## Chapter 1

# Variations in the Insect GABA Receptor, RDL, and Their Impact on Receptor Pharmacology

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The resistance to dieldrin (RDL) receptor is an insect γ-aminobutyric acid (GABA) receptor, characterized by the dieldrin resistance mutation that was pivotal to understanding target based insecticide resistance. RDL is the target for various non-competitive antagonists, including dieldrin and fipronil, as well as novel acting compounds such as the meta-diamides and isoxazolines. Therefore the RDL receptor has returned to center stage as a relevant and effective insecticide target. Our understanding of the function of RDL *in vivo* is still unfolding, with the discovery of species specific post-transcriptional modifications such as alternative splicing and RNA editing, modifications shown to influence the pharmacology of the receptor. Exposing these receptors to insecticides also evokes ever evolving mechanisms of mutagenesis, and a number of contributory mutations have been identified both in field and laboratory resistant insects, occurring in parallel to the dieldrin resistance mutation. We present an overview of these variations and discuss the impact on the pharmacology of GABA and various insecticides.

## Introduction

The targeting of insect neuronal receptors is one of the main mechanisms of insecticidal action. However, our understanding of these neuronal receptors and their functions *in vivo* are still unfolding, with the continuing discovery and functional analysis of post-transcriptional modifications such as alternative splicing and RNA editing. By the same token, exposing these receptors to insecticides evokes ever evolving mechanisms of mutagenesis by nature, contributing to insecticide resistance or offsetting the fitness costs of other mutations. An overview of these variations and their effects on receptor pharmacology are provided on the resistance to dieldrin (RDL) receptor, the most studied of the insect γ-aminobutyric acid (GABA) receptors and the target for both historic and novel non-competitive antagonists (NCAs) (*1*).

The RDL receptor is involved in rapid inhibitory synaptic transmission, utilizing GABA as its neurotransmitter. As the first invertebrate GABA receptor to be identified, most sequence and functional analysis has been conducted in the fruit fly model *Drosophila melanogaster,* in which the subunit was first identified and functionally expressed (*2*, *3*). The RDL receptor plays a key role in various processes, most notably the regulation of sleep (*4*), aggression (*5*) and olfaction (*6*, *7*), and in *D. melanogaster*, RDL is expressed both in the embryonic and adult central nervous system (*8*, *9*).

As a member of the cys-loop ligand-gated ion channel (cysLGIC) superfamily, the RDL receptor consists of a pentameric subunit structure, centered around a central pore. In turn, the RDL subunit is composed of extracellular N- and C-termini, four transmembrane domains (M1 – M4), the second of which lines the ion channel; and a large intracellular loop between M3 and M4 (*10*). The agonist binding site is located in the N-terminal extracellular domain and consists of distinct regions (loops A-F) (*11*). Also in the N-terminal domain is the dicysteine loop, which is characteristic of the cysLGIC superfamily. In addition to RDL, the cysLGIC superfamily contains other receptor targets for insecticides, including the glutamate gated chloride channels and nicotinic acetylcholine receptors (*1*, *12*, *13*).

GABA binds at the subunit interface, altering the conformation of the receptor and allowing the passage of chloride ions, which initiate the cascade of inhibitory action. By this function, the insect RDL receptor is most related to vertebrate $GABA_A$ receptors, though pharmacologically it does have some differences (*14*, *15*).

The RDL receptor was identified and characterized following the discovery of a mutation located in the ion channel pore forming M2 (amino acid position 301) of *D. melanogaster Rdl*. This mutation rendered the RDL receptor resistant to the antagonistic effects of 10 μM dieldrin, which is a cyclodiene insecticide (*3*). The *Rdl* subunit has since been cloned from several insect orders, including those of agricultural pests (e.g. red flour beetle *Tribolium castaneum* and planthopper *Laodelphax striatellus* (*16*, *17*)), pests afflicting domesticated animals (cat flea, *Ctenocephalides felis* (*18*)), human and animal disease vectors (house fly *Musca domestica*, and mosquitoes *Anopheles gambiae and Aedes aegypti* (*19–21*)) and beneficial species (miridbug *Cyrtorhinus lividipennis* and honeybee *Apis mellifera*

(*22*, *23*)). In most cases, an insect has only one *Rdl* gene, however, there are exceptions, such as the presence of two *Rdl* genes in the pea aphid (*Acyrthosiphon pisum*) (*24*) and the diamondback moth (*Plutella xylostella*) (*25*), while the silk worm (*Bombyx mori*) possesses three *Rdl* genes (*26*). The coding sequence of *Rdl* is remarkably conserved between diverse insect species, usually showing 70-90% identity at the amino acid level (*27*, *28*). The M2 mutation in the RDL subunit (commonly referred to as the A2′ mutation, which signifies an alteration in the second amino acid position of the ion channel domain) (*15*) has been extensively studied since its discovery, and is utilized as a diagnostic marker for resistance (*29*). However, several other RDL subunit mutations have recently been discovered in field and laboratory selected resistant insects, and interestingly always in parallel with the A2′ mutation (*30–32*).

