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Letter

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Synthesis and Biological Evaluation of Pyrroloindolines as Positive Allosteric Modulators of the α1β2γ2 $GABA_A$ Receptor

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ABSTRACT: γ-Aminobutyric acid type A ($GABA_A$) receptors are key mediators of central inhibitory neurotransmission and have been implicated in several disorders of the central nervous system. Some positive allosteric modulators (PAMs) of this receptor provide great therapeutic benefits to patients. However, adverse effects remain a challenge. Selective targeting of $GABA_A$ receptors could mitigate this problem. Here, we describe the synthesis and functional evaluation of a novel series of pyrroloindolines that display significant modulation of the $GABA_A$ receptor, acting as PAMs. We found that halogen incorporation at the C5 position greatly increased the PAM potency relative to the parent ligand, while substitutions at other positions generally decreased potency. Mutagenesis studies suggest that the binding site lies at the top of the transmembrane domain.

KEYWORDS: $GABA_A$ receptor, Cys-loop, positive allosteric modulator, ion channel, pyrroloindoline.

INTRODUCTION γ-Aminobutyric acid type A ($GABA_A$) receptors are key mediators of central inhibitory neurotransmission, and as such these receptors have been drug targets for numerous central nervous system (CNS) disorders.[1–3] The $GABA_A$ receptor is an anion-selective, pentameric, ligand-gated ion channel that is part of the larger Cys-loop receptor family. A functional receptor results from the assembly of five homologous subunits. A total of 19 homologous subunits exist, and they assemble into at least 30 different functional subtypes *in vivo*.[4] Some types, including those comprised of α1β2γ2 subunits, are predominantly expressed at the post-synaptic termini and mediate phasic inhibition, while others are located at extrasynaptic sites and mediate tonic inhibition.[4–6] The large diversity of subtypes and differential localization in the brain emphasize their importance, but also present a challenge, as current $GABA_A$ receptor therapeutics modulate a broad range of subtypes, which can result in adverse effects.

Each $GABA_A$ subunit consists of an N-terminal extracellular domain (ECD), a transmembrane domain (TMD) that comprises four transmembrane α-helices (M1-M4), an extracellular M2-M3 loop and C-terminus, and an intracellular domain composed predominantly of the M3-M4 loop.[7] Receptor activation occurs upon binding of an agonist to the orthosteric site, which is located in the ECD at the β+/α- subunit interfaces. This activation can be modulated by additional binding of other ligands to several allosteric sites on the pentameric complex.[8] Positive allosteric modulators (PAMs) potentiate the evoked response by an agonist, while negative allosteric modulators (NAMs) inhibit that response.[9] Over the years various modulators of $GABA_A$ receptors have been identified and several of the positive allosteric modulators are widely used to treat anxiety and panic disorders.[9,10]

Although $GABA_A$ receptor modulators have a proven therapeutic benefit, adverse effects remain a problem.[11,12] Additionally, elucidating functions of individual subtypes is crucial for a better understanding of the $GABA_A$ receptor's role in health and disease. Therefore, recent efforts have focused on finding subtype-selective modulators. Various novel modulators have been derived for the α+/β- interface.[13,14] For example, a series of pyrazolopyridinones developed by Blackaby *et al.* showed increased selectivity for α3β3γ2 over α1β3γ2.[15] Two different series of pyrazoloquinolinones exhibited selectivity for α6β3γ2 and β1-containing receptors, respectively.[16,17]

Physostigmine (**1**, Figure 1), also known as eserine or antilirium, is a reversible acetylcholinesterase inhibitor[18] that has been used to treat glaucoma and delayed gastric emptying.[19,20] In addition, it has been found to potentiate and inhibit nicotinic acetylcholine receptors, another member of the Cys-loop receptor family.[21–23]

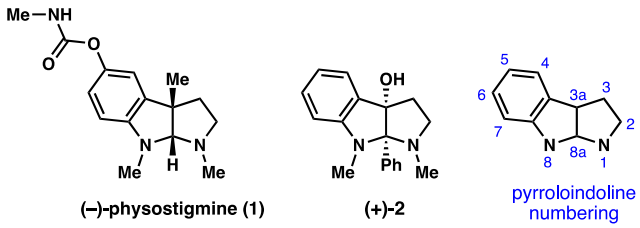
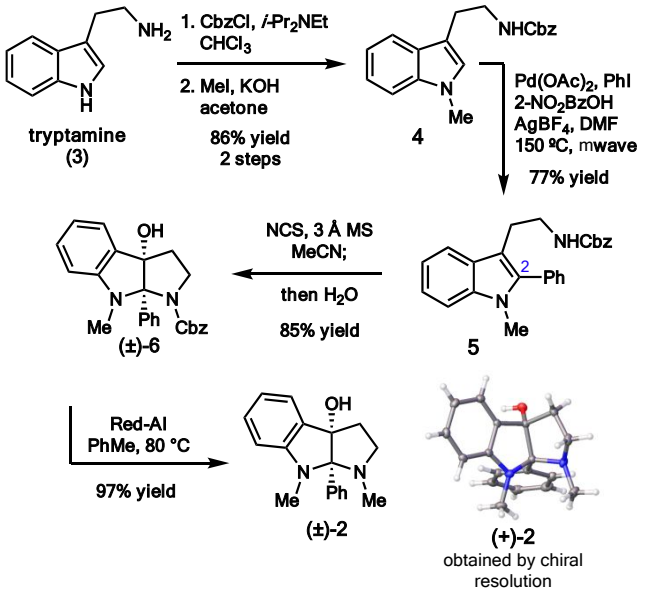


