Published in final edited form as:
Neuropharmacology. 2012 June ; 62(8): 2507–2514. doi:10.1016/j.neuropharm.2012.03.002.

The natural products magnolol and honokiol are positive allosteric modulators of both synaptic and extra-synaptic $GABA_A$ receptors

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Abstract

The National Center for Complementary and Alternative Medicine (NCCAM) estimates that nearly 40% of adults in the United States use alternative medicines, often in the form of an herbal supplement. Extracts from the tree bark of magnolia species have been used for centuries in traditional Chinese and Japanese medicines to treat a variety of neurological diseases, including anxiety, depression, and seizures. The active ingredients in the extracts have been identified as the bi-phenolic isomers magnolol and honokiol. These compounds were shown to enhance the activity of $GABA_A$ receptors, consistent with their biological effects. The $GABA_A$ receptors exhibit substantial subunit heterogeneity, which influences both their functional and pharmacological properties. We examined the activity of magnolol and honokiol at different populations of both neuronal and recombinant $GABA_A$ receptors to characterize their mechanism of action and to determine whether sensitivity to modulation was dependent upon the receptor's subunit composition. We found that magnolol and honokiol enhanced both phasic and tonic GABAergic neurotransmission in hippocampal dentate granule neurons. In addition, all recombinant receptors examined were sensitive to modulation, regardless of the identity of the α, β, or γ subunit subtype, although the compounds showed particularly high efficacy at δ-containing receptors. This direct positive modulation of both synaptic and extra-synaptic populations of $GABA_A$ receptors suggests that supplements containing magnolol and/or honokiol would be effective

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Animal use - All efforts were made to minimize animal suffering, to reduce the number of animals required, and to utilize in vitro alternatives.

anxiolytics, sedatives, and anti-convulsants. However, significant side-effects and risk of drug interactions would also be expected.

Keywords

GABA; anxiolytic; natural product; Magnolia

1. Introduction

Anxiety disorders are among the most common neurological conditions, with an estimated 15–26 million Americans suffering from anxiety-related symptoms annually (Greenberg et al., 1999). While benzodiazepines and other prescription medications remain the clinical standard for treatment, the potential anxiolytic properties of natural, plant-derived supplements have received increasing attention (Lakhan and Vieira, 2010). Nearly 40% of adults in the United States reported use of complementary and alternative medicine (CAM) in a 2007 survey conducted by the National Center for Complementary and Alternative Medicine (Barnes et al., 2008). In this survey, two of the top ten conditions treated with CAM for both adults and children were anxiety/stress and insomnia.

One promising source of neurologically active natural products is the bark of Magnolia trees (Johnson et al., 2006; Patočka et al, 2006; Lee et al., 2011). Extracts from the bark of *Magnolia officinalis* and other species have been used in traditional Chinese and Japanese medicine for centuries as sedatives and anxiolytics (Patočka et al, 2006; Lee et al., 2011). These extracts have anxiolytic activity in rodent models (Maruyama et al., 1998) and in two clinical studies were found to reduce temporary anxiety and improve sleep in humans (Mucci et al., 2006; Kalman et al., 2008). The isomers magnolol (5,5′-diallyl-2,2′-dihydroxybiphenyl) and honokiol (3,5′-diallyl-4,2′-dihydroxybiphenyl) are the principal active components of magnolia bark extract, typically making up 1–10% of the dried bark, depending upon the species and the isolation methods (Patočka et al, 2006; Lee et al., 2011). These two compounds have also been identified as neurologically active agents, with anxiolytic, sedative, neuroprotective and anti-convulsant actions in animal models (Watanabe et al., 1983; Maruyama et al., 1998; Kuribara et al., 1998; Kuribara et al., 2000; Lin et al., 2005; Ma et al., 2009; Chen et al., 2011; see review by Lee et al., 2011).

While these behavioral effects could be mediated through a variety of targets, many drugs used clinically to treat anxiety, insomnia, and seizures act by enhancing GABAergic neurotransmission, and both magnolol and honokiol have been shown to bind to and positively modulate $GABA_A$ receptors (Squires et al., 1999; Ai et al., 2001; Taferner et al., 2011). The pentameric $GABA_A$ receptors are structurally heterogeneous, and can be assembled from a combination of over sixteen different subunit subtypes (Whiting et al., 1999). Subunit expression levels vary throughout the brain and the subunit composition influences the functional and pharmacological properties of the receptor, as well as its trafficking and synaptic localization (Korpi et al., 2002). Post-synaptic $GABA_A$ receptors can mediate both a rapid phasic inhibition, and a long-lasting tonic inhibition (Brickley and Mody., 2012). The extra-synaptic receptor population responsible for tonic inhibition is a potentially important target for modulation of neuronal activity, and is characterized by a distinct subunit composition and pharmacological profile compared to synaptic receptors (Herd et al., 2007; Zheleznova et al., 2009; Brickley and Mody., 2012).

Since these natural products are widely available with limited regulatory oversight, it is important to determine if they can be used safely and effectively. In addition, the identification of compounds from medicinal plants which act through novel sites may

provide new treatment options for neurological disorders. The primary goals of this study were to compare the activity of magnolol and honokiol at synaptic and extra-synaptic $GABA_A$ receptor populations, to determine whether sensitivity to modulation is subunit-dependent, and to investigate their mechanism and site of action.

