Title:

Functional Characteristics of the Lepidopteran Ionotropic GABA Receptor 8916 Subunit Interacting with the LCCH3 or the RDL Subunit

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Running title: *GABA receptor 8916 subunit mediated ion channel*

ABSTRACT: The inotropic γ-aminobutyric acid (iGABA) receptor is commonly considered as a fast inhibitory channel and is an important insecticide target. Since 1990, RDL, LCCH3, and GRD were successively isolated and found to be potential subunits of the insect iGABA receptor. More recently, one orphan gene named as 8916 was found and considered to be another potential iGABA receptor subunit according to its amino acid sequence. However, little information about 8916 has been reported. Here, the 8916 subunit from *Chilo suppressalis* was studied to determine whether it can form part of functional iGABA receptors by co-expressing this subunit with CsRDL1 or CsLCCH3 in the *Xenopus* oocyte system. Cs8916 or CsLCCH3 did not form functional ion channels when expressed alone. However, Cs8916 was able to form heteromeric ion channels when expressed with either CsLCCH3 or CsRDL1. The recombinant heteromeric Cs8916/LCCH3 channel was a cation-selective channel, which was sensitive to GABA or β-alanine. The current of Cs8916/LCCH3 channel was inhibited by dieldrin, endosulfan, fipronil or ethiprole. In contrast, fluralaner, broflanilide and avermectin showed little effect on the Cs8916/LCCH3 channel (IC_{50}s > 10,000 nM). The Cs8916/RDL1 channel was sensitive to GABA, which were significantly different in EC_{50} and I_{max} for GABA to homomeric CsRDL1. Fluralaner, fipronil or dieldrin showed antagonistic actions on Cs8916/RDL1. In conclusion, Cs8916 is a potential iGABA receptor subunit, which can interact with CsLCCH3 to generate a cation-selective channel that is sensitive to several insecticides. Also, as Cs8916/RDL1 has a higher EC_{50} than homomeric CsRDL1, Cs8916 may serve to affect the physiological function of CsRDL1 and therefore play a role in fine-tuning GABAergic signaling.

KEYWORDS: *Chilo suppressalis*; *Xenopus* oocyte; 8916; GABA receptor; insecticide;

INTRODUCTION

Ionotropic γ-aminobutyric acid (iGABA) receptors mediate the fast-inhibitory neurotransmission in the nervous system of invertebrates. They are members of the cys-loop ligand-gated ion channel superfamily, where ion channels consist of five subunits surrounding a central ion channel. [1] In insects, iGABA receptors are involved in memory, sleep and locomotion, and most importantly are the molecular target of several effective insecticides including fipronil, fluralaner and broflanilide. [2] However, our knowledge about the structure and subunit composition of iGABA receptors in insects remains unclear. Several findings relating to the function of iGABA receptors have been observed in the nematode *Caenorhabditis elegans*, [3] which indicate that multiple types of GABA-mediated receptors exist in invertebrates. For example, EXP-1 is an excitatory GABA-gated cation channel whereas UNC-49 is an inhibitory GABA-gated anion channel. [3, 4]

To date, three candidate genes, RDL (resistance to dieldrin), LCCH3 (ligand-gated chloride channel homolog 3) and GRD (GABA and glycine-like receptor of *Drosophila*), are considered as iGABA receptor subunits in insects. [1, 2, 5] RDL has been studied from several insect species, such as the fruit fly *Drosophila melanogaster*, small brown planthopper *Laodelphax striatellus* and the honeybee *Apis mellifera*. [6-10] Due to amino acid sequence homology, it has been suggested that the 8916 gene is a putative GABA receptor subunit that has been identified in many insect species including *D. melanogaster*, *L. striatellus*, the silk worm *Bombyx mori*, rice stem borer *Chilo suppressalis*, parasitoid wasp *Nasonia vitripennis*, *A. mellifera*, and the red flour beetle *Tribolium castaneum*. [1, 11-15] In *D. melanogaster*, Dm8916 and DmLCCH3 are located on chromosome X, whilst DmGRD and DmRDL are found on chromosome 3. In *A. mellifera*, Am8916 and AmLCCH3 are also located together on chromosome LG9 whilst AmRDL and AmGRD are found on chromosomes LG7 and LG1, respectively. [16] It is worth noting that AAF48539 (GenBank No. NP_001162770.1) reported to encode for the Dm8916 subunit, [11] does not express functional channels, likely due to a missing N-terminus sequence. [17]

Different combinations of subunits can generate ion channels that exhibit distinct functional and pharmacological properties. For *D. melanogaster*, heterologously expressed DmRDL,

DmLCCH3 and DmGRD have been studied. [10, 17] DmRDL can generate a functional anion-selective homomeric iGABA-gated chloride channel whereas DmLCCH3 with DmGRD can generate a GABA-gated cation-selective channel. [17, 18] Coincidentally, while this manuscript was in preparation, heterologous expression of AmLCCH3 and AmGRD from *A. mellifera* was reported. [19] Native currents induced by GABA recorded from different cell types or developmental stages are diverse and present subtle differences, which suggest the existence of several subtypes of iGABA receptors. [20-22] As 8916 is considered to be a potential iGABA subunit, studying this subunit and its interactive effects with LCCH3 and RDL may enhance our understanding of the functional properties of insect GABA receptors.

Previously, we have described the cloning and functional expression of *C. suppressalis* RDL1, RDL2 and LCCH3. [12, 23] In this study, we report isolation of Cs8916 and functional studies of this subunit with CsLCCH3 or CSRDL1 expressed in *Xenopus laevis* oocytes.

MATERIALS AND METHODS

Ethical Statement. The use of *X. lavies* in the present study strictly followed the ethics of the China [2010-172] and Nanjing Agricultural University guidelines for the protection of animal welfare.

Insect and Chemicals. *Chilo suppressalis* were reared as previously reported. [24] Broflanilide, avermectin, and the plasmid vector pGH19 with/without fluorescence for cRNA expression in *X. laevis* oocytes were used as previously described. [25, 26] Fluralaner (purity ≥ 99.0%) was purified from Bravecto as previously described. [27] Fipronil (purity ≥ 98%), ethiprole (purity ≥ 98%), endosulfan (purity ≥ 98%) were purchased from J & K Scientific Ltd. (Beijing, China).

Isolation and Determination of the Genomic Location of *C. suppressalis* iGABA Receptor Subunits. The open reading frames (ORFs) of the iGABA receptor subunit genes, Cs8916, CsLCCH3 and CsRDL1 genes, were cloned by RT-PCR using total RNA extracted from *C. suppressalis* as described previously, [12] and were ligated into p*EASY*-T3 using the p*EASY*®-T3 Cloning Kit (TransGen Biotech, Co., LTD, Beijing, China).

