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The neonicotinoid imidacloprid, and the pyrethroid deltamethrin, are antagonists of the insect Rdl GABA receptor

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Abbreviations: GluCl, glutamate-gated chloride channel; nAChR, nicotinic acetylcholine receptor; Rdl, resistant to dieldrin; SOS, standard oocyte saline; TM, transmembrane domain; VGSC, voltage-gated sodium channel

Abstract

A mutation in the second transmembrane domain of the GABA receptor subunit, Rdl, is associated with resistance to insecticides such as dieldrin and fipronil. Molecular cloning of Rdl cDNA from a strain of the malaria mosquito, *Anopheles gambiae*, which is highly resistant to dieldrin revealed this mutation (A296G) as well as another mutation in the third transmembrane domain (T345M). Wild-type, A296G, T345M and A296G + T345M

homomultimeric Rdl were expressed in *Xenopus laevis* oocytes and their sensitivities to fipronil, deltamethrin, 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (DDT), imidacloprid and spinosad were measured using two-electrode voltage-clamp electrophysiology. Spinosad and DDT had no agonist or antagonist actions on Rdl. However, fipronil, deltamethrin and imidacloprid decreased GABA-evoked currents. These antagonistic actions were either reduced or abolished with the A296G and the A296G + T345M mutations while T345M alone appeared to have no significant effect. In conclusion, this study identifies another mutation in the mosquito Rdl that is associated with insecticide resistance. While T345M itself does not affect insecticide sensitivity, it may serve to offset the structural impact of A296G. The present study also highlights Rdl as a potential secondary target for neonicotinoids and pyrethroids.

Introduction

The insect γ-aminobutyric acid (GABA) receptor Rdl (resistant to dieldrin) plays a central role in neuronal signalling and is involved in various processes, including regulation of sleep (Liu *et al.*, 2014), aggression (Yuan *et al.*, 2014) and olfactory learning (Liu *et al.*, 2009). It is a member of the cys-loop ligand-gated ion channel superfamily and thus contains an N-terminal extracellular domain, where GABA binding occurs, and four transmembrane (TM) domains, the second of which lines the ion channel (Nys *et al.*, 2013). Rdl from several species can be expressed as functional homomeric receptors in expression systems, such as *Xenopus laevis* oocytes (Buckingham *et al.*, 2006), permitting the study of their pharmacological properties.

Rdl is also of interest as it is the target of highly effective insecticides (Raymond-Delpech *et al.*, 2005; Buckingham *et al.*, 2005). Studies on the model organism *Drosophila melanogaster* identified an alanine to serine mutation located in TM2 that underlies resistance to several insecticides, including dieldrin, picrotoxinin and fipronil (Ffrench-Constant *et al.*, 1993; Hosie *et al.*, 1995).This alanine to serine mutation, found also as alanine to glycine, has since been associated with insecticide resistance in varying species, ranging from crop pests (e.g. the planthopper *Laodelphax striatellus* (Nakao *et al.*, 2011)), pests afflicting livestock (the horn fly *Haematobia irritans* (Domingues *et al.*, 2013)), domesticated animals (the cat flea *Ctenocephalides felis* (Bass *et al.*, 2004)) and disease vectors (the malaria vector mosquito *Anopheles gambiae* (Du *et al.*, 2005)). In several cases, a second mutation has been observed (Feyereisen *et al.*, 2015). For example, in dieldrin resistant *Anopheles funestus* mosquitoes, the additional mutation V327I was found always with the TM2 A296S mutation (Wondji *et al.*, 2011). V327I is located in the large intracellular loop between TM3 and TM4, however the impact of this mutation on Rdl function and insecticide sensitivity has yet to be studied. In another case, Rdl from fipronil-

resistant *Drosophila simulans* had a mutation (T350M) in addition to the classical TM2 mutation (A301G) (Le Goff *et al.*, 2005). T350M is located in TM3 and two-electrode voltage-clamp electrophysiology was applied to *X. laevis* oocytes expressing *D. simulans* Rdl. Results showed that A301G and T350M individually decreased sensitivity to fipronil and picrotoxin, with the double mutant showing highest resistance to fipronil. It remains to be determined whether these second mutations are present in other species and so may reflect a common mechanism leading to insecticide resistance.

We report here the presence of alanine to glycine and threonine to methionine mutations (A296G and T345M) in Rdl of an *An. gambiae* strain that is resistant to dieldrin. The conservation of this double mutation in association with insecticide resistance is as previously identified in *D. simulans* (Le Goff et al., 2005). Using *X. laevis* oocytes, we present the first heterologous expression studies of an *Anopheles* Rdl to assess how the mutations affect sensitivity to fipronil. In addition, we studied the actions of other insecticides, specifically imidacloprid, spinosad, deltamethrin and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (DDT). Even though imidacloprid and spinosad are thought to target primarily nicotinic acetylcholine receptors (nAChRs) (Millar and Denholm, 2007), and deltamethrin is thought to target primarily voltage-gated sodium channels (VGSCs) (Soderlund, 2012), work on cultured cells and brain membranes suggests that they also may act on GABA receptors (Deglise *et al.*, 2002; Wakeling *et al.*, 2012; Kirst, 2010). We therefore took advantage of the *X. laevis* oocyte expression system to test for the first time whether these insecticides act directly on Rdl and whether any observed actions were affected by the A296G and T345M mutations.

Methods

Culturing of *An. gambiae* mosquitoes

Dieldrin resistant and susceptible *An. gambiae sensu stricto* mosquitoes were obtained from the Vector Control Reference Laboratory of the National Institute for Communicable Diseases, Johannesburg. Adult female laboratory cultured mosquitoes drawn from a colony originally collected from the Democratic Republic of Congo were selected for dieldrin resistance by exposure to filter papers containing 4% dieldrin for 2.5 hours. Subsequent phenotypic selection was conducted 24 hours post exposure and mosquitoes were stored in silica at -80^{o}C prior to analysis. Ethical clearance for the use of mosquitoes for research purposes has been obtained from the Research Ethics Committee (medical) of the University of the Witwatersrand, Johannesburg (ref: W-CJ-100510-1).

Materials

All chemicals, including insecticides, were purchased from Sigma Aldrich, unless otherwise stated.

