

***Title page***

**Insect GABA receptors: splicing, editing and targeting by antiparasitics and insecticides**

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## ***Running title page***

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Abbreviations:

5-HT3: 5-hydroxytryptamine

BIDN: 3,3-bis-trifluoromethyl-bicyclo-[2,2,1]heptane-2,2-dicarbonitrile

CACA: *cis*-amino crotonic acid

CNS: central nervous system

GABA: gamma-aminobutyric acid

GABAR: GABA receptor

GRD: GABA receptor of *Drosophila*

LCCH3: Ligand-gated chloride channel homolog 3

nAChR: nicotinic acetylcholine receptor

RDL: Resistant to Dieldrin

TM1: Transmembrane domain 1

## **Abstract**

Ionotropic  $\gamma$ -aminobutyric acid (GABA) receptors are abundant in both vertebrate and invertebrate nervous systems where they mediate rapid, mostly inhibitory synaptic transmission. A GABA-gated chloride channel subunit from *Drosophila melanogaster* (RDL - Resistant to DieLdrin) has been cloned, functionally expressed and found to exhibit many aspects of the pharmacology of native, bicuculline-insensitive, insect GABA receptors. RDL is the target of the commercially-important insecticide, fipronil. A point mutation in the channel-lining region of the RDL molecule is known to underlie most cases of resistance to insecticides acting on GABA receptors. RDL is widely distributed throughout the insect nervous system, but the subunit composition of RDL-containing *in situ* receptors is unknown. It is possible that in some instances RDL co-expresses with glutamate-gated chloride channel subunits. Other ionotropic receptor subunits, LCCH3 and GRD form GABA-gated cation channels when heterologously expressed. Interest in RDL as a model ligand-gated anion channel has been enhanced by the recent discovery of pre-mRNA A-to-I editing, which, together with alternative splicing, adds to the functional diversity of this GABA receptor subunit.

## ***Introduction***

Ionotropic  $\gamma$ -aminobutyric acid (GABA) receptor molecules (GABARs) are members of the dicysteine-loop ('cys-loop') superfamily of neurotransmitter receptors that also includes nicotinic acetylcholine receptors, type 3 5-hydroxytryptamine (5-HT<sub>3</sub>) receptors and glycine receptors (Karlin, 2002; Karlin and Akabas, 1995; Olsen et al., 2004). Ionotropic GABARs are pentameric proteins. Each polypeptide subunit possesses a long N-terminal extracellular domain which contains residues that contribute to the neurotransmitter binding site and 4 transmembrane regions (M1 - M4), the second of which (M2) provides many of the residues that line the integral chloride channel (Darlison et al., 2005; Kim et al., 2004; Whiting, 2003). Vertebrate ionotropic GABARs may be divided into two pharmacological categories: bicuculline-sensitive GABA<sub>A</sub> receptors, which are composed of  $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$  and  $\pi_{1-3}$  subunits and are allosterically modulated by benzodiazepines, barbiturates and pregnane steroids; and bicuculline-insensitive GABA<sub>C</sub> receptors, which are composed of the 3 known isoforms of the  $\rho$  subunit and are insensitive to the majority of modulators of GABA<sub>A</sub> receptors (Connolly and Wafford, 2004; McKernan and Whiting, 1996; Mustafa, 1995; Rudolph and Mohler, 2004; Sieghart, 1995; Whiting, 2003). This division into A and C subtypes is difficult to reconcile with recent findings on GABARs in brainstem neurones, which are composed of  $\rho_1$  subunits co-expressed with  $\alpha_1$  and  $\gamma_2$  subunits (Milligan et al., 2004) to yield receptors with properties of both GABA<sub>A</sub> and GABA<sub>C</sub> subtypes. Furthermore, GABA<sub>A</sub> and GABA<sub>B</sub> receptors, which differ structurally and in their signalling mechanisms, may be closely functionally coupled. This is supported by the recent finding that the GABA<sub>A</sub>  $\gamma_2S$  subunit forms a complex with GABA<sub>B</sub>R1 subunits (thereby enhancing GABA<sub>B</sub> receptor trafficking to the cell surface, which otherwise requires co-expression with the GABA<sub>B</sub>R2 subunit). The GABA<sub>A</sub>  $\gamma_2S$  subunit also forms a complex with both GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits to allow agonist-induced receptor internalization (Balasubramanian et al., 2004).

