Molecular Characterization of Binding Loop E in the Nematode Cys-Loop GABA Receptor

Ariel Kwaka, Mohammad Hassan Khatami, Joshua Foster, Everett Cochrane, Sarah A. Habibi, Hendrick W. de Haan, and Sean G. Forrester

Faculty of Science, University of Ontario Institute of Technology, Oshawa, Ontario, Canada

Received May 2, 2018; accepted August 30, 2018

ABSTRACT

Nematodes exhibit a vast array of cys-loop ligand-gated ion channels with unique pharmacologic characteristics. However, many of the structural components that govern the binding of various ligands are unknown. The nematode cys-loop GABA receptor uncoordinated 49 (UNC-49) is an important receptor found at neuromuscular junctions that plays an important role in the sinuoidal movement of worms. The unique pharmacologic features of this receptor suggest that there are structural differences in the agonist binding site when compared with mammalian receptors. In this study, we examined each amino acid in one of the main agonist binding loops (loop E) via the substituted cysteine accessibility method (SCAM) and analyzed the interaction of various residues by molecular dynamic simulations. We found that of the 18 loop E mutants analyzed, H142C, R147C, and S157C had significant changes in GABA sensitivity as its mutation to cysteine increased sensitivity to 5-aminovaleric acid (DAVA) to behave as a full agonist. Overall, this study has revealed potential differences in the agonist binding pocket between nematode UNC-49 and mammalian GABA receptors that could be exploited in the design of novel anthelmintics.

Introduction

Nematodes such as the model free-living nematode Caenorhabditis elegans and parasitic nematodes such as Haemonchus contortus contain a vast array of cys-loop ligand-gated chloride channels that respond to a variety of neurotransmitters including serotonin, tyramine, dopamine, GABA, and glutamate (Jones and Sattelle, 2008; Bartos et al., 2009; Laing et al., 2013). In addition, nematodes exhibit a unique class of acetylcholine-gated chloride channels (Putrenko et al., 2005) and GABA-gated cation channels (Beg and Jorgensen, 2003). However, like vertebrates, nematodes also exhibit anion selective receptors for GABA.

The nematode cys-loop GABA receptor that is the most characterized pharmacologically and functionally is the uncoordinated 49 (UNC-49) receptor (Bamber et al., 1999; Siddiqui et al., 2010). This receptor is expressed at the neuromuscular junctions of nematodes and contributes to the worm’s sinuoidal movement (Bamber et al., 2005). With regard to amino acid sequence, the UNC-49 receptor does not appear to be analogous to any mammalian GABA receptor, and it appears to exhibit unique pharmacologic features. One significant pharmacologic difference is the low sensitivity of the classic GABA receptor antagonist bicuculline (Bamber et al., 2003), which is a characteristic shared among invertebrate GABA receptors (Hosie et al., 1997). Differences have also been observed between the agonist profile of the UNC-49 receptor from H. contortus (Hco-UNC-49) and the mammalian receptors. Most notable is the insensitivity of the nematode receptor to sulphonated compounds such as taurine and P4S, which have been shown to exhibit efficacy at the mammalian GABAa receptor (Kusama et al., 1993; Woodward et al., 1993; del Olmo et al., 2000; Kaji et al., 2015).

The unique pharmacologic features of nematode cys-loop GABA receptors suggest that there are structural differences in the agonist binding pocket. However, compared with their mammalian counterparts, little is known about the structural elements that govern agonist binding to nematode GABA receptors.

The binding site of cys-loop receptors is made up of six discontinuous loops, designated by the letters A to F. The number of loops and their general position appear conserved across phyla (Breje et al., 2001; Hibbs and Gouaux, 2011). Loops A to C are found within the primary subunit, and loops D to F are in the adjacent subunit. The role of various binding

ABBREVIATIONS: cRNA, copy RNA; DAVA, 5-aminovaleric acid; MD, molecular dynamics; MS-222, 3-aminobenzoic acid ethyl methane sulphonate salt; MTSET, 2-[(trimethylammonium)methyl methanethiosulfonate; ND96 buffer, [96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES]; PS, pregnenolone sulfate; RDL, resistance to dieldrin; SCAM, substituted cysteine accessibility method; UNC, uncoordinated; WT, wild type.
loop residues in the function of mammalian GABA receptors have been examined through site-directed mutagenesis and the substituted cysteine accessibility method (SCAM).

