

## ORIGINAL ARTICLE

# The role of loops B and C in determining the potentiation of GABA<sub>A</sub> receptors by midazolam

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**Abstract**

Many benzodiazepines are positive allosteric modulators (PAMs) of GABA<sub>A</sub> receptors that cause sedation, hypnosis, and anxiolysis. Benzodiazepines bind GABA<sub>A</sub> receptors at the extracellular interface of the  $\alpha$  and  $\gamma$  subunits. Within the  $\alpha$  subunit, the benzodiazepine binding site is defined by three highly conserved structural loops, loops A-C. Although previous mutagenesis studies have identified His102 in Loop A as important for benzodiazepine modulation of GABA<sub>A</sub> receptors, the functional roles of many of the other conserved residues in loops A-C remain incompletely understood. In this study, we made single mutations in loops A-C of the benzodiazepine binding-site across all six  $\alpha$  subunits. We used whole-cell patch clamp recording to measure the functional effects of these mutations on midazolam potentiation. The results showed that mutating the threonine in loop B and serine in loop C (Thr163 and S206 in human  $\alpha$ 1) did not abolish the receptors' responsiveness to midazolam, as the  $\alpha$ 1(H102R) mutation did. The loop C mutations exhibited a novel array of  $\alpha$ -isoform specific effects on midazolam potentiation. The  $\alpha$ 3(S230I) and  $\alpha$ 5(S209I) mutations had the largest effect on midazolam potentiation, increasing the efficacy of midazolam. Novel benzodiazepines targeting loop C may represent a future direction for designing new drugs that specifically alter the activity of  $\alpha$ 3- and  $\alpha$ 5-containing GABA<sub>A</sub> receptors.

**KEYWORDS**

allosteric modulator, benzodiazepine, canonical loops, GABA(A) receptor

**1 | INTRODUCTION**

Benzodiazepines can induce sedation, anxiolysis, amnesia, seizure reduction, and muscle relaxation by enhancing inhibitory GABAergic neurotransmission through the  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptors.<sup>1,2</sup> GABA<sub>A</sub> receptors are cysteine loop ligand-gated ion channels

assembled from five subunits ( $\alpha$ 1-6,  $\beta$ 1-3,  $\gamma$ 1-3,  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\pi$ ,  $\rho$ 1-3) around a central pore.<sup>3</sup> GABA<sub>A</sub> receptors have a stoichiometry of two  $\alpha$  subunits, two  $\beta$  subunits and one auxiliary subunit (predominantly  $\gamma$  or  $\delta$ ) (Figure 1A). Each subunit has a different spatial, temporal and pharmacological profile in the brain.<sup>4,5</sup> Upon GABA binding, the receptor's anion channel opens, causing hyperpolarizing

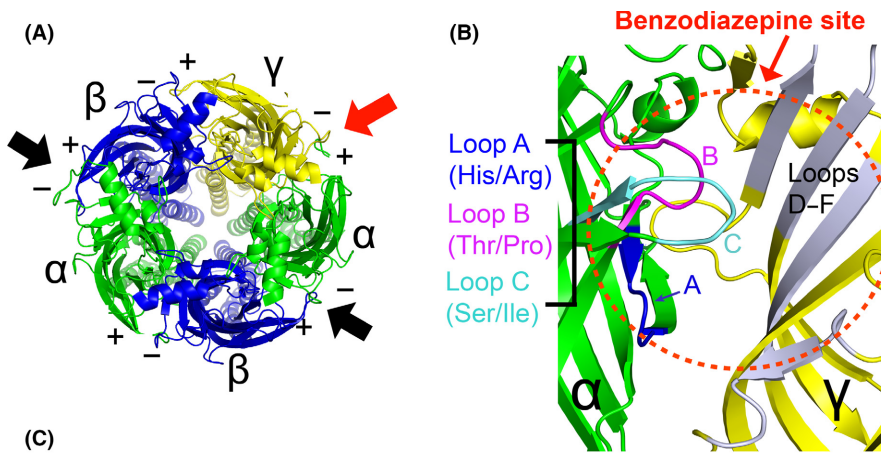
**Abbreviations:** cDNA, complementary DNA; EC<sub>50</sub>, 50% effective concentration; EGTA, ethylene glycol-bis ( $\beta$ -aminoethyl ether); GABA,  $\gamma$ -aminobutyric acid; GABA<sub>A</sub>,  $\gamma$ -aminobutyric acid type A; GFP, green fluorescent protein; HEK293T, human embryonic kidney cells expressing the SV40 T-antigen; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; N, N, N', N'-tetraacetic acid; PAM, positive allosteric modulator.

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Recommended section assignment: Neuropharmacology

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**FIGURE 1** The structural loops A-C within the  $\alpha$  subunit form the benzodiazepine binding site on the GABA<sub>A</sub> receptor. (A) The assembly of the  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptor with arrows pointing to the two GABA sites (black) and high-affinity benzodiazepine site (red). (B) The structural loops A-C (blue, magenta, cyan) on the  $\alpha$  subunit and loops D-F (grey) on the  $\gamma$  subunit form the benzodiazepine site (red dotted circle) on the  $\alpha_x\beta_2\gamma_2$  receptor. Target residues used in this study noted under loops. (C) The structural loops A-C are highly conserved across GABA<sub>A</sub> receptor  $\alpha$  subunits. The location of the residues of interest are highlighted in **bold** with the specific mutation numbers listed to the right. The numbering is based on the human mature peptide sequences not including the signal peptide (peptide sequences based on NP\_000797 ( $\alpha_1$ ), NP\_000798 ( $\alpha_2$ ), NP\_000799 ( $\alpha_3$ ), NP\_000800 ( $\alpha_4$ ), NP\_000801 ( $\alpha_5$ ), NP\_000802 ( $\alpha_6$ )). The mutations made in this study are referred to by the abbreviations “loop A”, “loop B” and “loop C” in subsequent figures and text

Subunit	Benzodiazepine-site loops			Mutations in this study		
	Loop A	Loop B	Loop C	Loop A	Loop B	Loop C
$\alpha_1$	FF <b>H</b> NG	GSYAY <b>T</b> R	<b>S</b> STGEYV	H102R	T163P	S206I
$\alpha_2$	FF <b>H</b> NG	GSYAY <b>T</b> R	<b>S</b> STGEYT	H101R	T162P	S205I
$\alpha_3$	FF <b>H</b> NG	GSYAY <b>T</b> R	<b>S</b> STGEYV	H126R	T187P	S230I
$\alpha_4$	FF <b>R</b> NG	GSYAY <b>P</b> K	<b>S</b> ITGEYI	R100H	P161T	I204S
$\alpha_5$	FF <b>H</b> NG	GSYAY <b>P</b> N	<b>T</b> STGEYT	H105R	P166T	S209I
$\alpha_6$	FF <b>R</b> NG	GSYAY <b>P</b> K	<b>S</b> NTGEYV	R100H	P161T	N204I

membrane potentials in the adult mammalian brain. One highly relevant class of positive allosteric modulators (PAMs) of GABA<sub>A</sub> receptors are benzodiazepines.

Benzodiazepines bind at the extracellular interface of the  $\alpha$  and  $\gamma$  subunits.<sup>6</sup> There are three structural loops (loops A, B, and C) on the  $\alpha$ -subunit and three loops on the  $\gamma_2$  subunit (loops D, E, and F) that form the structure of the benzodiazepine binding site (Figure 1B). Loops A-C form connectors between sequential  $\beta$ -strands. They are sometimes referred to as loop 5 (loop A), loop 8 (loop B) and  $\beta$ -sheet 10 (loop C), based on nomenclature for the acetylcholine-binding protein.<sup>7,8</sup> Loops A-C are highly conserved across GABA<sub>A</sub> receptor subunits and form a homologous GABA agonist binding site at the  $\beta$ +/ $\alpha$ - interface.<sup>9,10</sup>

A combination of mutagenesis with functional or binding assays has been used to determine the role of specific amino acids within the structural loops A-F of the benzodiazepine site.<sup>11-16</sup> The conserved histidine in loop A (His101 in rodents and His102 in bovine and human cDNAs) is important for the molecular and behavioral actions of diazepam using in vitro experiments<sup>6,11</sup> and knock-in mice.<sup>2,17</sup> Other residues in loops A-C have been studied, but most mutagenesis experiments were constrained to mutating less than three  $\alpha$  subunit isoforms. This limits the conclusions drawn. Many benzodiazepine ligands bind to multiple GABA<sub>A</sub> receptor assemblies, and a mutagenesis study across the six  $\alpha$  subunits is needed to determine the structural role of specific residues on benzodiazepine efficacy and potency.