To detect the location of iGABA receptor subunits on *C. suppressalis* chromosomes, genomic data was downloaded from InsectBase database (http://www.insect-genome.com/) and aligned with each subunit using Local BLAST with BioEdit software (IBIS Biosciences, USA).

Electrophysiological Expression of cRNA in *Xenopus Laevis* Oocytes. The p*EASY*-T3-CsLCCH3 and p*EASY*-T3-Cs8916 vectors were used as template to amplify CsLCCH3 or Cs8916 by PCR (see **Table 1** for primers used) with the following reaction conditions: 35 cycles of 98 ºC for 10 s, 55 ºC for 5 s and 72 ºC for 20 s; and final elongation at 72 ºC for 5 min. Subsequently, amplified Cs8916 and CsLCCH3 were ligated into pGH19-EYFP and pGH19-mRFP, respectively, using the ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China) to generate pGH19-CsLCCH3-mRFP and pGH19-Cs8916-EYFP. [25] The fluorescent tag was fused at the C-terminus of the iGABA receptor subunits. These plasmids were used to detect the expression and distribution of subunits expressed in *X. laevis* oocytes. The recombinant plasmids were transferred into the *Trans*1-T1 Phage Resistant Chemically Competent Cell (TransGen Biotech), sequenced by GenScript (Nanjing) and stored at -80 ºC until use.

Table 1. Primers used in construction of oocyte expression vectors with fluorescence protein tags

Primer name	Primer sequence (5′→3′)	Function
CsLCCH3-*Hind*III-F	GGGGATCCGAATTCG**AAGCTT***GCCACC*ATGAGCGCGCGTCGCA	Construction of pGH19-CsLCCH3-mRFP
CsLCCH3-*Hind*III-R	CCATGATATCTCGAG**AAGCTT**GGTCGAATATATAGAAGACC	
Cs8916-*Hind*III-F	GGGGATCCGAATTCG**AAGCTT***GCCACC*ATGTTCGCGGTCGACA	Construction of pGH19-Cs8916-EYFP
Cs8916-*Hind*III-R	CCATGATATCTCGAG**AAGCTT**GGTGGGAGAGACTATTCATTG	

Note: Nucleotide sequences matching the pGH19 vector are underlined, the restriction enzyme sites for *Hind* III are shown in bold, and the Kozak sequence is indicated in italics whilst the remaining sequences are gene-specific.

The capped RNA (cRNA) transcripts were synthesized from the plasmids pGH19-Cs8916-EYFP, pGH19-CsLCCH3-mRFP and pGH19-CsRDL1-EGFP using mMESSAGE mMACHINE® T7 Kit (Life Technologies, Carlsbad, CA). Briefly, the plasmid was linearized by *Not*I at 37 ºC for 4–5 h before *in vitro* transcription to generate cRNA at 37 ºC for 2 h in a reaction system including 2 μL 10 × Reaction Buffer, 10 μL 2 × NTP/CAP, 1 μg linearized plasmid, 2 μL Enzyme Mix, and nuclease-free water to adjust the total volume up to 20 μL. The cRNA was purified with isopropanol and 75% (v/v) ethanol, dissolved in nuclease-free water, quantified by NanoDrop 1000 UV-VIS spectrophotometer (Thermo Fisher Scientific, Inc.) before being diluted to 272 ng μL^{-1} and stored at -80 ºC until use.

African clawed frogs (*Xenopus laevis*) were immersed in ice for 30 min to be anesthetized for acquirement of oocytes. The ovary lobes were dissected from the *X. laevis* and subsequently treated with collagenase type 1A (2 mg mL^{-1}) in Ca^{2+}-free standard oocyte saline (SOS) solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6) at 18 ºC for 90 min in a shaking incubator at 60 rpm. Each oocyte, at stage V-VI, was injected with 5.0 ng of cRNA dissolved in nuclease-free water (18.4 nL), and then the oocytes were incubated at 16 ºC for 2 – 4 days in SOS solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6) containing penicillin-streptomycin (100 U mL^{-1}-100 μg mL^{-1}), 50 μg mL^{-1}gentamicin sulfate, 2.5 mM sodium pyruvate and 5% (v/v) HI horse serum (Life Technologies) before electrophysiological recordings.

Electrophysiological assays were performed using a two-electrode voltage-clamp setup on the Axoclamp 900A Microelectrode Amplifier Platform (Molecular Devices, CA) at a holding potential of -60 mV with a pipette puller resistance of 0.5 – 3 MΩ. Micropipettes were prepared from glass capillaries (Model P-97, Sutter Instrument Co., CA) with parameters: O.D.: 1.2 mm, I. D.: 0.69 mm, 10 cm length. GABA-induced current signals were recorded by the Axon Digidata 1440A Data Acquisition System (Molecular Devices). Experiments were performed at 20 ºC and oocytes were placed in a recording chamber and perfused using SOS solution with perfusion speed at 8 – 10 mL min^{-1}. GABA dissolved in SOS solution was applied to stimulate oocytes for 5 s, at intervals of 85 s. Dose-response curves of GABA were obtained by sequential applications of increasing

concentrations. Peak current amplitudes were plotted against GABA concentrations and the median effective concentration (EC_{50}) values were determined using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Insecticides were initially dissolved in dimethyl sulfoxide (DMSO) before being diluted in SOS solution with a final DMSO concentration less than 0.1% (v/v). DMSO at 0.1% had no effect on the response of oocytes. Insecticidal solution was perfused alone for 85 s after successive control applications of GABA at EC_{50}, and then GABA (EC_{50}) was co-applied with the insecticidal solution consecutively for the remainder of the experiment for 5 s at 85 s intervals during perfusion, until the inhibition of the response was constant. The median inhibition concentration (IC_{50}) values were determined from the mean of 3-10 replications using the non-linear regression program with GraphPad Prism 6.

Digital-imaging Fluorescence Microscopy. Fluorescence images were acquired from Nikon's SMZ25 stereomicroscope with exclusive Perfect Zoom System (Nikon Instruments Inc., Melville, NY). Laser confocal images of oocytes were captured using a Leica TCS SP8 with the Leica Application Suite X software platform (Leica Microsystems Inc., Buffalo Grove, IL). For labeling of different membrane compartments, oocytes were exposed to SOS solution. Peak maxima light excitation/emission for EYFP (514 nm/527 nm) and mRFP (561 nm /582 nm) was used. All images were processed by using Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA) with identical values for contrast and brightness.