Figure 1. Chemical structures of selected pyrroloindolines.

As a result of our interest in the synthesis of pyrroloindoline natural products, we have prepared a number of new, non-natural pyrroloindoline compounds.[24–26] Given their structural similarity to other modulators of Cys loop receptors, we screened a representative collection of these structures, and found that compounds bearing aryl substitution at C8a can act as PAMs of $GABA_A$ receptors.[27,28] Here, we report the synthesis of pyrroloindoline (+)-**2** (Figure 1) and modification of this scaffold by substitution at N1, C3a, C5 and C8a, yielding a novel series of $GABA_A$ receptor ligands. All of the compounds were tested for agonism and allosteric modulation properties at the human α1β2γ2 $GABA_A$ receptor, the most abundant $GABA_A$ subtype in the adult brain, expressed in *Xenopus laevis* oocytes via two-electrode voltage clamp electrophysiology. Additionally, we performed mutagenesis experiments to identify the binding site of these ligands.

RESULTS AND DISCUSSION The synthesis of the pyrroloindoline framework commenced with protection of tryptamine (**3**) to provide carbamate **4** (Scheme 1). Pd-catalyzed C2 arylation with iodobenzene under microwave conditions gave 2-phenyl tryptamine **5** in 77% yield.[29] Various approaches were investigated for effecting oxidative cyclization of **5**.[30] Although there are many examples of related cyclizations of tryptamine and tryptophan derivatives,[24,31–35] we found that many of these conditions were unsuitable for tryptamine **5**, presumably due to the phenyl substituent at C2. After extensive experimentation, it was found that oxidative cyclization of **5** by treatment with *N*-chlorosuccinimide followed by water afforded C3a-hydroxy pyrroloindoline (±)-**6** in 85% yield. Reduction of carbamate (±)-**6** with Red-Al provided the *N*1-methyl pyrroloindoline (±)-**2**.[31] Attempts to render the cyclization of **5** to **6** enantioselective have thus far been unsuccessful;[35] however, the enantiomers of both compounds (±)-**6** and (±)-**2** can be resolved using preparative SFC with a chiral stationary phase. X-ray crystallography confirmed the structure and absolute stereochemistry of (+)-**2**.

Scheme 1. Synthesis of the pyrroloindoline scaffold.



In a preliminary screen, five pyrroloindoline compounds including (±)-**2**, were tested for modulation of eight pentameric ligand-gated ion channels (pLGICs): muscle type nAChR, α4β2 nAChR, α7 nAChR, 5-HT_{3A} receptor, α1β2γ2 $GABA_A$ receptor, α1β2 $GABA_A$ receptor, GluR2, and the glycine receptor. This assay identified pyrroloindoline (±)-**2** as a potent PAM of the α1β2γ2 $GABA_A$ receptor (Table S1 and Figure S1).[27,28] Although no $GABA_A$ receptor activity has been previously reported for physostigmine, compound (±)-**2** appears to selectively potentiate α1β2γ2 $GABA_A$ receptors over other Cys-loop receptors.

Based on the selective PAM profile of (±)-**2**, we decided to further characterize this ligand. We set out to determine if both enantiomers are active at the α1β2γ2 $GABA_A$ receptor. Enantiomer-specific effects would imply a specific drug-receptor interaction, rather than some more generic effect such as altering membrane properties. To assess functional effects, we used a similar two-electrode voltage clamp protocol to one previously described by Marotta *et al*.[28] Briefly, the current responses of three identical EC_{50} doses of GABA were recorded, followed by a dose of the test-ligand at 40 µM. After a 30 s incubation, a dose was applied containing both GABA at its EC_{50} and the test-ligand at 40 µM. Finally, two doses of GABA EC_{50} were applied. The first three GABA doses establish a baseline of the GABA response at that concentration, and the purpose of the last two GABA doses is to verify proper functioning of the receptor post modulation and control for independent rise in current amplitude. Of the two (±)-**2** enantiomers, only (+)-**2** showed a meaningful potentiation of the EC_{50} GABA dose, with a mean of 16 ± 4.1%, as shown in Figure 2B.

