Pharmacological characterization of the excitatory 'Cys-loop' GABA receptor family in *C. elegans*

Running Title: Pharmacology of excitatory nematode GABA receptors

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Figures = 8

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Abstract

Background and Purpose

Ionotropic GABA receptors are evolutionarily conserved proteins that mediate cellular and network inhibition in both vertebrates and invertebrates. A unique class of excitatory GABA receptors has been identified in several nematode species. Despite well-characterized functions in *C. elegans*, little is known about the pharmacology of the excitatory GABA receptors EXP-1 and LGC-35. Using a panel of compounds that differentially activate and modulate ionotropic GABA receptors, we investigated the agonist binding site and allosteric modulation of EXP-1 and LGC-35.

Experimental Approach

We used two-electrode voltage clamp recordings to characterize the agonist, antagonist and allosteric modulation of EXP-1 and LGC-35 receptors expressed in *Xenopus laevis* oocytes.

Key Results

EXP-1 and LGC-35 exhibit a hybrid pharmacological profile that is unique from $GABA_A$ and $GABA_A$-ρ receptors. Both receptors are resistant to the competitive orthosteric antagonist bicuculline, and are also resistant to classical ionotropic

receptor pore blockers. The $GABA_A$-ρ specific antagonist, TPMPA, was the only compound tested that potently inhibited EXP-1 and LGC-35 current responses. Neurosteroids have nominal effect on GABA-induced currents in either EXP-1 or LGC-35 expressing oocytes. However, ethanol selectively potentiates LGC-35.

Conclusions and Implications

The pharmacological properties of EXP-1 and LGC-35 more closely resemble the ionotropic $GABA_A$-ρ family. However, EXP-1 and LGC-35 exhibit a unique profile that differs from vertebrate $GABA_A$ and $GABA_A$-ρ receptors, insect GABA receptors, and nematode GABA receptors. As a pair, EXP-1 and LGC-35 may be utilized to further understand the differential molecular mechanisms of agonist, antagonist and allosteric modulation at ionotropic GABA receptors, and may aid in the design of new and more specific anthelmintics that target GABA neurotransmission.

Tables of Links

TARGETS
Receptors[a]
$GABA_A$
$GABA_A$-ρ

LIGANDS	
alphaxalone	Mecamylamine
β-alanine	Muscimol
Bicuculline	THIP
GABA	TPMPA
Glycine	
Isoguvacine	

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d,e]Alexander et al., 2015a,b,c,d,e).*

Abbreviations

GABA (γ-aminobutyric acid); EXP-1 (expulsion defective); LGC-35 (ligand-gated ion channel)

Introduction

GABA (γ-aminobutyric acid) is the primary fast inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems (Hosie & Sattelle, 1996). Fast inhibition is produced when GABA binds and activates chloride-selective ionotropic GABA receptors. These receptors belong to the 'cys-loop' superfamily of ligand-gated ion channels, which include the nicotinic acetylcholine (nAChRs), glycine (GlyRs), and serotonin type 3 receptors ($5HT_3$Rs) (Bormann, 2000). Like all members in the superfamily, ionotropic GABA receptors consist of five subunits that pseudo-symmetrically arrange around a central ion-conducting pore (Johnston, 2005). Each subunit consists of a large extracellular N-terminal domain that contains consensus motifs that comprise the orthosteric agonist binding site, four transmembrane-spanning domains (M1-M4), and a large intracellular loop between M3-M4, which is thought to mediate receptor trafficking and subcellular localization (Michels & Moss, 2007; Thompson, Lester & Lummis, 2010). Within the receptor complex, each subunit is arranged such that the second transmembrane domain (M2) lines the ion channel pore and determines ion selectivity.

In vertebrates, ionotropic GABA receptors are divided into two classes based on their distinct oligomerization and pharmacological profiles: 1) $GABA_A$ receptors are heteropentameric (i.e. multiple different subunits) and inhibited by the competitive antagonist bicuculline; and 2) $GABA_A$-ρ receptors are homopentameric (i.e. single subunit) and bicuculline-insensitive (Bormann, 2000; Chebib & Johnston, 2000). In *Caenorhabditis elegans* (*C. elegans*), the ionotropic GABA receptor family consists of chloride-conducting inhibitory (UNC-49) and sodium-conducting excitatory receptors (EXP-1 and LGC-35) (Bamber, Beg, Twyman & Jorgensen, 1999; Beg & Jorgensen, 2003; Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015). Each of these receptors mediate

distinct and coordinated neuromuscular contractions. Specifically, the inhibitory UNC-49 receptor is required to reciprocally inhibit antagonistic body wall muscles to ensure coordinated locomotion (Bamber, Beg, Twyman & Jorgensen, 1999); whereas, the excitatory EXP-1 receptor is required for intestinal muscle contraction during defecation (Beg & Jorgensen, 2003). Expanding the unique role of excitatory GABA signaling in the *C. elegans* nervous system, LGC-35 was recently shown to act as a spillover receptor that modulates locomotor body bending and movement speed (Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015).

Although the inhibitory UNC-49 GABA receptor has been pharmacologically characterized in both free living and parasitic nematodes (Bamber, Twyman & Jorgensen, 2003; Kaji, Kwaka, Callanan, Nusrat, Desaulniers & Forrester, 2015), relatively little is known about the pharmacological profile of excitatory GABA receptors in *C. elegans*. Moreover, excitatory GABA receptor signaling not only regulates a wider variety of neuromuscular contractions that underlie specific behaviors in the nematode, but also utilizes a larger repertoire of characterized receptors (EXP-1 and LGC-35). Despite these observations, canonical inhibitory GABA signaling and pharmacological profiling of UNC-49 have been the primary focus of investigation in nematode species. Here, we characterize the pharmacological profiles of EXP-1 and LGC-35 utilizing a broad panel of agonists, antagonists and allosteric modulators that differentially regulate ionotropic $GABA_A$ and $GABA_A$-ρ receptors. We conclude that EXP-1 and LGC-35 more closely resemble the $GABA_A$-ρ receptor subfamily; however, they both exhibit unique pharmacological profiles despite their highly conserved orthosteric GABA binding sites. In addition, several characteristics distinguish these receptors from vertebrate $GABA_A$ and $GABA_A$-ρ receptors, insect GABA receptors, and nematode GABA receptors.

Taken together, our data reveal that inhibitory and excitatory GABA receptor signaling can be pharmacologically segregated, which may aid in the differential and specific modulation of nematode GABA receptor neurotransmission. Given the high amino acid sequence identity between EXP-1 and LGC-35, these receptors, as a pair, provide a useful framework to better understand the differential mechanisms of agonist, antagonist and allosteric modulation at ionotropic GABA receptors, and may aid in the design of new and more specific anthelmintic GABA mimetics.

Methods

Electrophysiology: The *C. elegans exp-1* and *lgc-35* cDNAs were isolated and cloned into the *Xenopus laevis* expression vector pSGEM (courtesy of M. Hollman) as previously described (Beg & Jorgensen, 2003; Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015). Briefly, plasmids were linearized with the restriction enzyme *MscI* and capped RNA (cRNA) was prepared using the mMessage mMachine kit (Life Technologies) following manufacturer guidelines. All animal care and experimental procedures followed the guideline from the National Institutes of Health and the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan. *Xenopus laevis* ovaries were obtained from *Xenopus1 (*Ann Arbor, MI). Briefly, female *Xenopus laevis* frogs were housed in a climate-controlled, light-regulated room. Frogs were anaesthetized by immersion in 0.2% tricaine until non-responsive to toe pinch. Subsequently, frogs were decapitated and ovarian lobes were harvested and defolliculated by incubation in 2mg/ml collagenase (Type I, Worthington, Lakewood, NJ). Defolliculated stage V-VI oocytes were sorted and injected with EXP-1 (50ng) or LGC-35 (12.5ng) cRNA. Injected oocytes were incubated for 2-5 days at 18°C in Barth's solution: 88mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, supplemented with 1mM Na^+-pyruvate, and 50mg/L gentamicin (pH = 7.4, NaOH). The

standard bath solution for dose-response experiments was Frog Ringer's: 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES (pH = 7.4, NaOH).

Electrophysiological recordings were performed 2-5 days after injection and were carried out at room temperature in Ringer's solution. Two electrode voltage clamp recordings were obtained using an OC-725C Oocyte clamp amplifier (Warner Instruments, Hamden, CT) using 3M KCl-filled microelectrodes (1-5 MΩ). All recordings were performed at a holding potential of -60 mV. Oocytes were exposed to varying concentrations of compounds using a custom-built gravity controlled perfusion system with a flow rate of ~8 mL/min. Dose-response curves were generated on single oocytes by applying compounds at increasing concentrations (low to high). Oocytes were continually perfused with control Ringer's for 2 minutes between each dose.

Data Analysis: The response of each compound dose was normalized to the maximal GABA response (3mM) of each individual recorded oocyte. To determine the overall efficacy of agonists, the maximal response of each drug tested was normalized relative to the maximal GABA response (3mM). Antagonists and allosteric modulator dose-response recordings were performed as follows. Individual oocytes were exposed to GABA alone (10μM), followed by GABA plus increasing concentrations of drug (10μM GABA + [Drug]), and control

responses were re-tested at the end of each recording with GABA alone (10μM) to confirm complete drug washout and cell health. Dose response curves from individual oocytes were normalized to the maximum values and averaged using Prism 7.0 software (GraphPad, San Diego, CA). Normalized data were fit to the four parameter Hill equation: $Y = Min + (Max\text{-}Min)/(1 + 10^{H(LogEC50 - X)})$, where *Max* is the maximal response, *Min* is the response at lowest drug concentration, *X* is the logarithm of agonist concentrations, EC_{50} is the half-maximal response, and *H* is the Hill coefficient.