Isolation of Rdl and GluCl from dieldrin resistant and susceptible *An. gambiae* mosquitoes

Total RNA was isolated by homogenising 3-5 adult female mosquitoes with a hand held homogeniser and the homogenate filtered through a column from a QIA shredder kit (Qiagen, Valencia, CA, USA). The eluate was then purified using an RNeasy Mini kit (Qiagen) to isolate the RNA. The RNA was reverse transcribed using the GoScript Reverse Transcription System (Promega, Madison, WI, USA) and the complete coding sequence of Rdl was amplified from the complementary DNA using Pfu polymerase (Promega), using the following flanking primers: N-terminal 5'-ATGTCGCTAACTATCGAAGTTC-3', C-terminal 5'-TTACTTCTCCTCGCCCAGC-3'. The full length *An. gambiae* GluCl was amplified using the primers 5'- ATGGCCTCGGGCCATTTC-3' and 5'- GTCCTCCTCCTCTTCGCG-3'. Amplification products were sequenced at Source BioScience (http://www.sourcebioscience.com/).

Site-directed mutagenesis and preparation of DNA

The amplified Rdl_{bd} variant with the A296G and T345M mutations was cloned into the pCI vector (Promega) using the PCR primers 5'-TTTTTTGAATTCATGTCGCTAACTATCGAAGTTC-3' and 5'-AAAAAAGCGGCCGCTTACTTCTCCTCGCC -3', which respectively have EcoRI and NotI restriction sites. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, CA, USA) to create wild-type and single mutant Rdl constructs. The mutagenesis primers used were as follows: G296A 5'-GCTACACCAGCACGTGTTG**C**ATTAGGTGTAACTACTGTC-3' and M345T 5'-CTGCTGGAGTACGCCA**C**GGTCGGTTACATGGCTAAG-3' (mutations are bold and underlined). The mutations were verified by DNA sequencing. The cloning generated four constructs for analysis: Rdl wild-type, A296G, T345M and double mutant (A296G+T345M). The splice variant and presumed RNA editing sites were kept consistent in each construct (Rdl_{bd}, edits: I176V+N183G).

Expression of Rdl in *X. laevis* oocytes and two-electrode voltage clamp electrophysiology

The functional effects of the Rdl mutations in *An. gambiae* were evaluated using the *X. laevis* expression system and two-electrode voltage clamp electrophysiology. Stage V and

VI *X. laevis* oocytes were harvested and rinsed with Ca^{2+} free solution (82 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.6), before defolliculating with 1 mg/ml type IA collagenase in Ca^{2+} free solution for 1 - 2 hrs at room temperature. Defolliculated oocytes were injected with 3.5 ng (23 nl) wild-type Rdl or mutant Rdl plasmid DNA into the nucleus of the oocyte and stored in standard Barth's solution (supplemented with 5% horse serum, 50 µg/ml neomycin and 10 µg/ml penicillin/streptomycin) at 17.5°C. Oocytes 2-7 days post-injection were placed in a recording chamber and clamped at -60 mV with two 3 M KCl filled borosilicate glass electrodes (resistance 0.5-5 MΩ) and an Oocyte Clamp OC-725C amplifier (Warner Instruments, CT, USA). Responses were recorded on a flatbed chart recorder (Kipp & Zonen BD-11E, Delft, Netherlands). Oocytes were perfused with standard oocyte saline (SOS; 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) at a flow rate of 10 ml/min and with 3 min between challenges to prevent the effects of desensitisation. Oocytes were selected for experiments if stable after three consecutive challenges of 1 mM GABA.

Concentration response curves were generated by challenging oocytes to increasing concentrations of GABA in SOS. Curves were calculated by normalising the 0.1 µM – 2 mM GABA responses to average control responses induced by 1 mM GABA before and after application.

Insecticide inhibition curves were generated by inhibiting 1 mM GABA in SOS with 0.001 – 500 µM fipronil, imidacloprid, deltamethrin, spinosad or DDT. The insecticides were initially diluted in dimethylsulphoxide (DMSO), before diluting to final concentrations in SOS. Final concentrations of 0.1% DMSO did not affect electrophysiological readings. Oocytes were initially incubated with a perfusion of the insecticide in SOS for 3 min (fipronil) or 6 min (imidacloprid, deltamethrin, spinosad and DDT) before challenging with 1 mM GABA plus insecticide, at the required concentrations. Inhibition curves were calculated by normalising the responses to the previous control response induced by 1 mM GABA.

Data analysis

Data are presented as mean ± SEM of individual oocytes from at least 3 separate frogs. The concentration of GABA required to evoke 50% of the maximum response (EC_{50}), the concentration of insecticide required to inhibit 50% of the maximal GABA response (IC_{50}) and the Hill coefficient (nH) were determined by non-linear regression using Graphpad Prism 4 (Graphpad Software, CA, USA). Statistical significance was determined by using one-way ANOVA or an unpaired t-test (Graphpad Software).

Results

Sequencing of the full length Rdl coding sequence from dieldrin sensitive and resistant *An. gambiae*

The Rdl receptor was characterised in *An. gambiae* samples that were either resistant or susceptible to dieldrin. Amplification of the Rdl cDNA from resistant mosquitoes revealed two mutations (Fig. 1), the well-established A296G mutation (Buckingham et al., 2005) (GCA → GGA) and another mutation in TM3, T345M (ACG → ATG). In an analysis of three Rdl RT-PCR amplifications from three corresponding *An. gambiae* RNA extractions, the two mutations always occurred together. In contrast, neither mutation was present in the amplifications from three susceptible mosquito RNA extractions. Two further substitutions were detected, I176V (ATC → GTC) and N183G (AAT → GGT). These involved adenosine to guanosine changes, which is consistent with RNA A-to-I editing (Keegan *et al.*, 2004). Since the two potential editing sites were present in the susceptible mosquitoes and therefore did not appear to be associated with insecticide resistance, they were not investigated further in this study. The Rdl cDNA that was amplified, cloned and used for functional studies was the bd splice variant (ffrench-Constant and Rocheleau, 1993), which is the predominant Rdl variant found in *D. melanogaster* (Jones *et al.*, 2009).

Since Rdl and the glutamate-gated chloride channel (GluCl) can be targeted by the same insecticide, such as fipronil (Raymond-Delpech et al., 2005), we also amplified and sequenced *An. gambiae* GluCl (Meyers *et al.*, 2015) from both dieldrin susceptible and resistant strains. When compared to the *An. gambiae* PEST strain genomic sequence (Accession CM000357 AAAB01000000), I157V substitution (ATT → GTT) was observed in both susceptible and resistant strains. The adenosine to guanosine change indicates RNA A-to-I editing. No resistance associated mutations were found.