SCAM analysis involves changing the predicted binding site residues to cysteines via site-directed mutagenesis. If the introduced cysteine is modified by a reducing agent such as 2-(trimethylammonium)ethyl methanethiosulfonate (MTSET) (see Fig. 1), GABA binding should be reduced if that cysteine residues is in the binding pocket. Binding pocket residues are confirmed through the use of agonists and antagonists, which show the rate of modification of the introduced cysteines by reducing agents (Kłoda and Czajkowski, 2007). However, it is important to note that modification of cysteines by molecules such as MTSET could have more complex effects on receptor activation, so the results should be interpreted with caution.

The detailed knowledge that we now have about the structure of the GABA binding pocket in mammalian GABA receptors has provided an opportunity for comparative analyses on the GABA receptors of more primitive organisms such as nematodes. Several residues in loops A to D have previously been examined in the Hco-UNC-49 receptor through site-directed mutagenesis, which revealed functional similarities when compared with mammalian GABA receptors (Accardi and Forrester, 2011). Binding loop E, on the other hand, is made up of 18 residues; the residues that play key roles in ligand binding have been mapped in both the GABAA1 and the GABAc1 subunits via SCAM analysis (Sedelnikova et al., 2005; Kłoda and Czajkowski, 2007). We aligned loop E from these two GABA receptor classes with the Hco-UNC-49 receptor, revealing 12 positions that are not completely conserved (Fig. 2).

Interestingly, residues such as H142 and F145 appear to be conserved among nematode UNC-49-like receptors. We wondered whether the unique residues present in loop E of nematode GABA receptors have any functional significance when compared with published reports on mammalian receptors and if they could possibly explain some of the pharmacologic characteristics observed. We conducted an examination of each residue in loop E from the H. contortus UNC-49 receptor using site-directed mutagenesis and SCAM, two methods that have been used to characterize the mammalian GABA binding site, to gain some insight into differences between mammalian and nematode GABA receptors. Of the residues we examined experimentally and via molecular dynamic simulations, some appeared to have similarity in function with mammalian receptors, but others could have some functional differences.

Materials and Methods

Site-Directed Mutagenesis—Primer Design. Amino acid alignments of Hco-UNC-49B and other GABA receptor subunits in the ligand-gated chloride channel (LGCC) family, were used to determine the location of loop E within the H. contortus sequence (Fig. 2). The primers were designed using Stratagene's web-based QuikChange Primer Design program (www.stratagene.com/sdmdesigner/default.aspx) to create 18 Hco-unc-49b (GenBank Accession EU939734.1) mutants: H142C, N143C, S144C, F145C, L146C, R147C, I148C, D149C, S150C, D151C, G152C, T153C, V154C, Y155C, T156C, S157C, Q158C, and R159C.

Each pair of mutagenic primers was designed to change each amino acid residue within loop E into cysteine residues by way of nucleotide introduction, resulting in singly mutated cysteine mutants.

Site-Directed Mutagenesis of Hco-unc-49b. Previous work in our laboratory found that the binding site for GABA in the Hco-UNC-49BC channel was at the interface of two adjacent UNC-49B subunits. However, the exact stoichiometry of the heteromeric channel is unknown (Accardi and Forrester, 2011). The template used in the site-directed mutagenesis of Hco-unc-49b was previously subcloned into a pTTVs transcription vector, in which Xenopus laevis β-globin untranscribed DNA was incorporated into the 5' and 3' ends of the Hco-UNC-49B subunit cDNA (Siddiqui et al., 2010).

A naturally occurring cysteine residue located at position 224 of the template amino acid sequence was removed through site-directed mutagenesis to prevent interference with the results obtained from the introduced cysteine residues. With no alteration in function (Fig. 3;
ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, temperature. After defolliculation, the oocytes were stored at 20°C in 5 mM HEPES, pH 7.5) for 2 hours with gentle rocking at room

For experiments using the negative allosteric modulator of GABA receptors, SF, oocytes expressing mutant or WT Hco-UNC-49BC

Table 1), the cysteine-less mutant (C224A) was used as template and a baseline from which all data obtained could be compared throughout.