Sequence Alignments: Protein sequences were aligned using the Clustal W algorithm in the MacVector software suite. The GenBank identifier (GI) numbers for the protein sequences used for comparative sequence analysis are as follows: EXP-1 (550540762), LGC-35 (71998246), GABRA1 (27808653), GABRB2 (292495010), GABRG2 (189083762), GABRR1 (223590210), RDL (635377460).

Compound preparation and testing: The following compounds were obtained from Tocris Bioscience (Minneapolis, MN): *trans*-4-aminocrotonic acid (TACA); (*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid sulfate (ZAPA); 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol hydrochloride (THIP); (1,2,5,6-

Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA); (3α,5α)-3-Hydroxypregnane-11,20-dione (Alphaxalone); (3α,5β)-3-Hydroxy-pregnan-20-one (Pregnanalone). The following compounds were obtained from Sigma-Aldrich: γ-aminobutyric acid (GABA); β-alanine; 5-aminomethyl-3-hydroxyisoxazole (Muscimol); aminoethanoic acid (Glycine); imidazole-4-acetic acid (IMA); piperdine-4-sulphonic acid (P4S); 4-piperidinecarboxylic acid (Isonipecotic acid); 5-aminovaleric acid (DAVA); 1,2,3,6-Tetrahydro-4-pyridinecarboxylic acid hydrochloride (Isoguvacine); 1(S),9(R)-(−)-Bicuculline methchloride (Bicuculline); Mecamylamine hydrochloride (Mecamylamine); 1,2,3,4,10,10-Hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-epoxy-1,4:5,8-dimethanonaphthalene (Dieldrin). All compound stocks (≥10mM) were dissolved in Ringer's solution and stored at -20°C. The water insoluble compounds alphaxalone and pregnanalone were dissolved in absolute ethanol. Due to their hydrophobicity, the maximum soluble concentration of these compounds in Ringer's was 30μM (Pregnanalone) and 100μM (Alphaxalone).

Homology modeling: The homology models of EXP-1 and LGC-35 were constructed based on the crystal structure of the homopentameric human β3 GABA receptor (PDB ID: 4COF) (Miller & Aricescu, 2014). The homology models were built using Modeller 9.17 software (Marti-Renom, Stuart, Fiser, Sanchez,

Melo & Sali, 2000). An initial twenty homology models were generated for EXP-1 and LGC-35, which were then further evaluated with Procheck software (Laskowski, Rullmannn, MacArthur, Kaptein & Thornton, 1996). The model with the lowest DOPE value and highest LG score was selected for molecular docking. To compare the differences in ligand binding, GABA was docked to the crystal structure of the dimeric human GABA receptor (Chain A and B) and the homology models of EXP-1 and LGC-35 using Autodock Vina software (Trott & Olson, 2010). The ligand box was centered in the ligand binding pocket of human GABA receptor with a dimension of 20x24x28 Å. The docked results were viewed and graphed using pymol software (http://www.pymol.org). Residues were numbered beginning at the start methionine for each receptor.

Statistical analysis: The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Statistical analyses were performed using Prism 7 (GraphPad, San Diego, CA). One-way ANOVA with Holm-Sidak post hoc test was used for comparison involving more than two groups. Data are reported as mean ± SEM. Statistical tests with $p < 0.05$ were considered significant.

Results

The *C. elegans* excitatory GABA receptors EXP-1 and LGC-35 are homopentameric 'cys-loop' ligand gated ion channel proteins that share 53% identity and 68% similarity across their entire protein coding sequences. Sequence alignments against human ionotropic $GABA_A$ and $GABA_A$-ρ1 receptor subunits reveal that EXP-1 and LGC-35 have highly homologous residues that comprise the orthosteric GABA binding site (Figure 1A). Previous data has demonstrated that the neurotransmitters acetylcholine, glycine, serotonin and glutamate exhibit no activity at EXP-1 or LGC-35 when expressed in *Xenopus laevis* oocytes (Beg & Jorgensen, 2003; Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015). Given the specific GABA-gating, we characterized a broad range of agonists, antagonists and modulators to determine the pharmacological profiles of EXP-1 and LGC-35 and to functionally classify the receptor family as $GABA_A$ or $GABA_A$-ρ like. We expressed each receptor in *Xenopus laevis* oocytes and used two-electrode voltage clamp to test the concentration-response of a variety of compounds known to differentially activate, inhibit or modulate $GABA_A$ and $GABA_A$-ρ receptors.

Agonist profiling of EXP-1 and LGC-35

Application of the endogenous neurotransmitter GABA produced robust and concentration-dependent current responses with a median effective concentration (EC_{50}) comparable to previously published data (Figure 2A, 3A, Table 1). Muscimol, a potent orthosteric agonist that differentially activates ionotropic GABA receptors, is a full agonist at vertebrate $GABA_A$ receptors, but only a partial agonist at vertebrate $GABA_A$-ρ receptors and nematode UNC-49 receptors (Johnston, 2014; Siddiqui, Brown, Rao & Forrester, 2010). Dose response curves revealed that muscimol exhibited partial agonist activity at both EXP-1 and LGC-35 with reduced efficacy and potency compared to GABA, comparable to that observed in nematode UNC-49 GABA receptors (Siddiqui, Brown, Rao & Forrester, 2010) (Figures 2, 3, and Table 1).

GABA is a flexible molecule thought to bind and activate $GABA_A$ receptors in a partially folded conformation (Krogsgaard-Larsen, Frolund & Liljefors, 2002). Supporting this model, a number of partially-folded conformationally-restricted GABA mimetics are potent agonists at $GABA_A$ receptors (Hosie & Sattelle, 1996; Kusama, Spivak, Whiting, Dawson, Schaeffer & Uhl, 1993; Woodward, Polenzani & Miledi, 1993). In contrast, these partially-folded analogs are weak partial agonists or antagonists at $GABA_A$-ρ receptors; whereas, extended and planar GABA analogs like *trans*-4-Aminocrotonic acid (TACA) exhibit high potency and efficacy at $GABA_A$-ρ receptors (Johnston, 2005). To classify the agonist profile of

EXP-1 and LGC-35 we determined dose-response relationships using a panel of conformationally-restricted analogs of GABA and muscimol that show differential potencies and efficacies at $GABA_A$ vs. $GABA_A$-ρ receptors (Figure 1B). Known agonists at $GABA_A$ receptors that displayed no current responses at maximal doses (3mM) in either EXP-1 or LGC-35 expressing oocytes were piperdine-4-sulphonic acid (P4S), imidazole-4-acetic acid (IMA) and 4,5,6,7-Tetrahydroisoxazolo(5,4-c)pyridin-3-ol (THIP). The muscimol analog Isonipecotic acid (3 mM) showed <5% activity at EXP-1 and LGC-35 and was not further characterized. Compounds that exhibited initial agonist activity were further characterized by dose-response relationships. The rank order of potency for EXP-1 was, TACA > GABA > DAVA > ZAPA > isoguvacine > muscimol > β-alanine (Figure 2A, Table 1). The rank efficacy order for EXP-1 was GABA > DAVA > TACA > muscimol > ZAPA > β-alanine > isoguvacine (Figure 2B, Table 1). TACA was equally potent as GABA and demonstrated strong partial agonist activity at EXP-1. DAVA was the only compound that showed full agonist activity at EXP-1, but was slightly less potent compared to GABA. All tested agonists produced fast-desensitizing currents that were similar to those elicited by GABA (Figure 2C). For LGC-35 the rank order of potency was similar to EXP-1, GABA > TACA > ZAPA > DAVA > isoguvacine > muscimol > β-alanine (Figure 3A, Table 1). The rank order of efficacy for LGC-35 was GABA, TACA, ZAPA >

DAVA > muscimol > β-alanine > isoguvacine (Figure 3B, Table 1). Maximal agonist concentrations produced slow-desensitizing currents that were similar to GABA responses (Figure 3C). The agonist profile of LGC-35 differed from that of EXP-1 in that both TACA and ZAPA showed full agonist activity, but their potencies were three and five-fold decreased compared to GABA (Table 1). Taken together, these data demonstrate that the agonist profiles of EXP-1 and LGC-35 more closely resemble $GABA_A$-ρ receptors.

Compared to inhibitory nematode UNC-49 GABA receptors, EXP-1 and LGC-35 exhibit a unique agonist profile. First, IMA (94% efficacy) and Isonipecotic acid (81% efficacy) are full and strong partial agonists at UNC-49, respectively, but fail to activate either EXP-1 and LGC-35 (Kaji, Kwaka, Callanan, Nusrat, Desaulniers & Forrester, 2015). Second, Isoguvacine is a strong partial agonist at UNC-49 (86% efficacy), but a very weak partial agonist at EXP-1 and LGC-35 (Table 1). Third, DAVA is a very weak partial agonist at UNC-49 (31% efficacy), but is a full agonist at EXP-1 and strong partial agonist at LGC-35 (Table 1). Fourth, ZAPA is an extremely weak partial agonist at UNC-49 (11% efficacy) (Kaji, Kwaka, Callanan, Nusrat, Desaulniers & Forrester, 2015), but exhibits differential activity as a partial agonist at EXP-1 (56% efficacy), and full agonist at LGC-35. Together, these data suggest that excitatory and inhibitory

GABA neurotransmission in *C. elegans* may be selectively activated using distinct GABA-mimetics.

Antagonist profiling of EXP-1 and LGC-35

EXP-1 is highly resistant to the orthosteric competitive $GABA_A$ antagonist bicuculline and the pore blocker picrotoxin (Beg & Jorgensen, 2003). The pore domain of EXP-1 is functionally analogous to the cation-conducting members of the 'cys-loop' family, yet EXP-1 is also highly resistant to the use-dependent nicotinic acetylcholine receptor blocker mecamylamine. Co-application of GABA (10μM) with increasing concentrations of bicuculline, picrotoxin and mecamylamine corroborated previous data showing that EXP-1 is strongly resistant to these classical antagonists (Figure 4A-C). Since the agonist profile of EXP-1 and LGC-35 more closely resembles $GABA_A$-ρ receptors, we tested the inhibitory effects of the selective and competitive $GABA_A$-ρ antagonist (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA). Current amplitudes of GABA (10μM) were dose-dependently inhibited by increasing concentrations of TPMPA (Figure 4, IC_{50} = 172 ± 7 μM). THIP (Gaboxadol) is widely used as a high efficacy partial agonist at $GABA_A$ receptors, but exhibits antagonist activity at $GABA_A$-ρ receptors (Krogsgaard-Larsen, Frolund & Liljefors, 2002). The lack of agonist activity at EXP-1 and LGC-35 suggested that THIP may be an

antagonist at these receptors. Application of increasing concentrations of THIP dose-dependently decreased GABA currents, but inhibition was no greater than bicuculline, picrotoxin or mecamylamine, which all reduced GABA (10μM) currents by ~50% (Figure 4A-C).

