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The antidepressant bupropion is a negative allosteric modulator of serotonin type 3A receptors

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Abstract

The FDA-approved antidepressant and smoking cessation drug bupropion is known to inhibit dopamine and norepinephrine reuptake transporters, as well as nicotinic acetylcholine receptors (nAChRs) which are cation-conducting members of the Cys-loop superfamily of ion channels, and more broadly pentameric ligand-gated ion channels (pLGICs). In the present study, we examined the ability of bupropion and its primary metabolite hydroxybupropion to block the function of cation-selective serotonin type 3A receptors (5-HT_{3A}Rs), and further characterized bupropion's pharmacological effects at these receptors. Mouse 5-HT_{3A}Rs were heterologously expressed in HEK-293 cells or *Xenopus laevis* oocytes for equilibrium binding studies. In addition, the latter expression system was utilized for functional studies by employing two-electrode voltage-clamp recordings. Both bupropion and hydroxybupropion inhibited serotonin-gated currents from 5-

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7. Author contributions

AP, HW, MPB and MJ developed the study concept and design. MJ was responsible for the overall project management and strategy. AP performed experiments, acquired and analyzed data and wrote first draft of the manuscript. ASP conducted electrophysiological experiments with anion-selective LGICs. All authors contributed to the final version of the manuscript. All of the authors approved the final version for publication.

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HT_{3A}Rs reversibly and dose-dependently with inhibitory potencies of 87 μ M and 112 μ M, respectively. Notably, the measured IC₅₀ value for hydroxybupropion is within its therapeutically-relevant concentrations. The blockade by bupropion was largely non-competitive and non-use-dependent. Unlike its modulation at cation-selective pLGICs, bupropion displayed no significant inhibition of the function of anion-selective pLGICs. In summary, our results demonstrate allosteric blockade by bupropion of the 5-HT_{3A}R. Importantly, given the possibility that bupropion's major active metabolite may achieve clinically relevant concentrations in the brain, our novel findings delineate a not yet identified pharmacological principle underlying its antidepressant effect.

Graphical Abstract

Keywords

antidepressant; bupropion; hydroxybupropion; 5-HT_{3A} receptors; pentameric ligand-gated ion channels

1. Introduction

Of the seven subclasses of 5-hydroxytryptamine/serotonin receptors (5-HT₁ – 5-HT₇R) identified to date, the 5-HT₃R is unique for being the only ionotropic ligand-gated ion channel (LGIC) (Derkach et al., 1989; Thompson and Lummis, 2013). This pentameric LGIC is one of the prominent members of the Cys-loop superfamily, which also includes the cation-selective excitatory nicotinic acetylcholine receptors (nAChR) as well as the anion-selective inhibitory γ -amino butyric acid type A (GABA_A) and glycine (Gly) receptors (Thompson and Lummis, 2006). The assembly of five 5-HT₃ subunits constitutes a central ion-channel which is permeable to the monovalent cations, Na⁺ and K⁺ (Yang, 1990), and the divalent cation Ca⁺⁺ (Hargreaves et al., 1994). The 5-HT₃R activation-induced influx of cations via its intrinsic ion channel results in a rapid depolarization of the neuronal cell membrane (Yakel and Jackson, 1988). 5-HT₃R mediated depolarization of the presynaptic nerve terminal modulates the release of a spectrum of neurotransmitters, including dopamine (DA), GABA, acetylcholine (ACh), glutamate and substance P (Fink and Gothert, 2007; Giovannini et al., 1998; Koyama et al., 2000; Wang et al., 1998), whereas postsynaptically, it evokes excitatory fast synaptic neurotransmission (Sugita et al., 1992).

The isolation of cDNA clones encoding for five distinct 5-HT₃ subunits (5-HT_{3A} – 5-HT_{3E}) has considerably augmented the diversity of 5-HT₃Rs (Davies et al., 1999; Niesler et al.,

2003). Notwithstanding, the architecture of these subunits is highly comparable; with the notable exception of two 5-HT_{3D} subunit variants where one is devoid of the majority of the N-terminal domain and the other lacks the signature Cys-loop (Lummis, 2012; Niesler et al., 2007). The 5-HT_{3A} subunit, when heterologously expressed in mammalian cells and *Xenopus laevis* oocytes, assembles to form functional homomeric pLGICs in the cell membrane (Hussy et al., 1994; Maricq et al., 1991). In contrast, the 5-HT_{3B} and the other subunits (5-HT_{3C-3E}) *per se* are not known to constitute functional homomeric receptors *in vitro*. Their co-assembly with at least one 5-HT_{3A} subunit is obligatory in order to form functional receptors, which are consequently heteromeric, e.g. 5-HT_{3AB}. (Morales and Wang, 2002; Niesler et al., 2008). Homomeric 5-HT_{3A} and heteromeric 5-HT_{3AB} receptors have been extensively studied in heterologous expression systems as well as in some native preparations, and exhibit distinct electrophysiological and biophysical properties (Dubin et al., 1999; Kelley et al., 2003).