2. Materials and Methods

2.1. Hippocampal slice recordings

Brain slices were prepared from isoflurane-anesthetized rats (20–25 days old). Transverse 300 μm thick slices were cut using a vibratome (Leica VT1000S, Nussloch, Germany) and were prepared in cold (4°C), oxygenated (95% O_2/5% CO_2), sucrose-based 'cutting' artificial cerebrospinal fluid (aCSF) that contained (in mM): 248 sucrose, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 0.5 $CaCl_2$ and 5 $MgS0_4$ (350 mOsm). Slices were then incubated for approximately one hour at room temperature in oxygenated (95% O_2/5% CO_2) aCSF containing (in mM): 125 NaCl, 2.7 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 0.5 $CaCl_2$, 7 $MgSO_4$, 0.02 D-APV and 1 kynurenic acid (pH 7.4; 305–312mOsm). For whole-cell patch-clamp recording, slices were placed in a submerged chamber perfused with warmed (30–32°C), oxygenated (95%O_2/5% CO_2) aCSF containing (in mM): 125 NaCl, 2.7 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$,10 glucose, 2 $CaCl_2$ and 1 $MgSO_4$ (pH 7.4; 305 mOsm). Recordings were made from dentate granule cells (DGCs) located in the infra-pyramidal blade of the dentate gyrus, visually identified with infrared-differential interference contrast optics. IPSCs were evoked by stimulation in *stratum moleculare* of the dentate gyrus near the recording electrode. Stimuli were 0.1ms, cathodal, monophasic, rectangular constant current pulses (10–100μA) delivered through monopolar, platinum-iridium stimulating electrodes (FHC Inc, Bowdoin, ME). IPSCs were recorded using borosilicate glass electrodes (5–8 MΩ) filled with an internal solution containing (in mM): 130 D-gluconic acid, 130 CsOH, 7 CsCl, 10 HEPES, 3 QX-314, 2 MgATP, 0.3Na_2GTP, yielding a chloride reversal potential of −42 mV. mIPSC and tonic recordings were performed using an internal solution containing (in mM): 140 CsCl, 10 HEPES, 3 QX-314, 4 MgATP, 0.3 Na_2GTP, yielding a chloride reversal potential of 0 mV. Cells were voltage-clamped at −20 mV for evoked IPSCs and −60 mV for tonic current and mIPSCs. Input and series resistance were monitored throughout the experiment and recordings in which either changed by more than 25% were discarded.

In all experiments IPSCs were pharmacologically isolated by blocking NMDA receptors using D-2-amino-5-phosphopentanoic acid (D-APV, 50μM), or MK-801 maleate (10 μM) and AMPA/kainate receptors using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 μM). Tonic currents and mIPSCs were isolated with the addition of tetrodotoxin (TTX, 1 μM). Bicuculline methochloride (20μM) was added at the end of each experiment to confirm that the recorded IPSC was entirely GABAergic. Drugs were perfused for a minimum of 20 minutes in order to obtain a stable baseline. Honokiol and magnolol (Sigma-Aldrich, St Louis MO) were prepared as stock solutions in DMSO and diluted into aCSF on the day of the experiment with a final concentration of DMSO no greater than 0.06%.

2.2. Transfected mammalian cells

Full-length cDNAs encoding mammalian $GABA_A$ receptors in pCMVNeo expression vectors were transfected (2 μg of each subunit) into the human embryonic kidney cell line HEK-293T (GenHunter, Nashville, TN) using calcium phosphate precipitation. Subunit clones were generously provided by Dr. Robert Macdonald (Vanderbilt University, Nashville TN) and Dr. David Weiss (University of Texas Health Sci. Center, San Antonio TX). For selection of transfected cells, 1 μg of the plasmid pHook™-1 (Invitrogen, Carlsbad CA) containing cDNA encoding the surface antibody sFv was also transfected into the cells.

Following a 4–6 hr. incubation at 3% CO_2, the cells were treated with a 15% glycerol solution in BBS buffer (50 mM BES(N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na_2HPO_4) for 30 sec. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The selection procedure for pHook expression was performed 44–52 hrs later (Chesnut et al., 1996). The cells were passaged by a 5 min. incubation with 0.05% trypsin/0.02% EDTA solution in phosphate buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH=7.3) and mixed with 3–5 μl of magnetic beads coated with antigen for the pHook antibody (approximately 6×10^5 beads). Following a 30–60 min. incubation to allow the beads to bind to positively transfected cells, the beads and bead-coated cells were isolated using a magnetic stand. The selected cells were resuspended into DMEM, plated onto glass coverslips treated with poly L-lysine and coated with collagen, and used for recordings 18–28 hrs. later.

For recordings from HEK-293T cells the external solution consisted of (in mM): 142 NaCl, 8.1 KCl, 6 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with pH = 7.4 and osmolarity adjusted to 295–305 mOsm. Recording electrodes were filled with an internal solution of (in mM); 153 KCl, 1 $MgCl_2$, 5 K-EGTA (ethylene glycol-bis (β-aminoethyl ether N,N,N′N′-tetraacetate), 2 MgATP and 10 HEPES with pH = 7.4 and osmolarity adjusted to 295–305 mOsm. GABA was diluted into external solution from freshly made or frozen stocks in water. Magnolol and honokiol were diluted from stocks in DMSO and the final DMSO concentration for these experiments was no greater than 0.01%. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments, Sarasota FL) on a two-stage puller (Narishige, Japan) to a resistance of 5–10 MΩ. For whole-cell recordings GABA was applied to cells using a stepper solution exchanger with a complete exchange time of <50 msec (open tip, SF-77B, Warner Instruments, Hamden CT). For macropatch recordings the 3-barrel square glass was pulled to a final size near 200 μm which provided 10–90% rise times at the open tip consistently faster than 400 μsec.

2.3. Construction of mutated subunit cDNAs

Point mutations were generated using the QuikChange procedure and products (Stratagene, La Jolla, CA). Oligonucleotide primers were synthesized and DNA sequencing was performed by the University of South Carolina DNA core facility (Columbia, SC). Numbering of mutation sites is based on the mature rat sequence.

2.4. Data analysis

Hippocampal brain slice—Whole-cell currents were recorded using a MultiClamp700B amplifier and filtered at 1 kHz. Responses were digitized using a Digidata 1440A A–D board and analyzed using the programs Clampfit (pClamp10.3 suite, Molecular Devices, Sunnyvale, CA) and Origin 7.5 (OriginLab, Northampton, MA). Potentiation of evoked IPSCs was assessed by measuring the 50% decay time and the peak IPSC amplitude, while mIPSCs were identified and analyzed using MiniAnalysis software (Synaptosoft, Inc., Fort Lee, NJ). The size of the tonic inhibitory current was determined by subtracting the holding current under baseline conditions from the holding current in the presence of bicuculline (20 μM). For experiments measuring tonic GABAergic current, any cell with a baseline tonic current < 15 pA was discarded.

Recombinant receptors—Currents were recorded with an Axon 200B patch clamp amplifier and analyzed using the programs Clampfit and Prism (Graphpad, San Diego, CA). Concentration-response data was fit with a four-parameter logistic equation (Current = [Minimum current + (Maximum current − Minimum Current)]/1+(10^(log EC_{50} – log

[GABA])*n) where n represents the Hill number. All fits were made to normalized data with current expressed as a percentage of the maximum response to GABA for each cell. Macropatch currents were digitized at 10 kHz and analyzed with the pClamp suite of programs. The deactivation rate was determined by fitting the decay current with the Levenberg-Marquardt least squares method with two exponential functions.