The RDL receptor is a target for a number of neuromodulatory and insecticidal compounds including lindane (*33*), picrotoxin (*34*), cyclodienes (e.g. dieldrin, endosulfan) (*2*), phenylpyrazoles (e.g. fipronil) (*35*) and macrocyclic lactones (e.g. ivermectin) (*36*). Recently, the RDL receptor has returned to center stage as a novel target for the isoxazolines (*37*, *38*), meta-diamides (*39*) and meroterpenoid chrodrimanins (*40*), that target different binding sites and have the distinct advantage of acting on RDL bearing subunits containing the M2 resistance mutation (*15*, *40*).

The study of RDL receptor is facilitated by the ability to express high levels of the homomeric receptor in *Xenopus laevis* oocytes and other expression systems (*41–44*), but the exact composition of the subunits *in vivo* remains unknown. These homomeric receptors maintain most of the pharmacological properties of native insect GABA receptors but their response to some benzodiazepines is different (*14*, *15*), suggesting that other subunits may co-assemble with RDL *in vivo*. This is supported by *in vitro* studies showing that RDL can co-express with the GABA receptor subunits GRD (GABA/glycine-like receptor of Drosophila) (*45*) and LCCH3 (ligand-gated chloride channel homologue 3) (*46*) as well as the glutamate gated chloride channels (GluCls) (*21*, *47*). However, there is other evidence to suggest that these subunits may not necessarily co-assemble *in vivo* (*48*, *49*).

The RDL receptor is likely to be far more complex than is suggested by the common perception that it is a homomeric. This is because alternative splicing and RNA editing have been found to increase the diversity of the *Rdl* transcriptome (*15*, *50*). In addition, evidence has come to light that these variations can affect the pharmacology of the receptor and influence the actions of insecticides (*51*, *52*).

In the following chapter we will consider the recent molecular variations identified in the insect RDL receptor and discuss the impact of these variations on the receptor pharmacology of GABA and various insecticides.

## Environmentally-Induced Variations: The Effects of RDL Resistance Mutations on Receptor Pharmacology

It is 24 years since the original dieldrin resistance mutation at position 301 (A2′S/G) was identified in an RDL subunit (*3*), and it is one of the most significant examples of direct target site resistance to date. Dieldrin was removed from the market several decades ago, but may persist in the environment (*53*). This combined with the potential for cross-resistance with currently used insecticides such as endosulfan and fipronil (*32*, *54*), may suggest why the dieldrin resistance mutation still persists in insect populations (*55*, *56*). With novel insecticides emerging that target the RDL receptor, albeit with different mechanisms of action (*57*), it seems timely to review the presence of novel insecticide associated mutations that have been discovered in recent years (summarized in Table 1).

The amino acid alanine in the channel forming M2 domain, corresponding to position 301 (A2′) in the *D. melanogaster* RDL subunit, is mutated most often to serine (*18*, *58*, *59*) or glycine (*58*, *60*) to confer resistance to channel blocking insecticides such as dieldrin and at varying levels to fipronil (*15*, *58*, *61*). It was recently found that the glycine mutation in fact facilitated a greater resistance to fipronil than the serine mutation (both *in vivo* and *in vitro*) in *D. melanogaster* (*62*). More recently, in 2010 and 2011, another mutation at the same site was observed in two fipronil resistant field populations of planthopper, in which alanine at the same position was mutated to asparagine (A2′N) (*44*, *63*).

The first mutation reported to co-exist with the A2′ mutation was T350M (Table 1), isolated from a laboratory selected *Drosophila simulans* population resistant to dieldrin, also showing a high level of resistance to fipronil (*31*). This mutation, located in the M3 domain, was always found in the presence of A2′G. Functional experiments with electrophysiology applied to expressed RDL subunit isoforms in *X. laevis* oocytes revealed that the T350M mutation contributed to fipronil resistance, as the $IC_{50}$ of the A2′G/T350M isoform for fipronil (221 nM) was significantly increased from that of A2′G alone (93 nM) and wild-type (31 nM) (*31*). Furthermore, expression of an RDL subunit isoform containing only T350M also showed reduced sensitivity to fipronil ($IC_{50}$ 215 nM), suggesting that the mutation may also individually contribute to resistance.

