Species specific RNA A-to-I editing of mosquito RDL modulates GABA potency and influences agonistic, potentiating and antagonistic actions of ivermectin

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ABSTRACT

The insect GABA receptor, RDL, is the target of several classes of pesticides. The peptide sequences of RDL are generally highly conserved between diverse insects. However, RNA A-to-I editing can effectively alter amino acid residues of RDL in a species specific manner, which can affect the potency of GABA and possibly insecticides. We report here that RNA A-to-I editing alters the gene products of *Rdl* in three mosquito disease vectors, recoding five amino acid residues in RDL of *Aedes aegypti* and six residues in RDLs of *Anopheles gambiae* and *Culex pipiens*, which is the highest extent of editing in RDL observed to date. Analysis of *An. gambiae Rdl* cDNA sequences identified 24 editing isoforms demonstrating a considerable increase in gene product diversity. RNA editing influenced the potency of the neurotransmitter, GABA, on *An. gambiae* RDL editing isoforms expressed in *Xenopus laevis* oocytes, as demonstrated by EC_{50}s ranging from 5 ± 1 to 246 ± 41 μM. Fipronil showed similar potency on different editing isoforms, with IC_{50}s ranging from 0.18 ± 0.08 to 0.43 ± 0.09 μM. In contrast, editing of *An. gambiae* RDL affected the activating, potentiating and inhibiting actions of ivermectin. For example, ivermectin potentiated currents induced by GABA at the EC_{20} concentration in the unedited isoform but not in the fully edited variant. Editing of a residue in the first transmembrane domain or the cys-loop influenced this potentiation, highlighting residues involved in the allosteric mechanisms of cys-loop ligand-gated ion channels. Understanding the interactions of ivermectin with molecular targets may have relevance to mosquito control in areas where people are administered with ivermectin to treat parasitic diseases.

Keywords: electrophysiology, fipronil, GABA receptor, ivermectin, mosquito, RNA editing

1. Introduction

The insect γ-aminobutyric acid (GABA) receptor RDL (resistant to dieldrin) plays central neuronal roles 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, which also includes nicotinic acetylcholine receptors (nAChRs) and glutamate-gated chloride channels (GluCls). RDL therefore contains an N-terminal extracellular domain where GABA binding occurs, the characteristic cys-loop motif consisting of two disulphide bond-forming cysteines separated by 13 amino acids, and four transmembrane (TM) domains (Nys et al., 2013).

RDL is also of interest as it is the target of highly effective insecticides (Buckingham et al., 2017). Studies on the model organism *Drosophila melanogaster* identified a mutation in genomic DNA resulting in an alanine to serine substitution located in TM2, which underlies resistance to several insecticides including dieldrin, picrotoxin and fipronil (Ffrench-Constant et al., 1993; Hosie et al., 1995).

Abbreviations: ADAR – adenosine deaminase that acts on RNA; GABA – γ-aminobutyric acid; nAChR – nicotinic acetylcholine receptor; RDL – resistant to dieldrin, an insect GABA receptor; TM – transmembrane domain

This alanine to serine mutation, also found as alanine to glycine or to asparagine, has since been associated with insecticide resistance in varying species, ranging from crop pests [e.g. the planthopper *Laodelphax striatellus* (Nakao, 2017)], pests afflicting livestock [the horn fly *Haematobia irritans* (Domingues et al., 2013)] or domesticated animals [the cat flea *Ctenocephalides felis* (Bass et al., 2004)], and disease vectors [the malaria mosquito *Anopheles gambiae* (Du et al., 2005; Taylor-Wells et al., 2015)]. Despite the emergence of insecticide resistance, RDL is still a potential target for insect control since novel compounds have been developed that are unaffected by the TM2 resistance mutation (Casida and Durkin, 2015).

RDL peptide sequences, as translated from genomic DNA, are highly conserved between diverse insect species, as shown by RDL sharing 69-90% identity (considering the whole subunit) in the honey bee (*Apis mellifera*), *D. melanogaster*, the parasitoid wasp (*Nasonia vitripennis*) and the red flour beetle (*Tribolium castaneum*) (Jones et al., 2010; Jones and Sattelle, 2006; Jones and Sattelle, 2007; Taylor-Wells et al., 2017). However, the diversity of RDL can be increased by RNA A-to-I editing. During this process, adenosine deaminases that act on RNA (ADARs) remove an amine group from certain adenosine residues in pre-mRNA to generate the rare nucleoside, inosine (Deffit and Hundley, 2016). Because inosine is interpreted by cellular machineries as guanosine, A-to-I editing produces transcripts with a nucleotide composition distinct from the corresponding genomic DNA. For example, RNA editing effectively alters four amino acid residues in the *D. melanogaster* RDL (Hoopengardner et al., 2003). In contrast, RDL from *A. mellifera*, *T. castaneum* and *N. vitripennis* were found not to undergo RNA A-to-I editing (Jones et al., 2010; Jones and Sattelle, 2006; Jones and Sattelle, 2007) whereas RDL 1 from the silk worm, *Bombyx mori*, has two potential RNA editing sites that differ to those found in *Drosophila* (Yu et al., 2010). Thus, RNA A-to-I editing generates RDL isoforms in a species specific manner.