In this study, we examined two residues within the conserved loops B and C across all six  $\alpha$  subunits. The conserved threonine in loop B (GSYAY**T**R) and serine in loop C (**S**STGEYV) have been reported to differentially affect the potency and efficacy of benzodiazepine-site ligands, including that of zolpidem, eszopiclone, flumazenil, and  $\beta$ -carbolines.<sup>12,18-21</sup> It is less understood how these specific residues affect the functional actions of nonspecific positive benzodiazepines across the six human  $\alpha$  subunits. In this study, we mutated the highly conserved histidine in loop A (His102 in  $\alpha_1$ ), threonine in loop B (Thr163 in  $\alpha_1$ ), and serine in loop C (Ser206 in  $\alpha_1$ ) in all six GABA<sub>A</sub>  $\alpha$  subunits. The  $\alpha_4$  and  $\alpha_6$  subunits have different residues (R100, P161, and I/N204) in these locations (Figure 1C) and form GABA<sub>A</sub> receptors insensitive to classic benzodiazepines, historically known as diazepam-insensitive receptors.<sup>22</sup> If midazolam acts as a canonical benzodiazepine then canonical mutations in  $\alpha_1$ -3 and  $\alpha_5$  to residues present in  $\alpha_4$  and  $\alpha_6$  should block its actions and *vice versa* in  $\alpha_4$  and  $\alpha_6$ . Whole-cell patch clamp recording was used to measure the actions of midazolam on mutated  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors. Midazolam was selected for this study because it is commonly used in the clinic to induce sedation,<sup>23</sup> it is easier to handle than other benzodiazepines (lower affinity for diazepam-sensitive receptors and higher solubility), and knowledge of its pharmacology could provide insight into designing novel sedatives with fewer side effects. We found that mutating the threonine and serine in loop B and loop C altered the efficacy of midazolam less than mutating the histidine in loop A across  $\alpha_1$ -6. Surprisingly, mutating the serine in loop C altered

the efficacy of midazolam potentiation in different directions depending on the  $\alpha$  isoform. These subunit-selective observations will be useful for the design of  $\alpha$ 3- and  $\alpha$ 5-selective benzodiazepines.

## 2 | MATERIALS AND METHODS

### 2.1 | cDNA plasmids and mutagenesis

Human (*Homo sapiens*) GABA<sub>A</sub> subunits ( $\alpha$ 1-6,  $\beta$ 2,  $\gamma$ 2s) were subcloned into pcDNA3.1+ vectors with a cytomegalovirus (CMV) promoter. The h $\beta$ 2 and h $\alpha$ 3 sequences were humanized rat (*Rattus norvegicus*) cDNA with amino acid substitutions made to match the human protein sequence. The  $\alpha$ 1-3,  $\alpha$ 5,  $\beta$ 2, and  $\gamma$ 2s subunits were a generous gift from Neil L. Harrison (Columbia University Medical Center, NY). The  $\alpha$ 4 subunit was obtained from GenScript (Piscataway, NJ), and the  $\alpha$ 6 subunit was a generous gift from Robert L. McDonald (Vanderbilt University, TN). All point mutations (listed in Figure 1C) were introduced using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions and were confirmed by sequencing (Eurofins MWG Operon, Louisville, KY).

### 2.2 | Cell culture and transfection

Human embryonic kidney cells containing the SV40 T-antigen (HEK293T) were acquired from American Type Culture Collection (ATCC<sup>®</sup>, Manassas, VA), catalogue number, CRL3216. HEK293T cells were maintained at 37°C and 5% CO<sub>2</sub> in Eagle Minimum Essential Medium (MEM) supplemented with 5% fetal bovine serum (Atlanta Biologicals Inc., Flowery Branch, GA), 40  $\mu$ M L-glutamine, 100 U/mL penicillin and 0.1 mM streptomycin. Cells were passaged regularly when they reached 70% confluency using trypsin. Cells were not passaged more than 22 times. New cells were revived from frozen stocks at passage 2-4. Cells used for in vitro electrophysiology experiments were grown on poly-D-lysine-coated glass coverslips (No.2, VWR, Radnor, PA) and transfected with X-tremeGENE (Roche Diagnostics, Indianapolis, IN) with the desired receptor subunit cDNAs at a 1:1:1 ratio to express  $\alpha_x\beta_2\gamma_{2s}$  receptors (2  $\mu$ g total cDNA) and with 0.5  $\mu$ g green fluorescent protein (GFP) as an expression marker. The  $\gamma$ 2s incorporation into receptors was tested with zinc inhibition assays regularly.<sup>24</sup> Patch clamp experiments were performed on cells at 24-72 hours post-transfection. All experiments were performed at 22°C. Experiments consisted of at least five cells recorded per day from at least two transfections across 3-4 days to control for cell health and transfection efficiency. At least three cells expressing wild-type receptors were recorded on days that mutant receptors were tested to provide a time-matched expression control. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

### 2.3 | In vitro electrophysiology

Wildtype and mutant GABA<sub>A</sub> receptors were characterized using whole-cell voltage-clamp electrophysiology of HEK293T cells

expressing  $\alpha_x\beta_2\gamma_{2s}$  receptors and GFP, similar to methods previously described.<sup>25</sup> Patch pipettes were created from thin-walled borosilicate glass (TW150F-4, World Precision Instruments, Inc., Sarasota, FL) using a horizontal puller (P97, Sutter Instruments, Inc., Novato, CA) to give a resistance of 2-8 M $\Omega$  when filled with intracellular solution (120 mM KCl, 2 mM MgCl<sub>2</sub>, 10 mM EGTA, 10 mM HEPES, and adjusted to pH 7.2 with NaOH, 315 mOsm). Extracellular solution contained 161 mM NaCl, 3 mM KCl, 1 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 10 mM HEPES, and 6 mM D-glucose, adjusted to pH 7.4 with NaOH (320-330 mOsm). GABA and midazolam (Hospira, Lake Forest, IL) were delivered using a rapid solution changer (RSC-160, BioLogics Science Instruments, Seyssinet-Pariset, France) connected to a 10-channel infusion pump (KD Scientific Inc., Holliston, MA). The perfusion system was controlled by protocols written in pClamp 9 (Molecular Devices, LLC., Sunnyvale, CA). Whole-cell currents were recorded at -60 mV, filtered at 100 Hz and sampled at 200 Hz with a MultiClamp 700B amplifier (Molecular Devices, LLC) and DigiData 1322A (Molecular Devices, LLC) digitizer. GABA concentration-response assays were performed by exposing each whole-cell patch to eight concentrations of GABA spread over a 3.5 logarithmic decade. Each GABA exposure was for 2 seconds with 8 seconds of washout between ligand application. GABA concentrations for  $\alpha_x\beta_2\gamma_2$  receptors were: 0.3-1000  $\mu$ M ( $\alpha$ 1), 0.1-300  $\mu$ M ( $\alpha$ 2 &  $\alpha$ 3), 0.03-100  $\mu$ M ( $\alpha$ 4), and 0.01-30  $\mu$ M ( $\alpha$ 5 &  $\alpha$ 6). Midazolam concentration-response assays were performed by exposing patches to two successive EC<sub>10</sub> (10% effective concentration) GABA exposures and then exposing the patches to ascending concentrations of coapplied midazolam (10, 50, 100, 500, 1000 nM) and GABA (EC<sub>10</sub>) (See Figure S1). Each midazolam drug exposure consisted of 3 seconds of coapplied GABA + midazolam and then 2 seconds of GABA at the end of each midazolam exposure before 5 seconds of washout in extracellular solution (see Figure S1 for waveform of drug exposure). GABA pre- and postcontrol runs were performed before and after each midazolam assay for each cell to verify a consistent EC<sub>10</sub> GABA response and full washout of midazolam. Control runs consisted of 3 seconds of GABA (EC<sub>10</sub>) and then 3 seconds of a saturating GABA concentration (100-300  $\mu$ M depending on the  $\alpha$  subunit) with 8 seconds of washout between ligand applications. Cells were recorded with the midazolam protocol no more than two times to avoid desensitization and incomplete washout or irreversible modulation.