The *Drosophila* T350 double resistance profile was also found to be conserved in RDL subunits of the malaria mosquito *An. gambiae,* that were highly resistant to dieldrin (*20*). Here, the M3 mutation T345M in the *An. gambiae* RDL subunit (Table 1), which is equivalent to T350M in *D. simulans*, was also found in the presence of the A2′G mutation (*20*). As the mosquitoes in this study were collected from wild populations showing phenotypic resistance to dieldrin, the mutations observed here may represent resistance mutations present in the field. In contrast with the double mutant RDL isoform from *D. simulans* however, the T345M mutation in *An. gambiae* did not appear to contribute to fipronil resistance, as a double mutation or individually (*20*). This supports observations in transgenic *D. melanogaster*, whereby fipronil resistance was not heightened by the presence of the T-M mutation, both alone and in combination with A2′G (*62*). It is speculated that the T-M mutation in *An. gambiae* may therefore play a structural rather than functional role, potentially offsetting the

fitness costs imposed by A2′G (*20*, *62*). Modeling of the receptor indicates that the two mutations are indeed in close proximity and hence capable of functional interaction (*62*). The $EC_{50}$ of A2′G (60 μM) for GABA was also found to be significantly lower than that of the A2′G/T345M double mutant (198 μM), indicating that the T-M mutation may serve to offset the heightened sensitivity to GABA, that would otherwise be detrimental to neuronal signaling (*20*).

A *D. melanogaster Rdl* subunit mutation, M360I, in the intracellular loop between M3 and M4, was observed alongside the A2′S mutation (Table 1) in a gene duplication that contained one wild type copy and one double mutant (A2′S/M360I) copy of *Rdl* (*64*). This duplication was associated with intermediate levels of dieldrin resistance and heat shock recovery, suggesting that the duplication may offset the temperature sensitive fitness cost associated with the A2′S mutation (*64*). Interestingly, M360 can be recoded to valine through RNA A-to-I editing (*51*). It was found that in the duplicated *Drosophila Rdl*, there were greater levels of RNA editing at I360 than at the wild-type M360 copy (*64*), which may suggest a link between RNA editing and the A2′S resistance mutation. However, the individual contribution of M360I to insecticide resistance has yet to be determined at a molecular/functional level.

In *Anopheles funestus Rdl*, a V327I substitution was found to be associated with the A2′S mutation in field collected samples of dieldrin resistant mosquitoes in Cameroon and Burkina Faso (Table 1) (*56*). The valine to isoleucine substitution is present in the loop between the M2 and M3 domains. Interestingly, the mutation was always found in conjunction with the A2′S mutation, but at a lower frequency than was observed for A2′S (*56*). The link between this mutated isoform and the resistance phenotype, as well as the functional consequences of this mutation, have however not as yet been investigated.

A number of different contributory mutations to the less common A2′N mutation have also been identified *Rdl* subunits from various species of planthopper (Table 1) (*65–67*). An R340Q mutation was found in the presence of A2′N in an RDL subunit of fipronil resistant *Sogatella furcifera* collected from a rice paddy in Japan (*65*). The mutation is present in the intracellular loop between the M3 and M4 domains, in relative close proximity to the T345M/T350M mutations found in *An. gambiae* and *D. simulans* (*20*, *31*). As with the V327I mutation in *An. funestus* RDL, the R340Q mutation was present at a lower frequency than A2′N, being present in only 9 of 17 cDNA clones carrying the A2′N mutation (*65*). Using a membrane potential assay and *Drosophila* Mel-2 cells stably expressing the homomeric RDL mutant isoforms, it was shown that the R340Q did not contribute significantly to fipronil resistance, both in combination with A2′N and alone. This result, combined with the location in the intracellular loop suggest, as with the T345M/T350M mutation, that this substitution may have a more subtle structural role and/or play a role in offsetting the fitness costs of the A2′N mutation.