Studies on animals and humans have revealed a widespread distribution of 5-HT₃Rs in neurons of the peripheral nervous system (PNS) and the central nervous system (CNS), as well as extra-neuronally in immune cells, synovial tissue and platelets (Fiebich et al., 2004; Stratz et al., 2008). Evidently, they are involved in a number of physiological functions and pathological states. In the PNS, 5-HT₃Rs exist on pre- and post-ganglionic autonomic neurons (Faerber et al., 2007), as well as on neuronal bodies of the sensory nervous system, and myenteric and submucosal plexuses of the human gastrointestinal tract (Bottner et al., 2010; Michel et al., 2005; Sakurai-Yamashita et al., 2000). Thus, in the PNS activation of these receptors modulates a myriad of sensory, sympathetic and parasympathetic functions including pain perception, gut motility, and peristalsis (Galligan, 2002; Kim et al., 2014). In addition, their presence on extra-neuronal cells is implicated in immunological and inflammatory processes which underlie diseases like atherosclerosis, rheumatic diseases, myofascial pain syndromes, osteoarthritis and fibromyalgia (Samborski et al., 2004; Spath et al., 2004; Stratz et al., 2001; Stratz and Muller, 2003). A radio-ligand binding study reported the first evidence of the presence of 5-HT₃-binding sites in the rodent CNS (Kilpatrick et al., 1987). Consequently, a series of elegant studies in humans and non-primate mammals, utilizing diverse techniques like immunohistochemistry, *in situ* hybridization and 5-HT₃-selective ligand binding, established the existence of 5-HT₃Rs in many brain areas including the hindbrain e.g. area postrema and nucleus tractus solitarius, spinal trigeminal nucleus and dorsal motor nucleus of the vagus nerve, as well as the forebrain e.g. the superficial layers of the cerebral cortex, entorhinal and temporal cortex, hippocampus, amygdala, nucleus accumbens, striatum, substantia nigra and ventral tegmental area (VTA) (Abi-Dargham et al., 1993; Barnes et al., 1989; Bufton et al., 1993; Chameau and van Hooft, 2006; Miquel et al., 2002; Tecott et al., 1993; Waeber et al., 1989). 5-HT₃Rs within these brain areas are implicated in the physiology of the vomiting reflex, cognition, associative learning and memory, social and emotional behavior, reward-related behaviors, response to stress, appetite control and the circadian rhythm, as well as importantly, in the etiopathogenesis of psychiatric disorders, such as anxiety and depression, schizophrenia, irritable bowel syndrome (IBS), addiction and substance abuse and cognitive dysfunction. Indeed, 5-HT₃Rs may represent prime targets for many therapeutic drugs used widely in clinic. In addition,

the development of new 5-HT₃-selective drugs could potentially provide a means for improved treatment outcomes in various neuro-psychiatric disorders.

5-HT₃Rs targeted pharmacotherapy has been largely the mainstay of management of cancer-chemotherapy induced, and post-operative emesis so far (Aapro, 1991; Leeser and Lip, 1991). However, owing to localization of 5-HT₃Rs in key brain structures involved in the pathophysiology of mood disorders, these receptors have emerged now as attractive targets for the pharmacotherapy of anxiety and depression. These ailments have been considered to be accounted for by an aberrant dopaminergic (Nestler and Carlezon, 2006), GABAergic (Sanacora and Saricicek, 2007), and more recently glutamatergic neurotransmission (Li et al., 2011; Li et al., 2013) within the mesolimbic dopaminergic pathway, hippocampus and the lateral habenula, respectively. Intriguingly, emerging evidence that neurotransmission in the neuronal networks of these brain structures can be modulated by native 5-HT₃Rs (Dorostkar and Boehm, 2007; Palfreyman et al., 1993; Ropert and Guy, 1991; Xie et al., 2016), reinforces a novel therapeutic relevance for 5-HT₃Rs in the treatment of depression. Historically, antidepressants have been well known for their classical mechanisms of action involving serotonin (5-HT), dopamine (DA) and norepinephrine (NE) reuptake transporters. Later on, some antidepressants have also been shown to directly interact with LGICs of the Cys-loop superfamily, e.g. nACh and GABA_A receptors (Rammes and Rupprecht, 2007). Interestingly, antidepressants fluoxetine (serotonin reuptake inhibitor) and reboxetine (norepinephrine reuptake inhibitor), which have initially been thought to primarily act on neurotransmitter reuptake transporters, dose-dependently antagonize the inward current mediated by 5-HT₃Rs in a non-competitive manner (Choi et al., 2003; Eisensamer et al., 2003).

Bupropion, a key 'atypical' antidepressant, has been in clinical use after it was introduced in the late 1980s in the United States (Stahl et al., 2004). Based on the initial landmark clinical trials (Hurt et al., 1997), bupropion has also been prescribed as a first line agent for the treatment of smoking addiction. Albeit, neurochemical mechanisms underlying bupropion's antidepressant effect are still elusive, its therapeutic efficacy may be attributed to the effects of bupropion and/or its active metabolite hydroxybupropion involving the inhibition of DA and NE reuptake transporters (Ascher et al., 1995; Ferris et al., 1982). We and others have also shown that bupropion additionally inhibits a number of nAChR-subtypes (Fryer and Lukas, 1999; Pandhare et al., 2012; Slemmer et al., 2000). Therefore, in addition to bupropion's currently known actions, we asked whether bupropion, analogous to effects of some atypical antidepressants, could modulate function of 5-HT₃Rs.

In the present study, we investigated the effects of bupropion and its active metabolite hydroxybupropion on the function of homomERICALLY expressed mouse 5-HT_{3A}Rs in *Xenopus laevis* oocytes. Here, we report the first evidence that both bupropion and hydroxybupropion dose-dependently inhibit inward currents of mouse 5-HT_{3A}Rs. The mechanism of bupropion's blockade of these receptors appears to be non-competitive in nature.

2. Materials and methods

2.1. Materials

[³H]-Granisetron (BRL-43694); 85.3 Ci/mmol; 1 μCi/μL) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA). Acetylcholine (ACh), glycine (Gly) and γ-aminobutyric acid (GABA) were purchased from Sigma-Aldrich (St. Louis, MO). Bupropion hydrochloride and hydroxybupropion were purchased from Toronto Research Chemicals, Inc. (North York, Canada); serotonin (5-HT; serotonin creatinine sulfate monohydrate) from Acros Organics (New Jersey, NJ), MDL-7222 from Sigma-Aldrich (St. Louis, MO); protease inhibitor cocktail set III from EMD (Calbiochem; Darmstadt, Germany) and trypsin (TPCK-treated) from Worthington (Lakewood, NJ). Dulbecco's modified Eagle's medium/Ham's F-12 50/50 mix (DMEM/Ham's F-12) was obtained from Mediatech, Inc. (Herndon, VA) and Geneticin (G-418.SULFATE) was purchased from A. G. Scientific, Inc. (San Diego, CA).