### 2.4 | Whole-cell analysis

Recordings were analyzed using MATLAB (Math Works, Inc., Natick, MA). GABA concentration-response relationship: Whole-cell peak currents (*I*) were measured from GABA concentration-response assays and fit using a nonlinear regression analysis based on the Hill equation:  $I = I_{\max} * [A]^{nH} / (EC_{50}^{nH} + [A]^{nH})$  where *I* was the peak current amplitude, *I*<sub>max</sub> was maximum current amplitude, EC<sub>50</sub> was the half-maximal GABA concentration, *A* was the agonist concentration, and *nH* was the Hill coefficient. The maximum peak current, EC<sub>50</sub> and Hill coefficient were estimated for assays from each cell. When Hill parameters are estimated from whole-cell recordings, the changes in

the parameters across receptor conditions can be ascribed to the following changes in receptor physiology. Changes in maximum current can be due to changes in the single-channel conductance or the rate of desensitization. Changes in cell surface receptor expression can cause minor changes in maximum current, but are unlikely to occur in the time course of our experimental protocol. Changes in the Hill coefficient can be due to changes in altered GABA cooperativity, the loss of a GABA binding site or altered channel desensitization. More often minor changes in the Hill coefficient are attributed to the altered homogeneity in the receptor population expressed by the HEK293T cell. For example, a shallower Hill coefficient could be caused by a shift in the population of receptors from mostly  $\alpha\beta\gamma$  receptors to a combination of both  $\alpha\beta$  and  $\alpha\beta\gamma$  receptors. Changes in GABA apparent-affinity can be due changes in GABA's binding affinity, gating or both for the receptor. Other explanations than the above are possible but less likely. *Midazolam concentration-response curves*: The midazolam potentiation (%) of each GABA-evoked response was calculated by the equation:  $Pot = (I_{MDZ} - I_G)/I_G \times 100\%$ , where  $Pot$  was potentiation (%),  $I_G$  was the average amplitude of peak currents from the two  $EC_{10}$  GABA responses, and  $I_{MDZ}$  was the amplitude of peak currents from co-applied GABA + midazolam. The potentiation measurements from midazolam concentration-response curves were fit using the Hill equation:  $P = P_{max} * [M]^{nH} / (EC_{50}^{nH} + [M]^{nH})$ , where  $P$  was potentiation,  $P_{max}$  was maximum potentiation,  $EC_{50}$  was the midazolam concentration producing the half-maximal potentiation response,  $M$  was midazolam concentration, and  $nH$  was the Hill coefficient. Concentration-response relationships that were not described by a sigmoidal function were not included in our analysis (eg: no response or a linear nonsaturating response). The Hill equation was fit to each individual cell's concentration-response curve data.

## 2.5 | Statistics

Optimal sample sizes ( $n \geq 10$  cells) were calculated beforehand from preliminary  $\alpha 1$  mutant data using G\*Power (Heinrich-Heine-Universität Düsseldorf, Germany) ( $\alpha = 0.05$  and  $\beta = 0.8$ ) for a one-way analysis of variance (ANOVA) test. Hill parameters (maximum response or potentiation, Hill coefficient,  $EC_{50}$ ) from concentration-response curves (GABA and midazolam each) were compared for significant differences within each  $\alpha$  subunit ( $\alpha 1-6$ ) and its loops A-C mutants using a one-way ANOVA at the significance threshold of  $\alpha = 0.05$ . Where the results of the ANOVA were significant ( $P < 0.05$ ), Dunnett's post-hoc analysis for multiple comparisons ( $\alpha = 0.05$ ) was performed. Statistical analysis was carried out using Prism 7.0 (Graphpad Software, Inc., La Jolla, CA).

## 3 | RESULTS

We hypothesized that mutating single residues in the conserved loops A-C of the benzodiazepine binding site (Figure 1C) would alter the modulation of GABA<sub>A</sub> receptors by midazolam. Whole-cell patch

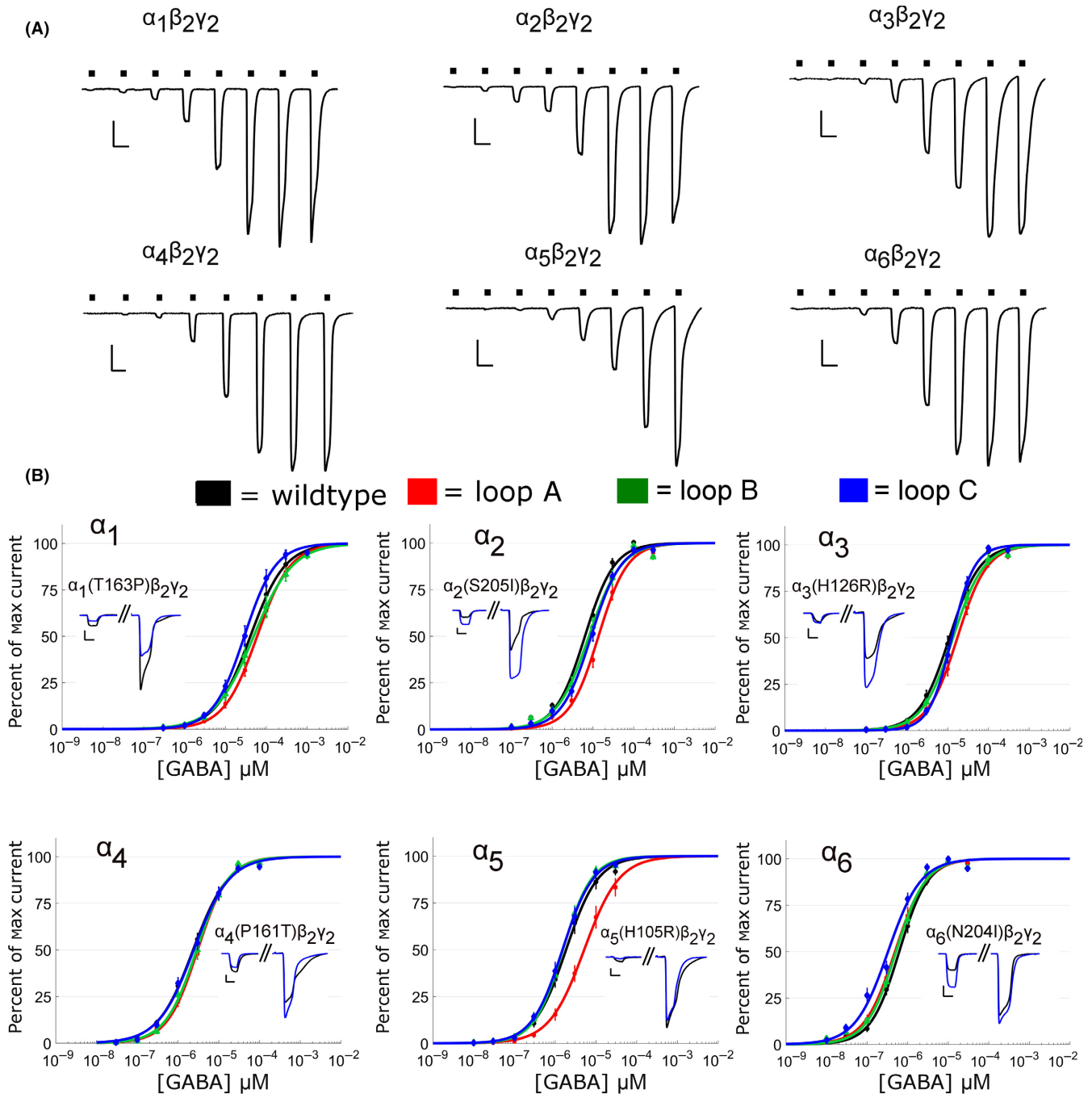
clamp recording of  $\alpha 1-6$ -containing  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors was used to measure the degree of potentiation by midazolam within the therapeutically relevant range of 10-1000 nM.<sup>26-28</sup> Midazolam potentiation was measured as the percent of enhancement in GABA-evoked currents. A 100% potentiation was a doubling in amplitude of the whole-cell current relative to the control  $EC_{10}$  GABA-response. Interestingly, we found that loop C mutations in  $\alpha 3$  and  $\alpha 5$  GABA<sub>A</sub> subunits increased the maximum potentiation by midazolam. However, single residue mutations in loop B and loop C did not alter, abolish or confer midazolam sensitivity as dramatically as the histidine-to-arginine exchanges in loop A.