LGC-35 was recently demonstrated to be an excitatory GABA receptor in *C. elegans*, but the receptor's pharmacological profile remains completely unknown (Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015). We tested a panel of antagonists against LGC-35. The antagonist profile of LGC-35 was similar to EXP-1 with some notable differences. First, bicuculline, picrotoxin and THIP all showed relatively similar antagonist activity at LGC-35, with each compound capable of blocking GABA (10μM) currents by ~75% (Figure 5A, B). Compared to EXP-1, these antagonists were not only more efficacious at blocking GABA-induced currents, but also exhibited greater potency. Interestingly, mecamylamine showed no difference in its antagonism at LGC-35 as compared to EXP-1. To further probe the pore-forming domain, we applied known pore blockers of ionotropic GABA receptors to EXP-1 and LGC-35 expressing oocytes. The insecticide dieldrin, β-lactam antibiotic penicillin G, and widely-used pro-convulsant pentylenetetrazol all failed to block GABA-induced currents (data not shown). Taken together, these data suggest that the nearly identical M2 pore forming domain (85% identity, 100% similarity) shared between

EXP-1 and LGC-35 is distantly related to other anion and cation conducting pores of the 'cys-loop' superfamily. Lastly, TPMPA was the only compound tested that was able to fully block LGC-35 GABA-induced currents (Figure 5, IC_{50} = 61 $\pm$ 4 μM). From a pharmacological perspective, the antagonist and agonist profile suggest that EXP-1 and LGC-35 more closely resemble the $GABA_A$-ρ receptor subfamily.

Homology modelling of EXP-1 and LGC-35

The crystal structure of the human homopentameric β3 $GABA_A$ receptor was used as a template for homology modelling of the nematode EXP-1 and LGC-35 receptors (Miller & Aricescu, 2014). GABA docked into the highly conserved aromatic-rich binding pocket that is located between the 'principal' and 'complementary' subunit interface (Figure 6A, E). In EXP-1, the amino group of GABA is positioned to hydrogen bond with the side chain of Glu 250 (Loop B), the backbone carbonyl groups of Ser 251 and Tyr 252 (both Loop B), and have potential cation-π interactions with Tyr 252 and Tyr 300 (Loop C), similar to the binding mode observed in the human GABA receptor (Figures 1A, 6A) (Miller & Aricescu, 2014). GABA also docked in a highly conserved manner in the LGC-35 model (Figures 1A, 6E). In both models, the agonists TACA, DAVA and β-alanine docked with their amine nitrogen in a similar pose to that observed for GABA

(Figure 6B-D, F-H). The binding energies (ΔG) for GABA, TACA, DAVA, β-alanine were -4.2, -4.4, -4.4, -3.5 kcal/mol for EXP-1, and -4.3, -4.4, -4.8, -3.6 kcal/mol for LGC-35, respectively. One of the major differences between the models is that a lysine residue, Lys 245 (Loop F), is located in the proximity of ligands in LGC-35, as opposed to a Glu 272 (Loop F) in the corresponding position in EXP-1 (Figures 1A, 6E-H). The resulting charge reversal orients the ligand carboxyl group in closer proximity to Lys 245, as opposed to Arg153 (Loop D) in EXP-1. Finally, compared to the other agonists, β-alanine fails to extend across the binding pocket, losing energetically favorable electrostatic interactions with 'complementary' subunit residues, which likely reduces binding energy and contributes to the weak partial agonist activity.

Neurosteroids do not modulate EXP-1 and LGC-35

Neuroactive steroids, metabolites of the stress hormone corticosterone and progesterone, can potently modulate (low nanomolar concentration) or directly activate (micromolar concentration) vertebrate and invertebrate ionotropic GABA receptors (Lambert, Belelli, Peden, Vardy & Peters, 2003). The endogenous neurosteroid pregnanalone and synthetic neurosteroid alphaxalone potently potentiate mammalian $GABA_A$ receptors. In contrast, vertebrate $GABA_A$-ρ receptors are differentially modulated by these neurosteroids, where alphaxalone

potentiates, but pregnanalone inhibits GABA-induced currents (Morris, Moorefield & Amin, 1999) . We tested if neurosteroids can modulate or directly activate EXP-1 and LGC-35. In the absence of GABA, application of high concentrations of pregnanalone (30μM) or alphaxalone (100μM) failed to directly activate either receptor, demonstrating that these neurosteroids have no agonist-like activity at EXP-1 or LGC-35. However, co-application of increasing concentrations of alphaxalone and pregnanalone with GABA weakly inhibited EXP-1 and LGC-35 GABA-induced currents, when compared to GABA alone (Figure 7A-B). Although weak, the neurosteroid inhibition of LGC-35 was markedly increased compared to EXP-1 (Figure 7C-D). Like EXP-1 and LGC-35, the inhibitory *C. elegans* $GABA_A$ receptor UNC-49 is only slightly inhibited by alphaxalone, but can be completely blocked by pregnanalone (Bamber, Twyman & Jorgensen, 2003). These data demonstrate that the site for neuroactive modulation is likely unique in that EXP-1 and LGC-35 receptors resemble neither the modulatory properties of $GABA_A$, $GABA_A$-ρ or nematode UNC-49 receptors.

LGC-35 is selectively potentiated by ethanol

Anesthetic drugs, including ethanol, can modulate cellular and network signal transduction by altering the function of a wide variety of ion channels, transporters, receptors and enzymes (Lobo & Harris, 2008). However, the

contribution of these molecular targets to the intoxicating and behavioral effects of ethanol remain unclear. $GABA_A$ receptors are thought to be key targets of ethanol action in the brain, and subunit composition is a critical determinant that mediates the potentiating effect of alcohol in heteropentameric $GABA_A$ receptors (Mihic et al., 1997). In contrast, the single subunit homopentameric ρ1 receptor is inhibited by alcohol. At a molecular level, key residues in transmembrane domain 2 and 3 (M2 and M3) have been shown to mediate the potentiating or inhibitory effects of alcohol in $GABA_A$ vs. $GABA_A$-ρ receptors, respectively. The 15' serine residue (S270) in M2 and alanine residue (A291) in M3 of the α1 $GABA_A$ receptor subunit were shown to critically regulate ethanol potentiation (Figure 8F) (Mihic et al., 1997; Ueno, Wick, Ye, Harrison & Harris, 1999). In the ρ1 receptor, the analogous isoleucine residue in M2 (I307) and asparagine residue in M3 (W328) confer resistance against the potentiating effect of ethanol (Figure 8F) (Borghese et al., 2016; Mihic et al., 1997). We used these distinct structural properties to determine if EXP-1 and LGC-35 are modulated by ethanol. Ethanol (expressed as % change of 10μM GABA responses) did not significantly potentiate or inhibit EXP-1 current responses at any concentration tested (Figure 8A). Although lower doses of ethanol (10mM and 30mM) did not significantly modulate LGC-35 current responses, higher doses (100mM, 200mM, 500mM) did significantly potentiate LGC-35 current response amplitudes

(Figure 8C). Stepwise ramps of increasing concentrations demonstrate the potentiating effect of alcohol in the presence of constant GABA exposure (Figure 8E).

Sequence alignments reveal that EXP-1 contains an identical isoleucine at the 15' position (I357), which is a critical determinant of alcohol resistance in the ρ1 receptor (Figure 8E). Interestingly, LGC-35 has a similar leucine at the 15' position (L330), yet the receptor is significantly potentiated by high concentrations of ethanol (Figure 8E). Both receptors contain a glycine residue in the M3 domain, which is similar to the alanine residue (A291) present in the α1 $GABA_A$ receptor subunit that confers alcohol sensitivity, but markedly different from the asparagine residue (W328) in the ρ1 subunit that confers alcohol resistance (Figure 8E). Although the potentiating concentrations of ethanol are likely not physiologically relevant, these data reveal that the differential effects of ethanol on EXP-1 and LGC-35 GABA-induced current is likely distinct from the previously identified residues that confer alcohol sensitivity to ionotropic GABA receptors. Together, these data may provide new insights into molecular determinants that contribute to alcohol modulation in ionotropic GABA receptors.

Discussion

EXP-1 and LGC-35 compose the excitatory GABA receptor family in *C. elegans*, and play important roles in distinct neuromuscular contractions that regulate defecation and locomotion, respectively (Beg & Jorgensen, 2003; Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015). Since their initial cloning and characterization, these receptors have been loosely classified as belonging to the $GABA_A$ receptor subtype family. Using a broad panel of pharmacological agonists, antagonists and modulators, we demonstrate this unique receptor family more closely resembles the $GABA_A$-ρ family. We discuss our findings in regards to the structure, function and pharmacology of 'cys-loop' ligand gated ion channels.