Reversal Potential of the Different Recombinant Receptors. The reversal potential of the different recombinant receptors was measured in a basic recording solution (100 mM NaCl, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.6) using voltage from -80 mV to +40 mV. For ion exchange experiments, 0%, 25%, 50% and 100% NaCl in basic recording solution was replaced by sodium acetate for chloride exchange or by TEACl for sodium exchange. The pH of NaCl solution used for exchange experiments should be finely adjusted to 7.6 with NaOH. [28] The differences between the reversal potential measured in the different solutions and that measured in basic recording solution (Erev shift) were plotted against the chloride or sodium concentration.

Statistical Analysis. Data were shown as mean ± standard error (SE). The EC_{50} and IC_{50} values were considered as significantly different if their 95% confidence interval (CI) did not overlap. [29, 30]

RESULTS

8916, LCCH3 and RDL1 Subunits Locate at Different Positions on Chromosomes 1 and 2. The ORFs of Cs8916, CsLCCH3 and CsRDL1 were successfully amplified and were found to consist of 1722, 1479 and 1458 nucleotides, respectively. [12, 23] Genomic analysis showed that Cs8916, CsLCCH3 and CsRDL1 genes respectively have 11 exons and 10 introns, 10 exons and 9 introns, 10 exons and 9 introns, and are located on chromosomal 1, chromosomal 1, and chromosomal 2, respectively (Figure 1).

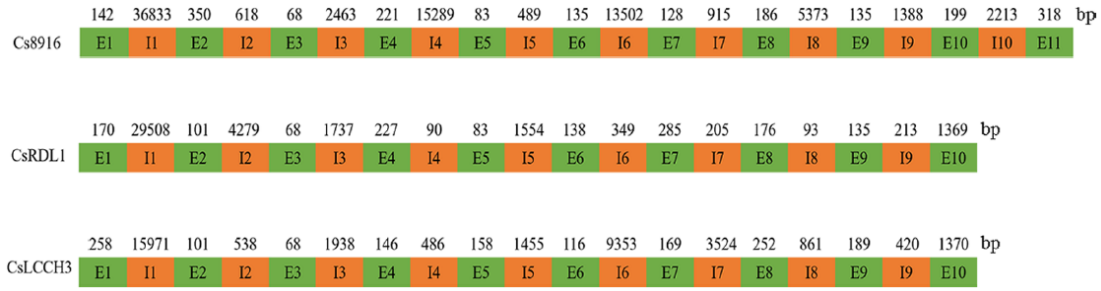
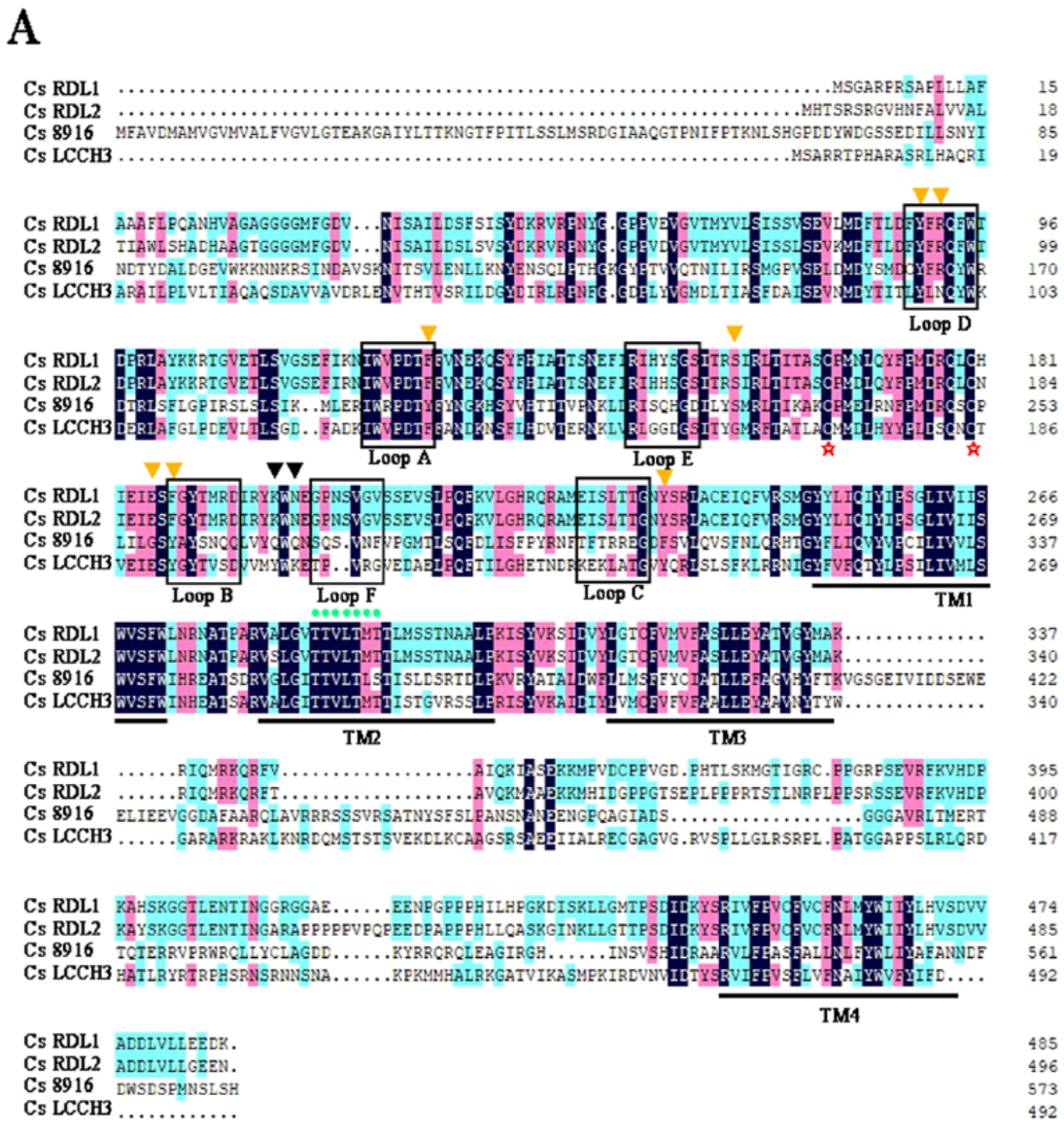


Figure 1. Genomic analysis of Cs8916, CsRDL1, and CsLCCH3. The labeled numbers represent the length of nucleotide sequences. The orange and green boxes represent the introns and exons, respectively

Cs8916 Exhibits High Identity with Other iGABA Receptor Subunits. The Cs8916 subunit shares 24.31% and 26.85% identity with CsRDL1 and CsLCCH3, respectively at the amino acid level. Seven amino acids (Y, R, F, S, E, F and Y) being responsible for GABA binding in DmRDL, [31-33] are all present in CsRDL1 and CsRDL2, whilst four (F/Y (Loop A), E/G (just before Loop B), F/Y (Loop B) and Y/F (near Loop C)) are changed in Cs8916 (**Figure 2A**).