2.2. Reagents

Stock solutions of bupropion (100 mM), serotonin (100 mM), ACh (1 M), Gly (1 M) and GABA (100 mM) were prepared in distilled water. Hydroxybupropion was dissolved in dimethyl sulfoxide (DMSO) to obtain a 100 mM stock. All stock solutions were diluted in oocyte Ringer's buffer (OR2) to desired concentrations just before each electrophysiological experiment. Final DMSO concentrations used were 1%, where 1% DMSO showed no effect on serotonin-induced currents (data not shown).

2.3. Preparation of RNA transcripts

The complementary DNA (cDNA) encoding the mouse 5-HT_{3A}R subunit containing the V5 epitope tag (GKIPNPLLGLDSTQ) near the N-terminus (5-HT_{3A}-V5) was engineered into the expression vector pGEMHE, previously (Jansen et al., 2008). Additionally, cDNAs encoding the human nACh α 7 (Peng et al., 1994), rat Gly α 1 (Beato et al., 2004) and human GABA ρ 1 receptor subunits in plasmids were used as described below. For *in vitro* transcription, all constructs were linearized by digestion with the appropriate restriction enzymes (*Nhe*I for the 5-HT_{3A}-V5-pGEMHE and Gly α 1-pXOON constructs; *Xba*I for the GABA ρ 1-pXOON and *Bam*HI for the nACh α 7-pMXT constructs). Subsequently, in each case mRNA was prepared by *in vitro* transcription using the T7 RNA polymerase (mMESSAGE mMACHINE[®] T7 Kit; Applied Biosystems/Ambion, Austin, TX). The resulting capped mRNA was purified using the MEGAclear[™] Kit (Applied Biosystems/Ambion, Austin, TX), and precipitated using 5M ammonium acetate. The mRNA was quantitated by measuring absorbance (NanoDrop[®], ND-1000 Spectrophotometer; NanoDrop Technologies, Inc., Wilmington, DE) and its quality was ascertained by a standard 1% agarose gel electrophoresis. The synthesized RNA was dissolved in nuclease-free water, divided into aliquots, and stored at – 80 °C.

2.4. *Xenopus laevis* oocyte microinjection and expression

Commercially available freshly defolliculated oocytes (EcoCyte Bioscience US LLC, Austin, TX) were thoroughly washed with oocyte Ringer's buffer (OR2; in mM: 82.5 NaCl,

2 KCl, 1 MgCl₂, 5 HEPES, pH 7.5 with NaOH) prior to microinjection with 10 ng of *in vitro* synthesized mRNA (0.2 ng/μL), using an automatic oocyte injector (Nanoject II™; Drummond Scientific Co., Broomall, PA). Injected oocytes were then maintained in standard oocyte saline medium (SOS; in mM: 100 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, pH 7.5) supplemented with a 1% antibiotic-antimycotic (100x) and 5% horse serum for 2–5 days at 16–18 °C before study.

2.5. Oocyte electrophysiology

Electrophysiological studies were performed 2–5 days after mRNA injection at room temperature. Briefly, a single oocyte was placed in a 250 μL bath chamber, which was gravity perfused continuously at a rate of 5–6 mL/min with OR2. Drugs were dissolved in the perfusion solution and applied by gravity perfusion. Currents were recorded from individual oocytes under two-electrode voltage-clamp conditions, with the oocyte transmembrane potential clamped at – 60 mV. A 3 M KCl/agar bridge connected the ground electrode to the bath. Glass microelectrodes filled with 3 M KCl had a resistance of <2 MΩ. Currents were amplified using a TEV-200A amplifier (Dagan Corporation; Minneapolis, MN), digitized using a Digidata® 1440A analog-to-digital converter (Molecular Devices; Sunnyvale, CA), and analyzed with pCLAMP (Clampex/Clampfit) version 10.3 software (Molecular Devices). The oocyte was washed with OR2 for a minimum of 6 min between successive drug applications. The variation of 10 – 15% between two consecutive agonist-induced current responses was indicative of a stable baseline current response. All experiments were conducted on at least 3 oocytes from two different batches of oocytes.

2.6. Cell culture and membrane isolation

HEK-293 cells stably transfected with mouse 5-HT_{3A}Rs with a C-terminal α-bungarotoxin (α-BgTx) pharmitope tag (HEK-α-BgTx-5-HT_{3A}Rs cells; (Sanghvi et al., 2009)) were grown in 140 mm culture dishes at 37 °C and 5% CO₂ in a humidified incubator (~ 100 dishes/week; Greiner Bio-One, Germany). The cells were maintained in a medium containing a 50/50 mix of DMEM and Ham's F-12 media, supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, 100 U/mL penicillin G, and 700 μg/mL G-418 as a selection agent. Once ~ 90% cell confluency was reached, growth media was aspirated from culture dishes and the cells were harvested by gentle scraping, and washed with ice-cold vesicle dialysis buffer (VDB: 100 mM NaCl, 0.1 mM EDTA, 0.02% NaN₃, 10 mM MOPS, pH 7.5) in the presence of protease inhibitor cocktail III (Calbiochem; 0.2 μL/mL). The cells were pelleted by centrifugation (210 *g* for 3 min at 4 °C) and the final cell pellet was either stored at – 80 °C or immediately used to isolate membranes.

For membrane isolation, HEK-α-BgTx-5-HT_{3A}Rs cell pellets (from 100 dishes) were homogenized in VDB in the presence of protease inhibitor cocktail III (1 μL/mL) using a motorized potter. The membrane fractions were separated by centrifugation (39,000 *g* for 1 h at 4 °C) and then resuspended in ice-cold VDB (~ 40 mL) enriched with protease inhibitor cocktail III (1 μL/mL). The total protein concentration was determined by Lowry protein assay (Lowry et al., 1951) and the membranes were stored at – 80 °C.