RNA A-to-I can have an impact on receptor function. For example, two-electrode voltage-clamp electrophysiology applied to *D. melanogaster* RDL isoforms expressed in *Xenopus laevis* oocytes showed that RNA editing modulates agonist potency and thus may fine-tune the sensitivity of the GABA receptor to its neurotransmitter (Jones et al., 2009). Furthermore, RNA A-to-I of RDL may affect the potency of insecticides as indicated by the finding that *Drosophila* RDL with an R122G editing variant was found to be less sensitive to fipronil (Es-Salah et al., 2008). It is therefore prudent to determine whether RDL undergoes RNA editing in pests as an instructive step in assessing whether there may be a species specific effect on insecticide potency.

Recently, it was observed that RDL from *An. gambiae* has at least two potential A-to-I RNA editing sites, I176V and N183G (Taylor-Wells et al., 2015), neither of which were found in *Drosophila* RDL. To understand further the molecular complexity of the GABA receptor in major disease vectors, we report here the identification of RNA A-to-I editing sites in RDL from not only *An. gambiae*, but also from *Aedes aegypti*, and *Culex pipiens*, mosquitoes that spread dengue fever and West Nile virus, respectively (World Health Organization a). In addition, we used two-electrode voltage-clamp electrophysiology applied to heterologously expressed mosquito RDL to measure the impact of RNA editing on the potency of GABA, fipronil and ivermectin.

2. Materials and methods

2.1. Materials

All chemicals, including the insecticides fipronil and ivermectin, were purchased from Sigma Aldrich (Dorset, UK), unless otherwise stated.

2.2. Identification of RNA editing sites in mosquito Rdl

Wild type adult mixed gender *An. gambiae* PEST strain mosquitoes, *Ae. aegypti* and *Cx. pipiens* were obtained from the National Institute of Health (MA, USA). Total RNA was extracted from groups of ten mosquitoes using Trizol reagent according to the protocol supplied. First-strand cDNA was synthesized as described previously (Jones et al., 2005) using M-MLV reverse transcriptase (Thermo Fisher Scientific, Loughborough, UK). The cDNA coding sequence of *Rdl* from the three mosquito species were amplified using PfuTurbo DNA Polymerase (Agilent Technologies, Stockport, UK) and primers listed in the Supplemental Material (Table S1). RNA editing was identified by Sanger sequencing (Source Bioscience, Oxford, UK) of the cDNA sequences and sequence chromatograms visualised by Chromas Lite (Technelysium, Brisbane, AUS). Frequency of editing was estimated by ratiometric A/G measurement from sequence chromatograms (Jepson and Reenan, 2007). *An. gambiae* genomic DNA present in the extracted total RNA, which was first treated with DNase-free RNase (Roche, West Sussex, UK), was amplified using primers recognizing introns (see Supplementary Material for primer sequences) before being sequenced.

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

The full length *Rdl* sequence was cloned into the pCI vector (Promega) with flanking primers containing EcoRI (TTTTTTGAATTCATGTCGCTAACTATCGAAGTTCCGC) and NotI (AAAAAAGCGGCCGCTTACTTCTCCTCGCC) (*Rdl* sequence underlined), and 123 clones were sequenced. Isoforms with single RNA edit sites not found *in vivo* were generated using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies) using oligonucleotides listed in the Supplementary Material (Table S2). The functional effects of the different *An. gambiae Rdl* RNA editing isoforms were evaluated using the *X. laevis* expression system and two-electrode voltage-clamp electrophysiology. *X. laevis* were purchased from Xenopus 1, Dexter, Michigan, USA and were handled strictly adhering to the guidelines of the Scientific Procedure Act, 1986, of the United Kingdom. Stage V and VI 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. Defolliculated oocytes were injected with 3.5 ng (23 nl) *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) or Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, 10 mM HEPES, pH 7.2) at a flow rate of 10 ml/min. Oocytes were selected for experiments if stable after three consecutive challenges of 1 mM GABA.