As is well-known, there are six loops, named as A, B, C, D, E, F, in each subunit of iGABA receptors (**Figure 2A**). Among these loops, loops A, B and C are on the principal subunit and loops D, E and F are on the complementary one, which are implicated in the agonist binding pocket located at the interface between extracellular domains of two subunits. [34] The amino acid sequences of the B and F loops of 8916 subunit from different organisms are more different when compared to other loops (**Figure S2**).

As shown in **Figure 2A**, four transmembrane segments, which are typical for cys-loop ligand-gated ion channel subunits, were observed in CsRDL, CsLCCH3, CsGRD and Cs8916. The first half of the second transmembrane segment (TM2), which includes most of the amino acids lining the channel pore (in particular the TTVLT motif), is highly conserved in Cs8916 subunits (**Figure 2B**).



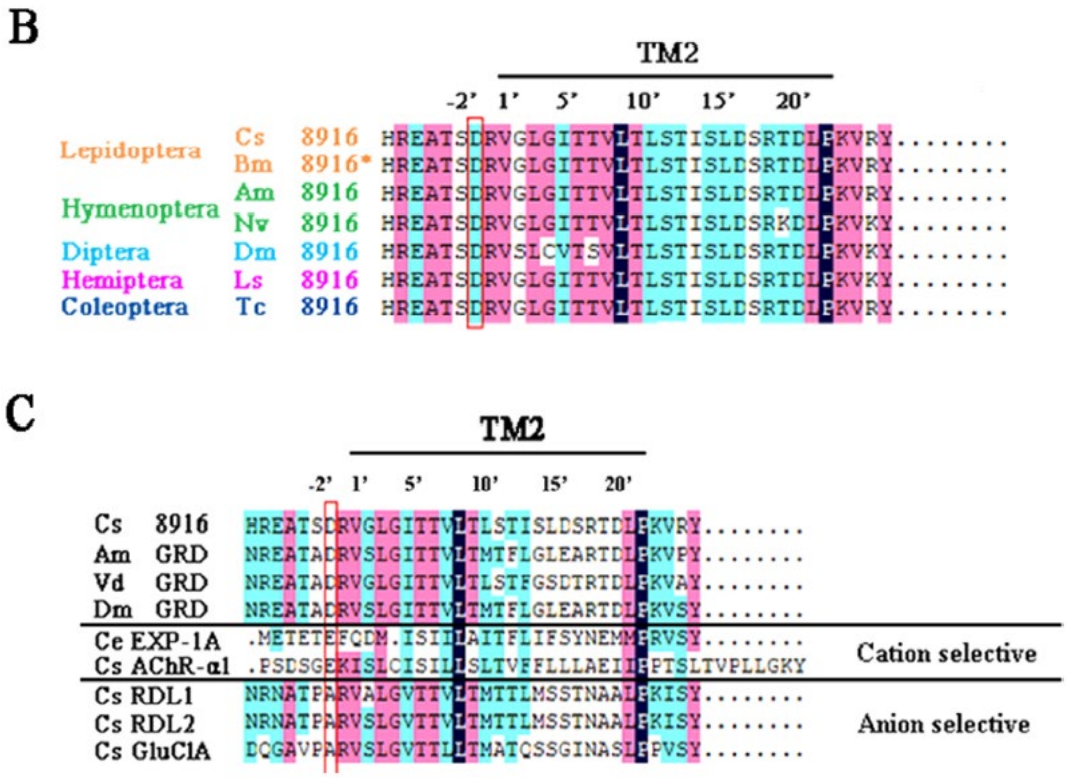


Figure 2. Amino acid sequence analysis of the iGABA receptor subunits

(A), Alignment of iGABA receptor subunits, including CsRDL1(ASY91961.1), CsRDL2(ASY91962.1), Cs8916(ASY91959.1) and CsLCCH3(ASY91960.1). Asterisk (☆) indicate the cysteine amino acid residues involved in the Cys-loop. The amino acids involved in GABA binding and implicated in first events of GABA binding are markered by yellow- and black-triangle, respectively. (B), Alignment of the TM2 segment of 8916 from various species. The accession number are follows: Cs8916 (ASY91959), Bm8916 ('*' referred to as BmGRD, NP_001182633.1), Am8916 (ABG75745), Nv8916 (XP_008203400), Dm8916 (NP_001162770), Ls8916 (AOO87784), Tc8916 (ABU63605). The aspartic acid labelled with red sequare is the key element of anionic or cationic conductance. (C), Alignment of subunits forming cation or anion channels. The accession number are as follows: AmGRD (AJE68942), DmGRD (CAA55144.1), VdGRD (AVY53073.1), CeEXP-1A (AAQ96594) *C. suppressalis* nicotinic acetylcholine receptor (CsAChR-α1, AKQ12739), CsRDL1(ASY91961.1), CsRDL2(ASY91962.1), *C. suppressalis* glutamate-gated chloride channel (CsGluClA). [35] The aspartic acid labelled with red sequare is the key element of anionic or cationic conductance.

Alanine (A) at the 2' position in TM2, which is a critical site for the action of insecticides, [2] was replaced by glycine (G) in most insect 8916 subunits from different species (**Figure 2B**). Also, the amino acids (P-2'; A-1'; T13' present in RDL and GluCl) required to be in an anion-selective channel were replaced in the 8916 subunits (S-2'; D-1'; T13'). [36] It is worth noting that Cs8916 possesses a negatively charge residue at position -1' (D-1'), which is a hallmark of cation selective ligand-gated ion channels, such as in CsAChR-α1 (E-1') (**Figure 2C**). To date, AmGRD, DmGRD and VdGRD have been reported to form cation selective receptor co-expressed with LCCH3. [17, 19] Therefore, our results provided the evidence from the amino acid level that Cs8916/LCCH3 could form cation selective receptor.

Loop C plays a role in gating for the GABA binding pocket, [37] however the loop C sequence varies among CsRDL1, Cs8916 and CsLCCH3 subunits (**Figure 2A**). In DmRDL, the 218th amino acid (arginine, R, equivalent to CsRDL1 position 199 just before Loop F) is considered as an essential amino acid for the first event of GABA binding. [33] At the equivalent position, Cs8916, CsRDL1 and CsLCCH3 bear Q (charged), N (uncharged) and K (charged) amino acids (**Figure 2A**), respectively, which is consistent for AmRDL and AmLCCH3.