Insect ionotropic GABARs do not readily fit the vertebrate GABA<sub>A</sub>/GABA<sub>C</sub> receptor categories. The majority are distinguished from the GABA<sub>A</sub> type of vertebrate receptors by their insensitivity to bicuculline and differ from GABA<sub>C</sub> receptors in that they are subject to allosteric modulation, albeit weak, by benzodiazepines and barbiturates (Sattelle, 1990). An insect ionotropic *Drosophila* GABAR subunit, RDL (Resistance to dieldrin), can be heterologously expressed to form functional homo-oligomeric receptors whose pharmacology closely resembles that of the majority of native insect GABARs, and so has proved to be of value in investigating GABAR physiology and pharmacology. Here we show that RDL very closely mimics the pharmacology of most insect native neuronal GABARs reported to date and is therefore a useful homomeric model insect GABAR, and as such facilitates the interpretation of site-directed mutagenesis experiments.

### **The insect GABA-gated chloride channel family**

The GABA-receptor *Rdl* gene was isolated from a naturally occurring dieldrin-resistant strain of the dipteran, *Drosophila melanogaster* (Ffrench-Constant et al., 1991; Ffrench-Constant and Rocheleau, 1993; Ffrench-Constant et al., 1993). Highly conserved *Rdl*-like receptor genes have since been identified in several other insect orders. *Rdl* subunits are distributed throughout the adult (Harrison et al., 1996) and embryonic (Aronstein and Ffrench-Constant, 1995) nervous system of *Drosophila*, but not on muscle (Harrison et al., 1996). Their expression pattern suggests roles in fast GABAergic synaptic transmission (Buchner et al., 1988; DiAntonio et al., 1993; Harrison et al., 1996) and learning (Harrison et al., 1996; Sattelle et al., 2000; Strambi et al., 1998) as well as visual and olfactory processing (Harrison et al., 1996). Recordings of spontaneous GABA-mediated currents in *Drosophila* embryonic neurones have provided electrophysiological evidence that RDL contributes to synaptic GABARs (Lee et al., 2003).

Other subunits in the *Drosophila* genome are predicted to have anion channel characteristics based on signature sequences in their predicted transcripts, among which there are GABAR subunit candidates. These include CG6927, CG7589, CG11340 and CG12344. Until functional expression data are available, these candidates are difficult to distinguish from other insect ligand-gated anion channels, which include histamine-gated chloride channels (Gengs et al., 2002), a glutamate-gated chloride channel  $\alpha$  subunit (Cully et al., 1996), candidate GABA-/glycine-gated ion channels GRD (Harvey et al., 1994) and CG7589 (identified in genome annotation), as well as CG8916, a protein identified in genome annotations as a GABA<sub>A</sub> receptor but so far not functionally expressed. Furthermore, although the GABAR-like sequences of LCCH3, another GABAR-like subunit, and GRD suggested possible anion channel activity, co-expression of these two subunits in *Xenopus* oocytes results in a cationic channel (Gisselmann et al., 2004). This finding counsels caution when interpreting annotations based on sequence data alone. RDL and LCCH3 co-express to form a picrotoxinin-insensitive, bicuculline-sensitive channel with pharmacology quite unlike native insect GABARs (Zhang et al., 1995). To date, coexpression of RDL with GRD has not been reported. Interestingly, RDL is not expressed on cockroach muscle (Harrison et al., 1996) despite the existence of GABA-gated chloride currents in these cells (Rauh et al., 1997a; Schnee et al., 1997). This suggests the existence of muscle GABA-gated chloride channels which do not contain RDL.