GABA concentration response curves were generated by challenging oocytes to increasing concentrations of GABA in Ringers solution, with 3 min between challenges. Curves were calculated by

normalizing the GABA responses to the mean of control responses induced by 1 mM GABA before and after application.

Fipronil and ivermectin inhibition curves were generated by inhibiting 1 mM GABA in SOS with 0.0001 – 10 μM fipronil/ivermectin, diluted initially in dimethyl sulphoxide (DMSO), then 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 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.

Ivermectin agonist and potentiating actions were measured by pre-incubating the oocytes with ivermectin in SOS for 3 minutes immediately followed by a combination of ivermectin and the respective EC_{20} GABA concentration (1 μM for the unedited, 15 μM for N183G, 10 μM for I278V and 80 μM for the completely edited isoforms) of the RDL construct for 12 seconds. This was followed by a wash step for 3 minutes and the above steps were repeated with increasing concentrations of ivermectin.

2.4. Data analysis

Data are represented as mean ± SEM of individual oocytes from ≥ 3 different batches of eggs. 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 nonlinear regression using Graphpad Prism 5 (Graphpad Software, CA, USA). Statistical significance was determined by using one-way ANOVA with Dunnett's multiple comparison post-hoc test or unpaired t-test (Graphpad).

3. Results

3.1. RDL transcripts of Ae. aegypti*,* An. gambiae *and* Cx. pipiens *undergo RNA A-to-I editing*

Sequence traces of cDNA PCR products were analysed to identify potential RNA editing sites in the *Rdl* coding region of *Ae. aegypti*, *An. gambiae* and *Cx. pipiens* (Fig. 1). Across all three mosquito species, a total of ten putative A-to-I editing sites were observed, as indicated by a mixture of A and G peaks in the sequence chromatograms or guanosine replacing the adenosine present in the genomic DNA (such as for N183G). Not all nine editing sites were found in a single species. Instead, six sites were seen in *Ae. aegypti Rdl*, eight in *Cx. pipiens* while the most with nine sites were in *An. gambiae Rdl*. Mixtures of adenosine and guanosine were not observed in sequence traces of *An. gambiae* genomic DNA (Fig. 1) and adenosine residues were present at equivalent sites in genomic DNA sequences of *Ae. aegypti* and *Cx. pipiens* (Giraldo-Calderon et al., 2015), confirming that the nucleotide changes occur at the RNA level. RNA editing at seven of the sites results in amino acid changes (Fig. 1), five of which (R119G, I162V, I176V, N183G and M240V) occur in the N-terminal extracellular domain where agonist binding occurs (Fig. 2), the sixth (I278V) in TM1 and the seventh (N289D) in the region between TM1 and TM2. Recoding of two of the amino acid residues (I278V and N289D) was also seen in *D. melanogaster* RDL (Fig. 2). To determine if identification of RNA editing sites is reproducible, we analysed *Rdl* sequences from five RT-PCR reactions, each from a different group of *An. gambiae* mosquitoes. We observed mixed A and G peaks in each sequence chromatogram with the average frequencies (± standard deviation) of

editing being: R119G = 49 ± 28%; I162V = 70 ± 11%; I176V = 63 ± 33%; N183G = 96 ± 7%; I278V = 56 ± 27%; N289D = 38 ± 31%.

3.2. Presence of different RNA editing profiles increases the complexity of Rdl *transcripts*

To identify RNA editing profiles present *in vivo*, 123 *An. gambiae Rdl* cDNA clones were sequenced. DNA sequencing revealed four variants (ac, ad, bc, bd) (Fig. 3) resulting from alternative splicing of exons 3 and 6 (ffrench-Constant and Rocheleau, 1993), which is highly conserved in diverse insect species (Jones et al., 2010; Jones and Sattelle, 2006; Jones and Sattelle, 2007; Yu et al., 2010). As is the case for *D. melanogaster* (Jones et al., 2009), the bd splice variant was the most predominant splice variant, present in 100 of the 123 clones (figure 2). This was followed by ad (19 clones), ac (3 clones) and bc (1 clone).

Across the four splice variants, a total of 24 RNA editing profiles were observed (Table 1). Of these, 22 profiles were identified in the bd splice background of which R119G+I162V+I176V+N183G+I278V was the most predominant, occurring in 28% of the clones. The I162V+N183G+I278V+N289D and unedited isoforms were not found in the bd splice background, instead being observed in ad and bc splice variants, respectively. The N183G edit (caused by an aat→ggt codon change) was the only profile found with a single edit *in vivo*. Another edit profile was observed, N183D+I278V, where the N183D edit was caused by an aat→gat codon change, showing that RNA A-to-I editing can generate two different amino acids at the same site.