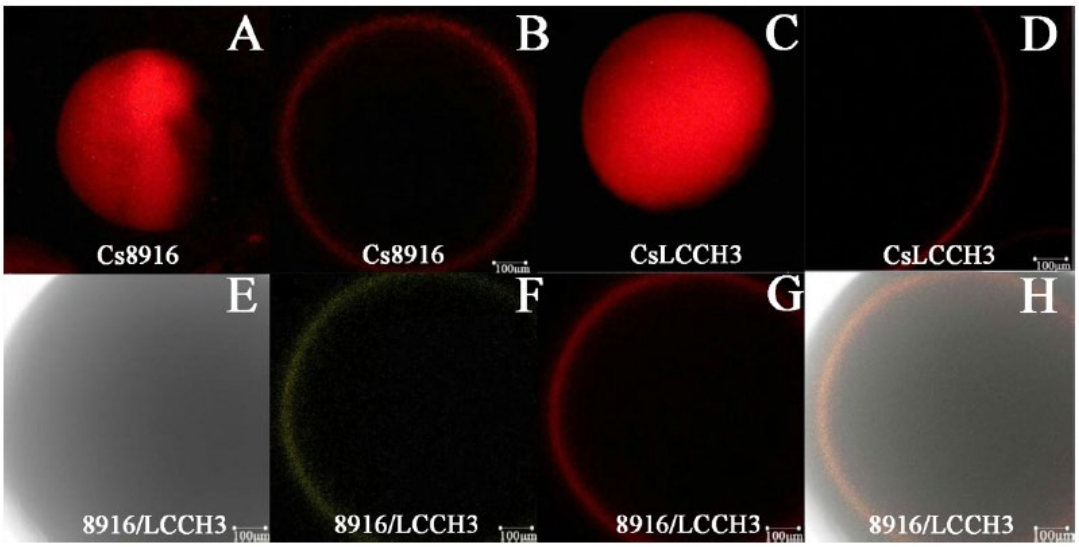


Figure 3. Fluorescence microscope images of *X. laevis* oocytes injected with iGABA receptor subunit(s). Raw images (A and C) and (B, D, E, F and G) were digitally acquired from Nikon's SMZ25 stereomicroscope and Leica TCS SP8, respectively. (A and C), Examples of whole oocytes injected with pGH19-Cs8916-EYFP or pGH19-CsLCCH3-mRFP cRNAs, respectively. (B and D), Laser focal image construction of *X. laevis* oocytes injected with mRFP-Cs8916 or

mRFP-*Cs*LCCH3 cRNAs. (E) Oocyte in bright field; (F) Yellow fluorescence at 514 nm; (G) Red fluorescence at 561 nm; (H) Merge of fluorescence of (F) and (G).

Heteromeric Channels Containing Cs8916 Respond to Agonists. In the present study, all fused fluorescence iGABA receptor subunits could be visualized using fluorescence and laser confocal microscopy in cRNA-injected *X. laevis* oocytes (**Figure 3**). The fluorescent results showed that the iGABA receptor subunit(s) could be successfully expressed in the *X. laevis* oocytes and fluorescence was distributed isotropically in the cellular membrane, either individually (Cs8916 or CsLCCH3) or in combination with a 1:1 stoichiometry (Cs8916/LCCH3) *in situ* (**Figure 3**).

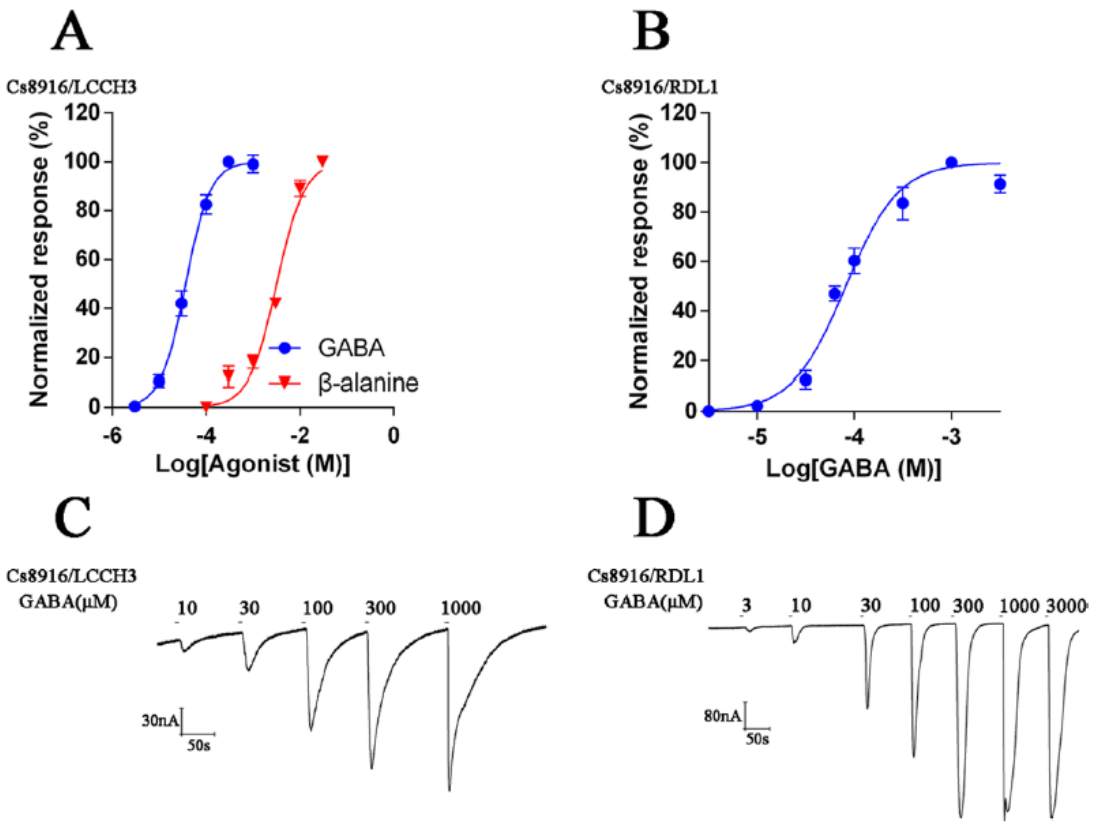
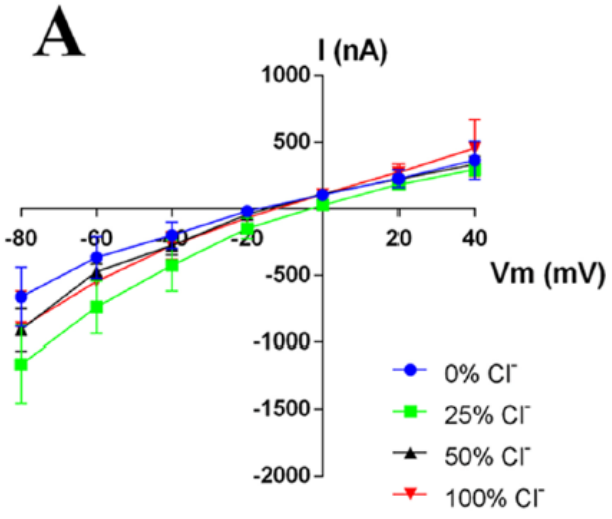