### **Alternative splicing of the *Rdl* gene**

By means of alternative splicing in two of its nine exons (exons 3 and 6), the RDL gene encodes 4 distinct polypeptides (Figure 1A), all of which are expressed (French-Constant and Rocheleau, 1993). Three splice variants (RDLac, RDLbd and RDLab) have been cloned and functionally expressed (Hosie and Sattelle, 1996a). Residues that vary in exon 3 lie upstream to loop D of the

GABA binding site and close to the equivalent positions of known determinants of agonist potency in GABA<sub>A</sub> receptors of vertebrates. Alternative splicing occurs in other ligand-gated ion channels (Villarroel, 1999) and has been shown to affect agonist sensitivity in glycine receptors (Miller et al., 2004). Interestingly, alternative splicing of the *Drosophila* nicotinic acetylcholine receptor subunit D $\alpha$ 6 (Grauso et al., 2002) also occurs in exon 3 which aligns to exon 3 of RDL. The exon 6 alternatively spliced residues of RDL lie in loops F and C (Figure 1B) and exon 6 splicing affects the expressed receptor's affinity for GABA (Hosie and Sattelle, 1996a) (Figure 1C). The agonist profile for one splice variant (RDLbc) remains to be determined. Nevertheless, two of the variant amino acid residues are of particular interest: the two splice variants containing the 'a' form of exon 3 (RDLac and RDLad) have V53 and L57 respectively, while those containing the 'b' form (RDLbc and RDLbd) have L53 and K57. These two residues are close to a valine (V46 in the nicotinic  $\alpha$  subunit of modified muscle cells in the electric organ of *Torpedo marmorata*), which is highly conserved amongst ligand-gated ion channels and in *Torpedo* is proposed to form the closest contact between the ligand-binding and the transmembrane domains (Miyazawa et al., 2003). This suggests that RDL exon 3 is involved in the coupling of ligand-binding to pore opening. Structural perturbation due to alternative splicing in exon 6 has been shown to account for differences in the potency of 3 glycine receptor agonists (Miller et al., 2004).

The 10 alternatively-spliced residues affected by exon 6 splicing are located in loops F and loop C of the RDL binding site on the external face of the vestibule (Figure 1B). Loop F is known to be involved in GABA binding and chloride channel gating in the vertebrate GABA<sub>A</sub>R  $\alpha$ 1 subunit (Newell and Czajkowski, 2003). Our comparative models of the RDL receptor suggest that exon 6 alternative splicing may influence the putative agonist binding site at the RDL subunit interface (pers. obs.).

The pharmacological diversity among vertebrate GABA<sub>A</sub> receptor subtypes is largely determined by different subunit isoforms that make up the receptor. Since the *Drosophila* genome only contains a small number of genes identified as GABAR subunits, two of which can be co-expressed to form GABA-gated cation channels, the alternative splicing of the *Rdl* gene in *Drosophila* may serve to increase functional diversity in the absence of a large number of GABAR subunits. This possibility is strengthened by findings from immunocytochemical studies that suggest that RDL is very widely distributed and hence a likely component of many insect nervous system GABARs (Aronstein et al., 1996; Harrison et al., 1996).

### **Pre-mRNA editing of RDL**

Pre-mRNA A-to-I editing is widespread in animal tissues (Athanasiadis et al., 2004). It involves substitution of an inosine for an adenosine, the result being interpreted by the translation machinery as a guanosine. RDL is edited at 4 sites which, on the basis of comparison with equivalent residues in other receptors, may potentially affect the receptor's responses to GABA. They are R122G in the N-terminal domain, I283V in the first transmembrane domain, N294D in the TM1-TM2 loop and M360V in the TM3-TM4 loop (Figure 2A). To date, the effects of these substitutions on receptor function have not been tested, but comparison with equivalent residues in other subunits suggests that at least some of these edited residues are at positions likely to affect agonist actions. The residue R122 is in loop A of the agonist binding site, adjacent to the equivalent residue (W93) in nicotinic acetylcholine receptors known to be critical in agonist binding (Figure 2B). The edited residue M360 is located in the large intracellular loop between TM3 and TM4, which has been shown to influence GABAR desensitization kinetics and GABA EC<sub>50</sub> (Fisher, 2004) and aligns with L277 of the human  $\alpha 2$  GABA<sub>A</sub> receptor subunit which, when mutated to an alanine, results in a 50-fold increase in the EC<sub>50</sub> for GABA (O'Shea and Harrison, 2000).



### **RDL as a model of native insect GABA receptors.**

In addition to its potential as a model for anionic ligand-gated ion channels, RDL may also provide a convenient model for insect GABARs, which are the targets of commercially-important insecticides, such as fipronil, used in animal health and crop protection applications. Although little is known of the pharmacology or physiology of native *Drosophila* GABA receptors, RDL shares a number of distinctive features with native GABA receptors of other insect species.