All site-directed mutagenesis was conducted using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Confirmation that the correct mutation was present was verified using DNA sequencing (Génome Québec, Montreal, Canada).

**cRNA Preparation—In Vitro Transcription.** Plasmid constructs containing either the mutated *Hco-unc-49b* and wild-type (WT) *Hco-unc-49c* (GenBank Accession EU049802.1) were linearized and used as a template (0.4–1.0 μg) to create capped *Hco-unc-49* copy RNA (cRNA). The cRNA was made using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). The cRNA was precipitated at e m p l a t e(0.4)

**TABLE 1**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>EC₅₀ ± S.E. (μM) (Hill Coefficient ± S.E.)</th>
<th>n</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C224A</td>
<td>32.56 ± 2.29 (1.37 ± 0.11)</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>H142C</td>
<td>2.70 ± 0.22 (1.44 ± 0.07)</td>
<td>7</td>
<td>0.1</td>
</tr>
<tr>
<td>N143C</td>
<td>199.69 ± 22.86 (1.33 ± 0.11)</td>
<td>7</td>
<td>6.1</td>
</tr>
<tr>
<td>S144C</td>
<td>133.76 ± 11.96 (1.31 ± 0.07)</td>
<td>7</td>
<td>4.1</td>
</tr>
<tr>
<td>F145C</td>
<td>383.53 ± 114.30 (1.40 ± 0.04)</td>
<td>7</td>
<td>11.5</td>
</tr>
<tr>
<td>L146C</td>
<td>361.39 ± 42.59 (1.30 ± 0.04)</td>
<td>7</td>
<td>11.1</td>
</tr>
<tr>
<td>R147C</td>
<td>90.32 ± 6.54 (1.82 ± 0.16)</td>
<td>8</td>
<td>2.8</td>
</tr>
<tr>
<td>I148C</td>
<td>19.37 ± 2.62 (1.53 ± 0.13)</td>
<td>8</td>
<td>0.6</td>
</tr>
<tr>
<td>D149C</td>
<td>39.79 ± 7.11 (2.15 ± 0.27)</td>
<td>7</td>
<td>1.2</td>
</tr>
<tr>
<td>S150C</td>
<td>54.39 ± 6.95 (1.78 ± 0.13)</td>
<td>7</td>
<td>1.7</td>
</tr>
<tr>
<td>D151C</td>
<td>36.11 ± 3.25 (1.43 ± 0.08)</td>
<td>7</td>
<td>1.1</td>
</tr>
<tr>
<td>G152C</td>
<td>54.91 ± 5.01 (1.48 ± 0.06)</td>
<td>7</td>
<td>1.7</td>
</tr>
<tr>
<td>T153C</td>
<td>122.21 ± 10.01 (1.71 ± 0.08)</td>
<td>6</td>
<td>3.8</td>
</tr>
<tr>
<td>V154C</td>
<td>104.65 ± 5.10 (1.51 ± 0.14)</td>
<td>7</td>
<td>3.2</td>
</tr>
<tr>
<td>L155C</td>
<td>111.49 ± 16.68 (1.64 ± 0.09)</td>
<td>7</td>
<td>3.4</td>
</tr>
<tr>
<td>T156C</td>
<td>628.48 ± 50.61 (1.71 ± 0.06)</td>
<td>6</td>
<td>19.3</td>
</tr>
<tr>
<td>S157C</td>
<td>3683.50 ± 413.66 (1.43 ± 0.09)</td>
<td>8</td>
<td>113.1</td>
</tr>
<tr>
<td>Q158C</td>
<td>35.44 ± 6.63 (1.44 ± 0.10)</td>
<td>7</td>
<td>1.1</td>
</tr>
<tr>
<td>R159C</td>
<td>3102.33 ± 130.43 (1.53 ± 0.03)</td>
<td>6</td>
<td>9.53</td>
</tr>
</tbody>
</table>

*Values statistically significantly different from C224A (P < 0.001).*

Fig. 3. (A) Representative electrophysiological tracings of Hco-UNC-49BC with mutated Hco-UNC-49B subunits H142C, C224A (cysteine-less mutant used as template for creation of other mutants), and S157C. (B) Dose–response curve of Hco-UNC-49B mutants showing differences in GABA sensitivity, with normalized currents. Each point represents the mean with bars of S.E. (n > 6).