GABA pharmacophores suggest EXP-1 and LGC-35 are activated by conformationally extended agonists

Decades of elegant research has demonstrated that vertebrate $GABA_A$ and $GABA_A$-ρ receptors are differentially sensitive to conformationally-restricted analogs of GABA (Johnston, 2005; Johnston, Chebib, Hanrahan & Mewett, 2010; Krogsgaard-Larsen, Frolund & Liljefors, 2002). Specifically, $GABA_A$ receptors tend to be preferentially activated by partially-folded forms of GABA; whereas, $GABA_A$-ρ receptors are activated by extended or near planar forms of GABA. Using a collection of conformationally-restricted analogs we conclude that both EXP-1 and LGC-35 more closely resemble the $GABA_A$-ρ family. We draw

several conclusions based on our data. First, the homo-oligomeric assembly of EXP-1 and LGC-35 supports their classification in the $GABA_A$-ρ family. Unlike $GABA_A$-ρ receptors, the vast majority of $GABA_A$ receptors are hetero-oligomers composed of three distinct subunits, with the $(\alpha_1)_2(\beta_2)_2(\gamma_2)_1$ combination being the most abundant in the human brain (Sigel & Steinmann, 2012). Moreover, EXP-1 and LGC-35 are not expressed in the same cells *in vivo* further supporting that these receptors do not act as hetero-oligomers (Beg & Jorgensen, 2003; Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015). Second, dose-response relationships reveal that most of the partially-folded GABA analogs tested either showed no agonist activity (nipecotic acid, IMA, P4S) or were weak partial agonists (muscimol, isoguvacine) at both EXP-1 and LGC-35. In contrast, the extended and non-aromatic analogs (TACA, DAVA) showed both higher potency and efficacy at EXP-1 and LGC-35. Third, carbon backbone length appears to be a key determinant of agonist potency and efficacy at EXP-1 and LGC-35. More specifically, increasing the carbon backbone length by one (DAVA) right shifts the potency in both EXP-1 and LGC-35 and decreases efficacy in LGC-35, but not in EXP-1. Reduction of the carbon backbone length by one (β-alanine) severely reduced both potency and efficacy, and reduction by two carbons (glycine) yields no activity at either receptor. Supporting these observations, TACA, which shares the same carbon backbone length as GABA, was the most

potent and efficacious compound tested at both receptors. A double bond between carbons 2 and 3 hold TACA in an extended conformation, supporting a model in which EXP-1 and LGC-35 are preferentially activated by extended ligands as opposed to folded forms. Fourth, although the pharmacological profile of EXP-1 and LGC-35 shares a resemblance to $GABA_A$-ρ receptors, there are notable exceptions that set these receptors apart as a family. ZAPA is a potent and full agonist at $GABA_A$ receptors, but is an antagonist at $GABA_A$-ρ receptors. We demonstrate that ZAPA is an agonist at EXP-1 and LGC-35 and not an antagonist as would have been predicted based on our pharmacological profiling. These data demonstrate that EXP-1 and LGC-35 exhibit a unique and hybrid pharmacological profile. Comparison of the ligand binding loops from EXP-1 and LGC-35 with ρ1 subunit demonstrates high conservation in residues implicated in the orthosteric binding site. Furthermore, EXP-1 and LGC-35 contain a single non-conserved amino acid difference in Loop C. Whether or not this single change accounts for the differential agonist activity at EXP-1 and LGC-35 remains to be determined.

EXP-1 and LGC-35 exhibit hybrid antagonist pharmacology

High affinity for GABA, bicuculline resistance and picrotoxin sensitivity are hallmark features of $GABA_A$-ρ receptors (Johnston, Chebib, Hanrahan & Mewett,

2010). From a pharmacological perspective, the agonist data suggests that EXP-1 and LGC-35 more closely resemble $GABA_A$-ρ receptors. In accordance with the agonist profile, both receptors were highly resistant to bicuculline as expected, but were also resistant to picrotoxin block. We previously characterized the molecular mechanisms of ion selectivity in the pore forming domain of both EXP-1 and LGC-35 (Beg & Jorgensen, 2003; Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015). Here, we investigated this unusual pore domain using a panel of well-characterized channel blockers at 'cys-loop" ligand gated ion channels. We failed to identify a single compound thought to act via pore blockade that could effectively inhibit GABA-induced currents. These data underscore that the pore-forming domain of EXP-1 and LGC-35 are highly divergent from any known $GABA_A$ or $GABA_A$-ρ receptors.

Supporting their $GABA_A$-ρ-like characteristics, both EXP-1 and LGC-35 were dose-dependently inhibited by the selective $GABA_A$-ρ antagonist TPMPA. Furthermore, THIP which differentially acts as a $GABA_A$ receptor agonist but a $GABA_A$-ρ antagonist, exhibited inhibitory activity at EXP-1 and LGC-35. These data suggest that EXP-1 and LGC-35 more closely resemble $GABA_A$-ρ receptors, although they exhibit a hybrid pharmacology that is not observed in either $GABA_A$ or $GABA_A$-ρ receptors.

Molecular profiling of neurosteroid resistance

$GABA_A$ receptors can be potently modulated by endogenous neurosteroids (Belelli & Lambert, 2005). Low nanomolar concentrations can potentiate GABA-induced currents (Belelli & Herd, 2003; Stell, Brickley, Tang, Farrant & Mody, 2003; Zhu & Vicini, 1997), and micromolar concentrations can directly activate receptors (Majewska, Harrison, Schwartz, Barker & Paul, 1986). Pregnanalone, an endogenous progesterone metabolite, and the synthetic neurosteroid alphaxalone, strongly enhance $GABA_A$-induced currents. In contrast, $GABA_A$-ρ receptors are differentially modulated by neurosteroids, where pregnanalone inhibits and alphaxalone potentiates ρ1 receptors (Morris, Moorefield & Amin, 1999). In comparison to $GABA_A$ receptors, which are enhanced in the nanomolar range, neurosteroid modulation of $GABA_A$-ρ receptors occur in the high micromolar range. We found that pregnanalone and alphaxalone exhibited almost no modulatory activity at EXP-1 (Figure 7A), but weakly inhibited LGC-35 only in the high micromolar range (Figure 7B). Previous studies have shown that the inhibitory nematode GABA receptor UNC-49 is strongly inhibited by pregnanalone, but is not modulated by alphaxalone (Bamber, Twyman & Jorgensen, 2003); whereas, the fly GABA receptor RDL is weakly potentiated by neurosteroids (Chen, Belelli, Lambert, Peters, Reyes & Lan, 1994). Chimeric studies using fly RDL and mouse α1 subunits identified two discrete residues in

transmembrane domain 1 that differentially contribute to direct neurosteroid activation and potentiation of GABA receptors (Hosie, Wilkins, da Silva & Smart, 2006). In the α1 subunit, a threonine (T236) in the transmembrane domain 1 was shown to be a major determinant for direct neurosteroid activation (Figure 7C). Substitution with an isoleucine present in RDL (T236I) markedly attenuated direct neurosteroid receptor activation without altering potentiation. Using the same strategy, a glutamine (Q241) in transmembrane 1 was found to be critically important for neurosteroid potentiation (Figure 7C). Substitution with an asparagine present in RDL at this position (Q241W) ablated neurosteroid potentiation. Comparative sequence alignments with RDL reveal that EXP-1 and LGC-35 contain a similar valine at that analogous position 236 and an identical asparagine at analogous position 241, which likely account for the lack of neurosteroid activation or potentiation (Figure 7C). Taken together, these data demonstrate that EXP-1 and LGC-35 exhibit a neurosteroid profile that does not resemble vertebrate $GABA_A$ or $GABA_A$-ρ receptors, or the inhibitory nematode GABA receptor UNC-49.

Does LGC-35 play a role in ethanol-mediated behaviors in C. elegans?

Ethanol produces profound behavioral effects in both humans and *C. elegans* at equivalent concentrations (McIntire, 2010). However, the relevant molecular

targets and biochemical pathways that mediate these effects remain unclear, as ethanol modulates a wide variety of receptors, ion channels and enzymes. The differential functional effects of ethanol on EXP-1 and LGC-35 is intriguing given the almost identical sequence conservation shared between the two proteins. Ethanol had no effect on EXP-1 GABA-induced currents, but significantly potentiated LGC-35 current responses at higher doses (100-500mM). From a behavioral perspective, acute exposure to ethanol produces dose-dependent changes in locomotor behaviors in *C. elegans* (McIntire, 2010) . Increasing concentrations of ethanol flatten the amplitude of body-bending and decreases speed of movement (Davies et al., 2003). We recently reported that LGC-35 regulates locomotor body bending and movement speed in *C. elegans* (Jobson, Valdez, Gardner, Garcia, Jorgensen & Beg, 2015). Specifically, deletion of LGC-35 results in exaggerated body bending and increases animals speed of movement. The specific ethanol potentiation of LGC-35, coupled with our cellular, molecular and behavioral data suggests that LGC-35 may be a relevant effector of ethanol's action in *C. elegans*; however, this remains to be tested.

In conclusion, the results from our studies expand the molecular repertoire for studying ionotropic GABA receptor structure-function relationships. The unique pharmacological profiles of EXP-1 and LGC-35 provide novel frameworks to differentially probe agonists, antagonists, and allosteric modulation at $GABA_A$

and $GABA_A$-ρ receptors. Furthermore, the homo-oligomeric assembly simplifies identifying critical amino acid residues and motifs, as the binding interfaces and ligand recognition sites are identical in each receptor complex. The highly conserved ligand-binding loops suggest the orthosteric GABA binding site is similar to vertebrate GABA receptors, but the hybrid pharmacology and differential sensitivities of EXP-1 and LGC-35 may provide a new tool for anthelmintic drug discovery and for the rationale design of GABA mimetics.

Author Contributions

G.C.B.N, H.Z. and A.A.B designed the research study and wrote the manuscript. G.C.B.N., A.J., R.W., H.Z and A.A.B performed the experiments and analyzed the data. H.Z. performed the homology modeling and docking. A.J. and R.W. prepared constructs, isolated and injected oocytes. G.C.B.N, A.J., R.W., H.Z. and A.A.B. contributed to the discussion and review of the manuscript. All authors read and approved the manuscript.

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Competing Interest

The authors declare no competing financial interests.