3.3 RNA editing of mosquito RDL generates a spectrum of sensitivities to GABA

To determine if RNA A-to-I editing of mosquito RDL modulates the potency of GABA, a total of 18 isoforms in the predominant bd splice background were used for functional analysis (Table 2). Seven of these isoforms reflect editing at each individual site. Since only one single edit isoform (N183G) was found *in vivo* (Table 1), the remaining six isoforms were generated by site-directed mutagenesis. Ten isoforms tested have editing in at least two sites forming profiles found *in vivo* (Table 1), including the three most abundant isoforms (R119G+I162V+I176V+N183G+I278V, I162V+N183G+I278V and I162V+I176V+N183G+I278V), the isoform with editing at every site (R119G+I162V+I176V+N183G+I278V+N289D) and more rare isoforms (N183G+N289D and R119G+N183G). A completely unedited isoform was also used for comparison, which was generated by site-directed mutagenesis since it was not found *in vivo* in the bd splice background (Table 1).

All 18 of the editing isoforms formed functional receptors when expressed in *Xenopus* oocytes, responding to GABA in a concentration-dependent manner as measured by two-electrode voltage-clamp electrophysiology (Fig. 4A). Two-electrode voltage-clamp electrophysiology also showed that RNA editing can affect the sensitivity of RDL to GABA. All editing isoforms observed *in vivo* increased the GABA EC_{50} when compared to the unedited subunit (Table 2). There was a significant difference between the GABA EC_{50}s of five of the editing profiles and the unedited RDL isoform (Table 2). Two of these significantly different edit profiles were the most prevalent (R119G+I162V+I176V+N183G+I278V) and completely edited (R119G+I162V+I176V+N183G+I278V+N289D) isoforms. The shift in sensitivity from the unedited isoform to the completely edited isoform is demonstrated in the concentration response curves in Fig. 4B.

3.4. RNA editing does not affect fipronil potency

Functional analysis was also conducted to determine whether RNA editing affects RDL sensitivity to the antagonist fipronil. Fipronil inhibition curves were generated for the unedited isoform as well as 11 selected edit isoforms found *in vivo* (Fig. 5, Table 2). As indicated by IC_{50} values, the sensitivity of RDL to fipronil was not significantly affected by RNA editing (Fig. 5).

3.5. RNA editing affects ivermectin potency

Two-electrode voltage-clamp electrophysiology was used to measure the actions of ivermectin on *An. gambiae* RDL. We found that ivermectin acts as an antagonist on *An. gambiae* RDL currents induced by a GABA concentration higher than the EC_{50} (Fig. 6A). In addition, the ivermectin IC_{50} of unedited RDL_{bd} (457 ± 118 nM) was significantly higher than the IC_{50} of the completely edited (R119G+I162V+I176V+N183G+I278V+N289D) isoform (50 ± 24 nM) (Table 2) (Fig. 6B), indicating that RNA editing can affect the potency of ivermectin. We also found that ivermectin alone, notably at concentrations of 0.01 μM and higher, elicited a sustained current in the unedited isoform whilst this irreversible agonist effect was significantly less in the fully edited variant (Fig. 7A and B). Similarly, 0.01 μM and 0.03 μM ivermectin potentiated currents induced by GABA at the EC_{20} in the unedited isoform but not in the fully edited variant (Fig. 7A and C). In order to determine whether RNA editing at individual sites can affect ivermectin action, we measured potentiation by ivermectin on the N183G and I278V single editing isoforms. For both isoforms, potentiation by ivermectin with GABA EC_{20} was abolished, similar to that of the fully edited variant (Fig. 8), indicating that editing at either site affects potency of ivermectin.

4. Discussion

We report here the identification of RNA A-to-I editing that can recode the genome to alter up to seven amino acid residues in the RDL GABA receptor of the mosquito species; *Ae. aegypti*, *An. gambiae* and *Cx. pipiens*. These findings confirm our previous suggestion that A-to-I editing generates I176V and N183G substitutions in *An. gambiae RDL* (Taylor-Wells et al., 2015). Two of the editing sites, I278V and N289D, are also found in *Drosophila* RDL (Fig. 2) (Hoopengardner et al., 2003) whilst none of these sites are shared with the two recoded amino acids in RDL1 of *B. mori* (Yu et al., 2010), highlighting that RNA editing generates species-specific isoforms. Editing at residue 162 of mosquito RDL recodes an isoleucine to a valine, whilst valine is already encoded for by the *Drosophila* genome (Fig. 2). A-to-I editing of sites which are genomically encoding guanosine in other species has also been observed for the insect α6 nicotinic acetylcholine receptor (nAChR) subunit. This lead to the suggestion that RNA editing might act as an evolutionary intermediate form between single nucleotide polymorphism sites, maintaining partial conservation at the protein and functional level, despite sequence divergence at the DNA level (Jin et al., 2007).