To determine activity at the α1β2 subtype and consequent involvement of the γ2 subunit in potentiation, we performed the same experiment for this subtype. For (±)-**2** a mean potentiation of 28 ± 5.2% was observed (Figure 2). Similar to the observations for the α1β2γ2 subtype, (+)-**2** showed increased potentiation over (–)-**2** with mean values of 17 ± 2.6% and 9.2 ± 1.1% respectively (Figure 2B and Table S2). These results demonstrate that the γ2 subunit is not required for potentiation of the α1β2γ2 receptor by (±)-**2.**

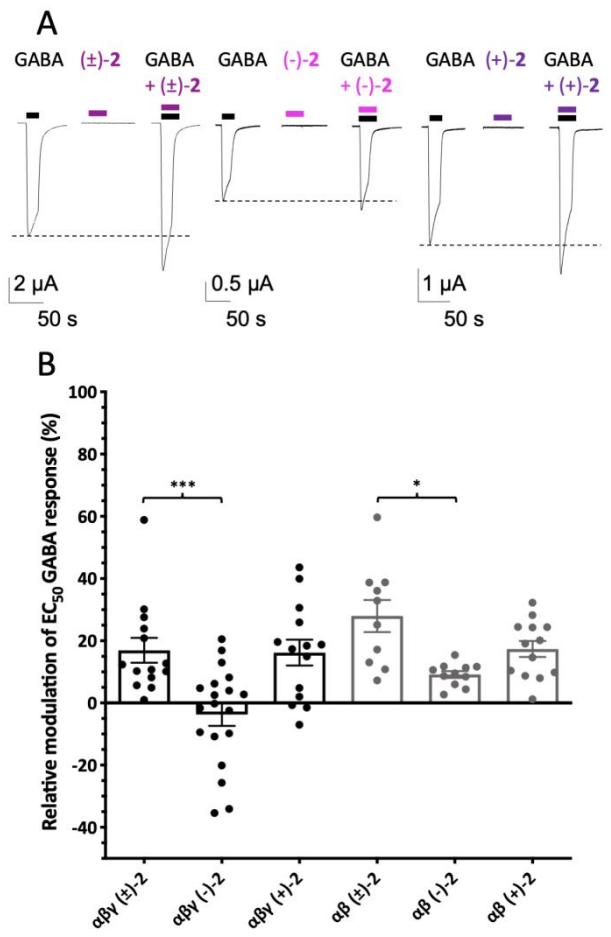


Figure 2. Functional effects of pyrroloindoline (±)-**2** on the α1β2γ2 and α1β2 $GABA_A$ receptor subtypes. A) Wave forms of the α1β2γ2 current responses from a GABA EC_{50} only dose, 40 μM (±)-**2** only dose, and co-application of GABA EC_{50} and 40 μM (±)-**2**. B) Relative modulation of a GABA EC_{50} response of the α1β2γ2 and α1β2 $GABA_A$ receptor subtypes by (±)-**2** and the individual enantiomers. *p <0.05; ***p <0.001 (one-way ANOVA).

The amplitude of potentiation is dependent on several factors, among which are both the PAM concentration and the GABA concentration at which we tested the modulation. Next, we determined the effect of 40 μM (±)-**2** on the GABA EC_{50} (ΔEC_{50}((±)-**2**)) at the α1β2γ2 receptor. The observed (±)-**2**-induced shift in GABA EC_{50} is 13 μM as shown in Figure 3A and Table S3. This shift is comparable to the induced shift seen for this subtype by the benzodiazepine Triazolam, 16-50 μM.[36] Moreover, we wanted to determine the potency of the pure enantiomer (+)-**2**. Well-studied modulators, such as flurazepam and zolpidem, have EC_{50}s in the nanomolar range when co-applied with GABA $EC_{2\text{-}5}$, being 270 nM and 340 nM, respectively.[37] The PAM tested here, (+)-**2**, appears to be less potent with an EC_{50} of 110 μM when co-applied with GABA EC_5, as shown in Figure 3B and Table S3.