Sensitivity of wild-type and mutant *An. gambiae* Rdl receptors to GABA and fipronil

Four Rdl constructs were generated for expression in *X. laevis* oocytes; wild-type, A296G only, T345M only and a double mutant (A296G + T345M). GABA concentration response curves were generated with concentrations ranging from 0.1 µM – 2 mM for each construct (Fig. 2). EC_{50} values were calculated for each mutant receptor (Table 1). The values for A296G and the double mutant were significantly different from one another.

The contribution of the T345M mutation to insecticide resistance was evaluated with fipronil, which is known to act on Rdl (Raymond-Delpech et al., 2005; Hosie et al., 1995). As previously shown (Le Goff et al., 2005), the A296G mutation conferred resistance to fipronil (Fig. 3), with a significant increase in IC_{50} (Table 1). This was also observed for the double mutant (A296G + T345M). The IC_{50} values for A296G and the double mutant were not

significantly different from each other. T345M resulted in no significant change in IC_{50} compared to wild-type suggesting that this mutation does not contribute to fipronil resistance.

Sensitivity of wild-type and mutant *An. gambiae* Rdl receptors to deltamethrin, DDT, imidacloprid and spinosad

Studies have indicated that imidacloprid, deltamethrin and spinosad act on GABA receptors (Deglise et al., 2002; Kirst, 2010; Wakeling et al., 2012), however their direct actions on Rdl have yet to be investigated. Since DDT can act on the same target (VGSCs) as deltamethrin (Hemingway *et al.*, 2004), we also measured its effect on Rdl.

Spinosad was evaluated as a potential agonist and/or antagonist of Rdl. Spinosad did not act as an agonist when applied at 100 µM and it did not reduce the 1 mM GABA response (Fig. 4A). Spinosad also did not act as an antagonist of Rdl when 100 µM was applied with 1 mM GABA following a 6 minute pre-incubation with 100 µM spinosad (Fig. 4B). A concentration range of 0.0001 – 100 µM spinosad was also tested using these conditions but none of the concentrations elicited a response (data not shown).

DDT was also evaluated as a potential agonist and/or antagonist of Rdl. Concentrations ranging from 0.0001 – 100 µM DDT were applied either alone or with 1 mM GABA following a 6 minute pre-incubation. No response was observed with DDT alone, and no reduction of the 1 mM GABA-evoked current was evident (data not shown).

We found that imidacloprid and deltamethrin inhibited the GABA induced currents elicited by *X. laevis* oocytes expressing wild-type Rdl (Figs. 5 and 6). A concentration range of 0.001 – 500 µM imidacloprid was used to generate an inhibition curve for wild-type Rdl, which had an IC_{50} of 109 ± 68 µM (Table 1 and Fig. 5B). Similar to fipronil, the A296G and double mutant (A296G + T345M) conferred resistance to imidacloprid, with no significant inhibition observed up to 500 µM (Fig. 5C). However, the T345M mutation did not significantly change the IC_{50} from that of the wild-type (Table 1).

A concentration of 50 µM deltamethrin was able to inhibit the 1 mM GABA response by 30 ± 5% in wild-type Rdl (Fig. 6). The maximum concentration of deltamethrin that could be used was 50 µM, owing to the lack of solubility at higher concentrations; therefore an inhibition curve and IC_{50} value could not be generated. In concordance with fipronil and imidacloprid, the A296G and double mutant significantly reduced the sensitivity to 50 µM deltamethrin ($P<0.01$) and inhibition of the 1 mM GABA response was negligible (Fig. 6C). The T345M mutation showed GABA inhibition with 50 µM deltamethrin comparable to that of the wild-type.

Discussion

Anopheles gambiae is a vector of the parasitic diseases malaria and lymphatic filariasis, which cause extensive morbidity and approximately 600 000 deaths each year (Hemingway *et al.*, 2006; World Health Organization, 2015). Disease vector control through the use of insecticides is one of the major strategies used to reduce malaria burden (Hemingway, 2014). However, resistance to insecticides threatens to undermine efforts to control malaria (Thomsen *et al.*, 2014).

Two major mechanisms give rise to insecticide resistance (Hemingway et al., 2004). One mechanism involves alteration in metabolism leading to heightened detoxification of foreign compounds, such as the elevated activity of P450 monooxygenase. The second mechanism involves alterations in proteins to which the insecticide directly interacts, which are commonly point mutations in the target site. Knowledge of mechanisms leading to insecticide insensitivity is crucial in detecting resistance in mosquito populations, which can then inform the use of alternative insecticide classes, as part of an effective resistance management program (Mnzava *et al.*, 2015). Certain point mutations in known insecticide target sites are associated with resistance. For example, the detection of the alanine to serine or to glycine mutation in TM2 of Rdl is used to identify resistance to dieldrin or fipronil (Kwiatkowska *et al.*, 2013). However, it is becoming more apparent that there are other mutations in Rdl that are associated with insecticide resistance (Feyereisen et al., 2015).

Here we report a second mutation in Rdl of *An. gambiae*, T345M, which was present with the well characterised A296G mutation (Buckingham et al., 2005). Both mutations were also found in fipronil resistant *D. simulans* (Le Goff et al., 2005), showing conservation of this resistance mechanism in two different species. Whereas resistance was induced in *D. simulans* in the laboratory, the mosquitoes we used were colonised from wild *An. gambiae* showing phenotypic resistance to dieldrin, suggesting that the genetic architecture conferring dieldrin resistance in the laboratory strain was inherited from the wild, and therefore indicating that these mutations have relevance in the field. Consistent with other studies (Le Goff et al., 2005), we found that the A296G mutation alone reduced the potency of fipronil (Table 1). T345M, on the other hand, did not affect sensitivity to fipronil and did not enhance the reduction in fipronil sensitivity beyond that conferred by A296G alone. This is in contrast to the equivalent mutation (T350M) in *D. simulans*, which significantly reduced sensitivity (Le Goff et al., 2005). Our results, however, are in line with a study using transgenic *D. melanogaster* that found the TM2 mutation (A301G) alone resulted in the greatest level of resistance to fipronil, which was not heightened by the addition of the T350M TM3 mutation, nor did T350M alone have any effect (Remnant *et al.*, 2014). This led to the suggestion that the TM3 mutation is a modifier, reducing the fitness cost associated with the TM2 mutation (Remnant et al., 2014). In this regard it is interesting to note that of the constructs tested,

A296G had the lowest GABA EC_{50} while A296G + T345M had a significantly higher value (Table 1). The addition of the T345M mutation may thus play a role in alleviating the heightened sensitivity to GABA resulting from A296G that would otherwise have a detrimental effect on neuronal signalling. A three-dimensional homology model of the *D. melanogaster* Rdl showed that A301 and T350 are in proximity to each other at the lower part of the ion channel indicating the potential for a functional interaction (Remnant et al., 2014). Furthermore, three-dimensional homology modelling and site-directed mutagenesis of the vertebrate $GABA_A$ receptor indicated that conformational changes in TM3 amino acid side chains can be detected by proximal residues in TM2 and that interactions between TM2 and TM3 play a role in receptor function (Gielen *et al.*, 2015). Residues implicated in these interactions are in the vicinity of the A296G and T345M mutations (Fig. 1). It may thus be possible that a structural perturbation caused by the TM2 mutation in Rdl is compensated for by the TM3 mutation.