Student's paired or unpaired t-tests, ANOVA and Tukey-Kramer multiple comparisons tests were performed using the Instat program (Graphpad) or Origin 7.5 (OriginLab, Northampton, MA) with a significance level of $p<0.05$. The logs of the EC_{50} measurements for GABA, magnolol and honokiol were used for statistical comparison.

3. RESULTS

3.1. Modulation of GABAergic neurotransmission

While there is evidence indicating that magnolol and honokiol can interact with $GABA_A$ receptors (Squires et al., 1999; Ai et al., 2001; Taferner et al., 2011), the effect of these compounds on GABAergic neurotransmission in neurons has not been reported. Therefore, we used whole cell recordings to measure the effect of honokiol and magnolol on $GABA_A$ receptor- mediated IPSCs and tonic current in dentate granule cells. Monosynaptic IPSCs were elicited by stimulation of GABAergic neurons at an intensity which evoked an IPSC that was 50% of maximal. These currents were completely blocked by 20 μM bicuculline (n = 10), indicating that they were mediated by $GABA_A$ receptors. The 50% decay time averaged 20.1 ± 1.7 msec (n = 10), in agreement with previous reports (Draguhn and Heinemann, 1996). Magnolol (30 μM) and honokiol (60 μM) were bath applied and their effect on the amplitude and decay time of the IPSC measured. Both magnolol (267.7±7.9%, $p<0.001$, n=5) and honokiol (299.5±16.6%, $p<0.001$, n=5) significantly slowed the decay time (Figure 1A). In contrast, the amplitude of the IPSC was not significantly affected by either compound (magnolol: 105.7±11.2%, $p>0.05$, n=5; honokiol: 113.3±25.0%, $p>0.05$, n=5; Figure 1A).

In dentate granule neurons, tonic inhibition is mediated by a population of extra-synaptic $GABA_A$ receptors separate from those that produce the IPSC (Farrant and Nusser, 2005). We examined whether the modulators could affect these $GABA_A$ receptors by analyzing their effect on tonic inhibition. Tonic GABAergic current was recorded in the presence of CNQX (50 μM), D-APV (50 μM), and TTX (1 μM) and averaged 36.1±4.7 pA (n=13). Both modulators caused a similar potentiation of this current (Figure 1B). Magnolol increased the tonic current by 402.5±61.7% ($p<0.001$, n=7), whereas honokiol enhanced the tonic current by 351.2±79.0% ($p<0.05$, n=6). These findings suggest that the modulators can potentiate extra-synaptic as well as synaptic $GABA_A$ receptors.

We also examined whether these compounds might affect GABAergic neurotransmission through presynaptic mechanisms. To investigate this possibility we analyzed the frequency of miniature IPSCs (mIPSCs) in the presence of 1 μM TTX. Both magnolol (476.2±108.5%, $p<0.01$, n=7) and honokiol (378.9±46.8%, $p<0.01$, n=6) significantly increased mIPSC frequency (Figure 1C). mIPSC amplitude was not affected, suggesting that the increase in mIPSC frequency reflects a presynaptic action of both modulators to increase GABA release. This presynaptic effect may contribute to the ability of the modulators to prolong IPSC decay and to enhance the tonic current, as action-potential dependent GABA release is known to contribute to ambient GABA levels in the hippocampus (Glykys and Mody, 2007; Holter et al, 2010).

3.2. Modulation of recombinant receptors

A wide variety of different mechanisms could produce the enhancement in phasic and tonic GABAergic neurotransmission observed in the brain slice recordings. To determine if magnolol and honokiol directly modulate $GABA_A$ receptors and to characterize any subunit dependence in their action, we examined their ability to modulate recombinant $GABA_A$ receptors expressed in mammalian cells. Synaptic and extra-synaptic $GABA_A$ receptor populations have distinct subunit compositions. Most synaptic receptors in dentate granule neurons contain the γ2 subunit, while a large proportion of extra-synaptic receptors contain the δ subunit (Stell et al., 2003). Therefore, we first compared modulation of recombinant α1β3γ2 and α1β3δ receptors.

Transiently transfected HEK-293T cells were voltage-clamped at −50 mV and magnolol or honokiol was co-applied for 5 sec. with a sub-maximal ($EC_{5–10}$) concentration of GABA (Figure 2). Each modulator enhanced the response to GABA at both γ2- and δ-containing receptors in a concentration-dependent manner. At α1β3γ2 receptors the potency and maximum potentiation were similar for both compounds, with average EC_{50}s (and maximum enhancement) of 1.24±0.21 μM (467.6±24.6%, n=5) for magnolol and 1.17±0.2 μM (483.4±64.4%, n=3) for honokiol (Figure 2A). The α1β3δ receptors also responded to both magnolol and honokiol (Figure 2B). Again, the two modulators were comparable to one another, with average EC_{50}s (and maximum enhancement) of 3.40±0.70 μM (1130.8±179.1%, n=3) for magnolol and 3.80±0.41 μM (1053.4±192.6%, n=5), for honokiol (Figure 2B). Compared to the α1β3γ2 receptors, the δ-containing receptors were significantly less sensitive to modulation ($p \leq 0.05$ for magnolol EC_{50} and $p \leq 0.01$ for honokiol EC_{50}), but exhibited substantially greater maximum potentiation, over twice that of the γ-containing receptors ($p \leq 0.05$ for both magnolol and honokiol). The ability of these modulators to increase the activity of both γ2- and δ-containing receptors is consistent with the enhancement of both phasic and tonic neurotransmission that we observed in hippocampal neurons (Figure 1).

3.3. Effect of magnolol and honokiol on responses to saturating GABA levels

While studies with low agonist concentrations represent appropriate conditions for extra-synaptic populations, synaptic receptors are likely to be activated by saturating levels of GABA. To determine the impact of magnolol and honokiol under these conditions, we examined the response of receptors to maximally effective GABA concentrations (Figure 3). We first compared the response of γ2- and δ-containing receptors in whole-cell recordings (Figure 3A, 3B). At α1β3γ2 receptors, 10 μM magnolol or honokiol had no effect on the peak amplitude of the response to 1 mM GABA. In contrast, they nearly doubled the peak response of α1β3δ receptors. This ability to increase the maximum response of δ-containing receptors is shared by several $GABA_A$ receptor modulators, including neurosteroids and general anesthetics (Bianchi and Macdonald 2003; Meera et al., 2009).