### 3.1 | Loop A mutations

The loop A mutation substituted the highly conserved histidine residue for an arginine residue (FFHNG) in the  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\alpha 5$  subunits. For the  $\alpha 4$  and  $\alpha 6$  subunits, the reverse arginine-to-histidine mutation was made. GABA concentration-response assays revealed only modest changes in loop A mutant receptors (Figure 2, Table S1). The presence of the arginine right-shifted the GABA concentration-response curves for  $\alpha 5$ (H105R)- and  $\alpha 2$ (H101R)-containing receptors. This caused a threefold increase in the GABA  $EC_{50}$  for  $\alpha 5$ (H105R) mutant receptors ( $\alpha_5$ (H105R) $\beta_2\gamma_2 = 9.84 \pm 3.29 \mu M$  ( $n = 10$ );  $\alpha_5\beta_2\gamma_2 = 3.18 \pm 0.71 \mu M$  ( $n = 10$ ),  $P = 0.0093$ ) and twofold increase in the GABA  $EC_{50}$  for the  $\alpha 2$ (H101R) mutant receptors ( $\alpha_2$ (H101R) $\beta_2\gamma_2 = 16.25 \pm 2.20 \mu M$  ( $n = 11$ );  $\alpha_2\beta_2\gamma_2 = 8.29 \pm 0.78 \mu M$  ( $n = 40$ ),  $P = 0.0003$ ). Midazolam assays showed that the  $\alpha 1$  (H102R),  $\alpha 2$ (H102R),  $\alpha 3$ (H126R),  $\alpha 5$ (H105R) mutations abolished the ability of receptors to respond to midazolam potentiation, and Hill fits could not be performed on this data (Table 1, see Table S2 for midazolam potentiation values). This is consistent with previous reports using diazepam.<sup>11</sup> The  $\alpha 4$ (R100H) and  $\alpha 6$ (R100H) mutations conferred the ability to receptors to respond to midazolam potentiation (midazolam  $EC_{50}$ :  $\alpha 4$ (R100H) $\beta_2\gamma_2 = 73.99 \pm 3.44$  nM ( $n = 8$ ) and  $\alpha 6$ (R100H) $\beta_2\gamma_2 = 41.88 \pm 6.02$  nM ( $n = 7$ ), Figure 3C). The wildtype  $\alpha 4\beta_2\gamma_2$  and  $\alpha 6\beta_2\gamma_2$  receptors showed no notable midazolam potentiation, and no meaningful Hill parameters could be estimated (Figure 3A-B, see Table S2 for values). Confirming the role of this histidine in loop A with midazolam provided a reference for how altering a key structural residue in a conserved region of the benzodiazepine binding site can maximally alter the amplitude of midazolam potentiation of the  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors.

### 3.2 | Loop B mutations

The loop B mutations consisted of mutating a threonine-to-proline (GSYAYTR) in  $\alpha 1-3$ , which we predicted would reduce the receptor's responsiveness to midazolam. The opposite mutation (proline-to-threonine) was made in the  $\alpha 4-6$  subunits. No significant ( $P < 0.05$ ) shifts in GABA apparent-affinity were seen for any  $\alpha 1-6$  loop B mutations (Figure 2, Table S1). The only significant ( $P < 0.05$ ) changes in GABA activation were modest changes in the amplitude of the maximum whole-cell current evoked by GABA for  $\alpha 1$ (T163P),



**FIGURE 2** Mutations in loops A-C across the  $\alpha_1$ -6 subunits generally had only subtle effects on the GABA concentration-response curves. (A) Example traces for wildtype  $\alpha_1$ -6-containing receptors were measured using whole-cell patch clamp recording of HEK293T cells expressing  $\alpha_x\beta_2\gamma_2$  receptors. GABA concentrations (black bars) were:  $\alpha_1 = 0.3$ -1000  $\mu\text{M}$ ,  $\alpha_2$  &  $\alpha_3 = 0.1$ -300  $\mu\text{M}$ ,  $\alpha_4 = 0.03$ -100  $\mu\text{M}$ , and  $\alpha_5$  &  $\alpha_6 = 0.01$ -30  $\mu\text{M}$ . Scale bars: 5 seconds, 500 pA. (B) GABA concentration-response curves of wildtype vs mutated receptors for each of the  $\alpha$  subunits. Line colors: wildtype (black), loop A mutation (red), loop B mutation (green), loop C mutation (blue). Loop A mutations are  $\alpha_1$ (H102R),  $\alpha_2$ (H101R),  $\alpha_3$ (H126R),  $\alpha_4$ (R100H),  $\alpha_5$ (H105R), and  $\alpha_6$ (R100H). Loop B mutations are  $\alpha_1$ (T163P),  $\alpha_2$ (T162P),  $\alpha_3$ (T187P),  $\alpha_4$ (P161T),  $\alpha_5$ (P166T),  $\alpha_6$ (P161T). Loop C mutations are  $\alpha_1$ (S206I),  $\alpha_2$ (S205I),  $\alpha_3$ (S230I),  $\alpha_4$ (I204S),  $\alpha_5$ (S209I), and  $\alpha_6$ (N204I). Insets within each subplot are example responses from the 4th and 8th GABA concentration exposures measured for wildtype receptors (black) and one selected loop mutation (in blue). Subplot GABA concentrations:  $\alpha_1$  (10  $\mu\text{M}$  and 1000  $\mu\text{M}$ ),  $\alpha_2$  (3  $\mu\text{M}$  and 300  $\mu\text{M}$ ),  $\alpha_3$  (3  $\mu\text{M}$  and 300  $\mu\text{M}$ ),  $\alpha_4$  (1  $\mu\text{M}$  and 100  $\mu\text{M}$ ),  $\alpha_5$  (0.3  $\mu\text{M}$  and 30  $\mu\text{M}$ ),  $\alpha_6$  (0.3  $\mu\text{M}$  and 30  $\mu\text{M}$ ). Scale bars are 5 sec, 500 pA. Sample sizes (cells per group) are:  $\alpha_1$  (10),  $\alpha_2$  (9-40),  $\alpha_3$  (11-16),  $\alpha_4$  (12-14),  $\alpha_5$  (10-12), and  $\alpha_6$  (7-15). Points are mean  $\pm$  SEM and where SEM is smaller than symbols, it is not visible

$\alpha_2$ (T162P), and  $\alpha_4$ (P161T) mutated receptors. The GABA  $EC_{50}$  values remained unaltered for these mutants ( $P > 0.05$ , Table S1). The receptors containing threonine-to-proline mutations failed to abolish

the receptors' response to midazolam for  $\alpha_1$ (T163P),  $\alpha_2$ (T162P), and  $\alpha_3$ (T187P) mutants. The midazolam  $EC_{50}$  values of  $\alpha_1$ (T163P),  $\alpha_2$ (T162P), and  $\alpha_3$ (T187P) mutants remained unchanged relative to the