An. gambiae RDL was chosen as a representative mosquito GABA receptor in order to further study the complexity of this insecticide target in important disease vectors. Analysis of 100 cDNAs of *Drosophila* RDL revealed eight isoforms with different RNA editing profiles (Jones et al., 2009). Considering that *An. gambiae* RDL possesses two additional amino acid residues that are affected by RNA editing (six residues compared to four in *Drosophila*), there is potential for greater diversity in the

mosquito *Rdl* transcriptome. In line with this, we found 22 different editing profiles in 100 cDNA clones of *An. gambiae* Rdl_{bd} (Table 1). This considerable increase in *Rdl* transcript diversity broadens the functional capabilities of the receptor, as demonstrated by the range of sensitivities to the agonist, GABA (Table 2), with the fully edited isoform (R119G+I162V+I176V+N183G+I278V+N289D) showing the greatest change in GABA EC_{50} compared to the unedited variant. This is in accord with most of the editing sites occurring within the N-terminal extracellular domain where agonist binding occurs (Fig. 2) and with findings supporting GABA forming many interactions with different residues (Ashby et al., 2012).

It has become apparent over the past decade that RNA editing may contribute to insecticide resistance. A reduction in *Adar* expression in cholinergic neurons and muscle of *D. melanogaster* reduced spinosad and imidacloprid sensitivity (Rinkevich and Scott, 2012). Higher levels of RNA A-to-I editing leading to N133D substitution in the β1 nAChR subunit were found in *Nilaparvata lugens* resistant to imidacloprid than in susceptible insects (Yao et al., 2009). In agreement with this, expression in *X. laevis* oocytes of nAChRs with the rat β2 subunit mimicking the N133D substitution resulted in reduced potency of imidacloprid (Yao et al., 2009). Another heterologous expression study found that the R122G edit in *D. melanogaster* RDL reduced the receptor's sensitivity to fipronil (Es-Salah et al., 2008). With the twelve isoforms studied here, we found that RNA editing of *An. gambiae* RDL had no effect on the potency of fipronil (Table 2). This included the fully edited isoform with six amino acid substitutions. This might be expected considering none of the editing sites are in the pore-lining TM2 region important for fipronil binding (Buckingham et al., 2005). In contrast, the fully edited isoform of *An. gambiae* RDL affected the agonistic, potentiating and antagonistic actions of ivermectin when compared to the unedited variant (Table 2) (Figs. 6 and 7), highlighting that ivermectin and fipronil have different modes of action on RDL. The triple action of ivermectin was also observed for *M. domestica* RDL (Fuse et al., 2016) where it was suggested that the number of orthosteric binding sites in a homo-pentamer occupied by GABA determines whether ivermectin is a potentiator or antagonist. Several studies have shown that residues in the transmembrane domains are important for interactions with ivermectin (Degani-Katzav et al., 2017; Fuse et al., 2016; Hibbs and Gouaux, 2011; Huang et al., 2017; Nakao et al., 2015; Wolstenholme, 2012; Zemkova et al., 2014). In line with this, we found that the single edit variant I278V, which is located in TM1 (Fig. 2), abolished the potentiating action of ivermectin (Fig. 8A). Interestingly, Lees *et al.* (Lees et al., 2014) reported that the predominant edit in *D. melanogaster* RDL, I283V, which is conserved with I278V in mosquito RDL, had no effect on the antagonistic action of ivermectin when high concentrations (100 μM) of GABA was applied. In contrast, our result suggests that I278V affects the allosteric action of ivermectin on RDL upon exposure to lower concentrations of GABA. Studies on the crystallized glutamate-gated chloride of *Caenorhabditis elegans*, with ivermectin bound or absent, have shown that parts of the N-terminal extracellular region come into contact with the transmembrane domain (Althoff et al., 2014; Hibbs and Gouaux, 2011). This enables the coupling of conformational changes between the two regions of the receptor, which is thought to be involved in carrying out the allosteric modulatory effects of ivermectin. One of these contacts includes an interaction between the characteristic cys-loop and the extracellular end of TM3 (Althoff et al., 2014). We found that the N183G edit isoform in mosquito RDL abolished potentiation by ivermectin (Fig. 8B), reinforcing findings suggesting that altering the conformation of the cys-loop can affect communication between the agonist binding regions and the transmembrane domain (Alcaino et al., 2017; Althoff et al., 2014; Lee et al.,

2008; Schofield et al., 2004). Further experiments are required to determine whether any of the remaining four edited amino acids in *An. gambiae* RDL also influence ivermectin actions.