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Figure Legends

Figure 1. The orthosteric binding site of EXP-1 and LGC-35 are highly conserved

(A) Sequence alignment of amino acid motifs (Loops A-F) that comprise the orthosteric GABA binding site. GABA binding occurs at the subunit interface of two adjacent subunits termed the 'principal' and 'complementary' subunit. The orthosteric binding site is formed from three loops on the 'principal' subunit (Loops A-C) and three β-sheets on the 'complementary' subunit (Loops D-F). Residue identity is indicated in dark grey and similarity in light grey highlights. Residues implicated in ligand binding in this study are bold-highlighted red (principal), and blue (complementary). Aligned protein sequences, *C. elegans* EXP-1 and LGC-35, human $GABA_A$ receptor subunits (α1, β2, γ2), and human $GABA_A$-ρ subunit (ρ1).

(B) Structures of known agonists at $GABA_A$ receptors that were assayed for agonist activity at EXP-1 and LGC-35. Compounds that directly activated are categorized as agonists, and those that elicited no activity are categorized as no response. γ-aminobutyric acid (GABA); *trans*-4-aminocrotonic acid (TACA); 5-aminovaleric acid (DAVA); β-alanine; (*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid sulfate (ZAPA); 5-aminomethyl-3-hydroxyisoxazole (Muscimol); 1,2,3,6-Tetrahydro-4-pyridinecarboxylic acid hydrochloride (Isoguvacine); aminoethanoic

acid (Glycine); 4-piperidinecarboxylic acid (Isonipecotic acid); imidazole-4-acetic acid (IMA); piperdine-4-sulphonic acid (P4S); 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol hydrochloride (THIP).

Figure 2. EXP-1 is differentially activated by GABA agonists.

(A) Dose-response curves comparing the responses of each agonist tested normalized to maximal GABA responses (3mM) for EXP-1. Oocytes were voltage clamped at -60mV and agonists were bath applied in series (1-3000 μM) for 5 seconds. Due to its low potency, β-alanine was applied in series at higher concentrations (3-300 mM). Each data point represents the mean ± SEM with n ≥ 10, and N = 4.

(B) Bar graph illustrating the efficacy of compounds tested relative to maximal GABA responses (3 mM). Each bar represents the mean ± SEM with n ≥ 10. One-way ANOVA, Holm-Sidak *post hoc*; n.s, not significant ($p \geq 0.05$), **$p \leq 0.01$.

(C) Representative current responses from an EXP-1 expressing oocyte with each compound tested in series at maximal dose (3mM; β-alanine = 300mM). Scale bar = 1μA, 10 seconds.

Figure 3. LGC-35 is differentially activated by GABA agonists.

(A) Dose-response curves comparing the responses of each agonist tested relative to maximal GABA responses (3mM) for LGC-35. Oocytes were voltage clamped at -60mV, and agonists were bath applied in series (1-3000 μM) to plateau. Due its low potency, β-alanine was applied in series (3-300 mM). Each data point represents the mean ± SEM with $n \geq 7$, and N = 3.

(B) Bar graph illustrating the efficacy of compounds tested relative to maximal GABA responses (3mM). Each bar represents the mean ± SEM with $n \geq 7$. One-way ANOVA, Holm-Sidak *post hoc*; n.s, not significant ($p \geq 0.05$), ****$p \leq 0.0001$.

(C) Representative current responses from an LGC-35 expressing oocyte with each compound tested in series at maximal dose (3mM; β-alanine = 300mM). Scale bar = 1μA, 30 seconds.

Figure 4. EXP-1 is resistant to ionotropic GABA receptor antagonists.

(A) Inhibitor dose-response curves were generated by applying GABA (10μM) alone followed by GABA plus increasing concentrations of antagonist (10 μM GABA + 1-1000 μM antagonist). TPMPA was the only compound that potently inhibited EXP-1 GABA-induced currents (IC_{50} = 172 ± 7 μM). Responses were normalized to maximal GABA (10μM) responses. Each data point represents the mean ± SEM with n ≥ 8, and N = 3.

(B) Bar graph illustrating the efficacy of compounds tested (10 μM GABA + 1mM antagonist) relative to maximal GABA only responses (10μM). Each bar represents the mean ± SEM with n ≥ 8, and N = 3.

(C) Representative current responses from an EXP-1 expressing oocyte with each compound tested in series at maximal dose (10μM GABA + 1mM antagonist). GABA responsiveness fully recovers (2-minute wash) after complete TPMPA inhibition. Scale bar = 0.5 μA, 10 seconds.

Figure 5. LGC-35 is sensitive to ionotropic GABA receptor antagonists.

(A) Inhibitor dose-response curves were generated by applying GABA (10μM) alone followed by GABA plus increasing concentrations of antagonist (10μM GABA + 1-1000μM antagonist). TPMPA was the most potent inhibitor of LGC-35 GABA-induced current (IC_{50} = 61 ± 4 μM). Responses were normalized to maximal GABA only responses. Each data point represents the mean ± SEM with $n \geq 7$, and N = 3.

(B) Bar graph illustrating the efficacy of compounds tested (10μM GABA + 1mM antagonist) relative to maximal GABA only responses (10μM). Each bar represents the mean ± SEM with $n \geq 7$, and N = 3.

(C) Representative current responses of individual LGC-35 expressing oocytes. GABA (10μM) was applied until a steady state non-desensitizing plateau was reached. Subsequently, increasing concentrations of antagonist (1-1000μM antagonist) were applied in series in the presence of constant GABA (10μM). The grey bars above each trace denote GABA application, and the colored bars illustrate antagonist concentration co-applications. GABA responsiveness fully recovers after the highest antagonist concentration block. Scale bar = 1 μA, 30 seconds.

Figure 6. Homology models of EXP-1 and LGC-35

(A-D) Molecular modeling of the binding modes of: (A) GABA, (B) TACA, (C) DAVA and (D) β-alanine in the homology model of EXP-1. The homology model of EXP-1 was constructed based on the crystal structure of human GABA receptor and the ligands were docked to the model with Autodock Vina software as described in Materials and Methods. Ligands are colored green with oxygen labelled red and nitrogen blue. Dotted lines represent potential hydrogen bonds between ligand and receptor residues. Solid light grey line represents potential electrostatic interactions of the ligand carboxyl group with Arg 126. Labeled distances are in angstroms. Unlabeled potential hydrogen bonds were less than 3.1 Å. Residues were numbered beginning at the start methionine.

(E-H) Molecular modeling of the binding modes of: (A) GABA, (B) TACA, (C) DAVA and (D) β-alanine in the homology model of LGC-35. The homology model of EXP-1 was constructed based on the crystal structure of human GABA receptor and the ligands were docked to the model with Autodock Vina software as described in Materials and Methods. Ligands are colored green with oxygen labelled red and nitrogen blue. Dotted lines represent potential hydrogen bonds between ligand and receptor residues. Solid light grey line represents potential electrostatic interactions of the ligand carboxyl group with Lys 245. Labeled

distances are in angstroms. Unlabeled potential hydrogen bonds were less than 3.2 Å. Residues were numbered beginning at the start methionine.

Figure 7. EXP-1 and LGC-35 are not modulated by neurosteroids.

(A) EXP-1 neurosteroid dose-response curves were generated by applying GABA (10μM) alone or GABA plus increasing concentrations of neurosteroids (10μM GABA + Alphaxalone = 1, 3, 10, 30μM; Pregnanalone = 1, 3, 10, 20, 30 μM). Responses were normalized to maximal GABA only responses. Each data point represents the mean ± SEM with $n \geq 6$, and N = 2.

(B) LGC-35 neurosteroid dose-response curves were generated by applying GABA (10μM) alone or with increasing concentrations of neurosteroids (Alphaxalone = 1, 3, 10, 30, 100μM; Pregnanalone = 1, 3, 10, 20, 30 μM). Responses were normalized to maximal GABA only responses. Each data point represents the mean ± SEM with $n \geq 7$, and N = 2.

(C) Comparative sequence alignment of transmembrane domain (M1), highlighting key residues that confer differential neurosteroid sensitivity (blue) and resistance (red) in the human α1 $GABA_A$ subunit and fly RDL subunit, respectively. EXP-1 and LGC-35 contain a similar valine and an identical asparagine present in the neurosteroid resistant RDL receptor. Dark grey shading marks amino acid identity and light grey shading identifies similarity.

Figure 8. LGC-35 is selectively potentiated by ethanol

(A) EXP-1 is neither significantly potentiated nor inhibited by ethanol. Current responses of co-applied ethanol (10μM GABA + 10-500 mM ethanol) were normalized relative to maximal GABA only responses (10μM). For each oocyte, an initial stable GABA response was first achieved. Subsequently, co-application of 10 μM GABA + increasing concentrations of ethanol were bath applied for 5 seconds, followed by a two-minute wash. Each bar represents the mean ± SEM with $n \geq 8$, and N = 3.

(B) Representative current responses from an EXP-1 expressing oocyte with co-application of increasing concentrations of ethanol. Scale bar = 0.5 μA, 10 seconds.

(C) LGC-35 is potentiated by high concentrations of ethanol. Current responses of co-applied ethanol (10μM GABA + 10-500 mM ethanol) were normalized relative to maximal GABA only responses (10μM). For each oocyte, an initial stable GABA response was first achieved. Subsequently, co-application of 10 μM GABA + increasing concentrations of ethanol were bath applied until a plateau was reached, followed by a two-minute wash. LGC-35 current responses are significantly potentiated by high concentrations of ethanol (100 –

500 mM). Each bar represents the mean ± SEM with $n \geq 8$, and N = 3. One-way ANOVA, Holm-Sidak *post hoc*; n.s, not significant ($p \geq 0.05$), *$p < 0.05$.

(D) Representative current responses from an LGC-35 expressing oocyte with co-application of increasing concentrations of ethanol. Note, GABA responsiveness is unchanged compared to initial GABA-only responses after increasing series of ethanol exposure. Scale bar = 0.5 μA, 30 seconds.