5 mM HEPES, pH 7.5) supplemented with 100 μg/ml gentamycin and 0.275 μg/ml pyruvic acid (Sigma-Aldrich). Supplemented ND96 solution was replaced twice every 24-hour period.

Clot-promoting injections of cRNA were performed on stage V and VI oocytes using a Drummond Nanoproject II (Drummond Scientific Company, Broomhall, PA) assisted by micromanipulators (World Precision Instruments, Sarasota, FL). Each oocyte was injected with 50 nl of a mixture of mutated Hco-unc-49b and WT Hco-unc-49c (equal amounts of 0.5 ng/ml cRNA of each). Electrophysiological recordings were taken after receptor expression, approximately 2 to 5 days after the cRNA injection.

**Compounds Tested and Their Preparation.** MTSET was obtained from Toronto Research Chemicals (Toronto, ON, Canada). GABA, 5-aminovaleric acid (DAVA), and pregnenolone sulfate (PS) were obtained from Sigma-Aldrich. All compounds were initially dissolved in ND96 except for PS, which was dissolved in dimethylsulfoxide for the preparation of a stock solution. Compounds were diluted in ND96 as working solutions. The structures of the compounds are found in Fig. 1.

**Two-Electrode Voltage Clamp Electrophysiology.** Using an Axoclamp900A voltage clamp (Molecular Devices, Sunnyvale, CA), two-electrode voltage clamp electrophysiology was used to observe and record the channel activity of the Hco-UNC-49BC/C channel. Glass electrodes were pulled from borosilicate capillaries using a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA), and filled with 3 M KCl (1–5 MΩ resistance). Each of the two electrodes were connected to Axon Instrument Headstages (Molecular Devices) using Ag|AgCl wires.

Oocytes were obtained from female *Xenopus laevis* (Nasco, Fort Atkinson, WI), which were housed in temperature-controlled rooms at the University of Ontario Institute of Technology (UOIT) according to the methodology outlined in Abdelmassih et al. (2017) and adhering to the guidelines of the UOIT Animal Care Committee and the Canadian Council of Animal Care. The frogs were anesthetized with 0.15% 3-aminobenzoic acid ethyl ester methane sulphonate salt (MS-222; Sigma-Aldrich, Oakville, ON, Canada). Lobes of the ovary were extracted, split into smaller pieces of 10–20 oocytes, and defolliculated in a treatment of 2 mg/ml collagenase-II (Sigma-Aldrich) and OR2 solution (82 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5) for 2 hours with gentle rocking at room temperature. After defolliculation, the oocytes were stored at 20°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂,
receptors were first exposed to EC$_{50}$ concentrations of GABA to ensure channel functionality. Oocytes were then exposed to a compaction of an increasing range (5–500 μM) of PS concentrations mixed with the corresponding EC$_{50}$ GABA concentration. Changes in current were recorded and inhibitory dose–response curves were generated to determine the IC$_{50}$ value for the mutant being tested.

**Substituted Cysteine Accessibility Method.** SCAM was used to characterize all Hco-UNC-49B mutants as well as the cysteine-less mutant (C224A) using the reducing agent MTSET. During testing, an oocyte was washed and hit with EC$_{20-50}$ concentrations of GABA in 5-minute intervals until the response stabilized within 10%. Once stabilized, MTSET (1 mM for 1 minute) was perfused over the oocyte, followed by 5 minutes of washing with ND96 solution. The EC$_{20-50}$ concentrations of GABA for the mutant being tested were reapplied, and currents were recorded (Sedelnikova et al., 2005; Kloda and Czajkowski, 2007).

**Statistical Analysis.** Dose–response curves were generated by Prism 5.0 (GraphPad Software, San Diego, CA) using the following equation in the set-up log (agonist) versus normalized response-variable slope:

$$I_{max} = \frac{1}{1 + (\frac{EC_{50}}{[D]})^k}$$

where $I_{max}$ is the maximal response, $[D]$ is the concentration of agonist, EC$_{50}$ is the concentration of agonist that is required to produce half-maximal current, and $k$ is the Hill coefficient. Responses used to produce dose–response curves were normalized as a percentage of the maximal current produced by the oocytes individual maximal response to GABA.