Although the modulators did not alter the peak whole-cell response of α1β3γ2 receptors, they did appear to slow the decay rate following agonist removal. To better quantify this effect on the kinetics of receptor deactivation, we used brief (5 msec) applications of 1 mM GABA to outside-out macropatches (Figure 3C). All current decays were fit with the sum of two exponential distributions and both magnolol and honokiol slowed deactivation. Co-application of 10 μM magnolol significantly increased the weighted mean deactivation rate by 171.8%, from an average of 60.7±10.6 msec to 104.3±16.2 msec (n=4 patches, $p \leq 0.05$, paired t-test). The slowing of the mean rate occurred through a combination of an increase in the slow τ (from 161.1±29.6 to 228.4±41.3 msec) and an increase in the % area of the slower component (from 31.5±5.0 to 41.8±8.1%). A similar effect was seen with co-

application of 10 μM honokiol, which increased the weighted mean by 147.2%, from 77.1±13.6 msec to 113.5±21.0 msec (n=3 patches, p≤0.05, paired t-test).

These results with 1 mM GABA are consistent with the effects we observed in hippocampal brain slice recordings, where magnolol and honokiol had no impact on the peak amplitude of the evoked IPSC, but did significantly slow the decay rate (Figure 1A). In addition, the ability of the modulators to increase the response of δ-containing (but not γ2-containing) receptors beyond that produced by saturating GABA alone could explain the observed difference in maximum potentiation at the α1β3γ2 and the α1β3δ receptors (Figure 2).

3.4. Effect of subunit composition of recombinant receptors

$GABA_A$ receptors are notable for their structural diversity, and the large number of subunits that can contribute to the formation of neuronal receptors (Whiting et al., 1999). For example, hippocampal dentate granule neurons produce a heterogenous population of $GABA_A$ receptors, and express the δ subunit along with all but one of the twelve α, β and γ subunit subtypes (Wisden et al., 1992). The subunit composition can substantially alter the pharmacological properties of the receptors (Korpi et al., 2002), and previous studies have suggested that magnolol or honokiol might preferentially modulate receptors containing either α2 (Ai et al., 2001) or α3 subunits (Taferner et al., 2011). We examined the response of recombinant receptors with varying subunit composition to determine if modulation by magnolol or honokiol is influenced by the α, β or γ subtype.

The six different α subtypes were expressed with β3 and γ2, the three β subtypes with α1 and γ2, and the three γ subtypes with α1 and β3. 10 μM magnolol or honokiol was co-applied with an $EC_{5–10}$ concentration of GABA for each isoform. All these receptor isoforms were potentiated to a similar degree, with no apparent selectivity among the subtypes (Figure 4). Since the δ subunit is commonly associated with the α4 subtype in hippocampal neurons, we also compared sensitivity of the α4β3δ receptors and found a large enhancement, similar to that of α1β3δ (Figure 4C). Because the γ and δ subunits conferred such substantial differences in the maximal potentiation, we examined the response of α1β3 receptors, and found that they responded to a level comparable to the γ-containing receptors (Figure 4C). This pattern is distinct from that of general anesthetics like etomidate and propofol, which have greater efficacy at δ-containing compared to γ-containing receptors, but enhance both αβ and αβδ receptors to a similar degree (Meera et al, 2009).

3.5. Effect of mutations that alter sensitivity to other $GABA_A$ receptor modulators

Several other classes of $GABA_A$ receptor modulators, including neurosteroids, alcohol, and general anesthetics, have also been shown to act at most receptors regardless of subunit composition and, like magnolol and honokiol, demonstrate particularly high efficacy at δ-containing receptors. To determine if magnolol and honokiol act through a previously identified allosteric site, we created point mutations in the α1 or β3 subunit that reduce sensitivity to other modulators (Figure 5A).

Positive modulation by neurosteroids such as tetrahydrodeoxycorticosterone (THDOC) and allopregnanolol could be eliminated through mutation of a conserved glutamine residue located in the first transmembrane domain of all the α subunits (Hosie et al., 2006; 2009). We found that $\alpha1_{(Q240W)}\beta3\gamma2$ receptors had increased GABA sensitivity (average EC_{50} = 1.8±0.6 μM, n=3, p≤0.001) compared to the wild-type receptor (EC_{50} = 12.5±0.8 μM, n=4), but that this mutation had no impact on modulation by either magnolol or honokiol (Figure 5).

Residues within the 2nd, 3rd, and 4th transmembrane domains of the α and β subunits contribute to the activity of a wide range of modulators, and have been proposed to form a large binding pocket shared by general anesthetics, alcohols, and other related molecules (Jenkins et al., 2001; see review by Garcia et al. 2010). We created mutations at residues within each of these domains (Figure 5A). Within the 2nd transmembrane domain, we examined the role of serine269 in the α1 subunit (homologous to S270 in the human sequence). Mutation of this residue to isoleucine decreases modulation by several inhaled anesthetics and ethanol (Mihic et al., 1997; Ueno et al., 1999; Krasowski et al., 2001), but does not affect the response to i.v. anesthetics such as propofol and etomidate. We also examined methionine286 within the 3rd transmembrane domain of the β subunit, which contributes broadly to the activity of general anesthetics. Mutation of this residue to tryptophan reduces or eliminates sensitivity to inhaled and i.v. anesthetics, ethanol, and menthol (Mihic et al., 1997; Krasowski et al., 2001; Watt et al., 2008). Finally, we examined a conserved tyrosine residue in the 4th transmembrane domain which contributes to sensitivity to both propofol and inhaled anesthetics (Jenkins et al., 2002; Richardson et al., 2007).

These mutations had a variety of effects on GABA sensitivity of the receptor. Both mutations in the β3 subunit significantly ($p \leq 0.001$) increased GABA sensitivity, with average EC_{50}s of 1.6±0.2 μM (n=3) for $\alpha1\beta3_{(M286W)}\gamma2$ and 2.9±0.8 μM (n=3) for $\alpha1\beta3_{(Y442W)}\gamma2$. The $\alpha1_{(S269I)}\beta3\gamma2$ receptors also showed significantly increased GABA sensitivity (EC_{50} = 2.2±0.2 μM, n=3, $p \leq 0.001$) while the $\alpha1_{(Y410W)}\beta3\gamma2$ receptors were not significantly different from wild-type (EC_{50} = 27.8±2.3 μM, n=3, $p > 0.05$). In all cases, however, the receptors retained wild-type potentiation in response to magnolol and honokiol (Figure 5).