2.7. Oocyte membrane preparation

X. laevis oocyte membranes expressing mouse wild-type 5-HT_{3A}Rs were isolated by homogenization and centrifugation as described elsewhere (de Oliveira-Pierce et al., 2009). Briefly, ~100 oocytes were homogenized in ice-cold VDB (100 µL/oocyte) in the presence of protease inhibitor cocktail III (2 µL/mL) using a motorized glass potter. The homogenate was centrifuged (800 *g* for 10 min at 4 °C) and the supernatant was collected. The resulting pellet was resuspended in ice-cold VDB (100 µL/oocyte) with protease inhibitor cocktail III (2 µL/mL), and centrifuged again (800 *g* for 10 min at 4 °C). Aliquots of supernatants thus obtained were combined, and centrifuged at a high speed (39,000 *g* for 1 h at 4 °C) to isolate the receptor-rich oocyte membranes. The membrane pellet was resuspended in VDB, the protein concentration was determined by Lowry assay, and appropriate aliquots were then stored at – 80 °C.

2.8. Radioligand binding assays

The effect of bupropion on the equilibrium binding of ligands ([³H]-granisetron/BRL-43694, competitive antagonist; [³H]-5-HT, agonist) which bind to the orthosteric binding site of the 5-HT_{3A}R, was examined by radioligand competition binding assays. Mouse 5-HT_{3A}Rs-rich oocyte membranes (~ 0.056 mg/mL; 7 pmol of binding sites/mg of total protein) or HEK-293 cell membranes (~ 0.5 mg/mL; 14 pmol of binding sites/mg of total protein) were mixed with 2.9 nM [³H]-granisetron or 10 nM [³H]-5-HT. The total volume was then divided into aliquots, and increasing concentrations of bupropion (0 – 1000 µM), from its stock solution in distilled water, were added to each tube and the membrane suspension allowed to incubate for 2 h at room temperature. After centrifugation (39,000 *g* for 1 h at 4 °C) of the samples, the [³H]-containing membrane pellets were resuspended in 200 µl 10% SDS and transferred to a scintillation vial with 3 ml Bio-Safe II scintillation cocktail. The bound fraction was determined by scintillation counting, with nonspecific binding determined in the presence of 100 µM MDL-7222.

2.9. Statistical/data analysis

All results are presented as mean ± SEM., with statistical significance assessed by employing student's *t* test or one-way ANOVA and Dunnett's multiple comparison test, where appropriate. A *p* value of < 0.01 was accepted as indicative of a statistically significant difference.

Dose-response (inhibition or activation) data were normalized to the maximal current in the absence of inhibitor recorded in the same oocyte. The concentration dependence of agonist stimulation or antagonist inhibition was fit using a variable-slope sigmoidal dose response curve in Prism 6 Software (GraphPad Prism®; La Jolla, CA) to the equations:

$$I/I_{\max} * 100 = 1 / (1 + 10^{((\text{LogEC}_{50} - X) * n_H)}) \quad (1a)$$

$$I/I_{\max} * 100 = 1 / (1 + 10^{((\text{LogIC}_{50} - X) * n_H)}) \quad (1b)$$

where, X is the agonist or antagonist concentration used respectively, EC_{50} is the agonist concentration that yields 50% of the maximal current (I_{max}), IC_{50} is the concentration of antagonist that produces 50% inhibition of the maximal agonist-induced current, and n_H is the Hill coefficient.

For radioligand competition binding, the data were normalized to the specific binding in the absence of inhibitor, and fitted by non-linear least squares analysis using the Prism software, where the corresponding IC_{50} values were calculated using the following single-site binding equation:

$$f(x) = f_0 / [1 + (x/IC_{50})] + f_{ns} \quad (2)$$

where $f(x)$ is the total radioligand bound in the presence of inhibitor concentration x , f_0 is the specific binding in the absence of inhibitor, f_{ns} denotes the nonspecific binding, and IC_{50} is the inhibitor concentration at which $\{f(x) - f_{ns}\} = 0.5$ (i.e. 50% bound).

3. Results

3.1. Inhibition of 5-HT_{3A}R function by bupropion or hydroxybupropion

We examined the actions of both bupropion as well as its major metabolite (Figure 1), hydroxybupropion, on the function of mouse 5-HT_{3A}Rs expressed in *Xenopus laevis* oocytes. We first substantiated that agonist, serotonin (5-HT), elicited responses from the expressed 5-HT_{3A}R with an EC_{50} value of $1.2 \pm 0.05 \mu M$ (Figure 3), comparable to EC_{50} values of 0.76 (Jansen et al., 2008) and 0.93 μM (Sessoms-Sikes et al., 2003) reported previously. The 5-HT concentration of 0.6 μM , used for the co-application with bupropion or hydroxybupropion, represents the effective concentration that elicits approximately 10% of the maximal response (EC_{10}). Bupropion or hydroxybupropion *per se* induced no current response in the absence of agonist, 5-HT (Figure 2C). However, when co-applied with agonist each compound dose-dependently inhibited 5-HT-induced responses. The inhibition was produced in the absence of any pre-incubation with bupropion or hydroxybupropion, and was fully reversible after a 6 min wash, as shown in the representative tracings of currents in Figures 2A and 2B for respective inhibitors. Blockade of 5-HT_{3A}R function was evident in the presence of increasing concentrations of bupropion or hydroxybupropion ranging from 3 – 1000 μM . The concentrations producing 50% inhibition (IC_{50}) were 87.1 ± 4.1 and $112.2 \pm 7.5 \mu M$ with Hill coefficients of 1.23 ± 0.05 and 1.07 ± 0.07 for bupropion and hydroxybupropion, respectively (Figure 2D). Notably, the potencies of bupropion and hydroxybupropion for inhibition of 5-HT_{3A}R function were not significantly different (Unpaired t test, $t(12) = 0.2030$, $p = 0.8425$).