Following up on findings that spinosad, imidacloprid and pyrethroids affect GABA responses in cultured neurons or may bind to GABA receptors in brain membranes (Kirst, 2010; Deglise et al., 2002; Wakeling et al., 2012), we tested to see whether they had direct actions on heterologously expressed Rdl. We found that spinosad had no observable effect on *An. gambiae* Rdl, either as an agonist or an antagonist (Fig. 4). Spinosad appeared to have an antagonistic effect on GABA responses in small-diameter cockroach neurons but had no such consistent effect on GABA responses in larger neurons, leading to the suggestion that there may be various GABA receptor subtypes in different neurons, showing distinct sensitivities to spinosad (Watson, 2001). Perhaps spinosad exerts its action on GABA receptors consisting of subunits other than Rdl, such as GRD or LCCH3 (Buckingham et al., 2005). In addition, we tested DDT as a possible agonist or antagonist of Rdl, but it also had no observable effect (data not shown).

We did, however, observe that the neonicotinoid, imidacloprid, and the pyrethroid, deltamethrin, act as antagonists of *An. gambiae* Rdl (Figs. 5 and 6). This is consistent with a study finding that imidacloprid reduced GABA-induced responses in cultured honey bee (*Apis mellifera*) Kenyon cells (Deglise et al., 2002). The antagonistic effects of imidacloprid and deltamethrin were abolished by the A296G mutation (Figs.5C and 6C), indicating that both insecticides are acting as ion channel blockers (Buckingham et al., 2005).This observation is in accord with results suggesting that deltamethrin and other type II pyrethroids bind the GABA receptor at the same site as picrotoxinin (Crofton *et al.*, 1987), the antagonistic action of which is also abolished by the TM2 mutation (Hosie *et al.*, 1996). While the effects of pyrethroids on GABA responses were initially attributed to action on GABA receptors (Wakeling et al., 2012), subsequent studies indicated that the pyrethroid effects were in fact indirect and downstream of the effects on sodium channels since they

were completely suppressed by tetrodotoxin (Eshleman and Murray, 1991; Wakeling et al., 2012). The findings reported here clarify that deltamethrin can indeed act directly on a GABA receptor.

Our results highlight that the widely-used insecticides, imidacloprid and deltamethrin, which are thought to target nAChRs and VGSCs respectively, may also act on GABA receptors with the Rdl subunit. This has the potential to broaden our perception of the mode of action of neonicotinoids and pyrethroids. It should be noted, though, that the concentrations of imidacloprid and deltamethrin required to elicit an effect on Rdl are considerably higher than those observed to affect nAChRs and VGSCs. For instance, imidacloprid acts as an antagonist of *An. gambiae* Rdl with an IC_{50} of 109 μM (Table 1) whereas it acts as an agonist with EC_{50} 0.07 μM on the α1/β2 *D. melanogaster*/*Gallus gallus* nAChR hybrid expressed in *X. laevis* oocytes (Dederer *et al.*, 2011). For deltamethrin, 50 μM was required to see an antagonistic effect on *An. gambiae* Rdl (Fig.6) whereas an EC_{25} of 0.01 μM on the *Aedes aegypti* sodium channel was observed (Hirata *et al.*, 2014). This suggests that Rdl would be a secondary target of these insecticides if it were to be relevant *in vivo*. The Rdl TM2 mutation is commonly seen in diverse insect species, which is attributed to resistance to certain classes of insecticides including fipronil and cyclodienes (Sparks and Nauen, 2015). In some instances, the Rdl mutation has been found along with mutations in sodium channels that result in pyrethroid resistance (Kwiatkowska et al., 2013). In these cases, it would be of interest to assess specifically whether mutations in both ion channels lead to a heightened resistance to pyrethroids. Similarly, it would be prudent to determine whether a combination of the Rdl mutation with alterations in nAChRs leads to a greater insensitivity to neonicotinoids.

The Rdl TM2 mutation has been found to persist in *Anopheles* field populations where cylcodienes are no longer used (Kwiatkowska et al., 2013; Wondji et al., 2011). This is surprising considering that dieldrin resistance confers a significant fitness cost (Platt *et al.*, 2015), thus in the absence of dieldrin and other insecticides with a similar mode of action a reversion of the resistance would be expected (Domingues et al., 2013; Wondji et al., 2011). The persistence of the Rdl mutation was therefore attributed to the agricultural use of insecticides for crop protection (Kwiatkowska et al., 2013; Wondji et al., 2011). In light of our findings, it is tempting to speculate that the widespread use of pyrethroids to control mosquitoes may play a role in selecting for the Rdl mutations in the population.

There are novel compound classes, such as isoxazolines (Ozoe *et al.*, 2010) and meroterpenoid chrodrimanins (Xu *et al.*, 2015), which are capable of acting on Rdl despite having the TM2 mutation, raising the prospect of the GABA receptor as a target of interest for future insecticide discovery (Casida, 2015). Heterologously expressed *An. gambiae* Rdl, such as that reported here, may therefore provide a useful tool to facilitate screening for

further novel compounds that act on the mosquito GABA receptor while being unaffected by mutations found in the field that confer resistance to currently used insecticides.

It is concluded that the T345M mutation in the *An. gambiae* Rdl is associated with insecticide resistance. While T345M itself does not affect insecticide sensitivity, it may serve to offset the structural impact of A296G. This maybe because A296 is a deleterious mutation, which can be counteracted by the development of small effect compensatory mutations with additive phenotypic effects. The present study also highlights Rdl as a potential secondary target for neonicotinoids and pyrethroids.