In the brown planthopper *Nilaparvata lugens*, another recent study revealed a mutation in combination with A2′S, a Q359E mutation between M3 and M4 of the RDL subunit (Table 1) (*30*). The mutations were observed when field collected insects were driven to resistance using the fiprole insecticide ethiprole. Ethiprole has particular relevance to *N. lugens*, as along with fipronil it has

replaced imidacloprid as a method of insect control in Asia, following resistance to neonicotinoids (*68*). There were differential effects of the A2′S mutation on the sensitivity of ethiprole and fipronil, a somewhat unexpected finding as the structures differ only by an ethylsulfinyl substituent in ethiprole, compared to a trifluoromethylsulfinyl moiety in fipronil (for structures see reference (*69*)). Both *in vitro* (homomeric RDL expressed in *X. laevis* oocytes) and *in vivo* (*D. melanogaster* insecticide bioassays) methods demonstrated that the A2′S mutation contributes to ethiprole resistance (*30*). This effect was most pronounced *in vivo*, whereby *D. melanogaster* with the A2′S mutation exhibited 4000-fold resistance in comparison to the wild-type strain. The Q359E mutation, however, did not contribute significantly to resistance *in vitro*, indicating that the A2′S mutation is the main mechanism of target site resistance. The authors speculate that the presence of the Q359E mutation may be owing to a structural linkage between the two mutations, or that the Q359E mutation confers a fitness advantage. It is difficult to envisage how Q359E may offset any fitness associated with A2′S considering that the mutation, both singly and as a double mutant, did not impact on the potency of GABA (*30*).

Secondary mutations were also found in the M2 domain of the RDL subunit from the planthopper *L. striatellus* (*66*). Sequencing of the *Rdl* gene from field collected insects driven to 87-fold resistance to fipronil revealed the A2′N mutation in combination with either an R305Q or R305W mutation (Table 1). The strain exhibited low cross-resistance to dieldrin and endosulfan and resistance was not affected by detoxification enzymes, suggesting that target site mutations are likely to be responsible for fipronil resistance (*66*).

In *N. lugens,* an R299Q mutation, also in the M2 domain of the RDL subunit, was recently identified in addition to the A2′S mutation (Table 1) (*67*). The A2′S/R299Q double mutant was found in insect populations from China, Thailand and Vietnam, with R299Q occurring at a lower frequency than A2′S. In addition, the R299Q mutation was identified during laboratory selection with fipronil (up to a resistance ratio of 237-fold) after the A2′S mutation had reached 100% penetrance. This suggests that R299Q is a secondary mutation potentially associated with persistent fipronil exposure. The mutations, as in previous studies, were only found in parallel, which the authors speculate may be because of the fitness costs associated with the R299Q mutation alone. Indeed, expression of the R299Q mutant subunit expressed as a homomeric receptor individually reduced the GABA potency almost 11 times ($EC_{50}$ = 413 μM) to that of wild type receptors (38 μM), suggesting a high fitness cost. This is a much larger reduction in potency than was observed with the A2′S mutation ($EC_{50}$ = 19 μM, 2 times lower). In contrast, the double mutant receptor restored the GABA potency to comparable levels with the wild-type ($EC_{50}$ = 54 μM), perhaps offsetting the fitness costs associated with increased sensitivity to GABA. In addition, the A2′S mutation conferred low levels of resistance to fipronil ($IC_{50}$ 45 nM compared to 20 nM in wild-type). However, in combination with R299Q, the sensitivity to fipronil was significantly decreased ($IC_{50}$ 96 nM), suggesting the double mutant also contributes to fipronil resistance. Therefore the A2′S and R299Q subunit mutations are likely to have both a compensatory and synergistic relationship; to offset fitness costs and increase fipronil resistance, respectively.

**Table 1. Resistance Mutations Found in Association with the A2′ Mutation in the RDL Subunit of Various Insect Species. The Mutations Are Listed in Order of Position within the Protein Sequence, with the Insect Species Detected, Resistance Ratio if Calculated and Geographical Location from Which They Were Isolated, unless They Were Obtained via Laboratory Selection.**

| *RDL mutation associated with A2′* | *Insect species* | *Resistance ratio* | *Geographical location isolated* |
|---|---|---|---|
| **M2 domain** | | | |
| A2′S and R299Q | *N. lugens* | Fipronil: 237 | China, Vietnam, Thailand, plus via laboratory selection (*67*) |
| A2′S and R305Q or R305W | *L. striatellus* | Fipronil: 87 | Laboratory selection (field collected strain from Japan) (*66*) |
| **Loop between M2-M3 domains** | | | |
| A2′S and V327I | *An. funestus* | Not determined | Africa (Burkina Faso and Cameroon) (*56*) |
| **M3 domain** | | | |
| A2′G and T345M | *An. gambiae* | Not determined | Laboratory selection (field collected strain from Democratic Republic of Congo) (*20*) |
| A2′G and T350M | *D. simulans* (*Eyguières 42)* | Fipronil: 20,000 | Laboratory selection (*31*) |
| **Intracellular loop between M3-M4 domains** | | | |
| A2′N and R340Q | *S. furcifera* | Not determined | Japan (*65*) |