The effect of MTSET modification was calculated using the following formula:

$$\left(\frac{I_{GABA-post}}{I_{GABA-pre}}\right) - 1$$

where $I_{GABA-post}$ is the current produced from GABA activation of the receptor after the application of MTSET, and $I_{GABA-pre}$ is the current produced from GABA activation before MTSET was applied (Kloda and Czajkowski, 2007).

The EC$_{50}$ values, $h$ values, and S.E. were determined using Prism 5.0 from a minimum of five oocytes from two different X. laevis. Statistical significance was derived from Student’s t test using the Bonferroni correction or a one-way analysis of variance with Dunnett multiple comparison test. $P < 0.001$ was considered statistically significant.

**Homology Modeling.** The C. elegans glutamate-gated chloride channel crystal structure (PDB 3RIF; Hibbs and Gouaux, 2011) was used as a template in MODELER 9.14 (Sali and Blundell, 1993) for the generation of a Hco-UNC49B extracellular domain homodimer (Supplemental Data PDB File). The most energetically favorable models were determined based on their DOPE score and PROCHECK Ramachandran plot analysis, as described in Kaji et al. (2015).

**Computational Agonist Docking.** The energetically reduced zwiterion form of GABA was obtained from the Zinc database (http://zinc.docking.org; Irwin et al., 2012). The GABA molecule was prepared for docking using AutoDock Tools (Morris et al., 2009) and was docked using AutoDock Vina (Trott and Olson, 2010). The center of the 30 × 30 × 30 Å search box located in the aromatic box of the agonist binding site was used for agonist docking. A maximum of 50 binding models, all within a range of 5 keal mol$^{-1}$ from the best scoring pose, were generated (Kaji et al., 2015).

**Molecular Dynamics Simulations.** The homology model of the protein we generated, containing the docked GABA molecule in the binding area, was used as the initial structure for the molecular dynamics (MD) simulations. The GROMACS 2016.4 software package (www.gromacs.org), employing the CHARMM36 force field (Best et al., 2012), was used to run the simulations. The CHARMM General Force Field (CGenFF) program, which is an automated method to provide parameters and charges by analogy (Vonr高新slaeghe et al., 2010, 2012; Vonr高新slaeghe and MacKerell, 2012; Yu et al., 2012; CGenFF interface at https://cgenff.paramchem.org), was used to acquire the CHARMM force field parameters for the GABA molecule. The simulation box was solvated with ~21,000 tip3p water molecules, along with Cl$^-$ counter ions to neutralize the total charge of the system.

To carry our the MD simulation, after a brief energy minimization, a 2 nanosecond equilibration simulation was performed under the NVT (number of particle, volume of the system, and temperature are constant) conditions at 300 K and a time step of 2 femtoseconds. The positions of the protein and the ligand were restrained to prevent any structural changes during the equilibration process.

In the production run, under the NPT (number of particle, pressure of the system, and temperature are constant) conditions, the system was kept at 300 K and 1 atm with a time step of 2 femtoseconds for 500 nanoseconds. The Parrinello-Rahman isotropic pressure coupling (with $\tau_p = 5$ picoseconds and compressibility $4.5 \times 10^{-5} \text{ bar}^{-1}$) and the Nose-Hoover thermostat (with a time constant of 0.1 picoseconds) were employed for these production runs. Long-range electrostatics were calculated with the particle-mesh Ewald method. The trajectory was printed every 10 picoseconds.

The gromacs utilities, such as gmx-mpi mindist, gmx_mpi gyrate, gmx_mpi rms, as well as the H-bond calculator plugin of VMD (Visual Molecular Dynamics) (Humphrey et al., 1996; http://www.ks.uiuc.edu/Research/vmd/), were used to calculate the results. The default values for distance ($d$) and angle ($\theta$) ($d < 3.0 \text{ Å}$ and $\theta < 120^\circ$) are used to determine the H-bonds. Ionic bonds between oppositely charged residues were determined at $d < 3.0 \text{ Å}$.