To investigate potential mechanisms of functional inhibition, 5-HT concentration-response profiles were obtained either alone or in the presence of various concentrations (IC_{85}) of bupropion. Functional blockade of the mouse 5-HT_{3A}R by bupropion was insurmountable even at a 5-HT concentration 10-fold greater than its EC_{100} (Figure 3), suggestive of a non-competitive nature of antagonism. Interestingly, we also observed rightward shifts in the 5-

HT concentration-response curves associated with respectively higher EC₅₀s as the concentration of bupropion was increased.

3.2. Effect of bupropion on the equilibrium binding of [³H]-granisetron or [³H]-5-HT

In nicotinic acetylcholine receptors (nAChR), bupropion is shown to inhibit the function by a non-competitive mechanism (Fryer and Lukas, 1999). We, in a photoaffinity labeling study, directly established two distinct allosteric binding sites for bupropion in the muscle-type *Torpedo* nAChR (Pandhare et al., 2012). Nonetheless, the observed effect of bupropion on the apparent affinity of 5-HT for the receptor in our functional studies lead to further probe the presence of a competitive component of block in 5-HT_{3A}Rs.

Here, we characterized binding interaction of bupropion with the antagonist/agonist binding site by examining its effect on the equilibrium binding of [³H]-granisetron (Figure 4A) or [³H]-5-HT (Figure 4B) to 5-HT_{3A}Rs overexpressed in HEK-293 cell membranes or oocyte membranes. Bupropion marginally inhibited [³H]-granisetron binding (IC₅₀s; HEK-5-HT_{3A}Rs, 1.5 mM; oocyte-5-HT_{3A}Rs, 1.4 mM) as well as [³H]-5-HT binding (IC₅₀; HEK-5-HT_{3A}Rs, 1.0 mM). For instance, at 100 μM 5-HT completely abolished the equilibrium binding of [³H]-granisetron, a known competitive and mutually exclusive interaction (Nelson and Thomas, 1989), however, at the same concentration bupropion inhibited [³H]-granisetron or [³H]-5-HT binding to HEK-5-HT_{3A}Rs by only ~10% or ~20%, respectively.

3.3. Allosteric blockade by bupropion of the 5-HT_{3A}R

To determine whether bupropion-mediated inhibition of 5-HT_{3A}Rs was a phenomenon dependent exclusively on channel opening, referred to as ‘use-dependence’ (Starmer et al., Am J Physiol 1986; Zaitsev et al., J Physiol 2011), we employed two different experimental approaches. At first, we coapplied 0.6 μM 5-HT and 100 μM bupropion without preincubation with bupropion to obtain a control response. Subsequently, the bupropion inhibition, normalized to the control response, was plotted as a function of preincubation time. During preincubation, oocytes were perfused with 100 μM bupropion for indicated time points: in min; 0.5 or 1 or 5 (Figure 5A). We observed a significant, time-dependent enhancement of blockade by bupropion following such preincubation (one-way ANOVA, $F(4, 23) = 31.44$, $p < 0.0001$). For example, after 5 min the peak current evoked by the coapplication of 5-HT and bupropion was reduced by ~38% of the control current, and after a 6 min wash, there was a 90% recovery of the control current.

In a second approach to examine use-dependence of bupropion inhibition of the 5-HT_{3A}R, oocytes were continuously perfused to equilibrate receptors with 0.6 μM 5-HT. The concentration of 5-HT elicited steady-state currents, which did not desensitize under the experimental conditions, when bupropion at various concentrations (3–1000 μM) was coapplied to the agonist-bound receptors presumably in the open state (Figure 5B). Following the coapplication, the 5-HT-evoked steady-state currents decayed until a bupropion concentration-dependent equilibrium block was attained. It is important to note that only a partial recovery of agonist-induced currents was observed when bupropion application was removed. The measured peak current amplitude at the equilibrium block was used to determine the potency of inhibition by bupropion of 5-HT_{3A}Rs, presumably in a

state of greater channel open probability (Figure 5C). The IC_{50} value thus calculated for the apparently open state ($69.4 \pm 4.7 \mu\text{M}$; $n_H = 1.25 \pm 0.1$; $n = 5$) of the receptor was not significantly different than that obtained from the experiments using simultaneous 5-HT and bupropion applications in the absence of agonist preapplication ($87.1 \pm 4.1 \mu\text{M}$; $n_H = 1.23 \pm 0.05$; $n = 5$). Unpaired t test, $t(12) = 0.0827$, $p = 0.9354$.

3.4. Effect of bupropion on the function of anion-conducting Cys-loop receptors

Blockade of cation-conducting currents of neuronal as well as muscle-type nicotinic acetylcholine receptors by bupropion has been previously studied (Pandhare et al., 2012; Slemmer et al., 2000). Here, we report similar effects of bupropion on the cation-conducting 5-HT_{3A}R. Therefore, the next logical step was to perform a preliminary evaluation of whether bupropion modulates the function of anion-conducting receptors from the same super-family, namely homopentameric GABA_A1 and Glyc_A1 receptors. As expected, at a dose (1 mM) greater than 15-fold of its previously known inhibitory potency (60 μM ; (Slemmer et al., 2000)), bupropion completely abrogated ACh-evoked currents in nACh_{A7} expressing oocytes (Figure 6A and 6B). However, bupropion at the same concentration showed no significant inhibition of GABA- or glycine-gated Cl⁻-currents from GABA_A1 (Figure 6C and 6D) or Glyc_A1 (Figure 6E and 6F) receptors, respectively.