**TABLE 1** Midazolam Hill fit parameters for GABAA receptors with loop A-C mutations in the benzodiazepine site of  $\alpha 1-6$ . Data points were taken from midazolam concentration-response relationships (10-1000 nM) measured with whole-cell patch clamp recording of HEK293T cells expressing  $\alpha\beta 2\gamma 2$  receptors

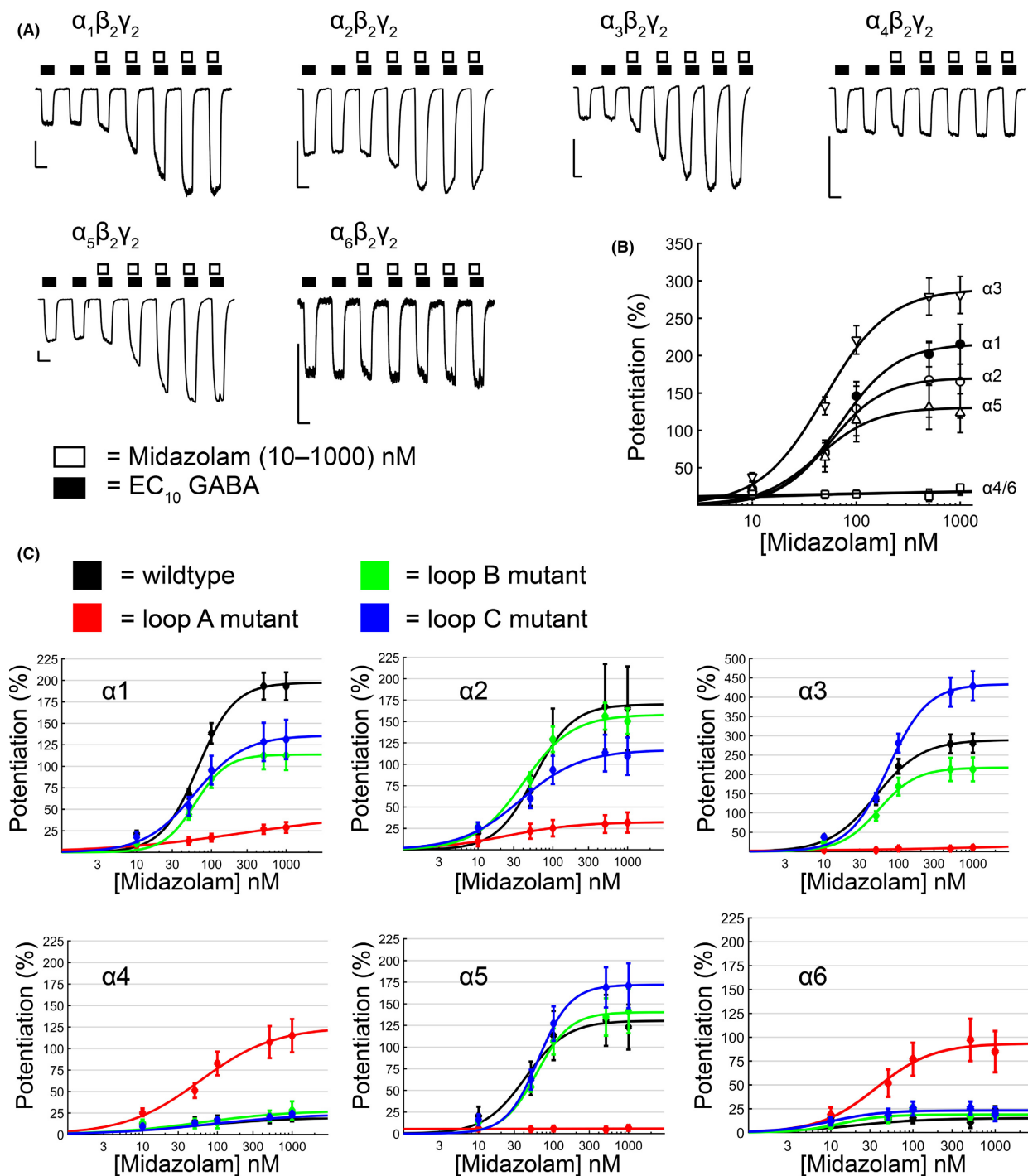
Conditions		Wildtype	Loop A	Loop B	Loop C
$\alpha 1$		$\alpha 1\beta 2\gamma 2$	$\alpha 1(H102R)$	$\alpha 1(T163P)$	$\alpha 1(S206I)$
	Max potentiation (%)	203.0 $\pm$ 17.6	h.n.f.	127.7 $\pm$ 16.0**	135.8 $\pm$ 23.78**
	Hill coefficient	1.765 $\pm$ 0.165	h.n.f.	2.113 $\pm$ 0.154	1.568 $\pm$ 0.199
	EC <sub>50</sub> (nM)	71.43 $\pm$ 5.80	h.n.f.	61.08 $\pm$ 3.72	59.77 $\pm$ 4.11
N	7	11	11	6	
$\alpha 2$		$\alpha 2\beta 2\gamma 2$	$\alpha 2(H101R)$	$\alpha 2(T162P)$	$\alpha 2(S205I)$
	Max potentiation (%)	169.6 $\pm$ 49.9	h.n.f.	158.2 $\pm$ 15.8	116.4 $\pm$ 23.0
	Hill coefficient	1.743 $\pm$ 0.133	h.n.f.	1.393 $\pm$ 0.073	1.362 $\pm$ 0.140
	EC <sub>50</sub> (nM)	50.90 $\pm$ 5.05	h.n.f.	42.03 $\pm$ 2.86	41.65 $\pm$ 4.99
N	7	7	6	8	
$\alpha 3$		$\alpha 3\beta 2\gamma 2$	$\alpha 3(H126R)$	$\alpha 3(T187P)$	$\alpha 3(S230I)$
	Max potentiation (%)	267.8 $\pm$ 20.3	h.n.f.	219.6 $\pm$ 32.3	436.0 $\pm$ 39.4**
	Hill coefficient	1.503 $\pm$ 0.117	h.n.f.	1.963 $\pm$ 0.224**	1.655 $\pm$ 0.061
	EC <sub>50</sub> (nM)	46.39 $\pm$ 7.44	h.n.f.	55.21 $\pm$ 2.91	73.56 $\pm$ 1.81**
N	7	6	6	7	
$\alpha 4$		$\alpha 4\beta 2\gamma 2$	$\alpha 4(R100H)$	$\alpha 4(P161T)$	$\alpha 4(I204S)$
	Max potentiation (%)	h.n.f.	113.8 $\pm$ 21.6	h.n.f.	h.n.f.
	Hill coefficient	h.n.f.	1.187 $\pm$ 0.150	h.n.f.	h.n.f.
	EC <sub>50</sub> (nM)	h.n.f.	73.99 $\pm$ 3.44	h.n.f.	h.n.f.
N	6	8	7	7	
$\alpha 5$		$\alpha 5\beta 2\gamma 2$	$\alpha 5(H105R)$	$\alpha 5(P166T)$	$\alpha 5(S209I)$
	Max potentiation (%)	107.9 $\pm$ 20.3	h.n.f.	140.7 $\pm$ 23.7	175.1 $\pm$ 26.6
	Hill coefficient	2.632 $\pm$ 0.329	h.n.f.	3.661 $\pm$ 1.897	2.232 $\pm$ 0.334
	EC <sub>50</sub> (nM)	52.84 $\pm$ 3.48	h.n.f.	53.28 $\pm$ 5.54	65.44 $\pm$ 2.76
N	7	7	7	6	
$\alpha 6$		$\alpha 6\beta 2\gamma 2$	$\alpha 6(R100H)$	$\alpha 6(P161T)$	$\alpha 6(N204I)$
	Max potentiation (%)	h.n.f.	93.27 $\pm$ 22.84	h.n.f.	h.n.f.
	Hill coefficient	h.n.f.	2.310 $\pm$ 0.56	h.n.f.	h.n.f.
	EC <sub>50</sub> (nM)	h.n.f.	41.88 $\pm$ 6.02	h.n.f.	h.n.f.
N	7	7	6	6	

Fits were performed on each cell's midazolam concentration response data. Midazolam concentration-response relationships not described by a sigmoidal function (h.n.f. = Hill Not Fit) were not included in our analysis (eg: no response or a linear nonsaturating response). Significance was determined using one-way ANOVA with Dunnett's post hoc analysis for each  $\alpha$ -subunit and its mutations. \*\* $P < 0.05$ . Multiple comparisons were made relative to the wildtype  $\alpha\beta 2\gamma 2$  receptor. The wildtype receptors containing  $\alpha 4$  and  $\alpha 6$  subunits lacked sigmoidal relationships and no statistics could be run to compare parameters from  $\alpha 4(R100H)$  and  $\alpha 6(R100H)$  datasets. Values are mean  $\pm$  S.E.M. from  $N$  number of cells.