**Results**

**Characterization of Loop E Cysteine Mutants in Hco-UNC-49BC.** The importance of loop E amino acid residues in the Hco-UNC49 receptor was first assessed by determining the impact of each cysteine mutation on the GABA activation of the channel. Each loop E mutant was exposed to increasing concentrations of GABA to determine the EC$_{50}$ values. Upon recording, good expressions of all receptors with mutated subunits were observed, all receptors were functional, and clean tracings were obtained (Fig. 3A). The cysteine-less mutant C224A was an appropriate control for this study as the GABA EC$_{50}$ (33 μM) was similar to WT (40 μM; Siddiqui et al., 2010), and this mutant was not sensitive to MTSET treatment.

Of the 18 mutants tested, 12 showed a substantial shift in GABA EC$_{50}$ compared with the C224A cysteine-less mutant from which they were derived (representative dose–response curves are seen in Fig. 3B). Of the mutants that displayed a different EC$_{50}$ from C224A (33 μM ± 2), H142C showed an increase in GABA sensitivity (2.7 μM ± 0.2). The remaining 11 mutants (N143C, S144C, F145C, L146C, R147C, T153C, V154C, Y155C, T156C, S157C, and R159C) had a decrease in GABA sensitivity, with S157C and R159C showing the greatest decrease (3683.50 μM ± 413.66 and 3102.33 μM ± 130.43, respectively). Finally, six of the cysteine mutants, I148C, D149C, S150C, D151C, G152C, and Q158C, showed minimal change in GABA EC$_{50}$. The EC$_{50}$ values that were statistically significantly different from C224A ($P > 0.001$) are shown in Table 1.

**Determination of Cysteine Accessibility.** SCAM was performed on all loop E Hco-UNC49B mutants to determine which were accessible to MTSET modification and the impact of modification on channel function. Overall, we found that...
most of the cysteine mutants were either not influenced or positively influenced by MTSET treatment. Specifically, out of the 18 mutants tested, only H142C, R147C, and S157C were significantly \( (P < 0.001) \) affected by MTSET modification (Fig. 4). Furthermore, only S157C had a decrease in the percentage change in \( I_{\text{GABA}} \) (~49.8% \pm 7.8%). Conversely, H142C and R147C all showed an increase in the percentage change in \( I_{\text{GABA}} \) (88% \pm 4% and 52.5% \pm 17%, respectively).

**Pharmacologic Characterization of H142C Mutant.**

Upon discovering significant hypersensitivity to GABA in H142C, we investigated two other compounds: DAVA and PS. Dose–response curves were created for both compounds (Fig. 5, A and C) with respect to the H142C mutant as well as the cysteine-less C224A baseline mutant. In addition, DAVA behaved as a partial agonist at the C224A receptor \( (I_{\text{max}} 21.2\% \pm 2.7\%) \), but as a full agonist at the H142C receptor \( (I_{\text{max}} 86.5\% \pm 2.8\%) \); Table 2). On the other hand, no significant difference was observed in the IC\(_{50}\) of PS between the C224A and H142C receptors (Fig. 5; Table 2). For the C224A receptor, the IC\(_{50}\) for PS was 47 \( \mu \)M compared with 70 \( \mu \)M for H142C.

**MD Simulations.**

As a further analysis of loop E residues we conducted molecular simulations. To ensure that the simulations provided a meaningful analysis of the role of loop E residues and their possible affect on GABA binding, we first examined the interaction of GABA with well-studied residues within the binding pocket. During the course of the simulation, the GABA molecule remained inside the initial binding pocket and interacted with key side chains previously implicated as essential for GABA binding in both mammalian (Newell et al., 2004) and the UNC-49 receptor (Accardi and Forrester, 2011; Kaji et al., 2015) (Fig. 6A). For example, the positive amino group of GABA forms ionic bonds with carboxylate of E185 (loop B) 94% of the time. Likewise, the carboxylate group of GABA forms ionic bonds with R87 (loop D) 99% of the time, and S236 (loop C) forms hydrogen bonds with GABA 35% of the time. These values were similar to MD simulations of the *Drosophila* resistance to dieldrin (RDL) receptor (Ashby et al., 2012).