*Continued on next page.*

**Table 1. (Continued). Resistance Mutations Found in Association with the A2′ Mutation in the RDL Subunit of Various Insect Species. The Mutations Are Listed in Order of Position within the Protein Sequence, with the Insect Species Detected, Resistance Ratio if Calculated and Geographical Location from Which They Were Isolated, unless They Were Obtained via Laboratory Selection.**

| *RDL mutation associated with A2′* | *Insect species* | *Resistance ratio* | *Geographical location isolated* |
|---|---|---|---|
| A2′S and Q359E | *N. lugens* | Under ethiprole selection:<br>Ethiprole: >14,000<br>Fipronil: >860 | Laboratory selection (field collected strain (Nl55) from India) (*30*) |
| A2′S and M360I | *D. melanogaster* | Dieldrin: >4000 | USA (*64*) |

## Naturally-Occurring Variations: Alternative Splicing and RNA Editing

Alternative splicing and RNA editing are post-transcriptional modifications that enhance the diversity of the transcriptome, thereby increasing the number of products from a single gene (*70*, *71*). Ion channel proteins functioning in the nervous system undergo a notably high level of RNA editing, commonly in functionally significant regions, which may serve as a mechanism for facilitating rapid neuronal signaling (*72*). The importance of alternative splicing and RNA editing is highlighted by their dysregulation, which results in neurodegenerative phenotypes (*73–75*). These mechanisms may also be particularly influential in insect genomes, serving to expand the repertoire of what would otherwise be a small complement of proteins. For example, insects possess less than 30 cysLGIC subunit genes, as opposed to 102 in *Caenorhabditis elegans* and 45 in humans (*27*, *76*, *77*).

### The Effects of Alternative Splicing on RDL Receptor Pharmacology

Alternative splicing is the process in pre-messenger RNA (mRNA), whereby introns are spliced out and various exons are introduced, removed or substituted to form the final processed mRNA. In this way, a single gene codes for multiple proteins, as a result of the alternatively spliced exons forming multiple protein products (*78*, *79*).

*D. melanogaster Rdl* is composed of nine exons, two of which, exons 3 (variants a and b) and 6 (variants c and d) (Figure 1) are alternatively spliced to produce subunit isoforms ac, ad, bc and bd, all of which are transcribed *in vivo* (*50*, *51*). Real time PCR and analyses of clones amplified from adult *D. melanogaster* and *An. gambiae* cDNA reveal that the $Rdl_{bd}$ variant is the most prevalent splice form *in vivo* (*51*, *80*). However the preference for the splice variant transcript appears to be different at the embryonic stage, with $Rdl_{bc}$ being the most abundant (*50*), indicating varying requirement for the different isoforms at different stages of development.

Alternative splicing of exons 3 and 6 is conserved in diverse insect species including the African malaria mosquito *An. gambiae* (*80*), the small brown planthopper *L. striatellus* (*81*), the silkworm *B. mori* (*26*), the honeybee *A. mellifera* (*28*), beetles *T. castaneum* and *Oulema oryzae* (*82*, *83*) and the parasitoid wasp *Nasonia vitripennis* (*27*) also revealed these common splice regions. In contrast, the white-backed planthopper *S. furcifera* has an additional splice variant at exon 3 (*65*). The diamondback moth, *P. xylostella*, has two *Rdl*-encoding genes, both of which are alternatively spliced at only exon 3 (*25*). This suggests that even within insect orders, the level of alternative splicing can differ, creating species specific RDL subunit isoforms.