Previous studies using radiolabeled ligands have suggested that magnolol and honokiol may act allosterically near the picrotoxin binding site (Squires et al., 1999). Therefore, we examined the effect of mutating the conserved threonine residue located at the 6′ position within the 2nd transmembrane domain, which has been shown to eliminate sensitivity to picrotoxin (Gurley et al., 1995). Mutation of this residue to phenylalanine did not significantly alter GABA sensitivity for $\alpha1_{(T260F)}\beta3\gamma2$ (average EC_{50} = 19.3±1.6 μM, n=3) or $\alpha1\beta3_{(T256F)}\gamma2$ (average EC_{50} = 16.4±2.7 μM, n=3) compared to the wild-type receptor and neither of these mutations had any impact on modulation by magnolol or honokiol (Figure 5).

These results suggest that the binding sites for magnolol and honokiol do not share common structural requirements with neurosteroids, anesthetics, ethanol or picrotoxin, and instead they appear to act through a unique and yet-undescribed site on the $GABA_A$ receptors.

4. Discussion

Given the widespread popularity of herbal supplements and other natural products in CAM, it is important to characterize their mechanism(s) of action so that they can be used safely and effectively. We examined the properties of magnolol and honokiol, the two isomeric compounds which are the neurologically active ingredients in magnolia bark extract and have been previously reported to modulate $GABA_A$ receptors. Our results from both neuronal and recombinant receptors show positive modulatory effects on the two major $GABA_A$ receptor populations. At synaptic receptors, which could contain a variety of α or β subtypes in combination with a γ2 subunit, the effect of magnolol or honokiol was to slow the deactivation rate without increasing the peak response. At the extra-synaptic population, which is activated by a submaximal GABA concentration and includes δ-containing receptors, these compounds produced a large enhancement of the current amplitude.

We found that modulation by magnolol or honokiol showed little dependence upon the α subtype of the receptor. These results are in contrast with two previous studies that examined recombinant $GABA_A$ receptors. Ai et al., (2001) examined enhancement of binding of radiolabeled muscimol or flunitrazepam to transfected cell membranes, comparing the response of receptors containing the α1, α2, α3 and α5 subtypes. In agreement with our results, they found that a γ subunit was not necessary for modulation, but they also found that the greatest enhancement was observed with the α2 subunit, and the lowest with the α5 subunit. Taferner et al. (2011) examined the activity of honokiol at recombinant $GABA_A$ receptors using electrophysiological recordings from *Xenopus* oocytes. They also compared the α1, α2, α3 and α5 subunits, but found honokiol showed greatest efficacy at α3-containing receptors, with little effect of subunit composition on potency. In both of these studies, the concentrations used to produce the effects were substantially larger than the EC_{50}'s of 1–3 μM that we found. In the report from Ai et al. (2001), the subunit-dependent differences became apparent only at concentrations greater than 100 μM, and the authors acknowledged that much higher concentrations were required to observe effects on muscimol binding than were required to enhance activity of recombinant α3-containing receptors when measured through electrophysiological recording. Similarly, EC_{50}s reported by Taferner et al., (2011) ranged from 23–60 μM. In our studies, we observed that at concentrations above 10 μM, both magnolol and honokiol had agonist activity, and could directly activate $GABA_A$ receptors in the absence of GABA. Therefore, it may be that the higher affinity modulatory site is not dependent upon the α subunit subtype, but that the lower affinity agonist site is influenced by subunit composition.

Our results showed that the largest impact of magnolol or honokiol may well be at the δ-containing receptors that contribute to the extra-synaptic tonic current in the hippocampus, thalamus and cerebellum (Zheleznova et al., 2009). These receptors have a number of unique functional and pharmacological characteristics, including insensitivity to modulation by benzodiazepines, and as such make promising drug targets. Disruption of tonic current is seen in a number of disorders, including epilepsy (Brickley and Mody., 2012). GABA is a high affinity but low efficacy agonist at δ-containing receptors (Keramidas and Harrison, 2008; Mortensen et al., 2010). Our results suggest that magnolol and honokiol are among the modulators that can increase GABA efficacy at these receptors and thereby produce a significant potentiation of the maximum response. Other clinically relevant or endogenous modulators with similar activity include the neurosteroids, ethanol, inhaled and i.v. anesthetics, and the pyrazolopyridines (Zheleznova et al., 2009; Brickley and Mody., 2012).

While it is reasonable to ascribe the anxiolytic, sedative and anti-convulsant actions associated with magnolol and honokiol to the enhancement of $GABA_A$ receptors, these compounds have also been shown to act at other sites throughout the nervous system (see review by Lee et al., 2011). Earlier studies reported that extracts containing magnolol and honokiol can modestly inhibit dopamine transporter activity and reduce binding to dopamine (D_5) and serotonin ($5HT_6$) receptors (Koetter et al., 2009). There is also evidence that magnolol and honokiol may inhibit glutamate receptors (Lin et al., 2005, 2009) and that honokiol derivatives can enhance the activity of $GABA_C$ receptors (Irie et al., 2001, Liu et al, 2005). In addition, our results showed that both magnolol and honokiol significantly increased the frequency of GABAergic mIPSCs in hippocampal brain slices, which would not be expected to result from enhancement of $GABA_A$ receptor activity. Interestingly, neurosteroids have also been reported to increase GABA mIPSC frequency, although the mechanism by which this occurs is not known (see review by Herd et al., 2007). Other studies have reported potential pre-synaptic effects, as honokiol enhanced evoked acetylcholine release in hippocampal slices (Tsai et al, 1995) and that both magnolol and honokiol increased acetylcholine release from rat hippocampus as measured with *in vivo* microdialysis (Hou et al., 2000). These other targets could contribute to the reported

beneficial effects of these compounds in animal models of depression (Nakazawa et al, 2003; Xu et al, 2008, Yi et al., 2009; Koetter et al., 2009), pain (Lin et al., 2009) and neurodegeneration (Chang et al,. 2003; Liou et al, 2003; Liu et al., 2005; Matsui et al., 2009; Lee et al., 2009) and could represent therapeutically important actions for these compounds distinct from their activity at $GABA_A$ receptors.