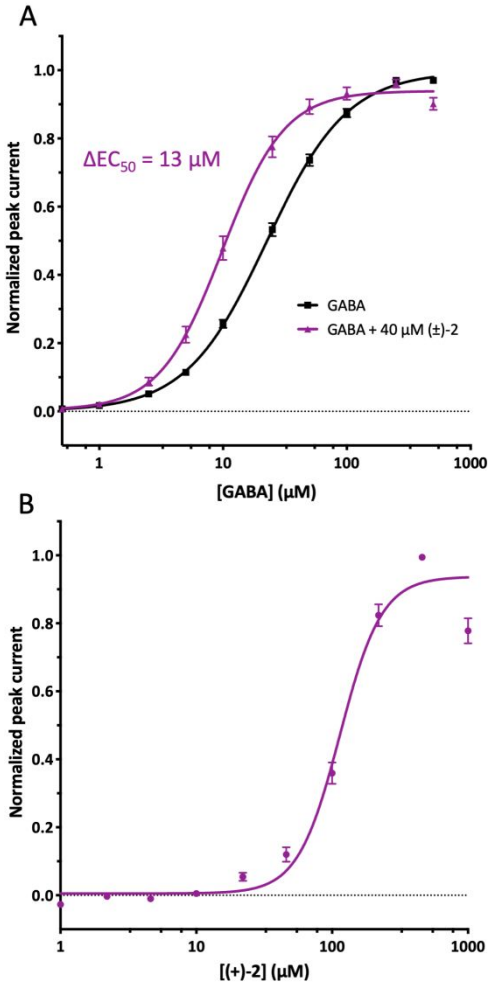


Figure 3. Functional characterization of pyrroloindoline (±)-**2** at the α1β2γ2 $GABA_A$ receptor. A) The (±)-**2**-induced shift in GABA EC_{50}. A 40 μM concentration of (±)-**2** was used here. B) (+)-**2** EC_{50} co-applied with GABA EC_5 doses. The peak current at GABA EC_5 was subtracted from all responses.

Having established that pyrroloindoline (+)-**2** acts as a PAM on the α1β2γ2 $GABA_A$ receptor, further potentiation experiments used the GABA $EC_{10\text{-}15}$ instead of EC_{50}. Using the $EC_{10\text{-}15}$ allows for a larger potentiation window than EC_{50}, which enables the detection of more subtle functional differences between $GABA_A$ mutants or pyrroloindoline analogues. Figures 2B and 4C illustrate this difference in modulation potency for (±)-**2**. For the α1β2γ2 subtype, (±)-**2** causes a 62% potentiation of the GABA EC_{10} response, while at the GABA EC_{50} this is only 17%.

GABA activates the $GABA_A$ receptor through binding in the ECD at the interface of the β+/α- subunits. Besides this orthosteric site, several allosteric binding sites have been established, of which the benzodiazepine site (BZ) in the ECD at the α+/γ- interface is the most well-known.[38] More recently, a distinct binding site in the ECD at the α+/β- interface has been identified for the ligand CGS9895.[39,40] In addition to binding sites in the ECD, several anesthetics and neurosteroids affect channel activity through binding in the TMD. Recent X-

ray crystal structures and cryo-EM structures have shed light on the TM residues involved in binding.[41,42]

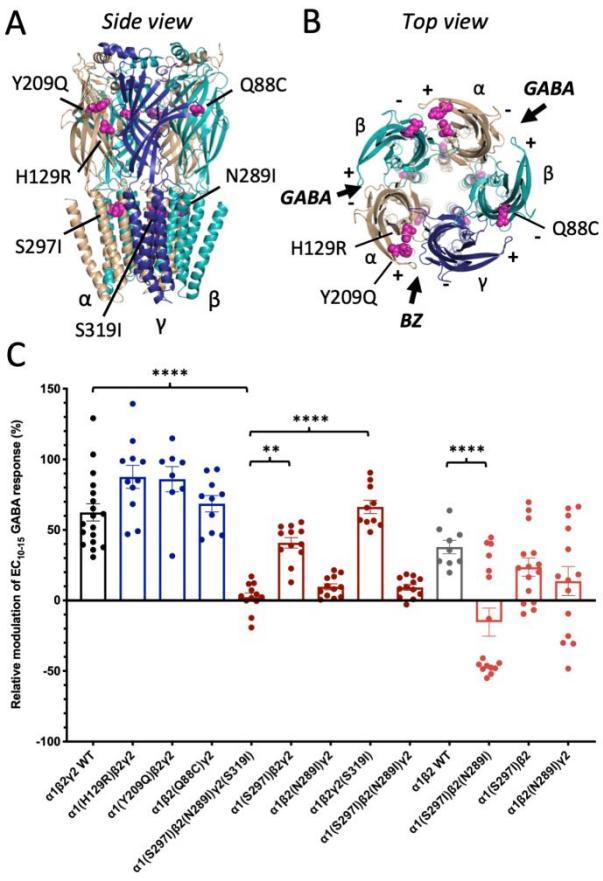


Figure 4. Functional effects of pyrroloindoline (±)-**2** on α1β2γ2 and α1β2 $GABA_A$ receptor mutants. A) Side view of the human α1β2γ2 $GABA_A$ receptor with the probed residues highlighted in pink (PDB ID: 6D6T). B) Extracellular view into the pore with GABA and BZ sites indicated with arrows. C) Relative modulation of $GABA_{10-15}$ responses by (±)-**2**. ECD mutants and TMD mutants of α1β2γ2 are in blue and dark red respectively. TMD mutants of α1β2 are in light red. **p <0.01; ****p <0.0001 (one-way ANOVA).