4. Discussion

For a long time, the blockade of DA and NE reuptake by the antidepressant bupropion within the hypofunctioning CNS monoamine systems has remained one of the pharmacological underpinnings for its therapeutic efficacy. The following studies uncovered its additional role as a non-competitive inhibitor, in the low to intermediate micromolar range, of both neuronal-type and muscle-type nAChRs, which are cation-selective LGICs of the Cys-loop superfamily (Fryer and Lukas, 1999; Pandhare et al., 2012; Slemmer et al., 2000). Our results for the first time substantiate that the range of LGICs that bupropion inhibits can be expanded to also include the 5-HT_{3A}R, another important cation-selective member of the LGIC superfamily. The results obtained from two-electrode voltage clamp recordings indicate that bupropion reversibly blocks inward currents of 5-HT_{3A}R expressed in *Xenopus laevis* oocytes in a concentration-dependent manner, with an inhibitory potency of 87 μM . In rodents, a major metabolite of bupropion, hydroxybupropion, is shown to contribute to the biological efficacy of bupropion (Bondarev et al., 2003). Moreover, an ⁸⁶Rb⁺ efflux study has established that hydroxybupropion antagonizes function of neuronal nAChRs in a non-competitive manner (Damaj et al., 2004). Therefore, we also measured the effect of hydroxybupropion on the 5-HT_{3A}R function. Indeed, we found that hydroxybupropion ($IC_{50} = 112 \mu\text{M}$) has functional inhibitory potency similar (Unpaired t test, $t(12) = 0.2030$, $p = 0.8425$) to bupropion at mouse 5-HT_{3A}R. The insurmountability of functional inhibition by bupropion with higher doses of agonist in electrophysiological studies suggests that the pharmacologic mechanism of bupropion blockade of 5-HT_{3A}R is non-competitive inhibition (Damaj et al., 2004). Although our radioligand binding studies display at best weak binding of bupropion to the agonist site, the overall binding interaction appears to be non-competitive in nature. Possibly, the very modest inhibition of orthosteric ligand binding at high bupropion concentrations is mediated rather through an indirect

allosteric pathway. Unlike its inhibitory actions at cation-selective members of the LGIC superfamily, bupropion at a higher dose, however, does not appear to block the function of anion-selective LGIC members; indicative of a possibility that bupropion's site(s) of action may not be conserved in the two groups of LGICs.

Next, we investigated the possibility whether the channel state influences the interactions of bupropion with 5-HT_{3A}Rs. Based on two different experimental approaches, we demonstrate that bupropion-mediated inhibition of 5-HT_{3A}Rs is non-use-dependent. For bupropion to qualify as a use-dependent blocker of the receptor function, it must not gain access to its binding site in the closed state. Therefore, preincubation with bupropion would exert inconsequential influence on the extent of bupropion blockade of 5-HT evoked peak currents following this incubation period. In contrast, we found a greater depression of the 5-HT_{3A}-mediated current with pre-application as opposed to co-application experiments. It was evident that the binding site for bupropion was accessible regardless of the absence of agonist binding. The observation that time-dependent incremental block develops with application prior to channel activation indicates that bupropion can bind to and inhibit the opening of the closed state of 5-HT_{3A}R channels. Thus, this particular component of bupropion inhibition of the 5-HT_{3A}R is non-use-dependent i.e. independent of channel opening. These findings also suggest that a reduction in peak current amplitude may represent the sum of the degree of block that occurs prior to the activation phase and that which occurs during the activation phase. It is plausible that the former effect may be due to bupropion reducing the energy barrier for 5-HT_{3A}Rs to favor a desensitized and/or resting/closed state by a mechanism previously proposed for nAChRs (Spivak et al., 2007), and/or due to a direct blockade of unliganded channels as observed with tricyclic antidepressants in the muscle-type nAChR (Gumilar et al., 2003).

Second, despite the conditions that favored the open state of the channel, this did not translate to enhanced inhibition of receptor function than that observed with co-application of agonist and bupropion. In other words, the continuous presence of agonist, 5-HT, before and throughout bupropion application had no pronounced effect on the inhibitory potency of bupropion at these receptors thereby implying a lack of preference for interaction with the open channel. However, a partial recovery of inward current at the end of bupropion application during drug wash-out suggests either increase in the desensitization rate and/or slow unblocking of the receptor-channel complex plausibly due to bupropion accumulation in the cell membrane (Orser et al., 1994; Papke and Oswald, 1989). Obviously, further studies focused on more detailed and precise investigation of bupropion modulation of receptor kinetics are warranted. Interestingly, the estimated n_H values from both conditions, pre-application and co-application of agonist with bupropion, were comparable and greater than unity which was indicative of bupropion inhibition of the receptor by a cooperative mechanism. We have previously identified two binding sites for bupropion, one high-affinity site within the channel transmembrane pore and one towards the extracellular end of the transmembrane segment $\alpha M1$, in the muscle-type *Torpedo* nAChR (Pandhare et al., 2012). However, the Hill coefficient was close to unity. Because of the pentameric assembly of identical or homologous subunits, positive cooperativity has been observed by binding to equivalent amino acids within different subunits (Bertrand et al., 2008; Bower et al., 2008). Here, we postulate that one of the bupropion sites within 5-HT_{3A}R may be either blocking

the channel or accelerating desensitization of the receptor (Dopico and Lovinger, 2009). Due to the lipophilicity of bupropion, access to a binding site may be provided via the lipid-bilayer to interact with the closed-state of the receptor-channel complex, similar to the actions of steroids, which non-competitively inhibit the *Torpedo* nAChR by interacting with residues at its lipid-protein interface (Blanton et al., 1999).