Ivermectin has been approved for human use in large scale drug administrations to treat diseases caused by parasitic nematodes such as river blindness in Africa (resulting from infection by *Onchocerca volvulus*) (Tekle et al., 2016) and lymphatic filariasis in over 50 countries (caused by *Wuchereria bancrofti, Brugia malayi* and *B. timori*) (Biritwum et al., 2017). Ivermectin can also treat other parasitic diseases. For example, a study has shown that ivermectin can reduce prevalence of strongyloidiasis (caused by *Strongyloides stercoralis*) in a human population in northwestern Argentina (Echazu et al., 2017) and there is increasing interest in the use of ivermectin to treat scabies (caused by the mite, *Sarcoptes scabiei var hominis*) (World Health Organization b). Interestingly, the survival of *Anopheles* mosquitoes was reduced when they fed on humans or cattle receiving ivermectin treatment (Chaccour et al., 2010; Kobylinski et al., 2010; Pooda et al., 2014). This raises the prospect of providing an additional route to reducing malaria transmission in areas where mass drug administration of ivermectin is used to treat parasites (Chaccour et al., 2017). Understanding mechanisms that may give rise to ivermectin insensitivity is crucial in delaying the emergence of resistance, thereby maximizing the potential of this approach, and studies are ongoing in understanding ivermectin susceptibility in *Anopheles* (Chaccour et al., 2017; Seaman et al., 2015). Our findings that RNA editing of *An. gambiae* RDL affects the potency of ivermectin may highlight a novel mechanism for increased tolerance to the drug. Whilst ivermectin is thought to act primarily on glutamate-gated chloride channels (Laing et al., 2017), it may have toxicological influence by acting on insect GABA receptors, given that activation, potentiation and antagonism of mosquito RDL can occur at nanomolar concentrations (Table 2, Fig. 7) and that similar concentrations of ivermectin (up to 40 nanomolar) have been measured in the plasma of patients who have taken a single oral dose of the drug (Chaccour et al., 2017; Elkassaby, 1991). It would, therefore, be of interest to determine whether any *Anopheles* mosquitoes showing differential sensitivity to ivermectin have altered levels of RNA editing of RDL.

The development of novel compounds, such as isoxazolines (Asahi et al., 2015; Ozoe et al., 2010), meta-diamides (Nakao et al., 2013) and meroterpenoid chrodrimanins (Xu et al., 2015), that can act on RDL bearing the TM2 resistance mutation (Buckingham et al., 2005) demonstrates that the insect GABA receptor is still a useful target for the development of novel insecticides. Heterologously expressed *An. gambiae* RDL, such as that reported here, may provide a useful tool for evaluating the effectiveness of compounds on isoforms particular to the mosquito GABA receptor.

In conclusion, the functional diversity of the mosquito GABA receptor, RDL, is broadened considerably by RNA A-to-I editing, which may serve to fine tune the response of the receptor to its neurotransmitter, GABA. This highlights that while the genomic DNA of *Drosophila*, *Aedes*, *Anopheles* and *Culex* translate to RDL peptide sequences that are almost identical, the genome can be recoded at the RNA level to give species-specific variants, even amongst different mosquito species. When studying RDL from a certain species, the whole coding sequence should be analysed to ascertain whether there is potential RNA editing, as this may have an impact on insecticide potency.

343 **Table 1** – List of edit profiles present in ac, bc, ad and bd splice variants of 123 cDNA clones of *An.*
344 *gambiae* RDL. The frequency of each profile is included. The edit profiles are arranged in decreasing
345 frequency found in the bd splice variant. * indicates isoforms used in functional analysis.

Edit profile	**bd splice variant**	**ad splice variant**	**ac splice variant**	**bc splice variant**
R119G+I162V+I176V+N183G+I278V	28*	1	0	0
I162V+N183G+I278V	11*	3	0	0
I162V+I176V+N183G+I278V	10*	0	0	0
N183G+I278V	9	3	1	0
R119G+I162V+I176V+N183G+I278V+N289D	6*	0	0	0
N183G	5*	5	0	0
I162V+N183G	5	0	1	0
I162V+I176V+N183G+I278V+N289D	4	0	0	0
I176V+N183G	3	0	0	0
N183G+I278V+N289D	2	1	0	0
R119G+I162V+I176V+N183G	2	0	0	0
I176V+N183G+I278V	2*	2	0	0
I162V+I176V+N183G	2*	0	0	0
I176V+N183G+I278V+N289D	2*	0	1	0
R119G+I162V+N183G+I278V	2	0	0	0
N183G+N289D	1*	1	0	0
I162V+N183G+N289D	1	0	0	0
R119G+I162V+N183G	1	0	0	0
N183D+I278V	1	0	0	0
R119G+I162V+N183G+I278V+N289D	1	0	0	0
R119G+N183G+I278V	1	1	0	0
R119G+N183G	1*	0	0	0
I162V+N183G+I278V+N289D	0	2	0	0
Unedited	0	0	0	1
Total clones sequenced	**100**	**19**	**3**	**1**