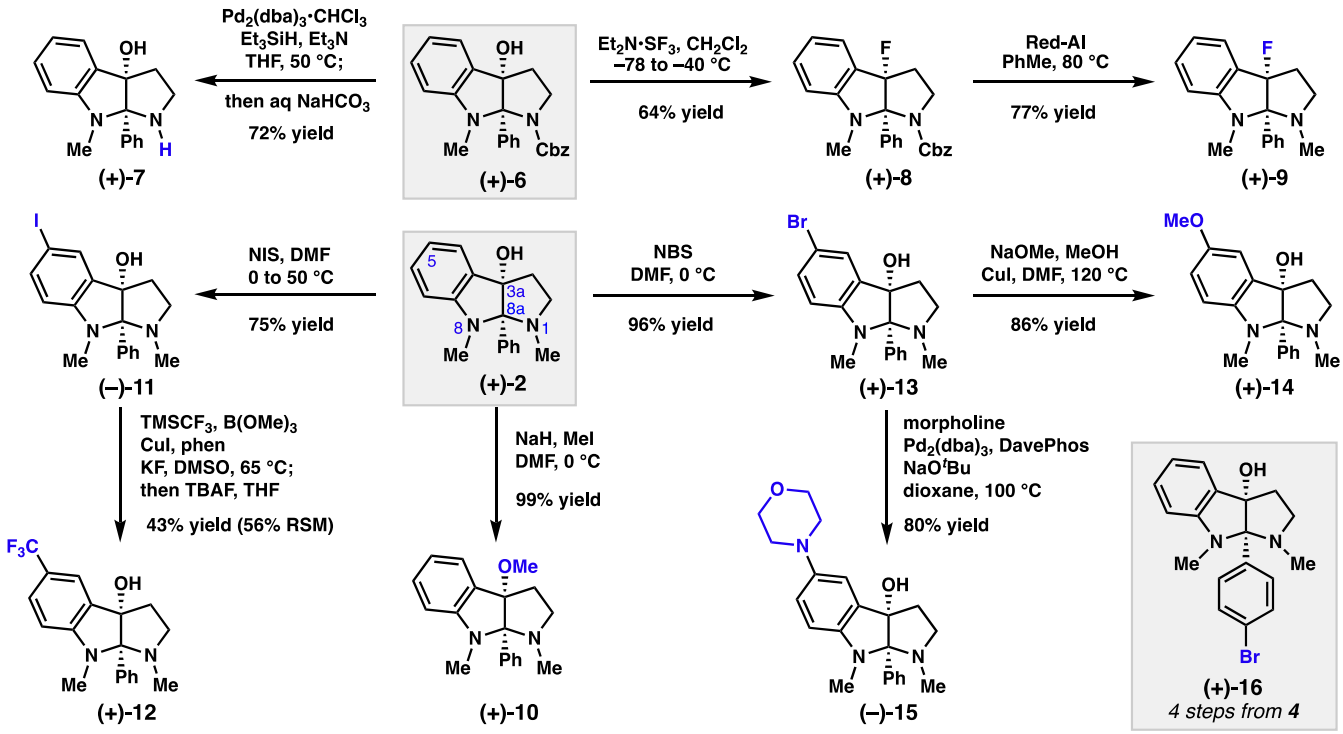
In order to determine the binding site for the PAM (±)-**2**, we performed mutagenesis on residues that have been implicated in binding of known modulators. For the first screen we selected α1(H129R)[38] and α1(Y209Q)[40,43] to probe the BZ-site, β2(Q88C) to probe the α+/β- site,[39] and triple mutant α1(S297I)β2(N289I)γ2(S319I) to probe for anesthetic sites in the TMD.[40,44] All three ECD mutants were potentiated to a similar extent as the WT receptor (mean 62 ± 6.1%). However, the triple TMD mutant was not affected by (±)-**2** (mean 1.0 ± 3.0%) as shown in Figure 4C and Table S4. These results indicate that (±)-**2** does not assert its potentiating affects through binding at the interfaces in the ECD, but on one or more interfaces in the TMD.