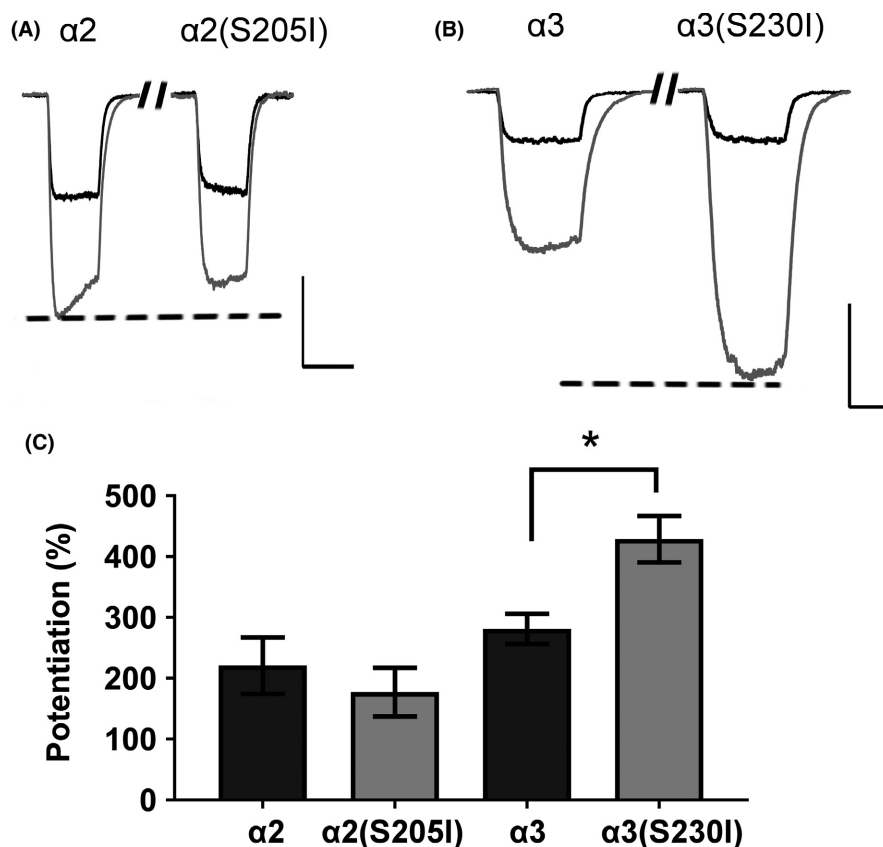
wildtype receptors ( $P > 0.05$ , Table 1). Only  $\alpha 1(T163P)\beta 2\gamma 2$  receptors had a significantly lower maximum potentiation compared to wildtype  $\alpha 1\beta 2\gamma 2$  receptors ( $\alpha 1(T163P)\beta 2\gamma 2$ : 133.8  $\pm$  19.51%,  $n = 11$ ;  $\alpha 1\beta 2\gamma 2$ : 203.0  $\pm$  17.6%,  $n = 7$ ,  $P = 0.0092$ ). The  $\alpha 5(P166T)$  mutation produced little change in midazolam potentiation, either maximum potentiation or midazolam EC<sub>50</sub> ( $P > 0.05$ ,  $n = 7$  per group). The presence of a threonine residue failed to confer midazolam responsiveness to  $\alpha 4(P161T)\beta 2\gamma 2$  and  $\alpha 6(P161T)\beta 2\gamma 2$  receptors (Figure 3C, potentiation values in Table S2). Overall, the presence of a proline in this location caused only subtle changes in both GABA-activation and midazolam potentiation.

### 3.3 | Loop C mutations

The loop C mutations (SSTGEYV) had little effect on GABA apparent-affinity but more noticeable effects on the magnitude of the midazolam potentiation of  $\alpha\beta 2\gamma 2$  GABA<sub>A</sub> receptors. Five of the six loop C mutations failed to significantly ( $P > 0.05$ ) alter the receptor's apparent-affinity for GABA, Table S1). The exception was  $\alpha 6(N204I)$  (EC<sub>50</sub>:  $\alpha 6(N204I)\beta 2\gamma 2 = 0.421 \pm 0.061 \mu\text{M}$  ( $n = 14$ );  $\alpha 6\beta 2\gamma 2 = 0.703 \pm 0.078 \mu\text{M}$  ( $n = 11$ ),  $P = 0.0001$ ) (Figure 2). As predicted, the  $\alpha 1(S206I)$  mutation decreased the amplitude of the maximum potentiation by midazolam by approximately 33% ( $\alpha 1(S206I)\beta 2\gamma 2 = 135.8 \pm 23.8\%$



**FIGURE 3** Mutations in loops A–C of the  $\alpha$  subunit alter the degree of midazolam potentiation measured at  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors. (A) Example traces of midazolam (white boxes, 10–1000 nM) potentiating EC<sub>10</sub> GABA (black boxes) responses in wildtype  $\alpha_x\beta_2\gamma_2$  receptors for  $\alpha_1$ – $\alpha_6$ . Whole-cell patch clamp recording was performed on HEK293T cells expressing  $\alpha_x\beta_2\gamma_2$  receptors. Scale bars: 5 seconds, 500 pA. (B) Midazolam concentration-response curves from wildtype  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors for 10–1000 nM midazolam. Potentiation (%) was measured as the percent of enhancement in peak current evoked by EC<sub>10</sub> GABA. A 100% potentiation would be a doubling in current of the EC<sub>10</sub> GABA control response. Legend:  $\alpha_1$  (●),  $\alpha_2$  (○),  $\alpha_3$  (▽),  $\alpha_4$  (\*),  $\alpha_5$  (Δ),  $\alpha_6$  (□). Points are mean  $\pm$  SEM and where SEM is smaller than symbols, it is not visible. N = 9–17 cells per group. (C) Midazolam concentration-response curves from  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors containing loop A–C mutations and compared to their wildtype  $\alpha_x\beta_2\gamma_2$  receptor counterparts. Potentiation (%) was measured as the percent of enhancement in peak current evoked by EC<sub>10</sub> GABA. Each line represents a different receptor condition: wildtype (black), loop A (red), loop B (green), and loop C (blue). Loop A mutations are  $\alpha_1$ (H102R),  $\alpha_2$ (H101R),  $\alpha_3$ (H126R),  $\alpha_4$ (R100H),  $\alpha_5$ (H105R), and  $\alpha_6$ (R100H). Loop B mutations are  $\alpha_1$ (T163P),  $\alpha_2$ (T162P),  $\alpha_3$ (T187P),  $\alpha_4$ (P161T),  $\alpha_5$ (P166T),  $\alpha_6$ (P161T). Loop C mutations are  $\alpha_1$ (S206I),  $\alpha_2$ (S205I),  $\alpha_3$ (S230I),  $\alpha_4$ (I204S),  $\alpha_5$ (S209I), and  $\alpha_6$ (N204I). Points are mean  $\pm$  SEM and where SEM is smaller than symbols, it is not visible. Sample sizes (cells per group) are: 9–14 ( $\alpha_1$ ), 7–10 ( $\alpha_2$ ), 6–17 ( $\alpha_3$ ), 6–9 ( $\alpha_4$ ), 7–11 ( $\alpha_5$ ), and 6–8 ( $\alpha_6$ )