In addition to neurological effects, magnolol and/or honokiol have also been widely studied for anti-inflammatory, anti-oxidant, and anti-cancer activity (see review by Lee et al., 2011). They have been shown to induce apoptosis and inhibit tumor growth and metastasis, and to reduce inflammation through inhibition of the NF-κB pathway or nitric oxide production. Some of these systemic effects could be mediated through activity at $GABA_A$ receptors, as an inhibitory action at lymphocytes was blocked by bicuculline (Munroe et al., 2010). The anti- cancer and anti-oxidant effects were typically observed at concentration ranges of 50–100 μM, while anti-inflammatory effects were seen at levels of 1–10 μM.

The use of CAM continues to be common among US adults and nearly $15 billion was spent for non-vitamin, non-mineral, natural products in 2007 (Barnes et al., 2008). Our results suggest that magnolia bark extracts or the purified active ingredients magnolol and honokiol would be effective sedatives and anxiolytics. However, as potent and effective modulators of $GABA_A$ receptors, these compounds also have the potential for serious safety concerns, including side-effects and drug interactions. Further studies are needed to determine the effects of co-administration of these compounds with alcohol and with prescription medications, such as the benzodiazepines, which are commonly used to treat insomnia and anxiety. The possibility that magnolol and honokiol may become physically addictive, induce cross-tolerance, or produce withdrawal symptoms after chronic use should also be carefully examined.

Acknowledgments

This work was supported by funds from NIH-NINDS (RO1-NS045950 to JLF), the University of South Carolina (Promising Investigator Research Award to DDM) and the Office of Undergraduate Research at the University of South Carolina (JLF and MA).

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- Magnolol and honokiol are positive allosteric modulators of $GABA_A$ receptors
- They enhance both phasic and tonic neurotransmission in hippocampal neurons
- Their activity shows little dependence upon $GABA_A$ receptor subunit composition
- The greatest enhancement is seen at δ-containing receptors

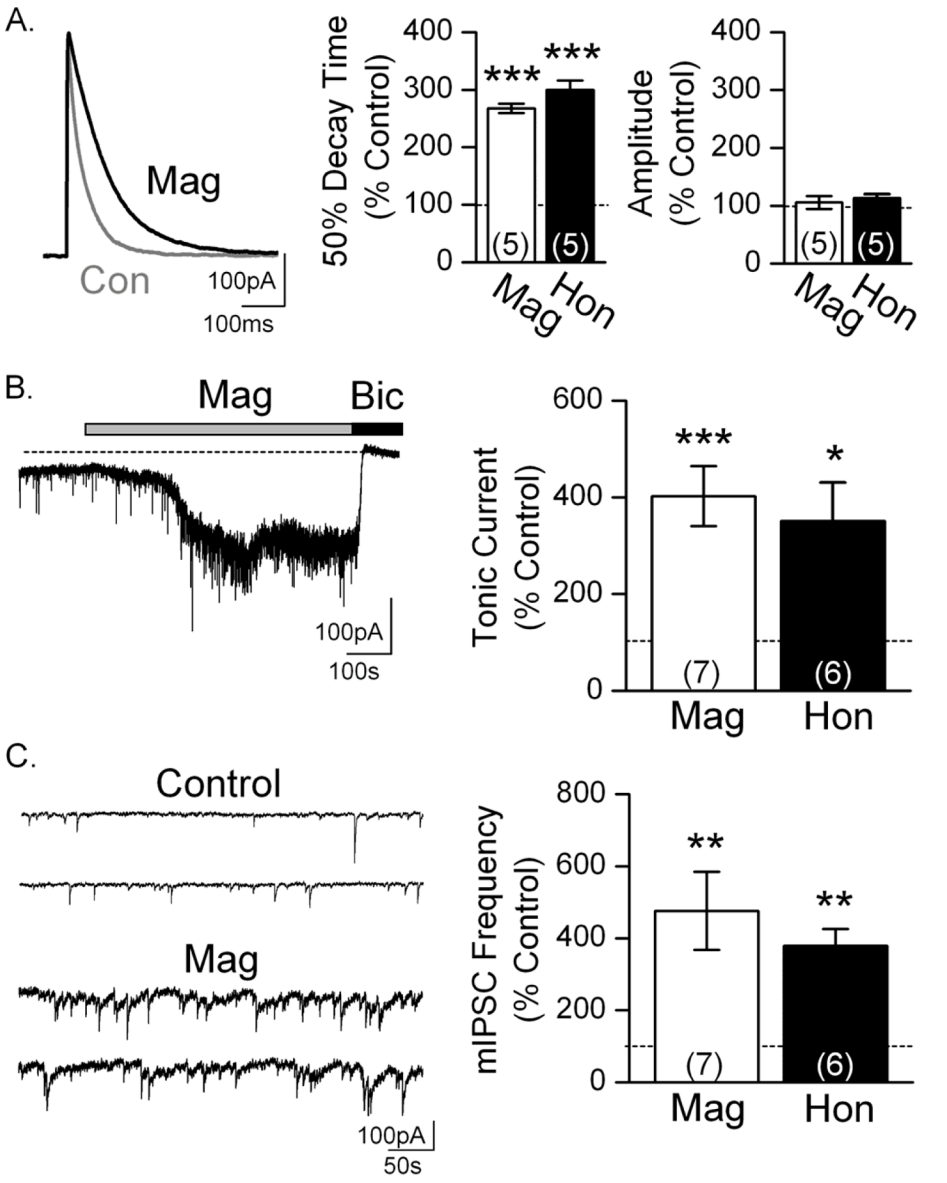

Figure 1. Magnolol and honokiol potentiate synaptic and extrasynaptic GABAergic currents in hippocampal dentate granule neurons

A. Whole-cell recordings of evoked monosynaptic IPSCs recorded in dentate granule neurons. *Left:* 30 μM magnolol (mag) prolonged the decay of the evoked IPSC, but did not alter its amplitude compared to a recording prior to magnolol treatment (Con, gray trace). *Right:* 30–60 μM Magnolol (*white bar, n=5*) or honokiol (*black bar, n=5*) slowed the 50% decay time, but not the amplitude of the evoked IPSC. ***B***. Whole cell recordings of the tonic current in dentate granule neurons. *Left:* 30 μM magnolol (Mag) strongly potentiated the tonic GABAergic current, which was completely blocked by 20 μM bicuculline (Bic). The dotted line indicates the baseline in the absence of tonic GABAergic current. *Right:* 30–60 μM magnolol (*white bar, n=7*) or magnolol (*black bar, n=6*) significantly potentiated the tonic GABAergic current. ***C***. Miniature IPSCs (mIPSCs) were measured prior to (Control) or during (Mag) treatment with magnolol or honokiol. *Left:* Sample traces showing the increase in mIPSC frequency produced by 30 μM magnolol. *Right:* 30–60 μM magnolol (*white bar, n=7)* or honokiol (*black bar, n=6*) significantly increased mIPSC frequency. All

bars show mean ± SEM with the number of cells indicated in parentheses. *p<0.05, **p<0.01, ***p<0.001 indicate a statistically significant difference compared to the control response prior to drug application.