To determine which specific interfaces are involved in binding in the TMD, we performed potentiation experiments for the single and double mutants of α1β2γ2, as well as the α1β2 subtype (Figure 4B). The mean potentiation in both mutants with single mutations in the α1 and γ2 subunits resembles that of the α1β2γ2 WT receptor. Only the single and double mutant receptors that contain a mutation in the β2 subunit demonstrate greatly reduced potentiation, suggesting involvement of the β2 subunit in binding. For the α1β2 subtype, greater variability among the mutants has been observed, but the same general trend appears. The β2(N289I) mutation is located in the TMD, close to the top of TM2, at the β+/α- interface (Figure 4A). This residue has been implicated in the binding of several anesthetics, such as etomidate, propofol, and loreclezole.[40,45]

We performed potentiation experiments on these $GABA_A$ mutants similarly as described in Figure 2, however here we co-applied the 40 μM PAM with an EC_{10-15} dose of GABA. To verify the EC_{10-15} values of the constructed mutant receptors, we determined the full dose-response relationships and found EC_{50} values similar those reported previously. (Table S5).[40,44]

Having characterized lead compound (+)-**2**, we aimed to optimize the potency of this ligand family by way of exploring substitutions at N1, C3a, C5, and C8a. Derivatization of the pyrroloindoline scaffold was undertaken with enantioenriched compounds **2** and **6** (Scheme 2). For each of the analogues synthesized, both enantiomers were prepared for evaluation of their ability to modulate $GABA_A$ receptors; however, for simplicity, the chemistry is depicted on the (+) enantiomers of **2** and **6** in Scheme 2. From carbamate (+)-**6**, Pd-catalyzed deprotection of the Cbz group afforded the *N*1–H pyrroloindoline (+)-**7**. Derivatization of C3a was examined to interrogate the possibility of the C3a hydroxyl group acting as a hydrogen bond donor. Deoxyfluorination of (+)-**6** using diethylaminosulfur trifluoride followed by carbamate reduction with Red-Al furnished tertiary fluoride (+)-**9**. Methylation of the C3a hydroxy group of (+)-**2** under standard conditions gave C3a-methoxy pyrroloindoline (+)-**10**.

Scheme 2. Derivatization of the pyrroloindoline framework.



Recognizing that physostigmine (**1**) possesses oxidation at C5, we also sought to derivatize this position of the pyrroloindoline framework with both electron-donating and electron-withdrawing substituents. Electrophilic aromatic substitution of (+)-**2** with *N*-iodosuccinimide afforded aryl iodide (–)-**11** (Scheme 2). Cu-catalyzed trifluoromethylation of the iodoarene gave (+)-**12**.[46] Bromination was also feasible using *N*-bromosuccinimide to furnish (+)-**13**. From the aryl bromide, Cu-catalyzed methoxylation provided methoxy analogue (+)-**14**.[47] Additionally, Buchwald-Hartwig coupling of aryl bromide (+)-**13** gave morpholine (–)-**15**.[48]

We also sought to introduce structural variations on the C8a aryl group. Using 1-bromo-4-iodobenzene, Pd-catalyzed C2 arylation of protected tryptamine **4** gave an aryl bromide analogue that was advanced to pyrroloindoline (±)-**16** (see Scheme S1 for synthetic details).

Functional evaluation of the series of pyrroloindoline derivatives was conducted using two-electrode voltage clamp electrophysiology as described earlier for (±)-**2.** General trends will be discussed first. Most of the derivatives do not demonstrate agonist behaviors, except for compounds (–)-**11** and (+)-**13**, which only activated the receptor with very low efficacy (Figure 6A). Generally, all derivatives demonstrate a similar activity pattern for the two enantiomers as we have observed for (±)-**2**, with only the *S,S*-enantiomer demonstrating activity. One exception to this is the aryl bromide **16**, for which both enantiomers show substantial potentiation. It is also worth noting that morpholines (+)-**15** and (–)-**15**, and aryl iodide (+)-**11** and (–)-**11**, have reversed signs for their optical rotations as compared to all other derivatives.