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Table 1 Effects of GABA on membrane currents from *X. laevis* oocytes expressing wild-type and mutant *Anopheles gambiae* Rdl receptors, with EC_{50} and hill coefficient (nH) displayed. Also included are the effects of fipronil and imidacloprid on GABA induced membrane currents, with IC_{50} values shown. Data are the mean ± SEM of n= 3-7 oocytes from 3-6 different frogs. [a] Indicates that the GABA EC_{50}s for A296G and A296G + T345M are significantly different from each other (one-way ANOVA Bonferroni's Multiple Comparison test, $P<0.05$). * Indicates values significantly different to wild-type, $P<0.05$ (unpaired t-test).

Mutant construct	GABA		Fipronil IC_{50} (μM)	Imidacloprid IC_{50} (μM)
	EC_{50} (μM)	nH		
wild-type	95 ± 32	1.35 ± 0.27	0.31 ± 0.12	109 ± 68
A296G	60 ± 14[a]	1.18 ± 0.11	4.43 ± 1.24*	No inhibition
T345M	169 ± 37	2.46 ± 0.60	0.36 ± 0.15	319 ± 192
A296G + T345M	198 ± 49[a]	1.48 ± 0.17	2.94 ± 0.79*	No inhibition

Fig. 1 Amino acid alignment of TM2 and TM3 from *An. gambiae* and *D. simulans* Rdl as well as the mammalian $GABA_A$ α1 receptor subunit. The alanine and threonine residues in Rdl that are mutated in association with insecticide resistance are indicated. For the $GABA_A$ α1 subunit, residues at the TM2-TM3 interface involved in receptor function (Gielen et al., 2015) are highlighted.

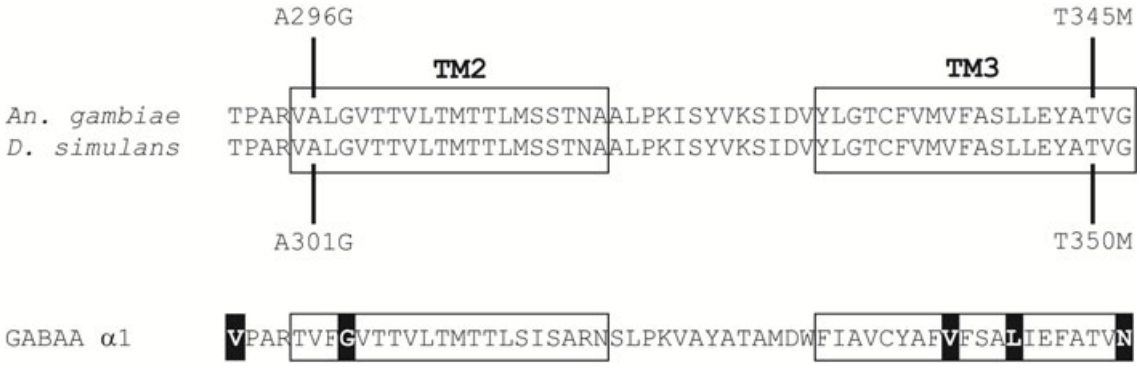
Fig. 2 Responses to GABA in *X. laevis* oocytes expressing the wild-type and mutant *An. gambiae* Rdl receptors. A) Representative current trace of a GABA concentration response curve showing responses to GABA from 10 μM – 2 mM. B) GABA concentration response curves obtained for wild-type, A296G, T345M and double mutant (A296G + T345M) Rdl receptors. Data is the mean ± SEM from n = 5-7 oocytes from 4-6 different frogs.

Fig. 3 Effects of fipronil on 1 mM GABA activated currents in *X. laevis* oocytes expressing the wild-type and mutant *An. gambiae* Rdl receptors. A) Representative current traces showing the effect of 1 μM fipronil on the 1 mM GABA response for the wild-type and double mutant (A296G + T345M). B) Fipronil inhibition curves for wild-type, A296G and double mutant Rdl, generated with concentrations from 0.0001 μM – 50 μM. Data are the mean ± SEM from n = 4-6 oocytes from 4 different frogs.

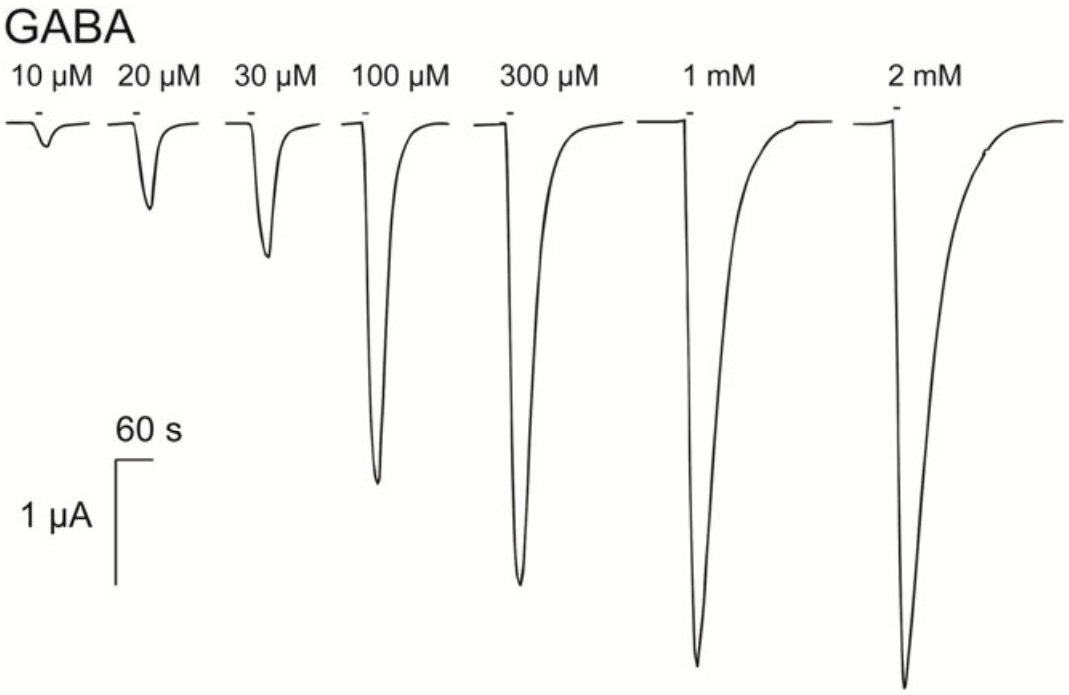
Fig. 4 Representative current traces showing the effects of spinosad on *X. laevis* oocytes expressing the wild-type *An. gambiae* Rdl receptor. A) Spinosad does not act as an Rdl agonist. 100 µM spinosad was applied 3 minutes after 1 mM GABA application and did not elicit a response. The subsequent 1 mM GABA application was not affected by the spinosad application. B) Spinosad does not act as an antagonist. A six minute pre-incubation of 100 µM spinosad was followed by a co-application of 100 µM spinosad with 1 mM GABA. The reduction in GABA response was negligible.