#### *The agonist profile and bicuculline insensitivity of RDL resembles the most abundant native insect GABARs*

RDL homomeric receptors are distinguished from vertebrate GABA<sub>A</sub> receptors by their insensitivity to bicuculline, their low sensitivity to 3-aminopropanesulphonic acid, and their high sensitivity to both isoguvacine (Hosie and Sattelle, 1996a) and muscimol (Buckingham et al., 1994a) (Figure 1C). They are distinguished from vertebrate GABA<sub>C</sub> receptors by the high efficacy of muscimol, CACA and isoguvacine. Such pharmacology is typical of many native insect GABARs (Lummis and Sattelle, 1985; Lummis and Sattelle, 1986; Sattelle et al., 1988). Unlike many *in situ* insect GABARs (Brotz and Borst, 1996), however, RDL is sensitive to CACA (cis-aminocrotonic acid), an agonist of vertebrate GABA<sub>C</sub>, but not of GABA<sub>A</sub> receptors (Hosie and Sattelle, 1996a). However, it should be noted that CACA does act on some insect GABARs, including those that mediate inhibition by identified filiform hair receptors of an identified projection interneuron (Gauglitz and Pfluger, 2001).

A feature of most insect GABARs is an insensitivity to bicuculline, a feature shared by extrasynaptic GABARs on the motor neuron, D<sub>f</sub> of *P. americana* (Buckingham et al., 1994b; Sattelle et al., 1988), synaptic GABARs on the giant interneuron 2 of *P. americana* (Buckingham et al., 1994b), GABARs that mediate inhibition by identified filiform hair receptors of an identified

projection interneuron (Gauglitz and Pflugger, 2001), motion-sensitive visual interneurons of the blowfly, *Calliphora erythrocephala* (Brotz and Borst, 1996), GABA-mediated IPSPs recorded in an identified locust (*Schistocerca gregaria*) interneuron (Watson and Burrows, 1987) and many, but not all, locust (*Locusta migratoria*) neuron ionotropic GABARs (Benson, 1988; Lees et al., 1987). However, it is clear that, in addition to bicuculline-insensitive receptors, a class of bicuculline-sensitive GABARs also exists in insects. For example, bicuculline blocks ionotropic GABARs of some adult (Waldrop et al., 1987) and larval *Manduca sexta* abdominal ganglion neurons (Sattelle et al., 2003) as well as electrically- or odour-evoked inhibitory post-synaptic potentials and GABA responses in projection neurons of antennal lobes of this species (Christensen et al., 1998a; Christensen et al., 1998b). It is clear, however, that RDL and its equivalent cloned from *H. virescens* (Wolff and Wingate, 1998) resemble, in their insensitivity to bicuculline, the majority of insect ionotropic GABARs.

*RDL provides a useful model of the native convulsant antagonist site*

The convulsant antagonist site is the target of insecticides, including fipronil (Figure3, although fipronil has also been shown to be a potent blocker of insect (Ikeda et al., 2003; Zhao et al., 2004) and nematode (Horoszok et al., 2001) L-glutamate-gated chloride channels at micromolar concentrations. Pharmacological differences between insect and vertebrate GABARs at this site have been shown to underlie the selectivity of these compounds for insects. RDL shares much of the convulsant pharmacology that distinguishes insect from vertebrate GABARs (Buckingham et al., 1996). The pharmacology of the convulsant site of RDL has been delineated in detail in a structure/function analysis of the sensitivity of RDL homomers to a range of picrodendrin analogues (Hosie et al., 1996). As is the case for *in situ* insect GABARs, RDL is also blocked by BIDN (3,3-bis-trifluoromethyl-bicyclo-[2,2,1]heptane-2,2-dicarbonitrile) (Buckingham et al., 1996; Hosie et al., 1995) (Figure4), an important new probe of the insect convulsant site (Rauh et al.,

1997a; Rauh et al., 1997b). While both BIDN and fipronil (Figure 3A, C) are potent blockers of RDL (Buckingham et al., 1994a; Buckingham et al., 1996; Grolleau and Sattelle, 2000; Rauh et al., 1997a) (Figure 4B and C) and *in situ* insect GABARs (Rauh et al., 1997a), single channel studies have revealed that they act at distinct, though possibly overlapping, sites (Grolleau and Sattelle, 2000) (Figure 3C and D). Indeed, up to four convulsant binding sites have been identified in housefly head membranes (Deng et al., 1993).