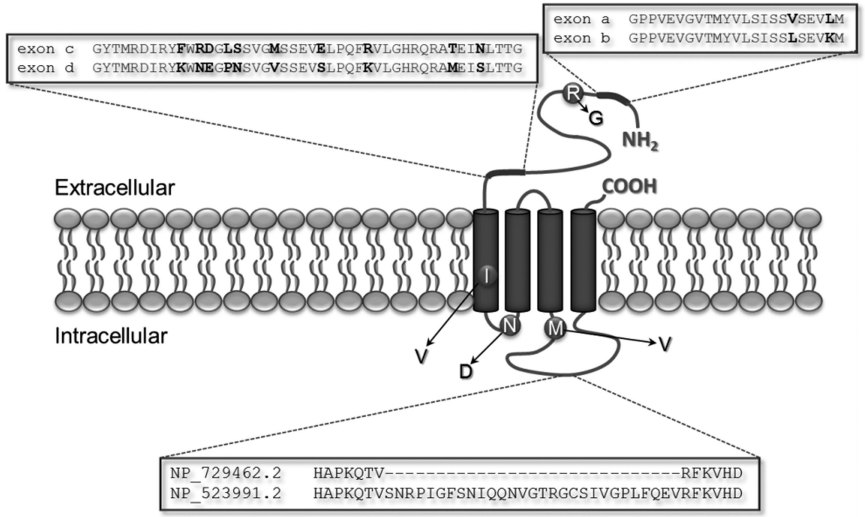


*Figure 1. Schematic illustration showing how post-transcriptional modifications can diversify the insect RDL subunit, in this case that of D. melanogaster. Alternative splicing yields two variants each for exons 3 (variants a and b) and 6 (variants c and d) changing amino acid residues in the N-terminal extracellular domain where agonist binding occurs. Residues that are different in alternative exons are shown in bold. Differential splicing generates M3-M4 intracellular loops of different lengths as shown by sequences with the accession numbers NP_729462.2 and NP_523991.2 (sequences directly submitted to the NCBI database). RNA editing leads to the R122G substitution in the N-terminal extracellular domain, I283V in M1, whereas N294D and M360V occur in intracellular domains.*

Exons 3 and 6 are located in the N-terminal extracellular domain, within proximity of agonist binding. In particular, exon 6 contains loops C and F, which contribute to the ligand-binding site (*15*). It was therefore not surprising to find that the splice isoforms in *Drosophila* differed in their sensitivity to GABA and various GABA analogues when expressed using the *X. laevis* expression system and two-electrode voltage clamp electrophysiology (*51*, *84–86*). The sensitivity to GABA was in the order bc>ac>ad>bd, ranging in $EC_{50}$ from 20 ± 0.2 μM ($RDL_{bc}$) to 152 ± 10 μM ($RDL_{bd}$) (*51*).

More recently it has been shown that different subunit isoforms of RDL can also arise from variation in the large intracellular loop between M3 and M4. Here, instead of using alternative exons, the use of different splice acceptor sites generates intracellular loops of varying lengths (Figure 1). For example, the miridbug *C. lividipennis* contains two RDL subunit isoforms differing by a 31 amino acid insertion in the intracellular loop (*23*, *28*). Two-electrode

voltage-clamp electrophysiology applied to *C. lividipennis Rdl* expressed in *X. laevis* oocytes showed that these two isoforms significantly differed in their sensitivity to fipronil, with the presence of the insertion significantly increasing the $IC_{50}$ from 6.47 ± 1.12 μM to 16.83 ± 2.30 μM. This suggests that diversity in this large intracellular loop may enhance the tolerance to fipronil or other insecticides. Another insect species, *A. mellifera,* has three differentially spliced M3-M4 isoforms of RDL (*22*, *28*). These variants, unlike those in *C. lividipennis,* did not differ in their sensitivity to fipronil (*22*). Interestingly, the site of insertion disrupts a putative protein kinase C phosphorylation consensus site, conserved in both *A. mellifera*, *C. lividipennis* and *D. melanogaster* RDL (*22*). Phosphorylation of the intracellular loop can influence events such as protein assembly, receptor desensitization and insecticide sensitivity (*87–90*). Therefore differential splicing has the potential to affect insecticide actions in a species specific manner.

The differential effects of species specific subunit isoforms on insecticide sensitivity described above are highly pertinent, since species such as *C. lividipennis* and *A. mellifera* are both beneficial insects. This is highly relevant considering that the use of fipronil has been restricted by the European Union (*91*) for its suspected negative effects on bees.

These findings hint at the complex level of species specific differential splicing that can occur in insects, which may impact on the response of receptors to insecticides. It remains to be seen whether differential splicing of the intracellular loop of RDL subunits are highly conserved in diverse insects, including pest species, and whether this presents another route to reducing sensitivity to insecticides.

**The Effects of RNA Editing on RDL Receptor Pharmacology**

RNA editing also diversifies the number of products produced from a single gene, but via single nucleotide substitutions, initiated by adenosine deaminases acting on RNA (ADAR) enzymes (*92*, *93*). In the most prevalent form of RNA editing, known as A-to-I editing, ADAR enzymes deaminate adenosine to inosine, which is then translated as guanosine (*94*). The result is a sequence transcript dissimilar to that of the genomic DNA, potentially changing amino acid residues that may affect protein structure and function.