Fig. 5 Effects of imidacloprid on 1 mM GABA evoked currents in *X. laevis* oocytes expressing the wild-type and mutant *An. gambiae* Rdl receptors. A) Representative current traces showing the effect of 500 µM imidacloprid on the 1 mM GABA response for the wild-type and double mutant (A296G + T345M). B) Imidacloprid inhibition curve for wild-type Rdl, generated with concentrations from 0.0001 µM – 500 µM. C) Effects of imidacloprid on the GABA response for the Rdl mutants. Data are the mean ± SEM from n = 3-4 oocytes from 3 different frogs.

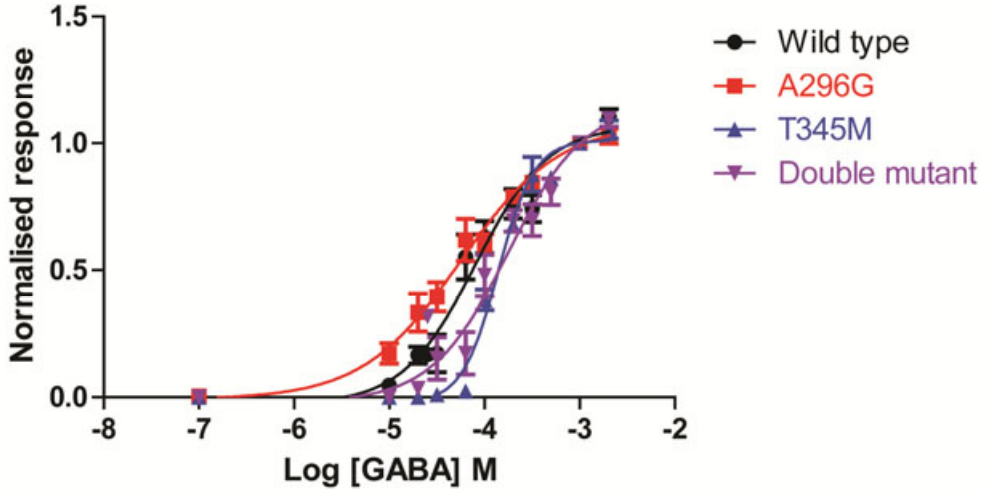
Fig. 6 Effects of deltamethrin on 1 mM GABA activated currents in *X. laevis* oocytes expressing the wild-type and mutant *An. gambiae* Rdl receptors. A) Representative current traces showing the effect of 50 µM deltamethrin on the 1 mM GABA response for the wild-type and double mutant (A296G + T345M). B) Effects of deltamethrin inhibition on the GABA response for wild-type Rdl, generated with concentrations from 0.0001 µM – 50 µM. C) Effects of 50 µM deltamethrin on the 1 mM GABA response for the Rdl mutants, expressed as a percentage current amplitude compared to the response without deltamethrin. None or negligible inhibition of the GABA response was observed for the A296G and double mutant. Data are the mean ± SEM from n = 3-5 oocytes from 3-4 different frogs. The A296G and double mutant responses are significantly different from the wild-type (* $P<0.01$, unpaired t-test).