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Table 2 – List of GABA EC_{50}s plus Hill coefficient (nH), fipronil and ivermectin IC_{50}s for the unedited, individual edits, completely edited (R119G+I162V+I176V+N183G+I278V+N289D) and 10 selected *An. gambiae* Rdl_{bd} edit profiles found *in vivo*. Values are the mean ± SEM and representative of 4-11 oocytes from ≥3 different batches of eggs. *GABA EC_{50} significantly different to the unedited isoform ($P<0.05$, 1 way ANOVA with Dunnett's multiple comparison test). ¥Ivermectin IC_{50} significant from the unedited isoform ($P<0.05$, un-paired t-test).

***An. gambiae* RDL edit profiles**	**GABA**		**Fipronil**	**Ivermectin**
	EC_{50} (μM)	**nH**	**IC_{50} (μM)**	**IC_{50} (nM)**
Unedited	10 ± 4	0.9 ± 0.1	0.41 ± 0.19	457 ± 118
Single edit profiles				
R119G	13 ± 5	1.2 ± 0.4	-	-
I162V	5 ± 1	0.7 ± 0.1	-	-
I176V	24 ± 9	1.2 ± 0.1	-	-
N183G	43 ± 7	1.4 ± 0.2	0.38 ± 0.17	-
N183D	13 ± 4	0.9 ± 0.1	-	-
I278V	25 ± 2	1.5 ± 0.1	-	-
N289D	20 ± 2	1.2 ± 0.2	-	-
Edit combination profiles				
I176V+N183G+I278V+N289D	42 ± 8	1.5 ± 0.2	0.31 ± 0.10	-
N183G+I278V+N289D	42 ± 10	1.6 ± 0.2	0.28 ± 0.06	-
N183G+N289D	43 ± 8	1.6 ± 0.2	0.17 ± 0.09	-
I162V+N183G+I278V	54 ± 16	1.8 ± 0.1	0.43 ± 0.09	-
R119G+N183G	62 ± 25	1.5 ± 0.2	0.32 ± 0.11	-
I162V+I176V+N183G+I278V	88 ± 17*	2.2 ± 0.2	0.13 ± 0.04	-
R119G+I162V+I176V+N183G+I278V	105 ± 13*	2.0 ± 0.2	0.29 ± 0.10	-
I162V+I176V+N183G	113 ± 25*	1.8 ± 0.1	0.18 ± 0.08	-
I176V+N183G+I278V	118 ± 55*	1.2 ± 0.2	0.21 ± 0.05	-
R119G+I162V+I176V+N183G+I278V+N289D	246 ± 41*	1.8 ± 0.18	0.23 ± 0.14	50 ± 24¥

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Author contributions: JT-W conducted most of the experiments, analysed the results and contributed to writing the paper. AS conducted some of the electrophysiology experiments and contributed to writing the paper. IB provided guidance on the electrophysiology experiments and critical appraisal of the manuscript. AKJ conceived the idea for the project, initially identified the RNA A-to-I editing sites in *Rdl* of the three mosquito species and contributed to writing the paper.

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Figure legends

Figure 1. Sequence chromatograms showing RNA A-to-I editing in RDL of *Ae aegypti*, *An. gambiae* and *Cx. pipiens*. Mixed adenosine/guanosine peaks in the cDNA sequence indicating RNA editing are highlighted and the resulting amino acid change is shown. The corresponding genomic DNA (gDNA) sequence of *An. gambiae*, which lacks the G signal, is also included.

Figure 2. RNA A-to-I edit sites in RDL are overlapping but not completely conserved between mosquito species and *D. melanogaster*. Amino acid sequence alignment of RDLs from *D. melanogaster* (top line) with *Ae aegypti*, *An. gambiae* and *Cx. pipiens* (bottom line). Regions involved in agonist binding (loops A-F), the cys-loop and the first transmembrane domain (TM1) are highlighted. RNA editing sites resulting in an amino acid change are identified with an arrow and correspond by symbol to *An. gambiae* (filled circle), *Ae. aegypti* (dotted circle) and *Cx. pipiens* (open circle).