With respect to loop E residues, the molecular simulations revealed only two residues forming hydrogen bonding with GABA. GABA most frequently formed H-bonds with S157 (74.25% of the time) and TYR155 (53.36% of the time) (Fig. 6, A and D). ARG147 did not interact with the GABA molecule, except for during the first few nanoseconds of the simulation (Fig. 6B; Fig. 7A). Alternately, the R147 side chain mainly associated with the D149 side chain (Fig. 7A) via an ionic bond (35%...
Discussion

A comparative analysis of residues between mammalian and invertebrate GABA receptors can provide some valuable information on ligand-binding sites and receptor function. Indeed, a comparative mutagenesis approach was key to identifying the neurosteroid binding site in cys-loop GABA receptors (Hosie et al., 2006). Our study describes the characterization of the 18 residues found in binding loop E of the Hco-UNC-49 receptor.

In this investigation we determined which residues, when mutated to cysteine, affected the sensitivity of the Hco-UNC-49 receptor to GABA and other agonists. Moreover, exposure of the introduced cysteines to the reducing agent MTSET revealed which residues were accessible to MTSET modification (e.g., in an aqueous environment) and affected GABA sensitivity. However, the SCAM results should be interpreted with caution because molecules such as MTSET have been shown to have more complex effects, such as acting as tethered partial allosteric modulator.

This study revealed some loop E residues within Hco-UNC-49 that may exhibit similar properties as those found in human receptors, but it also revealed several with possible distinct functions in the nematode receptor. Overall, it appears that the loop E of the Hco-UNC-49 receptor is more tolerant to cysteine mutagenesis than the GABA_A receptor (Kłoda and Czajkowski, 2007).

Another distinction in the overall results of this study was that only one of the mutations (S157C) resulted in a decrease in I_GABA when modified by MTSET whereas others exhibited either no change or an increase in current. Studies on the human GABA_C (which also used MTSET) receptor appeared to show the opposite trend (Sedelnikova et al., 2005). The results presented here may indicate an overall difference in the structure of loop E and the role it may play in nematode cys-loop GABA receptors when compared with their mammalian counterparts.

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>% Maximal GABA (S.E.)</th>
<th>n</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>% Maximal GABA (S.E.)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAVA</td>
<td>1353.2 ± 246.2 (1.15 ± 0.16)</td>
<td>86.5 ± 2.8 (1.44 ± 0.07)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
<td>2464 ± 107.2 (2.18 ± 0.1)</td>
<td>21.2 ± 2.7</td>
<td>5</td>
</tr>
<tr>
<td>GABA</td>
<td>2.7 ± 0.22 (1.44 ± 0.07)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>7</td>
<td>32.56 ± 2.29 (1.37 ± 0.11)</td>
<td>100</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values statistically significantly different from C224A (P < 0.001).

---

![Fig. 6. Molecular simulation snapshots of the Hco-UNC-49B homodimer with GABA docked. E185 is in loop B, S236 is in loop C, R87 is in loop D, and H142, S144, R147, D149, Y155, and S157 are in loop E.](Image)
In this study six mutants (I148C, D149C, S150C, D151C, G152C, and Q158C) did not affect the functionality of the receptor, exhibiting EC_{50} values close to those obtained from C224A (the cysteine-less mutant displaying WT function). According to our model (Fig. 2), I148 to G152 are at the top of loop E and therefore positioned ∼13–17 Å away from GABA. Interestingly, the UNC-49 G152 residue, which is conserved among all GABA receptors, appears to be much more tolerant to a change to cysteine (1.7-fold decrease), compared with mammalian receptors where this change resulted in either a 42-fold reduction in GABA sensitivity or nonfunctional receptors (Sedelnikova et al., 2005; Kloda and Czajkowski, 2007).

R147 appears to be conserved across many phyla (Fig. 2). We found a small 2.7-fold change in EC_{50} when this arginine was mutated to a cysteine; when MTSET was applied, there was a significant increase in GABA current. These results are very different from what was found for the GABA_A α1 subunit, there was a 260-fold decrease in EC_{50} compared with that of the WT (Kloda and Czajkowski, 2007), and the same mutation in GABA_C ρ1 resulted in a receptor that was expressed too low for further analysis (Sedelnikova et al., 2005). However, in both receptors it was determined that this arginine does not directly interact with GABA.