**FIGURE 4** Although  $\alpha_2$ - and  $\alpha_3$ -containing  $\alpha_x\beta_2\gamma_2$  receptors had similar GABA apparent-affinities ( $\alpha_2$ :  $EC_{50} = 8.29 \pm 0.78 \mu\text{M}$ ;  $\alpha_3$ :  $EC_{50} = 15.53 \pm 2.55 \mu\text{M}$ ), they showed different degrees of midazolam potentiation when the conserved serine in loop C of the  $\alpha$  subunit was mutated to an isoleucine. (A-B) Example traces of whole-cell responses to  $EC_{10}$  GABA (black) and  $EC_{10}$  GABA +  $1 \mu\text{M}$  midazolam (gray). (A) Example trace for  $\alpha_2\beta_2\gamma_2$  and  $\alpha_2$ (S205I) $\beta_2\gamma_2$  receptors. Scale bar is 5 seconds, 500 pA. (B) Example trace for  $\alpha_3\beta_2\gamma_2$  and  $\alpha_3$ (S230I) $\beta_2\gamma_2$  receptors. Scale bar is 5 seconds, 320 pA for  $\alpha_3\beta_2\gamma_2$  and 5 seconds, 500 pA for  $\alpha_3$ (S230I) $\beta_2\gamma_2$  receptors. The dotted line marks the highest degree of midazolam potentiation for each example trace. (C) Quantifying the amplitude of maximum potentiation in the presence of  $1 \mu\text{M}$  midazolam for  $\alpha_2\beta_2\gamma_2$ ,  $\alpha_2$ (S205I) $\beta_2\gamma_2$ ,  $\alpha_3\beta_2\gamma_2$  and  $\alpha_3$ (S230I) $\beta_2\gamma_2$  receptors. \* $P < 0.05$  significance was determined using a two-way ANOVA with Sidak's post hoc analysis. Bars are mean  $\pm$  SEM from  $n = 7$ -8 cells per group

( $n = 6$ );  $\alpha_1\beta_2\gamma_2 = 203.0 \pm 17.6\%$  ( $n = 7$ ),  $P = 0.0403$ ). The  $\alpha_2$ (S205I) mutation reduced the maximum midazolam potentiation by approximately 31% ( $\alpha_2$ (S205I) $\beta_2\gamma_2 = 116.4 \pm 23.0\%$ ,  $n = 8$ ) compared to wild-type receptors ( $\alpha_2\beta_2\gamma_2 = 169.6 \pm 49.9\%$ ,  $n = 7$ ), but this result was not significant ( $P = 0.416$ ). The  $\alpha_3$ (S230I) mutation had the largest alteration in midazolam potentiation (Figure 4). It enhanced the degree of maximum midazolam potentiation by approximately 63% ( $\alpha_3$ (S230I) $\beta_2\gamma_2 = 436.0 \pm 39.4\%$  ( $n = 7$ );  $\alpha_3\beta_2\gamma_2 = 267.8 \pm 20.3\%$  ( $n = 7$ ),  $P = 0.0004$ ), and it increased the midazolam  $EC_{50}$  by approximately 63% ( $\alpha_3$ (S230I) $\beta_2\gamma_2 = 73.6 \pm 1.8 \text{ nM}$ ;  $\alpha_3\beta_2\gamma_2 = 46.4 \pm 7.4 \text{ nM}$ ,  $P = 0.0014$ ). Similarly, the  $\alpha_5$ (S209I) mutation increased the maximum degree of midazolam potentiation by approximately 63%, although this difference was not statistically significant ( $\alpha_5$ (S209I) $\beta_2\gamma_2 = 175.1 \pm 26.6\%$  ( $n = 6$ );  $\alpha_5\beta_2\gamma_2 = 107.9 \pm 20.3\%$  ( $n = 7$ ),  $P = 0.1067$ ). The  $\alpha_4$ (I204S) and  $\alpha_6$ (N204I) mutations failed to convey any notable midazolam potentiation to the receptors and no meaningful Hill parameters for midazolam concentration-response curves could be estimated (Table 1). On the whole, loop C mutations showed that  $\alpha_1$ (S206I) $\beta_2\gamma_2$  and  $\alpha_2$ (S205I) $\beta_2\gamma_2$  receptors had a decreased maximal midazolam potentiation and the  $\alpha_3$ (S230I) $\beta_2\gamma_2$  and  $\alpha_5$ (S209I) $\beta_2\gamma_2$  receptors had an increased maximal potentiation.

Overall, mutating the threonine (loop B) and serine (loop C) residues failed to dramatically abolish the ability of  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors to be modulated by midazolam, as has been established for the critical histidine in loop A. Mutations in loop C had a novel array of effects on midazolam efficacy, particularly for  $\alpha_3$ - and  $\alpha_5$ -containing GABA<sub>A</sub> receptors.

## 4 | DISCUSSION

Midazolam is a benzodiazepine used to induce sedation and anaesthesia.<sup>23</sup> The therapeutically relevant range of midazolam measured from plasma is 75 ng/mL (207 nM, postoperative drowsiness) to 350 ng/mL (966 nM, anesthetized state).<sup>26-28</sup> PAM benzodiazepines were initially thought to enhance the activity of GABA<sub>A</sub> receptors by altering the GABA binding steps,<sup>29</sup> but more recent models have focused on gating mechanisms.<sup>30,31</sup> The structural loops, loops A-C within the  $\alpha$  subunit, define half of the benzodiazepine site on GABA<sub>A</sub> receptors. Understanding how different parts of the benzodiazepine site interact with modulators will help us better define the precise molecular mechanisms of these drugs.

In this study, we examined the role of the histidine in loop A, threonine in loop B, and serine in loop C within the  $\alpha$  subunit and how these residues affected the allosteric potentiation of the GABA<sub>A</sub> receptor by midazolam. The histidine-to-arginine loop A mutation provided an example of how a single residue mutation can dramatically alter the efficacy of midazolam potentiation. The loop B threonine and loop C serine are highly conserved across  $\alpha$  subunits, except in  $\alpha_4$  and  $\alpha_6$  subunits which are generally insensitive to classic benzodiazepines.<sup>22,32</sup> We predicted that the presence of a proline in loop B and isoleucine in loop C would decrease the degree of potentiation of the  $\alpha_x\beta_2\gamma_2$  GABA<sub>A</sub> receptors by midazolam. Overall, the mutation of the conserved threonine-to-proline in loop B had subtle effects on midazolam potentiation. The serine-to-isoleucine mutation in loop C altered the efficacy of midazolam potentiation, especially for  $\alpha_3$ - and  $\alpha_5$ -containing receptors.



Across the 18 mutations made in loops A-C within the benzodiazepine site, only subtle changes were seen in GABA apparent-affinity. Since the mutation was away from the GABA binding site, it is unlikely the mutations caused a structural rearrangement of the extracellular domain that affected the channel's activation. The  $\alpha 6$  (N204I) mutant increased the GABA's apparent-affinity, but this was not sufficient to make the receptor any more responsive to midazolam than the wildtype  $\alpha 6$ -containing receptors. On the whole, our results were consistent with mutations that had minimal effects on GABA's normal actions at the mutated receptor.