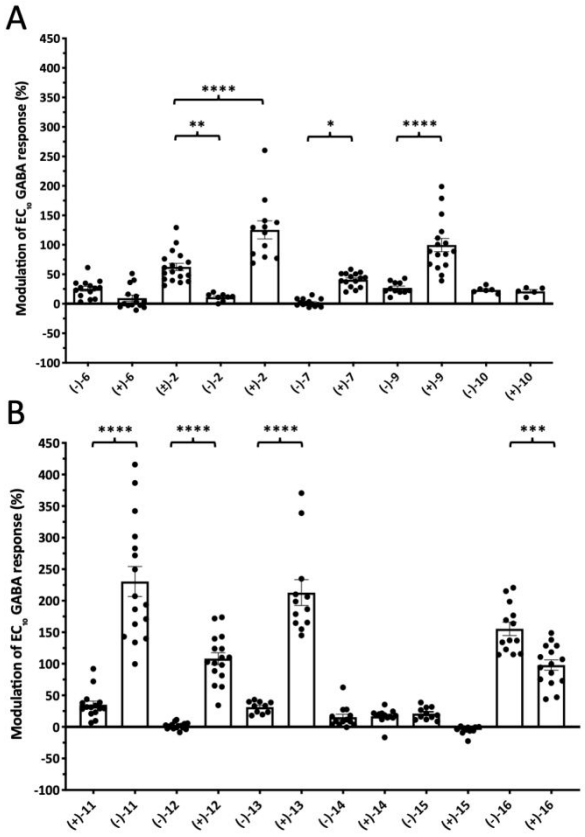


Figure 5. Functional effects of pyrroloindoline derivatives at the α1β2γ2 $GABA_A$ receptor. A) N1, C2, C3-substituted derivatives. B) C5-substituted derivatives. *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001 (one-way ANOVA).

Changes at *N*1 resulted in decreased potentiation relative to the enantiomerically pure parent ligand (+)-**2** (125 ± 15%), with the *N*1-protio compound (+)-**7** and the *N*1-Cbz compound (+)-**6** giving potentiation values of 41 ± 3.0% and 9.6 ± 5.2%, respectively (Figure 5A and Table S6). Substitution of the C3a hydroxyl with fluorine ((+)-**9**) gives similar potentiation (100 ± 11%) to (+)-**2**, whereas methylation of the hydroxyl group ((+)-**10**) results in a substantial reduction in activity (21 ± 3.0%).

Next, we looked at the C5-substituted derivatives (Figure 5B and Table S6). The presence of a methoxy ((+)-**14**) or morpholino ((+)-**15**) substituent at C5 drastically reduced the potentiation efficacy to 17 ± 3.2% and –4.8 ± 1.8% respectively. Potentiation by trifluoromethyl compound (+)-**12** resembled that of (+)-**2** at 108 ± 9.5%. Surprisingly, introduction of a halogen (Br or I) at C5 greatly increased potentiation with a modulation of 213 ± 21% for (+)-**13** and 231 ± 24% for (–)-**11** respectively (Figure 5B and 6A). This structure-activity relationship could indicate the presence of a halogen bonding binding interaction. These two ligands appear to have the largest potentiation effects on the α1β2γ2 $GABA_A$ receptor at 40 μM of all the derivatives evaluated here. Therefore, we attempted to determine a full dose-response relationship for these two PAMs, however solubility problems at concentrations greater than 100 μM prevented this. (Figure S2) Additionally, we determined the GABA ΔEC_{50} shift due to 40 μM (–)-**11** or (+)-**13** at the α1β2γ2 receptor; we observed a 9 μM and 16 μM shift for (–)-**11**- and (+)-**13** respectively (Figure 6B and Table S2). These values are similar to that observed for (±)-**2.**

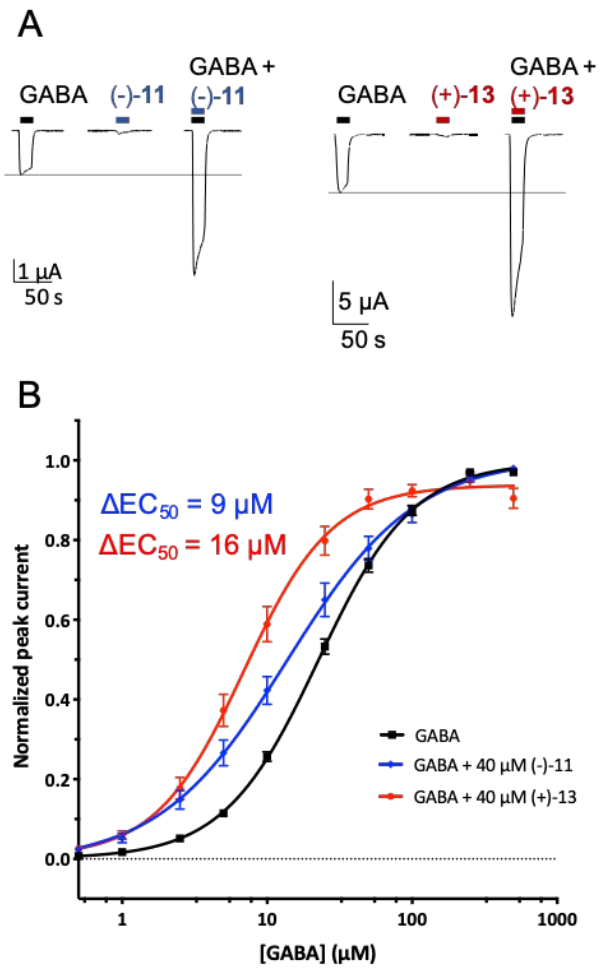


Figure 6. Functional characterization of pyrroloindoline (–)-**11** and (+)-**13** at the α1β2γ2 $GABA_A$ receptor. A) Wave forms of the α1β2γ2 current responses from a GABA EC_{10} only dose, 40 μM (±)-**11** or (+)-**13** only dose, and co-application of GABA EC_{10} and 40 μM PAM. B) PAM-induced shift in GABA EC_{50}. A 40 μM concentration of (±)-**11** and (+)-**13** was used here.