For example, four RNA editing sites that alter amino acid residues were identified in clones amplified from *D. melanogaster Rdl* cDNA (*51*, *72*) (Figure 1). In an analysis of >100 clones, the edit sites R122G, I283V, N294D and M360V were found in 16 different combinations, predominantly in the bd splice variant background (*51*). These amino acid substitutions are in structurally significant regions, making it likely that they will affect receptor function. For instance, R122G is located between ligand binding loops D and A, suggesting that it may influence agonist binding. Es-Salah *et al.* confirmed this finding by showing that when the arginine is replaced with glycine the GABA $EC_{50}$ is significantly increased (*52*). Functional expression of the different editing isoforms also generated a range of sensitivities to GABA in *D. melanogaster* RDL ($EC_{50}$s of 3-193 μM), showing that singly and in combination, the edit sites directly affect agonist potency (*51*).

Interestingly, the choice of splice variant arising from the alternative use of exons 3 and 6 influenced the potency of GABA in addition to RNA editing. This was shown by significantly different GABA $EC_{50}$s for the ad and bd splice variants with the same edit combination, suggesting that editing and splicing may act in concert to further broaden the functional diversity of the RDL receptor (*51*).

Resistance mutations in RDL subunits were also investigated in combination with RNA editing. The N-terminal R122G edit was investigated in comparison with an A2′G/T350M mutated RDL receptor isoform (*52*). The sensitivity to both GABA and fipronil was significantly reduced by the R122G addition, suggesting that RNA editing is able to affect insecticide sensitivity in RDL receptor isoforms bearing resistance mutations. The authors speculate that as fipronil acts preferentially on agonist-bound receptors and R122G reduces the potency of GABA, the reduction in the proportion of agonist bound receptors may affect fipronil binding (*52*). Lees *et al.* also investigated the effect of the RNA edit site I283V on fipronil sensitivity in A2′S mutant homomeric *D. melanogaster* RDL and found that this edit site did not affect GABA binding or fipronil sensitivity (*36*). This difference in effect may be due to the position of I283V in the M1 domain of the subunit, as it is not in close proximity to locations of agonist or antagonist binding.

RNA editing can generate isoforms that are highly species specific. For example, one of the two *Rdl* genes (RDL 1) from *B. mori* generates transcripts with two potential RNA editing sites that alter two amino acid residues at the C-terminal end, a region completely different to those altered in *D. melanogaster* RDL (*26*). Recently, RNA editing sites were also found in the RDL subunit of various mosquito species, which are not completely conserved with *D. melanogaster* (*80*). In this study, a comprehensive sequence analysis was conducted on the RDL subunit from the mosquito disease vectors; *An. gambiae*, *Culex pipiens* and *Ae. aegypti*. Nine putative RNA editing sites were observed in *Rdl* cDNA sequences; five in *Ae. aegypti*, seven in *Cx. pipiens* and eight sites in *An. gambiae*. Two of these, I278V and N289D, were conserved in *D. melanogaster* (*51*). Therefore the remaining sites were not only mosquito specific, but also in some cases mosquito species specific. The functional effects of different RNA editing isoforms in RDL subunits from *An. gambiae* were investigated in the bd splice variant background (*80*). As with *D. melanogaster*, the edit combination generated a spectrum of potencies to GABA, with $EC_{50}$s ranging from 5 ± 1 to 246 ± 41 μM in the 18 isoforms tested. Interestingly, the $EC_{50}$ increased in line with an increase in the number of RNA edited sites. RNA editing did not however have an effect on the sensitivity of fipronil. This is perhaps unsurprising as none of the editing sites are in the pore-lining M2 region important for fipronil binding (*15*).

The macrocyclic lactone ivermectin also acts on the GABA receptor, but the exact mechanisms of action are not clear. Ivermectin has been found to potentiate the GABA response of vertebrate GABA receptors (*95*) and the *D. melanogaster* RDL receptor (*96*) using patch clamp electrophysiology applied to cell lines and the FLIPR membrane potential assay. For *M. domestica* homomeric RDL receptors expressed in *X. laevis* oocytes, ivermectin was also found to potentiate currents induced by low concentrations of GABA ($EC_5$) (*97*). However,

ivermectin has been shown to act as an antagonist on *An. gambiae* (*80*), *D. melanogaster* (*36*) and *M. domestica* (*97*) RDL currents, as homomeric receptors expressed in *Xenopus* oocytes, when induced by a GABA concentration higher than the $EC_{50}$.