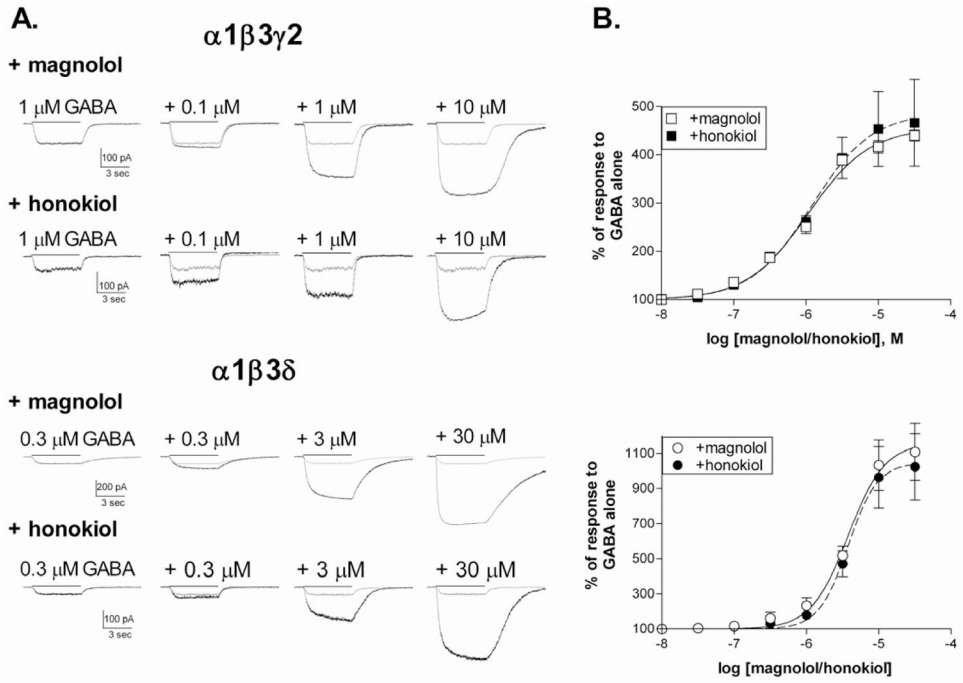

Figure 2. Magnolol and honokiol potentiate recombinant $GABA_A$ receptors

A. Responses to GABA were recorded from transiently transfected HEK-293T cells voltage clamped at −50 mV. 0.3 μM (α1β3δ) or 1 μM (α1β3γ2) GABA was co-applied for 5 sec as indicated by the solid line with varying concentrations of magnolol or honokiol. The response to GABA alone (gray trace) is overlaid by the response to GABA + modulator (black trace).

B. Concentration-response relationships were constructed by dividing the peak response to GABA plus 0.01 – 30 μM of magnolol or honokiol by the response to GABA alone for each cell. Averaged data (n=3–5 cells) were fit with a sigmoidal dose-response curve indicated by the solid (+ magnolol) or dashed (+ honokiol) lines.

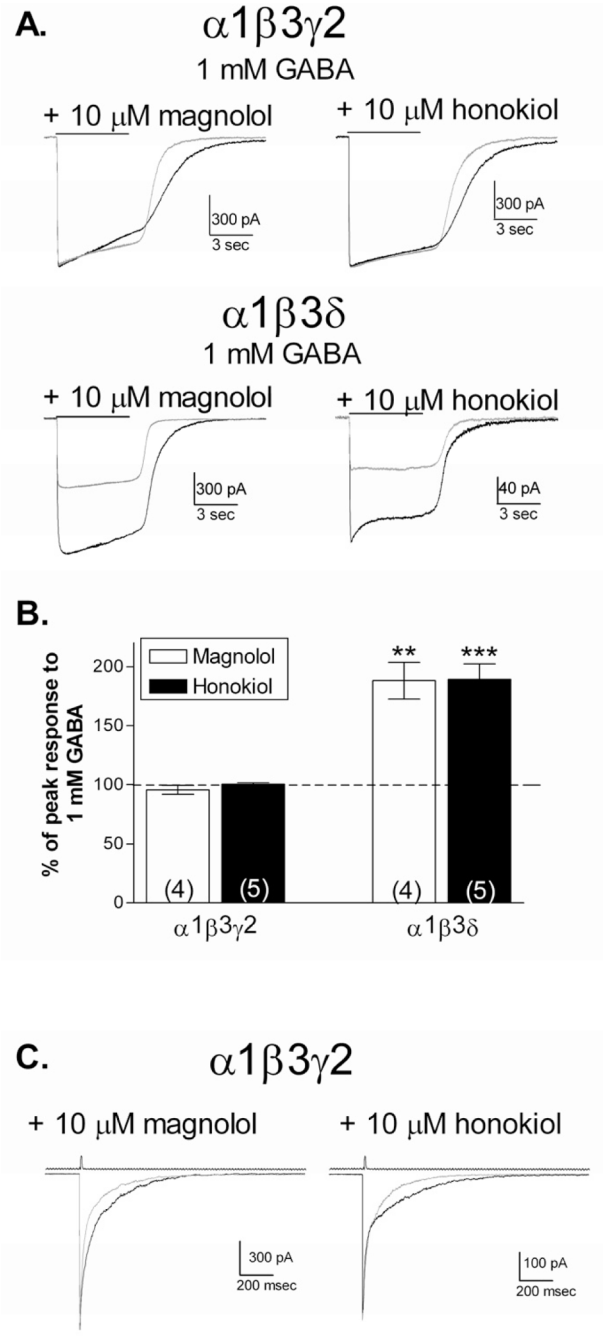

Figure 3. Modulation of the response of recombinant $GABA_A$ receptors to a saturating GABA concentration

A. Representative current traces from recombinant receptors in response to a 5 sec application of 1 mM GABA (gray) or 1 mM GABA co-applied with 10 μM honokiol or magnolol (black). Cells were voltage-clamped at −50 mV

B. The peak response to 1 mM GABA + magnolol or honokiol was divided by the response to GABA alone. Bars show the mean ± SEM and the n-value is given by the number in parentheses. The dashed line indicates the response to GABA alone (100%). **($p<0.01$) and ***($p<0.001$) indicate a significant difference in peak amplitude from the response to GABA alone (paired Student's t-test).