Comparing the different effects of C5 substitution and structural variations at the C8a aryl group, recall that methoxy analogue (+)-**14** did not exhibit any PAM properties, nor did methoxyarene (±)-**SI-5** (Table S5). However, bromide (+)-**13** demonstrated increased PAM properties relative to (+)-**2**. Considering the spatial positioning of the bromine at C5, we asked whether a ligand with a 4-Br-Ph at C8a would also possess PAM properties. Indeed, both aryl bromides (+)-**16** (98 ± 8.3%) and (–)-**16** (155 ± 11%) showed comparable potency to (+)-**2** (Figure 5B and Table S6). It is surprising that both enantiomers of **16** are active, and we hypothesize that the *R,R*-enantiomer (–)-**16** might be able to bind in an "upside down" orientation, in which the bromine occupies the same position as the bromine of ligand (+)-**13**. Figure 7 depicts an overlay of the two chemical structures, (+)-**13** and (–)-**16,** to illustrate this. (–)-**16** is shown at a slightly rotated orientation (looking down C3a and C8a instead of C2) to mimic the orientation of (+)-**13**, which indeed resembles this structure. These results indicate that not only is the *para*-position of the C8a-phenyl substituent permissive to halide substitution, but it is possible that (–)-**16**, the *R,R*-enantiomer, is able to fit the

binding pocket in the upside down orientation, unlike the other ligands tested in this study.

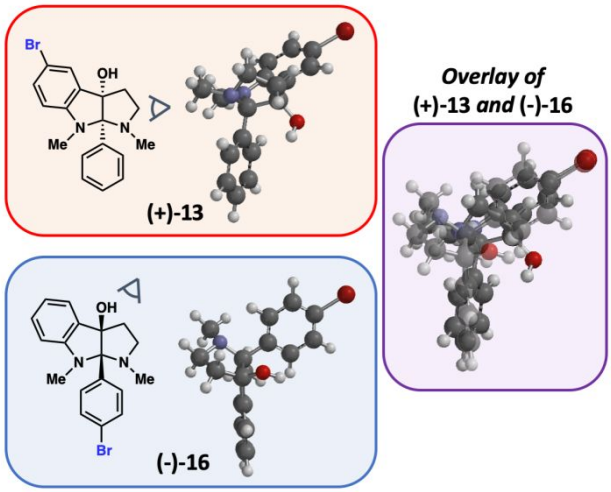


Figure 7. Overlay of chemical structures of (+)-**13** and (–)-**16**.

CONCLUSION In this work, we described the synthesis of a series of pyrroloindoline compounds and functional evaluation for positive allosteric modulation at the α1β2γ2 $GABA_A$ receptor. First, we characterized the lead positive allosteric modulator (±)-**2**, which has an EC_{50} of 110 μM and causes a 13 μM shift in GABA EC_{50}. Second, we performed mutagenesis studies to elucidate the binding site of this PAM. We found that the TMD triple mutant α1(S297I)β2(N289I)γ2(S319I) completely lost sensitivity to (±)-**2**, while ECD mutants displayed no meaningful change. This strongly suggests that the binding site is located in the TMD at the top of TM2.

Next, we explored substitution of **2** at various positions to increase efficacy. Like the parent compound, most ligands demonstrated PAM properties only for the *S,S*-enantiomer. The most potent PAMs tested here contain a bromine or iodine at C5, (+)-**13** and (–)-**11** respectively. Ligand (±)-**16** demonstrated increased potentiation efficacy relative to the parent ligand (±)-**2** for both enantiomers. We suspect that the (–) enantiomer, which is generally inactive for other compounds, may be able to fit into the binding pocket in an upside-down orientation due to its 4-Br-Ph substitution at C2.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, compound characterization data, ^1H and ^{13}C NMR spectra. The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

$GABA_A$, γ-aminobutyric acid type A; PAM, positive allosteric modulator; pLGIC, pentameric ligand-gated ion channel; BZ, benzodiazepine; SEM, standard error of the mean.

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Synthesis and Biological Evaluation of Pyrroloindolines as Positive Allosteric Modulators of the α1β2γ2 $GABA_A$ Receptor

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