It is well-established that the conserved histidine present in loop A (FFHNG) of the  $\alpha$  subunit is important in determining the molecular<sup>6,11,33</sup> and behavioral<sup>1</sup> effects of benzodiazepines. This histidine is present in the  $\alpha$  subunits sensitive to positive benzodiazepines, but in  $\alpha 4$  and  $\alpha 6$  isoforms that are insensitive, an arginine is present that sterically inhibits benzodiazepines from interacting properly with the receptor.<sup>15,22</sup> In our study, the histidine-to-arginine mutations in  $\alpha 1$ -3 and  $\alpha 5$  abolished midazolam potentiation, consistent with prior studies using diazepam.<sup>11</sup> Conversely, mutating the conserved arginine-to-histidine in  $\alpha 4$  and  $\alpha 6$  conferred midazolam potentiation capabilities to  $\alpha 4$ (R100H)- and  $\alpha 6$ (R100H)-containing  $\alpha_3\beta_2\gamma_2$  receptors. These results provided an example of how a single residue mutation could dramatically alter the efficacy of midazolam potentiation across receptors containing  $\alpha 1$ -6 isoforms.

One difficult aspect of measuring midazolam potentiation is choosing a drug application time that is sufficient to reach peak activation but avoids excessive desensitization and incomplete washout between applications. The 3 second midazolam exposure time used here, tried to balance these concerns while staying consistent across all receptor combinations used. Although incomplete peak responses at middle midazolam concentrations could result in a slightly underestimated  $EC_{50}$ , the conclusions drawn here focus on the maximum potentiation measured at saturating midazolam concentrations as an estimation of drug efficacy.

In this study, the threonine (GSYAYTR, loop B) and serine (SSTGEYV, loop C) mutations had more subtle effects on midazolam potentiation than the  $\alpha 1$ (H102R) mutation. Our loop B results showed that only the  $\alpha 1$ (T163P) mutation decreased the maximum amplitude of midazolam potentiation as predicted. Of the other loop B mutations,  $\alpha 3$ (T187P) only slightly decreased the maximum potentiation, while  $\alpha 5$ (P166T) slightly increased it. Our results were consistent with the threonine in loop B conferring slightly higher midazolam efficacy to the receptor than the proline. This is consistent with a proline-to-threonine mutation in  $\alpha 5$  and  $\alpha 6$  that moderately increased zolpidem<sup>21</sup> and diazepam<sup>15</sup> binding affinities in previous studies.

The loop C mutation had more obvious changes in the efficacy of midazolam potentiation. The wildtype  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\alpha 5$  subunits all contain the homologous Ser206 (human  $\alpha 1$ ) that we predicted would reduce midazolam potentiation when mutated to an isoleucine. Surprisingly, the results did not follow the predicted pattern. In the  $\alpha 1$ (S206I) and  $\alpha 2$ (S205I) mutants, the isoleucine decreased midazolam's maximum potentiation by 31-33%, but in  $\alpha 3$ (S230I) and  $\alpha 5$

(S209I), it increased midazolam's potentiation by approximately 63%. Only  $\alpha 3$ (S230I) significantly ( $P < 0.05$ ) altered midazolam's  $EC_{50}$ . In the case of an allosteric modulator, an altered  $EC_{50}$  might be caused by changes in the modulator's ability to bind and interact with the receptor or the modulator's ability to alter GABA's binding and gating of the channel.<sup>34</sup> As mentioned above, only modest changes in GABA apparent-affinity were seen for loop C mutations, suggesting that any changes in midazolam potentiation were more likely caused by an altered midazolam-receptor interaction and not global alterations in structure that transmitted to the GABA binding site.

Loop C is important for ligand binding because it has more mobility than the other loops<sup>35</sup> and may affect benzodiazepine ligand selectivity.<sup>36</sup> Previous studies found that the  $\alpha 6$ (Asn204) and  $\alpha 4$ (Ile203) residues (both homologous to human  $\alpha 1$ (Ser206)) were important for distinguishing the binding of negative benzodiazepines.<sup>19</sup> Ser206 also physically interacts with diazepam in  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 5$ , suggesting a critical role in benzodiazepine action.<sup>37</sup> However, a neighboring mutation, homologous to  $\alpha 1$ (T207C), specifically altered benzodiazepine efficacy and not binding.<sup>12</sup> We propose that the homologous Ser206 in loop C may provide an important point of contact between the ligand and benzodiazepine site that affects the coupling of the benzodiazepine site to GABA activation, thereby affecting the benzodiazepine's efficacy. Because the effect of mutations in  $\alpha 3$  and  $\alpha 5$  were most dramatic, this serine may be more appropriately positioned in these subunits to alter midazolam's efficacy.

The  $\alpha 3$  and  $\alpha 5$  subunits have specific expression profiles in the brain that reflect their roles in cognitive- and limbic-related pathways. The  $\alpha 3$  subunit is expressed in the cortex, amygdala, olfactory bulb, and thalamic reticular nucleus, where  $\alpha_3\beta_2/\beta_3\gamma_2$  receptors mediate phasic inhibition. The  $\alpha 5$  subunit is most highly expressed in the pyramidal hippocampal cells but also in the cortex and hypothalamus.<sup>4,38</sup> The  $\alpha_5\beta_3\gamma_2$  receptors contribute to tonic inhibition in the hippocampus<sup>39</sup> and have increasingly been studied for their role in cognition<sup>40,41</sup> and anesthetic-induced neurotoxicity.<sup>42</sup>

In our results, the greatest increase in midazolam's efficacy was seen with the  $\alpha 3$ (S230I) loop C mutation. The wildtype  $\alpha 3$ -containing receptors were the most sensitive to modulation by midazolam with the lowest midazolam  $EC_{50}$  and highest maximum potentiation relative to the other  $\alpha$  subunits. This is consistent with previous studies where diazepam and flunitrazepam potentiated  $\alpha_3\beta_1\gamma_2$  receptors more than  $\alpha_1\beta_1\gamma_2$  receptors.<sup>43,44</sup> Even with the higher wildtype levels of midazolam potentiation, the  $\alpha 3$ (S230I) loop C results were still notable. The  $\alpha 3$ (S230I) mutation in loop C dramatically increased the efficacy of midazolam potentiation compared to  $\alpha 2$ (S205I) (Figure 4) despite both  $\alpha_2\beta_3\gamma_2$  and  $\alpha_3\beta_3\gamma_2$  wildtype receptors having similar GABA apparent-affinities (Figure S2). This novel finding underlines the importance of better understanding the differences in allosteric modulation of GABA<sub>A</sub> receptors expressing  $\alpha 3$  compared to other  $\alpha$  subunits. For example, nonhypnotic drugs targeting the  $\alpha 2$  and  $\alpha 3$  subunits have been studied for their anxiolytic and analgesic effects.<sup>41,45</sup> However, creating ligands that distinguish these two subunits remains difficult, as shown when an

“ $\alpha$ 3-specific” PAM (SB-205384) was found to potentiate  $\alpha$ 6-containing GABA<sub>A</sub> receptors even more strongly than  $\alpha$ 3.<sup>46</sup> Another way to distinguish different GABA<sub>A</sub> receptor subtypes is through the  $\gamma$  subunit. Although other  $\gamma$  subunits can form benzodiazepine-sensitive receptors, the  $\gamma$ 3 subunit is less prevalent (~14% of receptors),<sup>47</sup> and the  $\gamma$ 1 subunit notably reduces the benzodiazepine affinity of the receptor.<sup>48</sup> The  $\gamma$ 2 subunit is the major  $\gamma$  isoform expressed in native GABA<sub>A</sub> receptors,<sup>49</sup> and thus  $\alpha$ <sub>3</sub> $\beta$ 2 $\gamma$ 2 receptors provide a reasonable estimate of benzodiazepine efficacy in the brain. Based on our results, loop C might be a potential target for developing novel drugs that specifically modulate  $\alpha$ 3- and  $\alpha$ 5-containing GABA<sub>A</sub> receptors using PAMs targeting the allosteric benzodiazepine site.

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## AUTHOR CONTRIBUTIONS

Participated in research design: O.A. Moody, A. Jenkins. Conducted experiments and data analysis: O.A. Moody. Wrote or contributed to the writing of the manuscript: O.A. Moody, A. Jenkins.

## DISCLOSURES

None declared.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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