C. Outside-out patches were excised from cells transfected with α1β3γ2 and voltage-clamped at −70 mV. 1 mM GABA was applied for 5 msec either alone (gray trace) or co-applied with 10 μM magnolol or honokiol (black trace).

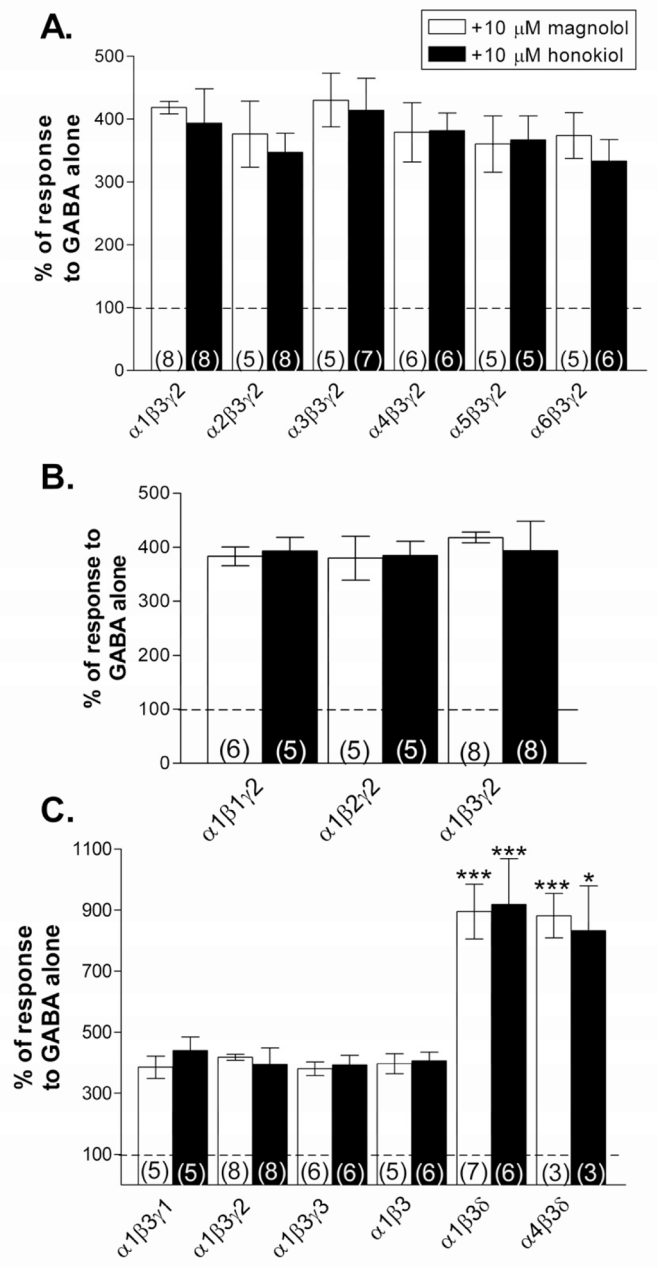

Figure 4. Effect of the subunit subtype on modulation by magnolol and honokiol

Cells were transiently transfected with the subunit combinations indicated and voltage-clamped at −50 mV in the whole-cell recording configuration. GABA was co-applied for 5 sec with 10 μM magnolol or honokiol. Recombinant receptors were expressed with different subunit compositions as indicated, varying **(A)** the α subtype, (**B**) the β subtype, or **(C)** the tertiary subunit (α β alone or with δ or γ subtypes). The peak response was measured and divided by the response to GABA alone. Bars show the mean ± SEM and the n-value is given by the number in parentheses. The dashed line indicates the response to GABA alone (100%). *($p \leq 0.05$) and ***($p \leq 0.001$) indicate a significant difference from the response of α1β3γ2 (Tukey-Kramer multiple-comparisons test).

A.

TM1

α1 - …IGYFVIQTYLPCIMTVILS**Q**$_{240}$VSFW…
β3 - …IGYFILQTYMPSIMITILSW VSFW…

TM2

α1 - …TVFGV**T**$_{260}$TVLTMTTL**S**$_{269}$ISA…
β3 - …VALGI**T**$_{256}$TVLTMTTIN THL…

TM3

α1 - …WFIA VCYAFVFSALIEFATVN…
β3 - …MYL**M**$_{286}$GCFVFVFLALLEYAFVN…

TM4

α1 - …IAFPLLFGIFNLV**Y**$_{410}$WAT…
β3 - …IVFPFTFSLFNLV**Y**$_{442}$WLY…

B.

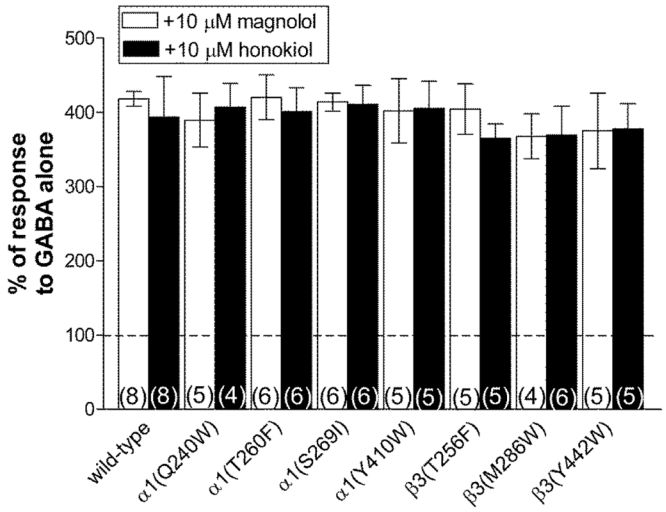

Figure 5. Effect of point mutations at known allosteric regulatory sites

A. Targeted residues within the four transmembrane domains (TM1–TM4) are indicated by bold type. Amino acid sequence and numbering are from the mature rat α1 and β3 subunits. B. The peak response was measured and divided by the response to GABA alone. Bars show the mean ± SEM and the n-value is given by the number in parentheses. The dashed line indicates the response to GABA alone (100%). There were no significant differences compared to the response of the wild-type α1β3γ2 receptor ($p>0.05$, Tukey-Kramer multiple-comparisons test).