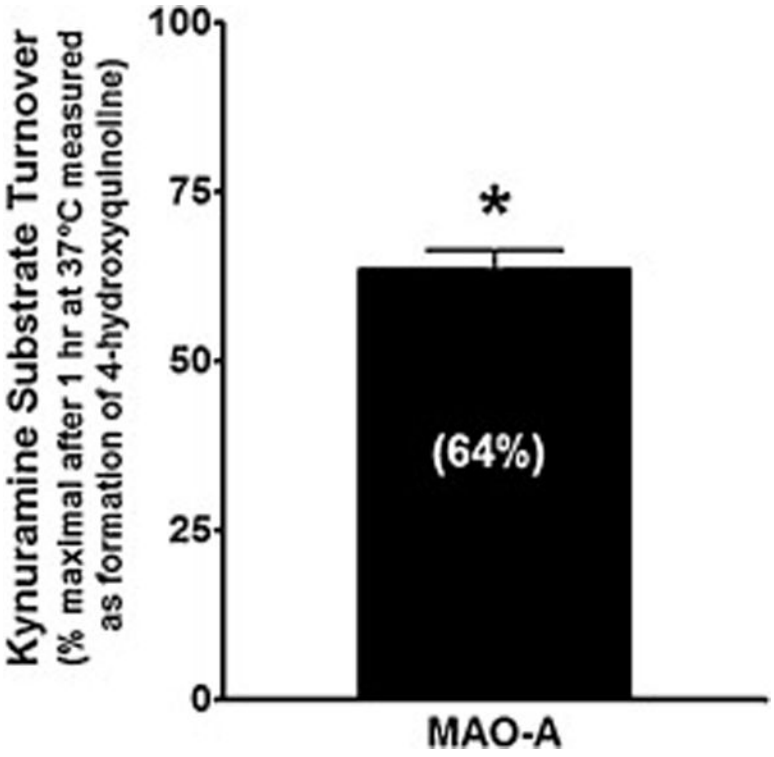

Figure 11. Efavirenz modestly inhibits monoamine oxidase-A

Efavirenz (10 μM) decreases the activity of MAO-A by 36 ± 1.7%. The mean value from multiple experiments ± SEM is plotted. Student's t-test was used to determine statistical significance ($P < 0.05$).

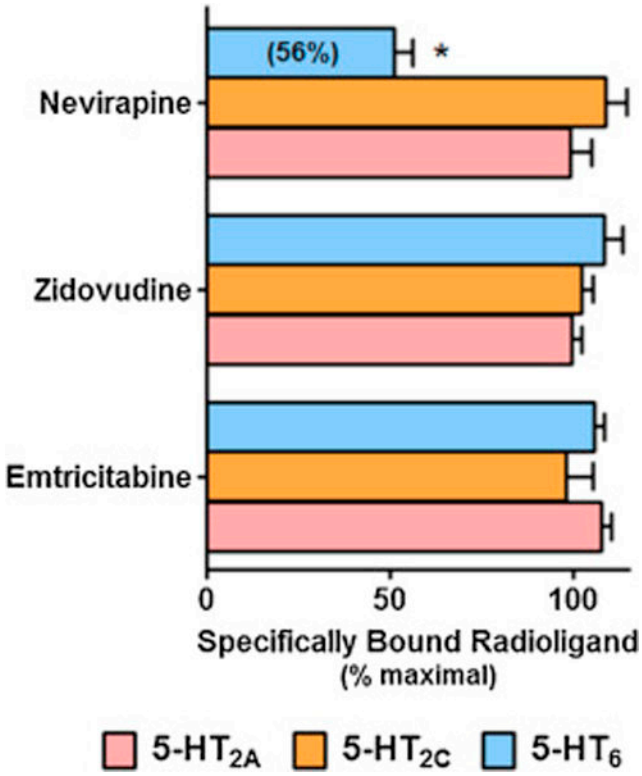

Figure 12. Nevirapine, but not emtricitabine or zidovudine, interacts with the cloned 5-HT$_6$ receptor

All of the ARV drugs were tested at the highest concentration (60 μM) that would stay in solution under the assay conditions. All data are presented as averaged values from multiple experiments ± SEM. A two-way ANOVA followed by a Bonferroni post-hoc indicated that interaction of nevirapine with the 5HT$_6$ receptor is significantly less than its vehicle control ($P < 0.001$).