Homology modeling of the Hco-UNC-49 receptor suggests that R147 faces the binding pocket but does not interact with GABA, based on our MD simulations. However, the positive charge of MTSET (when bound to the introduced cysteine) may stabilize the negative carboxyl group of GABA, which may explain the increase in GABA current we observed after application. In the insect RDL receptor, mutations at this position (i.e., R166) with either an A or G produced non-functional channels. However, it has been suggested that this residue was not likely to be essential for GABA binding (Ashby et al., 2012).

It is possible that R147 has a different role in nematode UNC-49 receptors compared with mammalian and possibly even Drosophila receptors. We found evidence for a molecular interaction between the side chains of R147 and D149 (Fig. 7A). In the GABA_A α1 and the GABA_C ρ1 receptors the residues in the analogous positions as D149 are nonionic polar residues (Fig. 2). Therefore, the potential interactions between R147 and other residues may provide some unique features of the nematode GABA receptor.

H142C produced a hypersensitive channel. Treatment with MTSET revealed that the introduced cysteine was accessible for modification, and the resulting modification increased the GABA current. Our MD simulations suggests that H142 does not face the binding pocket, so it may affect the binding site in other ways. Indeed, the mutation H142C not only had a positive effect on GABA, but also affected the Hco-UNC-49 receptor partial agonist DAVA, converting it to a full agonist. This mutation had minimal effect on the sensitivity of the neurosteroid PS, a negative allosteric modulator shown to bind at a site away from the agonist binding site (Wardell et al., 2006). Because histidine is an amino acid with a positively charged bulky side group, it is possible that its removal results in structural changes that may positively
functions of the nematode UNC-49 receptor will be focus of
in GABA binding. Whether it contributes to some unique
other GABA receptors is a nonpolar leucine. We anticipate,
H-bond with the carboxyl group of GABA (Ashby et al., 2012).
Simulations also revealed high occurrences of H-bonds be-
2007) where they form an H-bond with GABA. The molecular
both cases these residues were confirmed to lie within the
future drug target.

Acknowledgments

We thank Mieah Callanan for assistance with the molecular
modeling.

Authorship Contributions

Participated in research design: Kwaka, de Haan, Forrester.
Performed experiments: Kwaka, Hassan Khatami, Foster, Cochrane, Habibi.
Contributed new reagents or analytic tools: de Haan, Forrester.
Data analysis: Kwaka, Hassan Khatami.
Wrote or contributed to the writing of the manuscript: Kwaka, Hassan Khatami, Forrester.

References


Accardi MV and Forrester SG (2011) The Haemonchus contortus unc-49b subunit possesses the residues required for GABA sensitivity in homomeric and hetero-


Bamber BA, Ayling AA, and Jorgensen EM (1999) The Caenorhabditis elegans unc-49 locus encodes multiple subunits of a heteromeric GABA re-


Del Olmo N, Bustamante J, Del Río RM, and Solís JM (2000) Taurine activates GABA (A) but not GABA(B) receptors in rat hippocampal CA1 area. Brain Res 864:
329–337.


Hosie AM, Wilkins ME, da Silva HM, and Smart TG (2006) Endogenous neuro-


Kaji MD, Kwaka A, Callanan MK, Nusrat H, Desaulniers JP, and Forrester SG (2015) A molecular characterization of the agonist binding site of a nematode cys-

Kloda JG and Czajkowski C (2007) Agonist-, antagonist-, and benzodiazepine-


Sedelnikova A, Smith CD, Zakharkin SO, Davis D, Weiss DS, and Chang Y (2005) Mapping the rho1 GABA(C) receptor agonist binding pocket. Constructing a com-


field for drug-like molecules compatible with the CHARMM all-atom bi-

Vannomneslaeghe K and Mackrell AD Jr (2012) Automation of the CHARMM General Force Field (CGenFF) II: assignment of bonded parameters and par

Downloaded from jrn.org as part of the JRN by [Your Institution] on September 23, 2023. molpharm.aspetjournals.org Downloaded from morp.org at September 14, 2023.


**Address correspondence to:** Dr. Sean G. Forrester, University of Ontario Institute of Technology, 2000 Simcoe Street North, Oshawa, ON L1H 7K4, Canada. E-mail: sean.forrester@uoit.ca