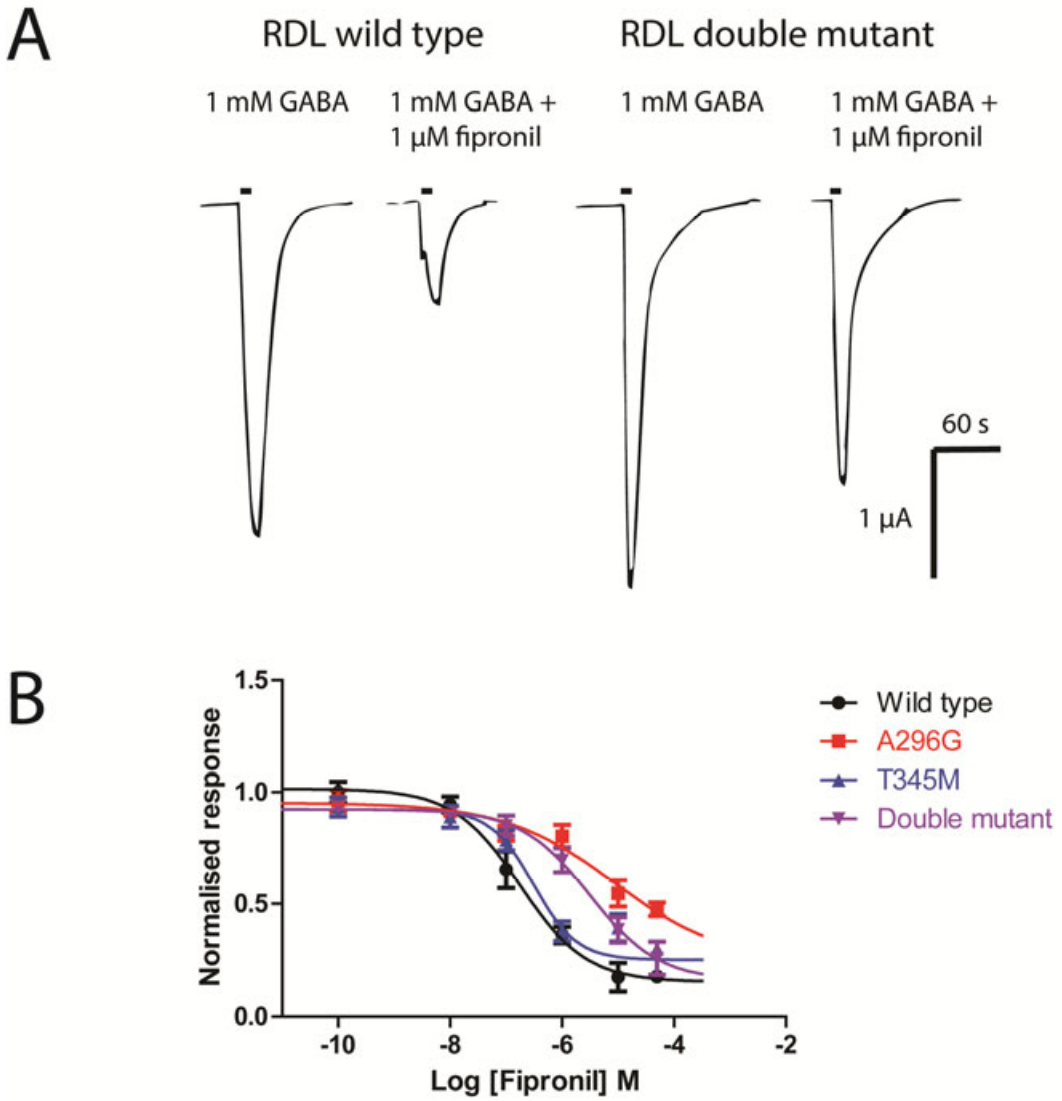

A296G
T345M
TM2
TM3
An. gambiae TPARVALGVTTVLTMTTLMSSTNAALPKISYVKSIDVYLGTCFVMVFASLLEYATVG
D. simulans TPARVALGVTTVLTMTTLMSSTNAALPKISYVKSIDVYLGTCFVMVFASLLEYATVG
A301G
T350M
GABAA α1 VPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAVCYAFVFSALIEFATVN