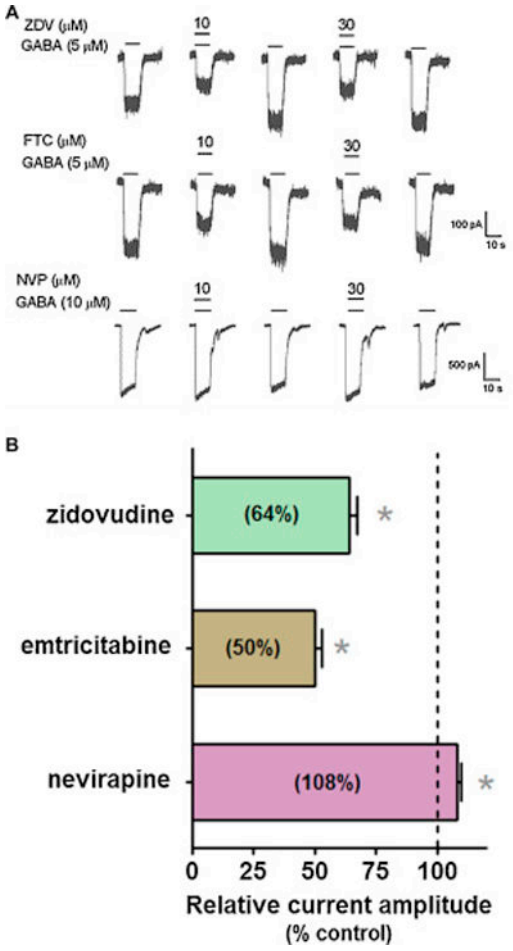

Figure 13. The ARV drugs zidovudine (ZDV), emtricitabine (FTC), but not nevirapine (NVP), inhibit cloned $GABA_A$ receptor currents

A, Representative traces showing whole cell currents activated by an EC_{30} concentration of GABA (5 μM) in HEK293 cells stably expressing $\alpha_1\beta_2\gamma_2GABA_A$ receptors. Efavirenz was co-applied with GABA for 10 sec. Note that ARV drug-induced action was rapid and reversible. **B**, Summary data of the modulatory effect induced by ZDV, FTC and NVP (30 μM). All currents were normalized to the GABA control response (GABA alone). *P < 0.05, paired t-test, compared to the control, n=6–8. Data are presented as means ± SEM.

Table 1

Conditions for measuring efavirenz's ability to displace radioligands specifically bound to the serotonin receptor subtypes. Cloned receptors expressed in cell lines served as the source of all subtypes, except for the 5-HT_4 receptor. The letter(s) in front of the receptor subtype designate the species: h = human, m = mouse, r = rat, gp = guinea pig.

Receptor Subtype	Cell type for expression	Radioligand	Drug for defining non-specific binding	Binding buffer and conditions
h5-HT_{1A}	HEK293	[^{3}H]MPPF	5 μM NAN-190	Buffer A; 90 minutes at 25°C
h5-HT_{2A}	HEK293	[^{3}H]MSP	5 μM mianserin	Buffer A; 90 minutes at 25°C
h5-HT_{2B}	HEK293	[^{3}H]LSD	30 μM mianserin	Buffer C; 90 minutes at 25°C
h5-HT_{2C}	HEK293	[^{3}H]mesulergine	5 μM mianserin	Buffer A; 90 minutes at 25°C
m5-HT_3	HEK293	[^{3}H]BRL-43694	10 μM mianserin	Buffer A; 90 minutes at 25°C
gp5-HT_4	Striatum	[^{3}H]GR113808	30 μM serotonin	Buffer A; 30 minutes at 25°C
h5-HT_{5A}	CHO-K1	[^{3}H]LSD	100 μM serotonin	Buffer B; 60 minutes at 37°C
r5-HT_6	HeLa	[^{3}H]LSD	30 μM chlorpromazine	Buffer C; 60 minutes at 37°C
h5-HT_7	CHO	[^{3}H]LSD	10 μM serotonin	Buffer B; 120 minutes at 25°C

Binding Buffer A = 50 mM Tris, pH = 7.4

Binding Buffer B = 10 mM $MgSO_4$, 0.5 mM EDTA, 50 mM Tris, pH 7.4

Binding Buffer C = 5 mM $MgCl_2$, 250 μM sodium ascorbate, 50 mM Tris, pH = 7.4

Approximate concentrations of the radioligands used in the displacement assays were 0.5 nM in all cases, except for [^{3}H]GR113808 which was tested at 0.7 nM, [^{3}H]GR113808 which was tested at 1.7 nM, and [^{3}H]LSD which in the case of $5HT_{2B}$ was tested at 0.25 nM and in the case of $5HT_7$ at 4 nM.