Several studies on animal models and clinical studies have reported the potential role of 5-HT₃R antagonists in mood and anxiety disorders (Faris et al., 2006; Hewlett et al., 2003; Jones et al., 1988; Olivier et al., 2000). The influence of these receptors on the neurobiology of anxiety and depression stems from their significant presence in related neuronal networks. For instance, GABA release in the synapse from the GABAergic presynaptic nerve terminals located in the amygdala is significantly facilitated by 5-HT₃Rs that contribute to the regulation of anxiety (Koyama et al., 2000). In addition, GABAergic dysfunction has been observed in animal model of depression (Sanacora and Saricicek, 2007). In the rodent neocortex, at least 90% of the cells expressing 5-HT₃Rs represent GABAergic interneuron population (Morales and Bloom, 1997). Notably, in the prefrontal cortex, only the 5-HT_{3A}R subunit appears to be expressed (Ferezou et al., 2002). Moreover, ~ 30% of cortical GABAergic interneurons contain 5-HT_{3A} receptors-expressing neurons which constitute the predominant interneuron population in supragranular cortical layers (Lee et al., 2010). The serotonergic activation of this population, mediated by 5-HT_{3A}Rs, has the ability to alter cortical activity through an enhancement of GABAergic neurotransmission (Lee et al., 2010). Therefore, modulation of these native 5-HT_{3A}Rs by its antagonists, such as bupropion and its active metabolite hydroxybupropion, has a potential to affect different behavioral states in mood disorders.

The concentrations of hydroxybupropion effective at 5-HT_{3A}Rs seem to be within the realm of clinically relevant concentrations. Bupropion is reported to concentrate in many tissues, with a brain to plasma ratio of 25:1 (Schroeder, 1983). Therefore, in the brain, bupropion concentrations can reach up to ~ 20 µM (Vazquez-Gomez et al., 2014). Importantly, here we report that in addition to bupropion, its major metabolite, hydroxybupropion, also inhibits 5-HT_{3A}Rs with comparable affinity. It is believed that the effect of hydroxybupropion in the CNS may be critical for bupropion's antidepressant activity (Damaj et al., 2004). Given the 10 to 100 times greater plasma levels of hydroxybupropion than those of the parent drug, as well as its long-half life (Damaj et al., 2004; Findlay et al., 1981; Hsyu et al., 1997), the resultant significant increase in concentration of this metabolite in the brain may compensate for its relatively low affinity for 5-HT_{3A}Rs in the micromolar range, leading to clinically-relevant inhibition of these receptors at therapeutic dosages.

5. Conclusions

The present findings indicate that, in addition to their established effects of blocking DA and NE reuptake transporters and nAChRs, bupropion and its active metabolite hydroxybupropion also inhibit the function of heterologously expressed homomeric mouse 5-HT_{3A}Rs. Importantly, in particular hydroxybupropion inhibits 5-HT_{3A}R at clinically relevant concentrations. Therefore, the negative allosteric modulation of the function of

native 5-HT_{3A}Rs by bupropion and its hydroxymetabolite may constitute a novel pharmacological basis for its antidepressant effects.

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Abbreviations

5-HT_{3A}R	5-hydroxytryptamine type 3A receptor
5-HT	5-hydroxytryptamine
ACh	acetylcholine
α-BgTx	α-bungarotoxin
CNS	central nervous system
DA	dopamine
GABA	γ-amino butyric acid
Gly	glycine
HEK	human embryonic kidney
LGIC	ligand-gated ion channel
nAChR	nicotinic acetylcholine receptor
NE	norepinephrine
OR2	oocyte Ringer's buffer
PNS	peripheral nervous system
SOS	standard oocyte saline medium
VTa	ventral tegmental area
VDB	vesicle dialysis buffer

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- Bupropion non-competitively blocked function of 5-HT_{3A}Rs in *X. laevis* oocytes.
- Bupropion inhibition at these receptors was non-use-dependent.
- The metabolite hydroxybupropion also inhibited function of 5-HT_{3A}Rs in oocytes.
- Our study establishes the 5-HT_{3A}R as a hitherto unidentified molecular target of bupropion.
- The study further provides a novel pharmacological basis for bupropion's antidepressant effect.

Figure 1. Chemical structures of A, bupropion and B, hydroxybupropion

Note that the presence of the hydroxyl in hydroxybupropion leads to the formation of an intramolecular ketal (morpholinol) with the phenylketone moiety.

Figure 2. Bupropion or hydroxybupropion dose-dependence for functional blockade of 5-HT₃ARs expressed in *Xenopus* oocytes

A standard two-electrode voltage clamp at a holding potential of -60 mV was employed to measure serotonin (5-HT)-evoked currents in a single oocyte expressing mouse 5-HT₃ARs. 0.6 μM 5-HT ($\sim \text{EC}_{10-20}$ dose) was applied for 60 s to elicit a control current. Once a stable baseline response was obtained, subsequent responses were recorded when 5-HT (0.6 μM) was applied concomitantly for 60 s in the absence or presence of increasing concentrations of bupropion (**A**, 3 – 1000 μM) or hydroxybupropion (**B**, 3 – 1000 μM), and in each case representative tracings of currents are shown. Functional blockade by bupropion or hydroxybupropion was reversible, as evident by washout of respective blockade observed with the final application of 5-HT alone 6 min later. **C**, in oocytes expressing 5-HT₃ARs, bupropion or hydroxybupropion alone did not elicit a response. **D**, after normalizing currents to the 0.6 μM 5-HT response, non-linear least-squares analyses of the concentration-response curves for bupropion ($\bullet$) or hydroxybupropion (Δ) yielded IC_{50} values of 87.1 ± 4.1 μM and $n_H = 1.23 \pm 0.05$ ($n = 5$ oocytes; means $\pm$ S.E.M.) or 112.2 ± 7.5 μM and $n_H = 1.07 \pm 0.07$ ($n = 3$ oocytes; means $\pm$ S.E.M.) respectively. Where not shown, the error bars were smaller than the size of the symbols.