RNA editing of *An. gambiae* RDL isoforms influences the potency of ivermectin, where it was shown to significantly reduce the $IC_{50}$ of the unedited receptor from 457 ± 118 nM to 50 ± 24 nM in the completely edited isoform (*80*). Ivermectin was also found to directly activate the *An. gambiae* RDL receptor as well as potentiate the GABA-induced responses at concentrations below the $EC_{50}$ (*80*). Differences between the edited and unedited isoforms were also observed under these experimental conditions. Concentrations of 0.01 μM and 0.03 μM ivermectin potentiated currents induced by GABA at the $EC_{20}$ in the unedited receptor isoform, but not in the edited isoform (*80*). The triple actions (agonistic, potentiating and antagonistic) of ivermectin were also observed for the *M. domestica* RDL receptor (*97*) where the authors speculated that the number of orthosteric binding sites in the pentamer occupied by GABA, may determine whether ivermectin is a potentiator or antagonist. It remains to be seen whether RNA editing sites in *An. gambiae* RDL subunits contribute individually or in conjunction to elicit changes in the sensitivity to ivermectin. Of particular interest would be the N183G edit site of the RDL subunit from *An. gambiae*, which is within the cys-loop, and therefore could influence communication between the GABA binding regions and the transmembrane domain (*98*). This information may have relevance to controlling vector-borne diseases as ivermectin has been shown to reduce the longevity of *An. gambiae* mosquitoes (*99*).

Intriguingly, not all insect species contain an RDL receptor that is RNA edited. For instance, RNA A-to-I editing sites have not been detected in RDL from the honey bee (*A. mellifera*), the red flour beetle (*T. castaneum*) and the parasitoid wasp (*N. vitripennis*) (*27*, *28*, *82*). RNA editing, therefore, can recode the highly conserved genomic sequence for *Rdl* in a species-specific manner. Why RDL subunits of some insect species undergo RNA editing and others do not remains a mystery. The finding that RNA editing generates numerous receptor isoforms of RDL in *An. gambiae* suggests that the mosquito GABA receptor requires a higher level of plasticity than that of the honey bee.

## Conclusions and Future Prospects

Identification of the dieldrin resistance mutation in the RDL receptor was pivotal to understanding the mechanism of target site resistance in insects. Dieldrin is no longer in use, however fipronil, which also shows varying levels of cross-resistance to the RDL receptor, is used worldwide in various applications ranging from the control of agricultural pests to the treatment of various parasitic diseases (*100*). Therefore understanding variations in the structure and function of the RDL receptor is relevant to understanding mechanisms of resistance in the field, as well as a target for emerging novel classes of insecticides (*39*, *40*).

Future investigations of the RDL receptor will likely continue to reveal further resistance associated mutations. The A2′S/G/N are the common variants of the classically conserved RDL mutation in M2, although a number of additional mutations have recently been identified in highly insecticide resistant insects (Table 1). In all instances, the second mutations were associated in parallel with the A2′ mutation. Identification of these mutations may indicate a gradual adaptive response to changing levels of insecticides in the environment. However, it may also be possible that these mutations have been present in populations for some time, but were not previously identified as the entire coding region of the *Rdl* gene is commonly not sequenced. To presume that the A2′ mutation is central to RDL receptor-based insecticide resistance may encourage diagnostic checks for resistance to concentrate solely on the M2 domain. This has the danger to miss other mutations that may be important (*32*).

There is increasing evidence that post-transcriptional modifications can impact on RDL receptor pharmacology, providing another reason to sequence the whole coding region to identify subunit isoforms that may arise from alternative splicing and RNA editing. There is no doubt that in the future novel splicing and editing isoforms of the RDL receptor will continue to be identified as more cDNA clones are investigated. It would be additionally advantageous to further investigate the effects of splicing and editing in pest species in order to investigate their contribution as potential factors in altering the tolerance to insecticides. Future experiments with potentially new insecticides that act on the RDL receptor could include elucidating whether splicing and RNA editing affect the potency of these novel compounds. Can the findings of species-specific splicing and RNA editing of RDL subunits be exploited? Perhaps future insecticide discovery efforts may identify compounds preferentially acting on RDL receptor isoforms found only in pest species. This is considerably timely since the use of fipronil and neonicotinoids have been restricted amidst fears that they are having a detrimental effect on non-target organisms (*100*).

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