*RDL mimics the barbiturates and steroid insensitivity of native insect GABA receptors*

GABARs of insects are only weakly sensitive to pregnane steroids and to pentobarbital, which potently enhance vertebrate GABA<sub>A</sub> receptors (Lummis and Sattelle, 1986). Phenobarbital and pentobarbital (10 μM – 1mM) enhanced the GABA responses of RDL homomers (Buckingham et al., 1996). The pregnane steroid 5α-pregnan-3 α-ol-20-one (10 μM), yielded a moderate enhancement of the GABA-response of RDL homomers (Millar et al., 1994), reflecting the low steroid sensitivity of native insect GABARs, a finding confirmed in binding studies. The potency of pentobarbital and phenobarbital on RDL homomers (Hosie and Sattelle, 1996b) is similar to that observed on native locust GABARs (Lees et al., 1987) and less than seen on vertebrate GABA<sub>A</sub> receptors. The steroid site on vertebrate GABARs remains elusive, but, once detected, will be of interest to compare it with the equivalent regions of RDL, which is much less sensitive to these compounds.

*Benzodiazepines are less potent on RDL homomers than on native insect GABA receptors*

Although RDL mimics well the agonist, competitive antagonist and convulsant sites of insect GABARs, differences have emerged between RDL and known *in situ* insect GABARs in their sensitivity to benzodiazepines. For example, whereas the agonist responses of certain bicuculline-insensitive insect GABARs are enhanced by micromolar concentrations of flunitrazepam (Sattelle

et al., 1988), the GABA responses of RDL homomers (Millar et al., 1994) or its *H. virescens* equivalent (Wolff and Wingate, 1998) are unaffected by flunitrazepam (100 $\mu$ M). More importantly, insect *in situ* GABARs and RDL homomers are both enhanced by lower concentrations of 4'-chlorodiazepam (Ro5-4864) (it inhibits *in situ* receptors at higher concentrations, R. Higashino and D.B. Sattelle, unpublished data), although RDL receptors were approximately 100-fold less sensitive to this compound than native insect receptors (Buckingham et al., 1996; Hosie and Sattelle, 1996b; Sattelle et al., 1988).

Thus, RDL homomers do not match closely the benzodiazepine or barbiturate pharmacology of known insect GABARs. It is therefore probable that *in situ* GABARs of insects are composed of RDL co-expressed with other, as yet unidentified subunits.

### **Co-assembly of RDL with Glutamate-gated chloride receptor subunits?**

One possible explanation for the difference between RDL homomers and native receptors at the benzodiazepine site may be that *in situ* insect GABARs are not RDL homomers, but include other subunit types or are a mix of RDL splice variants. Indeed, there is evidence that the GABARs present at the neuromuscular junction of *C. elegans* are heteromultimers composed of two splice variants encoded by the *unc-49* gene (Bamber et al., 2005). In heterologously expressed vertebrate GABA<sub>A</sub> receptors, the efficacy of benzodiazepines is highly dependent upon receptor subtype composition (Buhr and Sigel, 1997; Smith, 2001). Indeed, the single channel properties of RDL-containing receptors on cultured *Drosophila* neurons differ from those of RDL homomers (Zhang et al., 1995). Thus, native RDL-containing receptors are likely to be hetero-oligomers of RDL and as yet unknown subunits. Heterologous expression studies provide evidence that co-expression of RDL with other insect putative GABAR subunits results in receptors with distinct pharmacologies (Table 1). In a recent study (Ludmerer et al., 2002), antibodies to RDL and to a *Drosophila*

glutamate-gated chloride channel subunit both immunoprecipitated the entire fraction of receptors for the insecticide (nodulosporic acid), suggesting that RDL co-assembles *in vivo* with a glutamate receptor subunit - the first evidence for co-assembly of subunits from different subclasses of ligand-gated ion channels. Since RDL staining is not observed in muscle, although GABA-gated chloride channels are present in insect muscle, such co-expression must be confined to certain regions of the nervous system.