Figure 3. Serotonin concentration-response profiles in oocytes expressing 5-HT_{3A}Rs at different concentrations of bupropion

Measurements of currents in oocytes expressing mouse 5-HT_{3A}Rs were performed in the presence of 5-HT alone (▲) at the indicated doses to produce the 5-HT concentration-response curves. Additional 5-HT concentration-response curves were generated in the presence of 0.3 mM (■), 1 mM (◇), 3 mM (▼) or 10 mM (△) bupropion. The concentration response curve in the presence of bupropion was plotted after normalizing each individual 5-HT concentration-specific current response to the maximal control. The EC₅₀s generated for each of the curves were (in μ M) 1.2 ± 0.05 , 2.7 ± 0.4 , 6.0 ± 0.3 , 22.6 ± 1.0 and 54.1 ± 6.9 , respectively. Oocytes were voltage-clamped at a holding potential of -60 mV. Data are means $\pm$ S.E.M. ($n = 3-5$ oocytes).

Figure 4. The effect of bupropion on specific binding of $[^3\text{H}]\text{-granisetron}$ (BRL-43694) or $[^3\text{H}]\text{-5-HT}$ to $\alpha\text{-BgTx-5-HT}_3\text{A}$ Rs expressed in HEK-293 cells or to $5\text{-HT}_3\text{A}$ Rs expressed in *X. laevis* oocyte membranes

A, isolated receptor-rich cell membranes (HEK-293 cell membranes; closed symbols or oocyte membranes; open symbols) were equilibrated (2 h) with $[^3\text{H}]\text{-granisetron}$ (~2.93 nM) at room temperature (R.T.) in the presence of increasing concentrations of either bupropion ($\circ$, $\bigcirc$; 0 – 1000 μM) or 5-HT, an agonist as a reference compound ($\blacktriangle$, $\triangle$; 0 – 100 μM), and **B**, $\alpha\text{-BgTx-5-HT}_3\text{A}$ Rs expressed in HEK-293 cells were equilibrated (2 h) with $[^3\text{H}]\text{-5-HT}$ (~10 nM) at R.T. in the presence of increasing concentrations of bupropion ($\circ$; 0 – 1000 μM). Membranes were centrifuged and the radioactivity present in the pellets was measured as described in the Materials and Methods. Nonspecific binding was determined in the presence of 100 μM MDL-7222 ($\square$), and specific binding was calculated by subtracting nonspecific from total binding. For each experiment, the data were normalized to the specific binding in the absence of the competitor. 1% ethanol (∇) displayed a <10% effect on specific binding of $[^3\text{H}]\text{-granisetron}$ or $[^3\text{H}]\text{-5-HT}$ in the absence of the competitor. Each smooth curve drawn is the nonlinear least-squares fit to a single binding site, and is the average of *three* different experiments for bupropion and *two* different experiments for 5-HT. Data points are means $\pm$ S.E.M. Where error bars not shown, they were smaller than the symbol size.

Figure 5. Non-use-dependent allosteric inhibition of the 5-HT_{3A}R function by bupropion

A, the extent of bupropion inhibition of 5-HT-evoked currents was influenced by the pre-incubation time when an oocyte was preincubated with 100 μM bupropion (30 s or 1 min or 5 min), followed by the co-application of 100 μM bupropion and 0.6 μM 5-HT. A stable response elicited by the co-application of 100 μM bupropion and 0.6 μM 5-HT without a prior incubation with bupropion constituted the control current. Statistically significant difference, in comparison with the control, was determined by one-way ANOVA ($F(4, 23) = 31.44$, $p < 0.0001$) and Dunnett's multiple comparison test (multiplicity adjusted p values, **, $p = 0.0013$; ****, $p < 0.0001$; ns, not significant: $p = 0.1624$). **B**, a trace representative of

the test current when a range of bupropion concentrations (3 – 1000 μM), in this case 300 μM , was applied after the current induced by 0.6 μM 5-HT reached the peak. *C*, the data obtained were fit to generate a dose-response relationship for bupropion inhibition of the 5-HT_{3A}R, presumably during the open state of the channel (○). The IC₅₀ values calculated for the receptor with greater channel open probability vs that for the receptor with co-application of bupropion and 5-HT (°) were not significantly different (IC₅₀ (open state) = 69.4 $\pm$ 4.7 μM ; n_{H} = 1.25 $\pm$ 0.1; n = 5 oocytes; IC₅₀ (agonist-bupropion coapplication) = 87.1 $\pm$ 4.1 μM ; n_{H} = 1.23 $\pm$ 0.05; n = 5 oocytes). Unpaired *t* test, *t*(12) = 0.0827, *p* = 0.9354.

Figure 6. Effects of bupropion on nACh α 7, GABA ρ 1, and Gly α 1 receptors function

The effect of 1 mM bupropion on the function of nACh α 7, GABA ρ 1 or Gly α 1 receptors was measured, under two-electrode voltage-clamp conditions at -60 mV holding potential, when bupropion was co-applied with $\sim EC_{20-50}$ concentration of acetylcholine (177 μ M) or GABA (0.5 μ M) or glycine (76 μ M), respectively. Representative current traces (A, C, E) as well as bar graph representations (B, D, F) of bupropion's effect are shown.

Table 1Functional studies in *Xenopus laevis* oocytes expressing 5-HT_{3A}Rs

Agonist	pEC₅₀ (mean ± S.E.M.)	EC₅₀ (μM)	n_H (mean ± S.E.M.)	n
5-HT	5.89 ± 0.02	1.2	2.4 ± 0.2	3
Antagonists	pIC₅₀ (mean ± S.E.M.)	IC₅₀ (μM)	n_H (mean ± S.E.M.)	n
Bupropion	4.06 ± 0.02	87.1	1.23 ± 0.05	5
Hydroxybupropion	3.95 ± 0.03	112.2	1.07 ± 0.07	3