(E) Representative current responses from an LGC-35 expressing oocyte. GABA (10μM) was applied until a steady state non-desensitizing plateau was reached. Subsequently, increasing concentrations of ethanol (100-500 mM) were applied in series in the presence of constant GABA (10μM). The grey bars above each trace denote constant GABA exposure, and the colored bars illustrate ethanol concentration co-applications. Scale bar = 1μA, 60 seconds.

(F) Comparative sequence alignment of transmembrane domains 2 and 3 (M2 and M3), highlighting key residues that confer differential ethanol sensitivity (blue) and resistance (red) in the human α1 and ρ1 receptor subunits, respectively. Dark grey shading marks amino acid identity and light grey shading shows similarity.

Figure 1

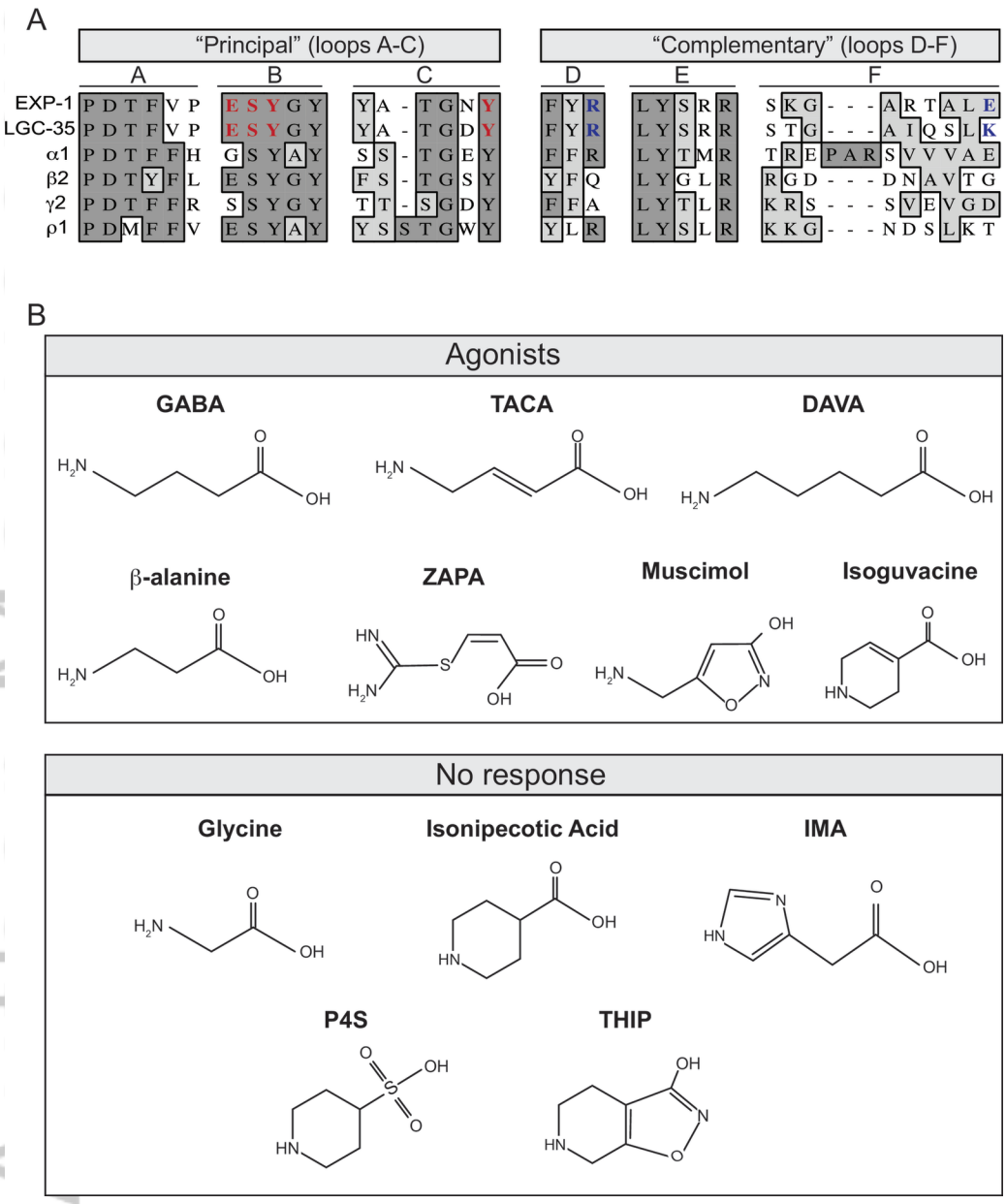