538 **Figure 3.** Alternative splicing of exons 3 and 6 in *An. gambiae* RDL. Equivalent alternative exons of *An.*
539 *gambiae* and *D. melanogaster* RDLs are aligned. *Anopheles* residues that differ in spliced exons are
540 underlined. *Anopheles* residues, which differ from those of the orthologous *Drosophila* exon, are
541 highlighted in bold.
542
543 **Figure 4.** RNA A-to-I editing in *An. gambiae* RDL can affect sensitivity to GABA. A) Representative
544 electrophysiological trace showing responses to GABA (from 1 μM – 2 mM) in *X. laevis* oocytes
545 expressing the most prevalent Rdl_{bd} edit isoform (R119G+I162V+I176V+N183G+I278V). B) GABA
546 concentration response curves of unedited Rdl_{bd} and the completely edited isoform
547 (R119G+I162V+I176V+N183G +I278V+N289D). The two curves have significantly different EC_{50}s
548 ($P<0.05$, 1 way ANOVA with Dunnett's multiple comparison test) (Table 2). Data are normalised to the
549 maximal response (1 mM GABA). Data are the mean ± SEM from 5-6 oocytes from 3 different batches
550 of eggs.
551
552 **Figure 5.** Effects of RNA editing in *An. gambiae* RDL on receptor sensitivity to fipronil. A)
553 Representative electrophysiological trace showing the effects of increasing concentrations of fipronil on
554 the 1 mM GABA response, in *X. laevis* oocytes expressing the most prevalent Rdl_{bd} edit isoform
555 (R119G+I162V+I176V+N183G+I278V). Concentrations of fipronil (0.0001 μM – 10 μM) were co-
556 applied with 1 mM GABA following a 3 min pre-incubation with the selected fipronil concentration. B)
557 Fipronil inhibition curves for the unedited, completely edited
558 (R119G+I162V+I176V+N183G+I278V+N289D) and further 10 selected RDL isoforms occuring *in vivo*.
559 Each data point was normalized to the response to 1 mM GABA. IC_{50} values are not significantly
560 different. Data are the mean ± SEM and representative of 3-4 oocytes from ≥3 different batches of eggs.

561 **Figure 6.** Effects of RNA editing in *An. gambiae* RDL on the antagonistic actions of ivermectin. A)
562 Representative electrophysiological trace showing the effects of increasing concentrations of ivermectin
563 on the 1 mM GABA response, in *X. laevis* oocytes expressing the completely edited
564 (R119G+I162V+I176V+N183G+I278V+N289D) RDL isoform. Concentrations of ivermectin (0.0001
565 μM – 10 μM) were co-applied with 1 mM GABA following a 3 min pre-incubation with the selected
566 ivermectin concentration. B) Ivermectin inhibition curves for the unedited and completely edited RDL
567 isoforms. IC_{50} values are significantly different ($P<0.05$, un-paired t-test). Each data point was
568 normalized to the response to 1 mM GABA. Data are the mean ± SEM and representative of 4-6 oocytes
569 from ≥3 different batches of eggs.
570
571 **Figure 7.** Effects of RNA editing in *An. gambiae* RDL on the activation and potentiation actions of
572 ivermectin. A) Representative electrophysiological trace showing the agonistic and potentiating effects of
573 ivermectin on GABA response in *X. laevis* oocytes expressing the completely edited and unedited
574 isoforms. B) Curves representing the change in base line (agonist activity) of the completely edited and
575 unedited RDL isoforms during the preincubation step. * indicates that the amplitude of response to 0.03
576 μM ivermectin in the unedited and completely edited isoforms were significantly different (p-value <0.05,
577 un-paired t-test). Data are the mean ± SEM and representative of 5-6 oocytes from ≥3 different batches of

eggs. C) GABA response curves of completely edited and unedited RDL isoforms after preincubation with ivermectin. Each data point was normalised to the GABA EC_{20} values obtained before the first ivermectin preincubation step (value of 1). Data are the mean ± SEM and representative of 5-6 oocytes from ≥3 different batches of eggs.

Figure 8. Effect of N183G or I278V editing on the potentiation action of ivermectin. A) GABA response curves of completely edited, unedited RDL and N183G isoforms after preincubation with ivermectin. B) GABA response curves of completely edited, unedited RDL and I278V isoforms after preincubation with ivermectin. Each data point was normalised to the GABA EC_{20} values obtained before the first ivermectin preincubation step (value of 1). Data are the mean ± SEM and representative of 3-6 oocytes from ≥3 different batches of eggs.