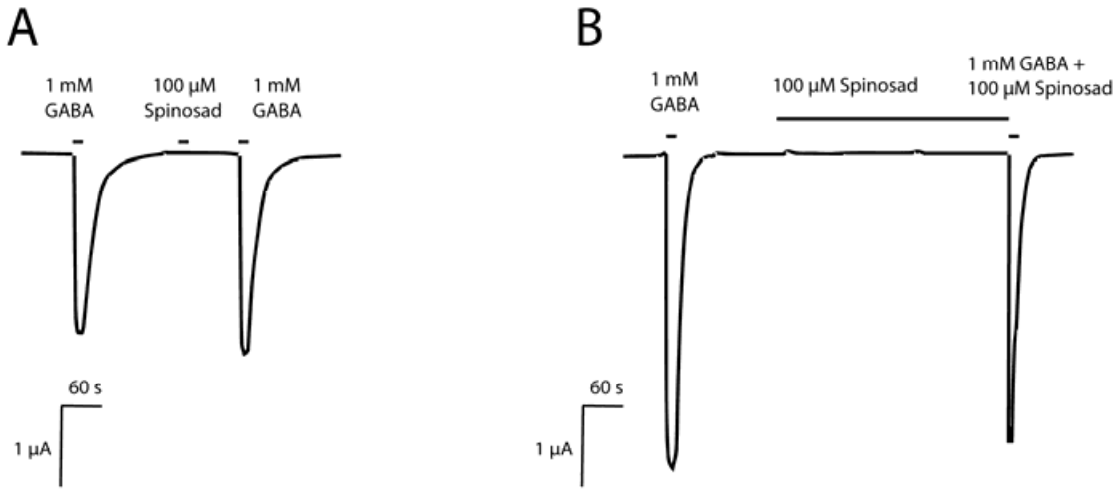
A

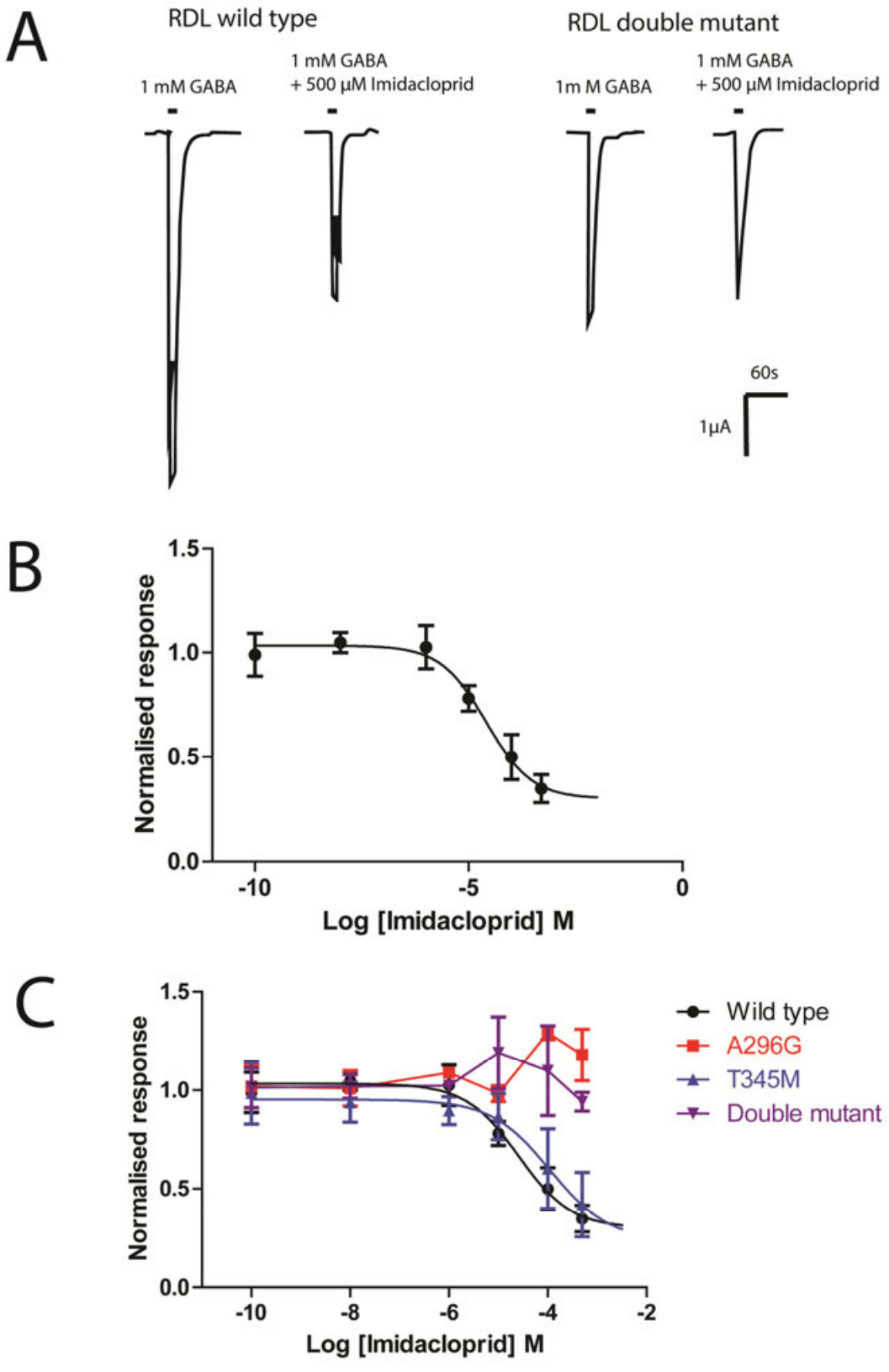

GABA
10 µM
20 µM
30 µM
100 µM
300 µM
1 mM
2 mM
60 s
1 µA

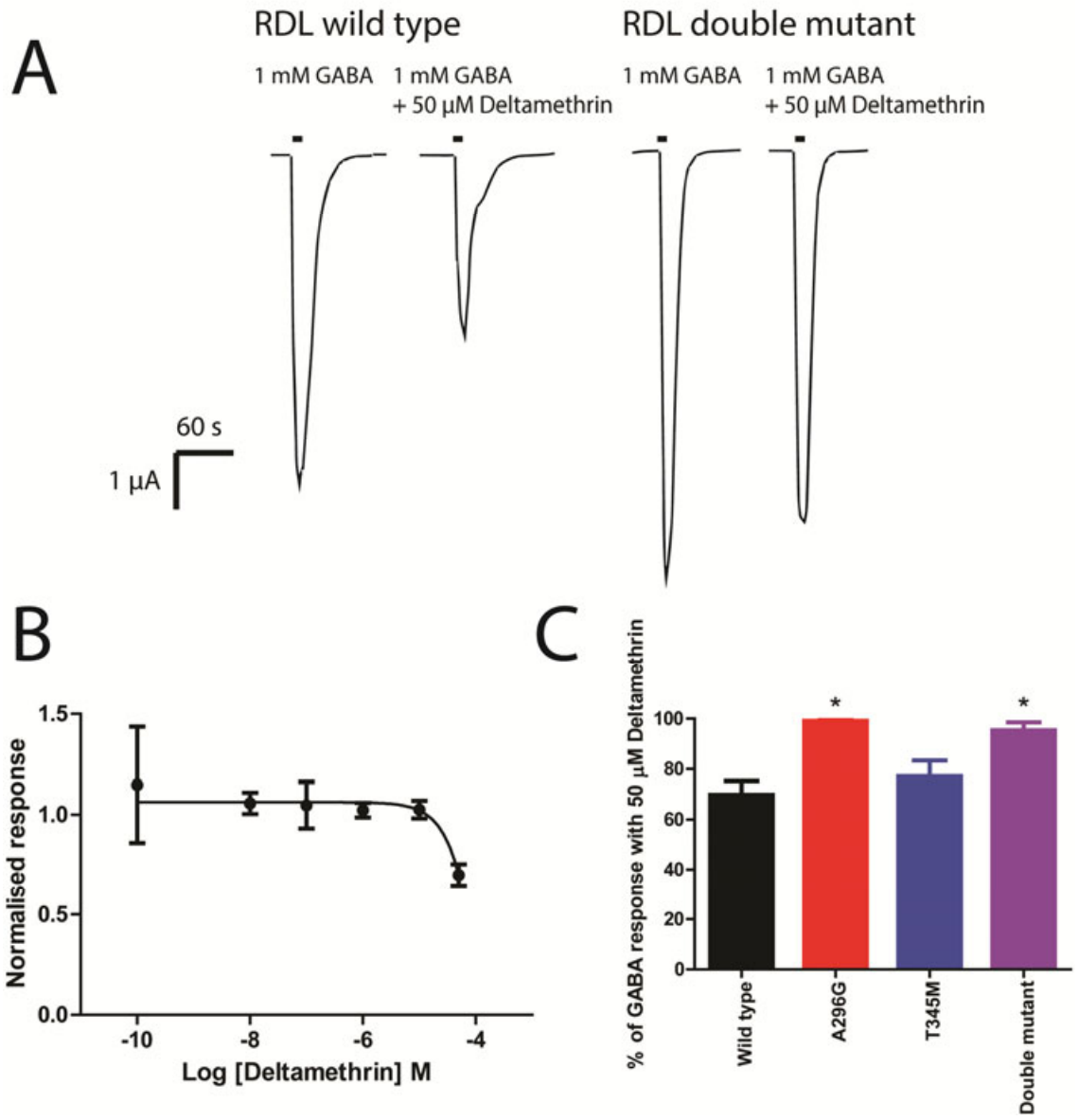
B


Normalised response
1.5
1.0
0.5
0.0
-8
-7
-6
-5
-4
-3
-2
Log [GABA] M
Wild type
A296G
T345M
Double mutant


A
RDL wild type
RDL double mutant
1 mM GABA
1 mM GABA +
1 µM fipronil
1 mM GABA
1 mM GABA +
1 µM fipronil
60 s
1 µA
B
Normalised response
1.5
1.0
0.5
0.0
-10
-8
-6
-4
-2
Log [Fipronil] M
Wild type
A296G
T345M
Double mutant


A
1 mM
GABA
100 µM
Spinosad
1 mM
GABA
60 s
1 µA
B
1 mM
GABA
100 µM Spinosad
1 mM GABA +
100 µM Spinosad
60 s
1 µA


A
RDL wild type
1 mM GABA
1 mM GABA
+ 500 µM Imidacloprid
RDL double mutant
1m M GABA
1 mM GABA
+ 500 µM Imidacloprid
60s
1µA
B
Normalised response
1.5
1.0
0.5
0.0
-10
-5
0
Log [Imidacloprid] M
C
Normalised response
1.5
1.0
0.5
0.0
-10
-8
-6
-4
-2
Log [Imidacloprid] M
Wild type
A296G
T345M
Double mutant


A
RDL wild type
1 mM GABA
1 mM GABA
+ 50 µM Deltamethrin
RDL double mutant
1 mM GABA
1 mM GABA
+ 50 µM Deltamethrin
60 s
1 µA
B
Normalised response
1.5
1.0
0.5
0.0
-10
-8
-6
-4
Log [Deltamethrin] M
C
% of GABA response with 50 µM Deltamethrin
100
80
60
40
20
0
*
*
Wild type
A296G
T345M
Double mutant