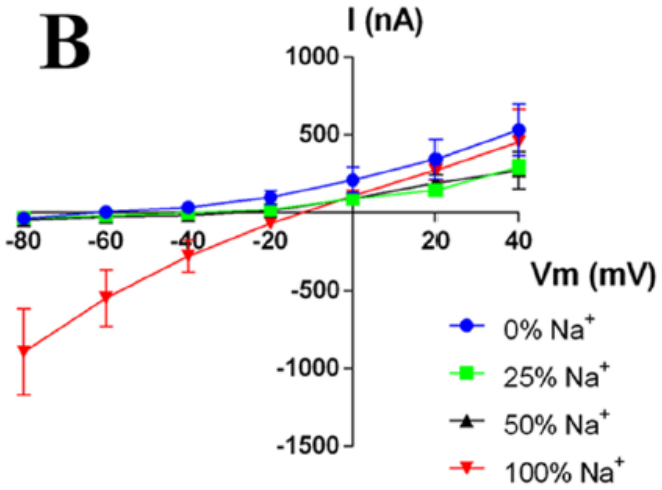
Figure 4. Concentration-response curves of GABA and β-alanine induced currents in *X. laevis* oocytes co-injected with EYFP-Cs8916/mRFP-CsLCCH3 (A and C) or EYFP-Cs8916/ EGFP-RDL1 (B and D).

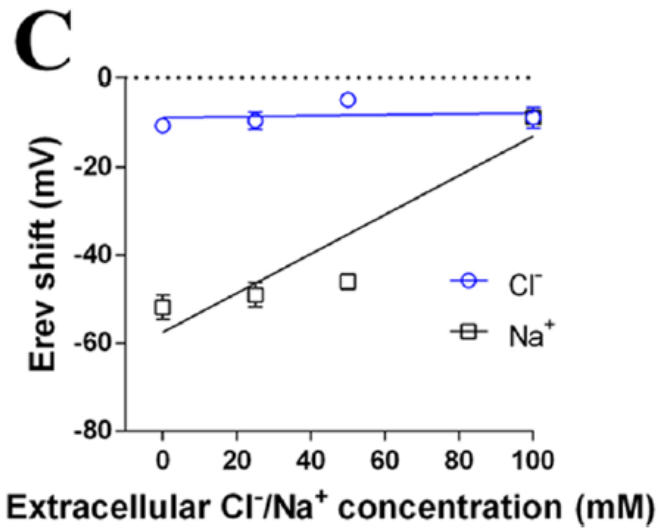
Xenopus laevis oocytes were injected with either CsRDL1, Cs8916 or CsLCCH3. CsRDL1 alone formed functional ion channels gated by GABA with an EC_{50} of 50.85 ± 6.29 μM and

maximum current (I_{max}) of 1605.75 ± 431.83 nA (n = 4). Cs8916 or CsLCCH3, on the other hand, did not elicit currents with GABA or β-alanine when injected alone (data not shown). However, the co-expression of Cs8916/LCCH3 or Cs8916/RDL1 resulted in heteromeric channels that responded to GABA or β-alanine (**Figure 4**). In the Cs8916/LCCH3 channel, GABA and β-alanine could both stimulate the inward currents in a dose-dependent manner with EC_{50} values of 37.00 ± 2.36 μM (n = 10) and 3217.00 ± 238.50 μM (n = 5), and with I_{max} of -309.36 ± 60.60 nA and -58.73 ± 8.62 nA, respectively (**Figure 4A and C**). Compared to β-alanine, GABA activates the Cs8916/LCCH3 channel with greater efficacy, and the EC_{50} of Cs8916/LCCH3 is 2.20-fold lower than that of Cs8916/RDL1 **(Figure 4A and B)**. In the Cs8916/RDL1 channel, GABA could induce I_{max} (**Figure 4D**) and the EC_{50} value at -1022.04 ± 315.33 nA and 81.56 ± 5.75 μM (n = 6) (**Figure 4B and D**), respectively.

Heteromeric Channel of Cs8916/LCCH3 Is a Cation Channel. We first measured the reversal potential of the Cs8916/LCCH3 channel currents in *X. laevis* oocytes (**Figure 5**). Changing voltage (-80 to + 40mV) was applied to continuous 37.00 μM GABA (EC_{50}) applications in basic recording solution (100 mM NaCl, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.6). For ion exchange experiments, 25%, 50% and 100% NaCl were replaced by sodium acetate for chloride exchange and by TEACl for sodium exchange. Under these conditions, the reversal potential of Cl^- and Na^+ for Cs8916/LCCH3 were between -4.73 ~ -10.56 mV (**Figure 5A**) and between -8.80 mV~-51.83 mV (**Figure 5B**).







Current-voltage relationship of *X. laevis* oocytes co-injected with pGH19-CsLCCH3-mRFP and pGH19-Cs8916-EYFP under varying external Cl^- (A) and Na^+(B) conditions. (C) Reversal potential shifts for ionic substitution experiments.

Reversal potential analysis of current-voltage curves were obtained by applying -80mV/+40 mV voltage ramps in the presence of 37.00 µM GABA. The reversal potential obtained for the Cs8916-containing GABA receptors was highly dependent on changes in external Na^+ concentrations, but insensitive to changes in external Cl^- concentrations. Reversal potentials of Cs8916/LCCH3 were not sensitive to increased Cl^- concentration but only sensitive to increased Na^+ concentrations (**Figure 5C**).