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Figure 2

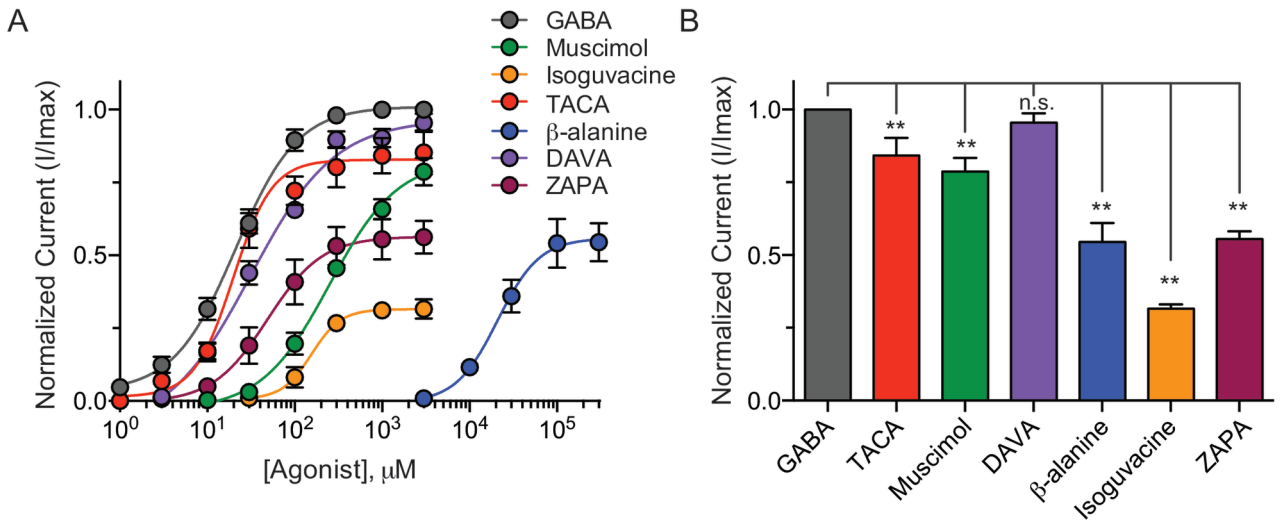

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Figure 3

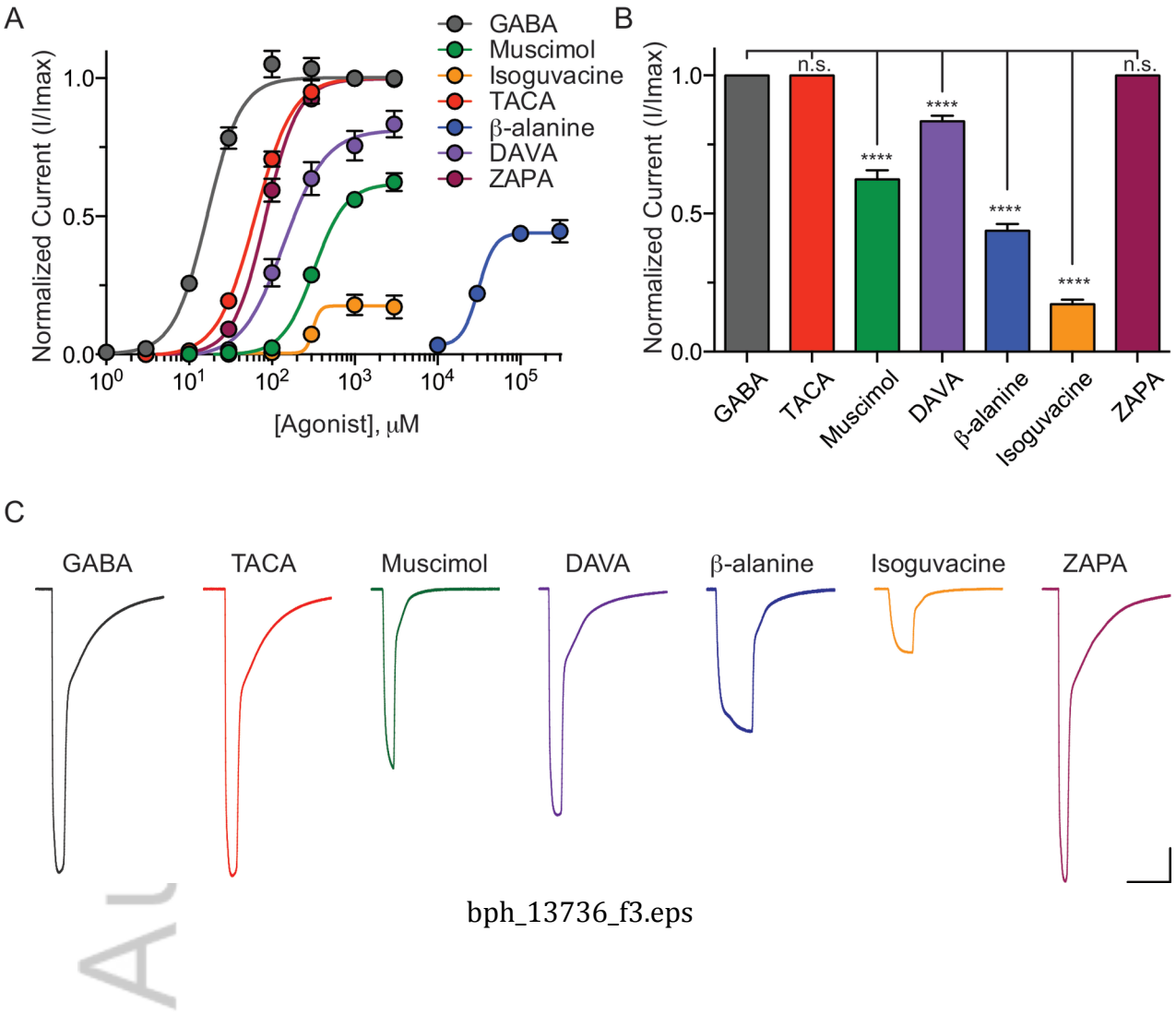

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Figure 4

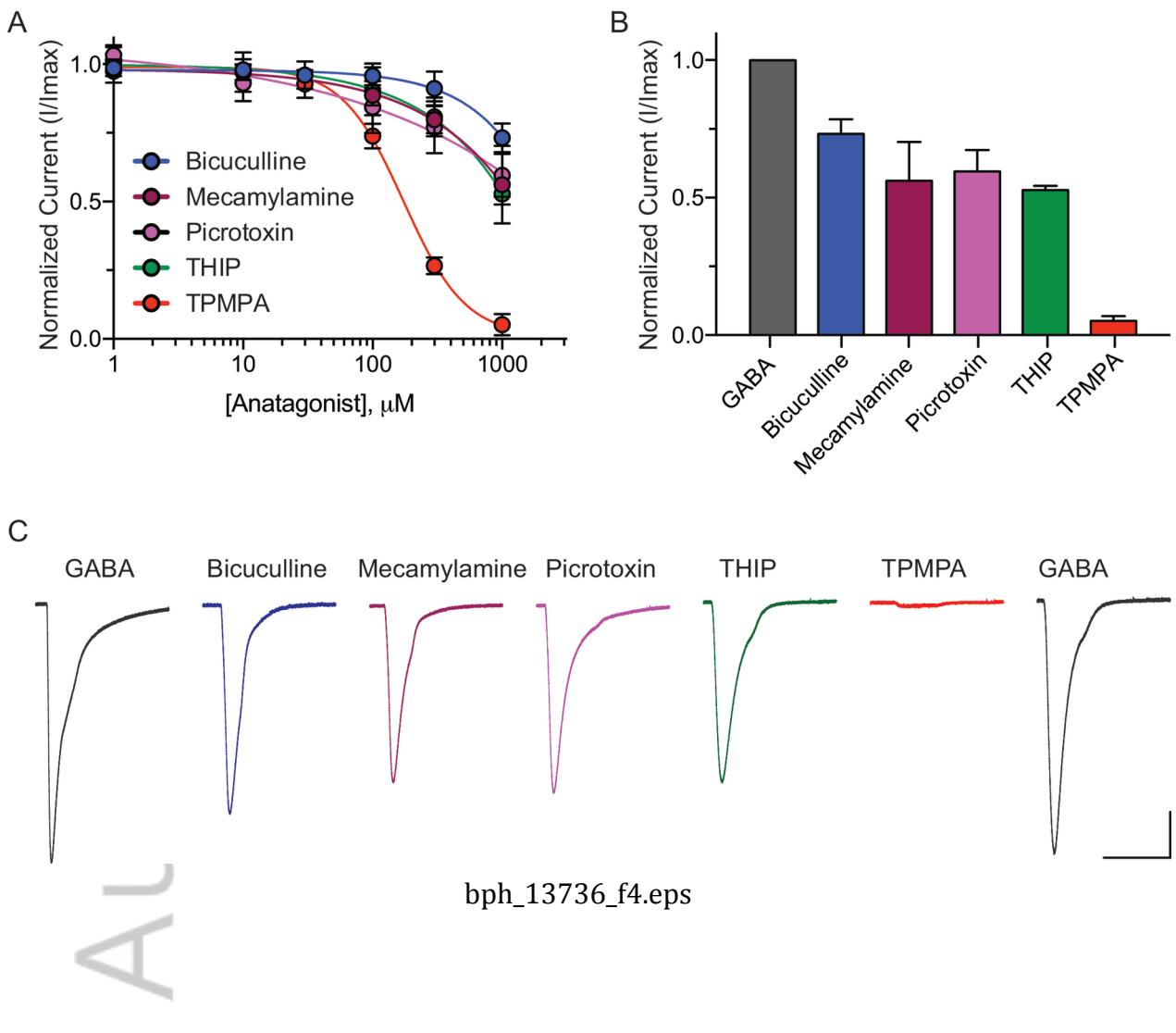

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Figure 5

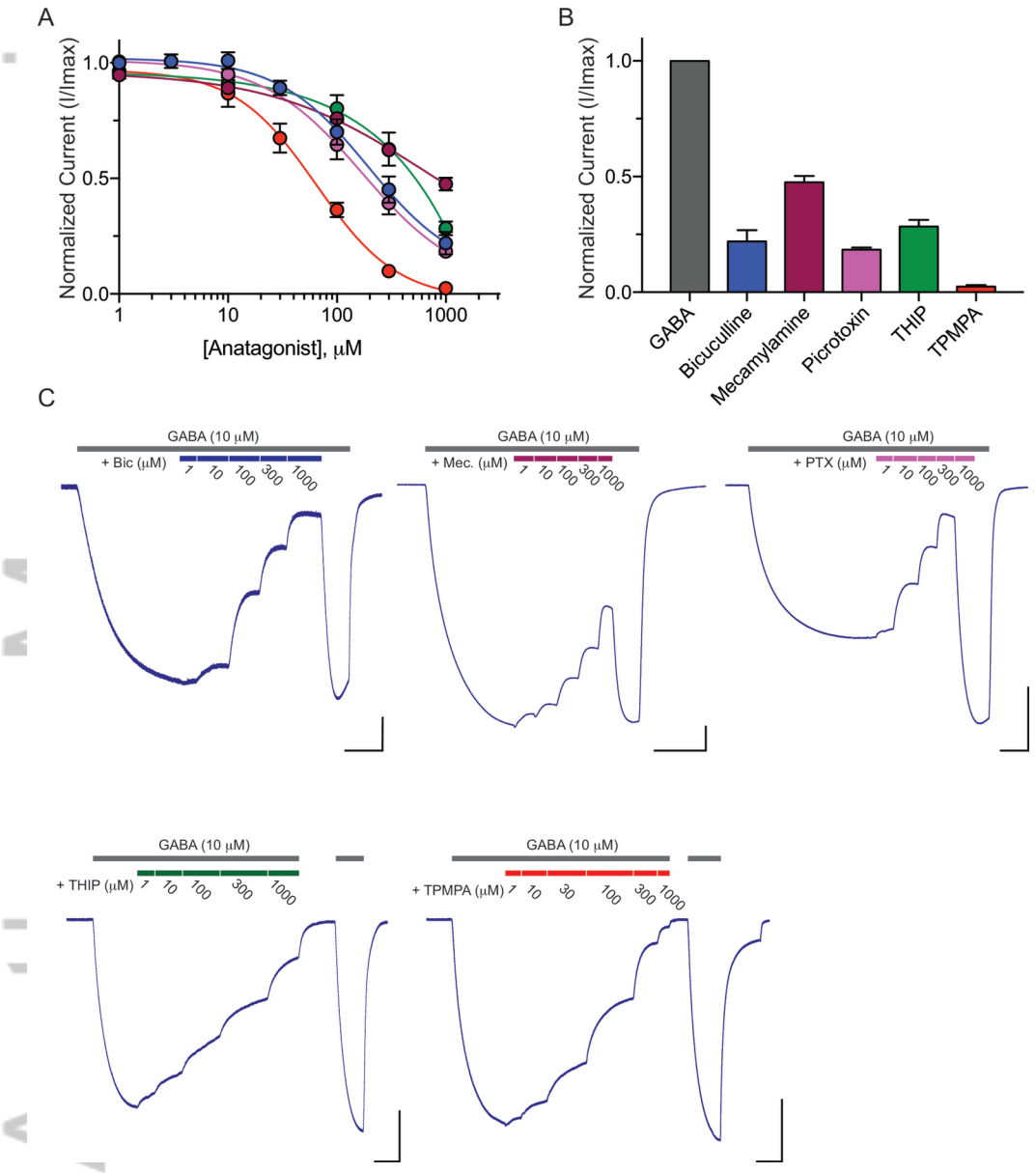

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Figure 6

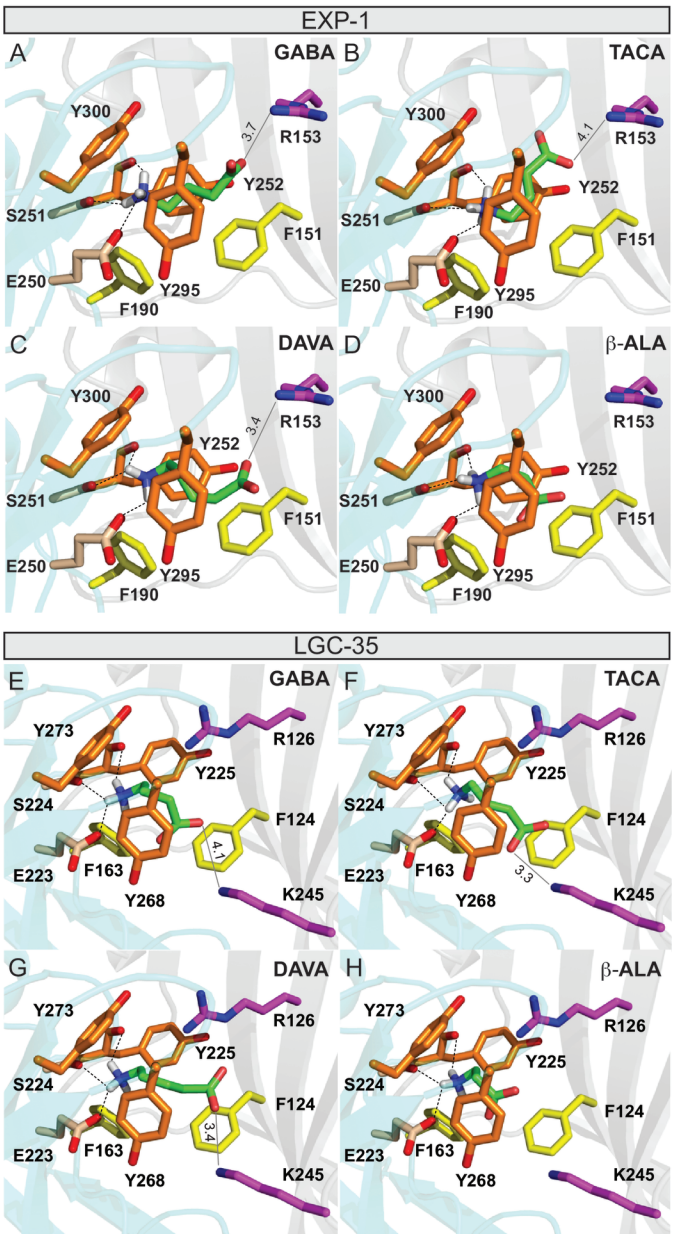

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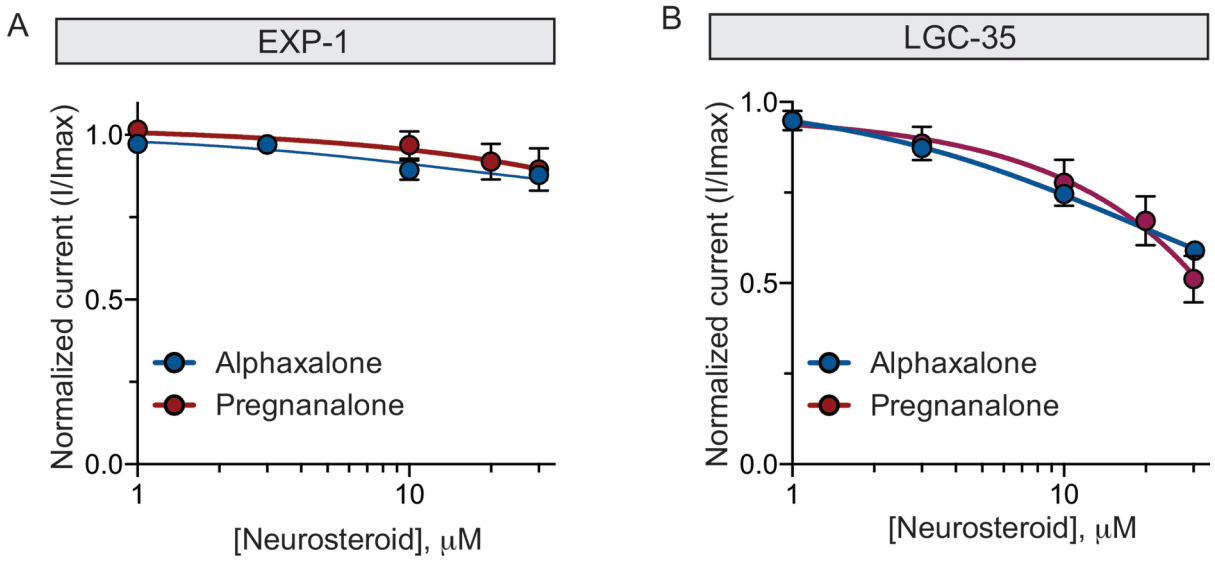
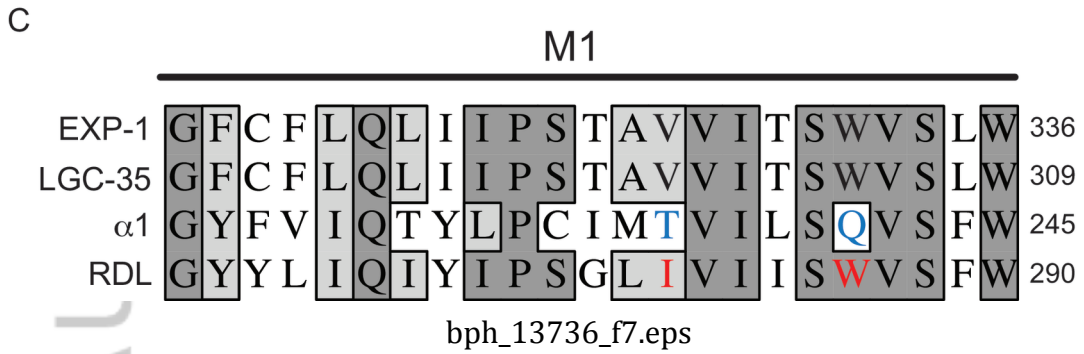
Figure 7





bph_13736_f7.eps

Figure 8

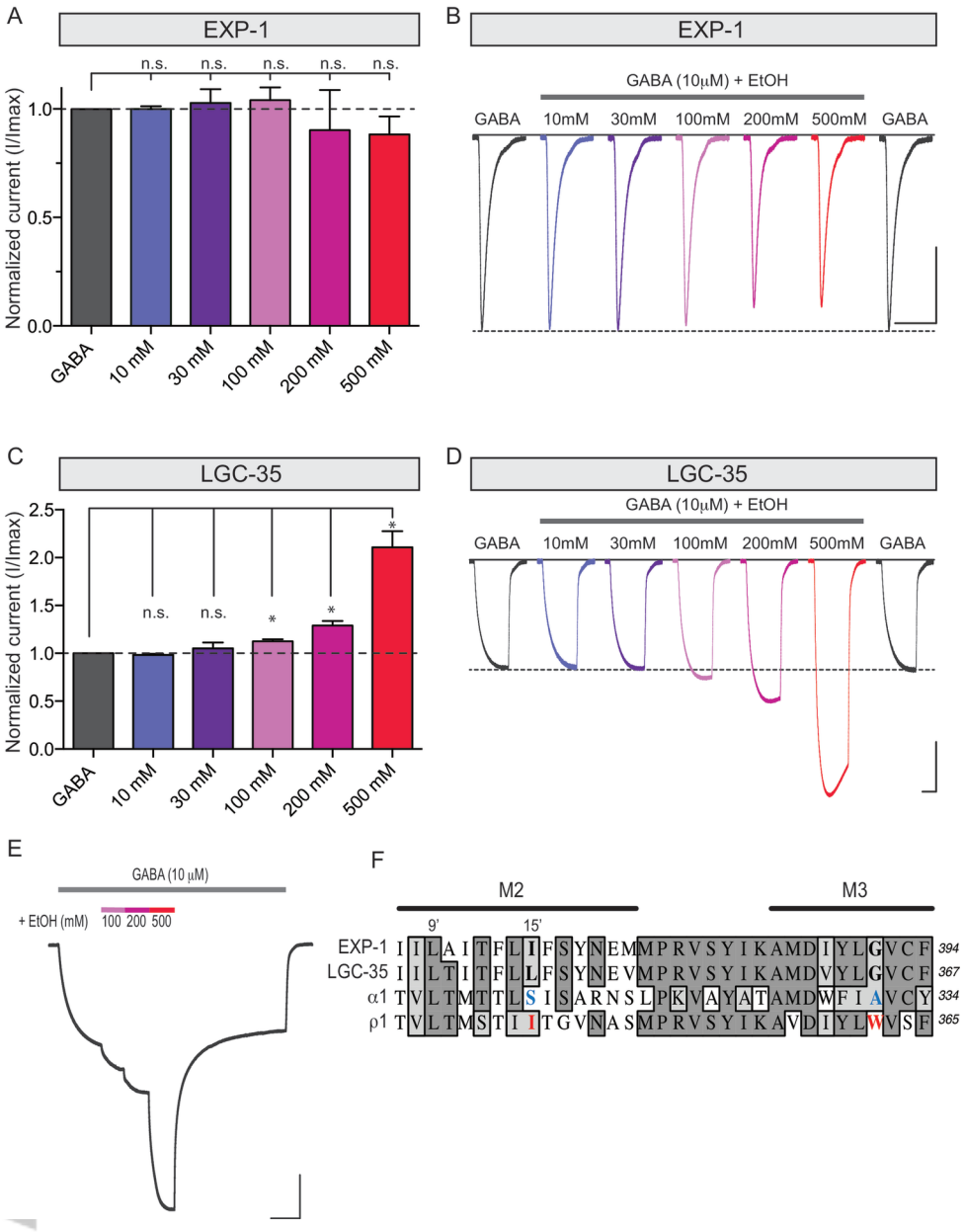