Heteromeric Channels were Differentially Inhibited by Insecticides. Seven insecticides were tested for their capacity to block the recombinant heteromeric *C. suppressalis* GABA-gated ion channels (**Figure 6A**). Four of the tested insecticides, dieldrin, endosulfan, fipronil and ethiprole, inhibited GABA-induced currents from Cs8916/LCCH3 channels expressed in *X. laevis* oocytes, and their IC_{50} values were 4.01 ± 1.12, 62.01 ± 27.22, 10.15 ± 2.56 and 12.92 ± 4.09 nM, respectively (**Figure 6B**). The current of Cs8916/LCCH3 was only slightly inhibited by fluralaner, broflanilide and avermectin at 10,000 nM with inhibition efficiency of 23.46 ± 3.78%, 24.75 ± 9.44% and 28.71 ± 6.03%, respectively (**Figure 6B)**. In contrast, Cs8916/RDL1 was considerably more sensitive to fluralaner. Fluralaner, fipronil and dieldrin showed antagonistic actions on Cs8916/RDL1, with IC_{50} values of 7.74 ± 2.47, 7.00 ± 1.43, 2.92 ± 1.35 nM, respectively (**Figure 6C**).

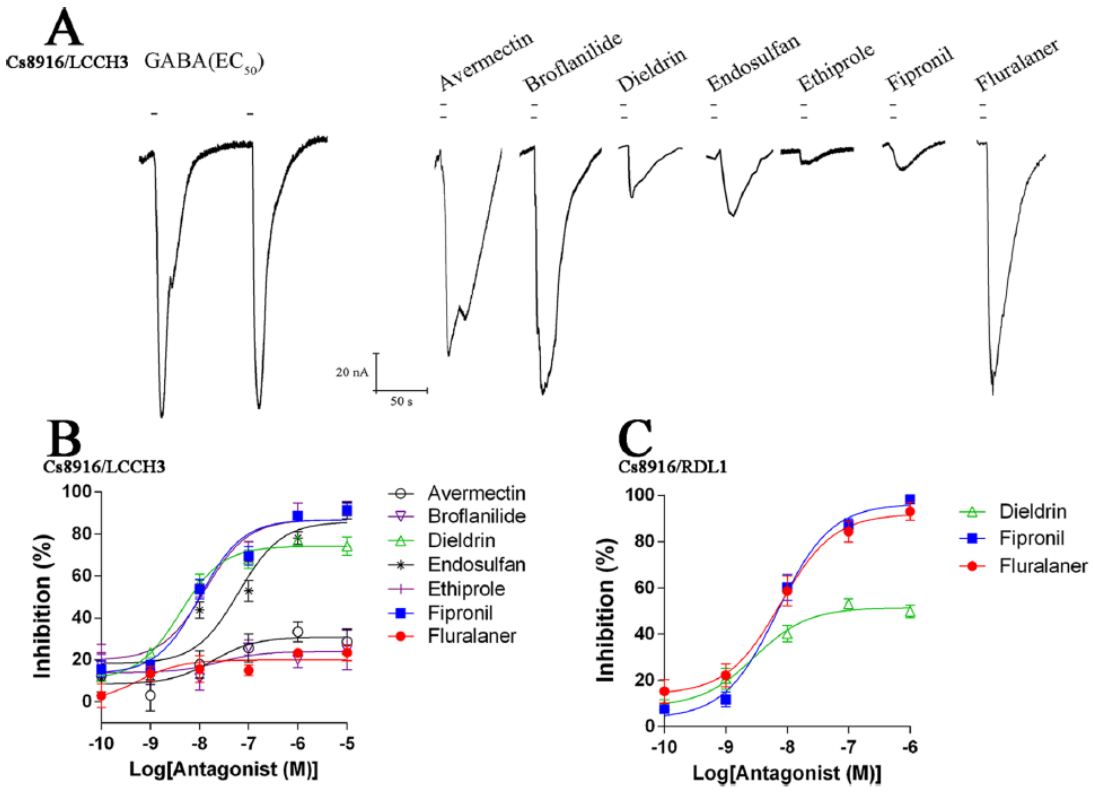


Figure 6. Concentration-response relationship of the blockade of GABA (EC_{50})-induced currents by insecticides

DISCUSSION

Insect iGABA receptors are of interest as they are important molecular targets of insecticides. [38, 39] In our previous study, four iGABA receptor subunits from *Chilo suppressalis* CsRDL1, CsRDL2, CsLCCH3 and Cs8916 were identified. [12, 23] The physiological and pharmacological function of RDL, LCCH3 and GRD in other insects, such as *Apis mellifera* and *Drosophila melanogaster* have been studied, [9, 10, 19, 40, 41] but the potential function of 8916 remains unclear. Recently, the Bm8916 ortholog has been shown to be expressed in Crz neurons and participate in progeny diapause induction. [42] We report here the first study describing the functional and pharmacological properties of the 8916 subunit as part of heteromeric GABA-gated ion channels when co-expressed with either CsRDL1 or CsLCCH3 in *X. laevis* oocytes.

The four types of iGABA receptor subunits, DmRDL, DmLCCH3, DmGRD and Dm8916 (CG8916), exist as separate genes in the *D. melanogaster* genome. [15, 43] DmLCCH3 and Dm8916 are both located on the sex chromosome 10, whilst DmGRD and DmRDL are located on chromosome 3. In *A. mellifera*, the Am8916 and AmLCCH3 genes are both found on chromosome LG9, whilst AmRDL and AmGRD are located on chromosomes LG7 and LG1, respectively. In *L. striatellus*, LsRDL, LsLCCH3, LsGRD and Ls8916 have also been identified.[11, 44] However, their chromosomal locations remain to be determined. In *C. suppressalis*, Cs8916 and CsLCCH3 are also encoded for by separate genes that are both located on the same chromosome whilst the CsRDL1 gene is on a different chromosome (**Figure 1**). [12]

To date, there is no strong evidence to clarify the combination of subunits forming insect iGABA receptors *in vivo*, even if some neurons have been shown to co-express RDL and LCCH3. [18, 45] In *Drosophila*, the GABACls/GluCls heteromer was reported by Ludmerer et al. (2002), [46] whereas negated afterward by Zhao et al. (2004). [47] Therefore, the homomeric and heteromeric channels of Cs8916 with CsLCCH3 or CsRDL1 were expressed in *X. laevis* oocytes to detect the potential physiological and pharmacological properties of Cs8916 and other iGABA receptor subtypes *in vitro*. When injected alone, Cs8916 or CsLCCH3 were unable to form a functional iGABA receptor (**Figure 3**), while only CsRDL1 could form a functional homomeric channel. [23]However, heterologous expression of Cs8916 or CsLCCH3 with a fluorescent tag in *X. laevis* oocytes showed these subunits were expressed on the surface (**Figure 3**), which is consistent with

other reports. [41, 48] It is necessary to state that because Cs8916/LCCH3 and CsRDL1 could successfully express in *Xenopus* oocytes to generate the functional channel, therefore the fluorescence images of Cs8916/RDL1 expressed in *Xenopus* oocytes were not captured (**Figure 3**). A previous study clarified that lacking two key amino acids (R111 and E204 in DmRDL, and the equivalent positions of amino acid acids in CsRDL1 are R92 and E185), which could produce an ionic interaction with the zwitterion GABA within the GABA binding pocket of DmGRD and DmLCCH3, prevented the generation of functional channels. [31] Therefore, we speculated that the failure of Cs8916 and CsLCCH3 to individually form function channels was also due to the absence of residues equivalent to R111 and E204 in DmRDL.

In a previous study, *D. melanogaster* 8916 (CG8916) alone or in combination with DmGRD or DmLCCH3 did not form any detectable GABA receptors when expressed in *X. laevis* oocytes, but this cannot rule out the possibility that the ORF of Dm8916 was incomplete and thus coded for a nonfunctional protein. [17] In **Figure 3**, the fluorescence images showed that Cs8916 and CsLCCH3 are localized on the entire oocyte membrane. Even though Cs8916 and CsLCCH3 did not form a functional receptor alone, however, both subunits together can form a functional ion channel. We therefore speculated that no functional channel and current can be detected without the formation of heteromer. In addition, previous studies on heteromeric GABA-gated channel, including DmGRD/LCCH3 [17], VdGRD/LCCH3 [49], AmGRD/LCCH3 [19], were also defined according to the electrophysiological method. Interestingly, we also found that injected of Cs8916 with CsLCCH3 formed a cationic channel and that GABA could elicit a response with an EC_{50} of 37.00 ± 2.36 μM (**Figure 4**), which is similar to DmGRD/LCCH3 [17] and AmGRD/LCCH3. [19] To date, there are indications from physiological studies in invertebrates that GABA mediates excitation by activating cation currents. [50, 51] Such excitatory GABA has been described in multiple physiological situations, for example, during immature rodent neuron development and synaptogenesis. [52-54] In the stomatogastric ganglion of the crab *Cancer borealis*, GABA could evoke excitatory currents. [51] The CeEXP-1 and CeLGC-35 cys-loop LGIC subunits from the nematode *C. elegans* can generate the GABA-gated cation channels in neurons and muscles, and have been implicated in physiological functions such as defecation. [3, 55, 56] We therefore speculated that Cs8916/LCCH3 receptors, as cation channels, may perform excitatory conductance in insect

372 cells.

373 In *C. suppressalis*, two RDL isoform (CsRDL1 and CsRDL2) were isolated, which very
374 highly resembled each other at the amino acid level, with the biggest difference between both
375 subunits being the 2nd amino acid in TM2. [23] Even though other species, such as aphids, also
376 possess two RDL subunits, most insects possess only one *Rdl* gene. [57] Thus, in the present study,
377 we chose CsRDL1 as the representative RDL subunit to determine whether it can assemble with
378 8916. We found that Cs8916/RDL1 generated an anion-selective heteromeric channel with Cl^-
379 conductance. In the Cs8916/RDL1 channel, it thus appears that the presence of RDL seems to be
380 decisive for the selectivity filter, [58] with the addition of Cs8916 reducing the sensitivity of the
381 heteromeric channel to GABA (**Figure 3**). The EC_{50} Cs8916/RDL1 is 1.60-fold higher and
382 significantly different to that of the CsRDL1 homomeric receptor whilst the maximum current
383 induced by Cs8916/RDL1 channel was decreased by 36% compared to that of CsRDL1 alone. [23]
384 In contrast to Cs8916/RDL1, functional data for homomeric CsRDL1 was generated where the
385 subunit was not fused to fluorescent tags, however, this was found not to affect the
386 electrophysiological properties of the expressed receptors. [25]

387 The iGABA receptor is one of the most important target of insecticides, including fipronil,
388 avermectin, fluralaner and broflanilide (desmethyl-broflanilide). [2, 5, 59, 60]. The pentameric RDL
389 GABA receptor so far is the only one considered in assessing the toxicity of insecticides directed
390 against insect iGABA receptors. [21, 61, 62] Our findings that Cs8916 affects sensitivity to insecticides,
391 such as Cs8916/RDL1 being more sensitive to dieldrin but less sensitive to fluralaner when
392 compared to homomeric CsRDL1 (**Figure 6** and **reference** 23) In addition, Cs8916/LCCH3 is
393 sensitive to fipronil, dieldrin and endosulfan highlights this heteromeric receptor as being another
394 molecular target for these insecticides, mutations in which may result in resistance. Also,
395 Cs8916/LCCH3 may represent a novel target for the development of insecticides. It is therefore
396 pertinent to determine whether Cs8916/RDL1 receptors exist *in vivo*.

397 In conclusion, the novel iGABA receptor subunit Cs8916 can generate a heteromeric cationic
398 channel with CsLCCH3, and influence the CsRDL subunit. The identification of heteromeric
399 GABA receptors with cation selectivity expands our view of iGABA receptors in insects. It can be
400 speculated that heteromeric receptors composed of proteins with 8916 and LCCH3 may form the

molecular basis of the excitatory action of GABA in some invertebrates. Functional expression studies will help us further characterize the pharmacological properties of this new class of iGABA receptor and assess their potential as targets for novel insecticides. However, it should be noted that the Cs8916/LCCH3 channel arose from co-expression in a heterologous system *in vitro* and the potential function of 8916 and involvement of this subunit in insecticide sensitivity *in vivo* should be verified using techniques such as RNAi or CRISPR/Cas9.

ASSOCIATED CONTENT

Supporting Information

Figure S1. GABA-evoked currents from Cs8916/LCCH3 expressed in *Xenopus laevis* oocytes by applying -80 mV/+40 mV voltage ramps

Figure S2. Comparison of iGABA receptor GRD and 8916 subunit sequences from various insects

Figure S3. Phylogenetic tree of iGABA receptor subunits generated by EvolView

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Notes

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‡ Both authors (QTH and CWS) contribute equally to this work.

ABBREVIATIONS USED

Cs*RDL*, RDL gene of *Chilo suppressalis*; CsRDL, protein of RDL of *Chilo suppressalis*; RDL,

resistance to dieldrin gene; GABA, γ-aminobutyric acid; GRD, GABA and glycine receptor-like subunit of *Drosophila*; LCCH3, ligand-gated chloride channel homolog 3; LGICs, ligand-gated ion channels

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