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Table 1. EC50, Hill slope and maximal current response efficacy for agonists at EXP-1 and LGC-35 receptors.

Agonist activity at EXP-1 and LGC-35. EC_{50} (μM), efficacy (% maximal GABA), Hill slope, and replicate values (*n*) are shown as mean ± SEM.

	EXP-1				LGC-35			
Compound	**EC_{50} (μM)**	**Efficacy**	**Hill**	***n***	**EC_{50} (μM)**	**Efficacy**	**Hill**	***n***
GABA	21 ± 0.69	100	0.91 ± 0.06	15	17 ± 0.71	100	2.1 ± 0.10	12
Muscimol	250 ± 22	79	1.2 ± 0.16	11	348 ± 17	62	2.1 ± 0.32	8
TACA	20 ± 2	84	1.7 ± 0.19	11	63 ± 3	100	1.9 ± 0.11	8
DAVA	31 ± 6	95	0.99 ± 0.12	11	142 ± 8	83	1.7 ± 0.03	7
Isoguvacine	155 ± 8	32	2.5 ± 0.53	10	316 ± 9	17	2.9 ± 0.21	7
β-alanine	26074 ± 2739	55	1.4 ± 0.10	11	37275 ± 1058	44	1.7 ± 0.08	11
ZAPA	51 ± 4	56	1.4 ± 0.07	12	86 ± 6	100	2.1 ± 0.12	10