### **GABA-gated cation channels**

It was recently reported (Gisselmann et al., 2004) that GRD and LCCH3 form GABA-gated cation channels when co-expressed in *Xenopus* oocytes. These channels are bicuculline-insensitive but are blocked by picrotoxin. Unlike RDL, they are insensitive to dieldrin and diazepam. It would be of interest to pursue further the expression patterns of RDL and these two subunits – RDL expression is not known to overlap with that of LCCH3 (Aronstein et al., 1996), but the expression patterns of GRD have not yet been reported and so the prediction that the expression patterns of LCCH3 and GRD overlap remains untested. A GABA-gated cation channel (EXP-1) has also been cloned from *C. elegans* (Beg and Jorgensen, 2003). No native GABA-gated cation channel has so far been recorded from any insect neuron, so if such receptors exist in insects they may be rare.

Studies on other homomeric receptors from the LGIC superfamily offer some insight into the residues that define the charge-selectivity filter of the RDL receptor. In the case of both the  $\alpha 7$  nicotinic ACh receptor (Corringer et al., 1999a; Corringer et al., 1999b) and the 5HT<sub>3</sub> receptor (Gunthorpe and Lummis, 2001) selected residues in the TM1-TM2 loop and within TM2 dictate if the receptors are cationic or anionic. An alignment of RDL with LCCH3, GRD and the  $\alpha 7$  nAChR suggests that GRD at least shares similarities with the cationic  $\alpha 7$  receptor at these key residues (Figure 5A).

### **Molecular target for insecticides and a point mutation in RDL that accounts for resistance**

As GABARs are prevalent in the nervous systems of insects, they are the targets of naturally-occurring (e.g. picrotoxinin and a wide range of picrotoxin-like molecules of plant origin) as well as man-made (e.g. dieldrin, fipronil) insecticides. Historically dieldrin, the structure of which can be related to picrotoxin, was a major insecticide, but it has been banned because it persists in the environment and because its intensive use in the post-WWII years resulted in many examples of resistance to insecticide. Fipronil is the first of the phenylpyrazole group of chemicals to be introduced for pest control and is now a major pesticide in use with crops and as an antiparasitic (flea and mite control) with an estimated world market of \$150M US. The usefulness of an insecticide is often limited by the development of resistance, which is common in field populations of many insect species (Georghiu, 1986). In the case of dieldrin resistance in *Drosophila*, insecticide resistance arises from the substitution of a single amino acid: A→S at the 2' position in the lumen of the GABA receptor's channel (Bass et al., 2004; ffrench-Constant et al., 2000; Hosie et al., 1997) (Figure 5B). In the peach aphid, *Myzus persicae*, cyclodiene resistance results from an A→G mutation (Anthony et al., 1998). Fipronil resistance in the RDL equivalent in *Drosophila simulans* has also been shown to arise from the two mutations, A→G similar to the equivalent mutation in *D. melanogaster*, and a T350M mutation in the third transmembrane domain (Le Goff et al., 2005). The mutation at T350 has not been described in any field populations of dieldrin resistant *D. melanogaster* or in any mosquito populations. Resistance raised in the laboratory (Le Goff et al., 2005) may arise from a different selection pressure to that in the field resulting in different mutations. In functional expression studies comparing the wild-type and resistant forms of *D. melanogaster* RDL, 2' A→S confers resistance to a variety of insecticides, such as fipronil, dieldrin, picrotoxinin, and picrodendrin-O, which act allosterically as non-competitive antagonists of insect GABARs. This substitution also renders RDL homomers resistant to the antagonists

TBPS, lindane and BIDN (Buckingham et al., 1994a; Buckingham et al., 1996; Hosie et al., 1995; Sattelle, 1990) (Figure 2D), although these compounds interact non-competitively in radioligand binding studies suggesting that they have distinct binding sites. The ability of RDL to form functional homomers allows a more convenient study of the effects of the A302S/G mutation upon single channel properties of the channel, as well as simplifying the interpretations of such experiments. For example, the single channel properties of dieldrin-resistant RDL stably expressed in a *Drosophila* cell line revealed provided the first evidence that the two insecticides, fipronil, and the new convulsant site probe, and BIDN, act at separate sites (Grolleau and Sattelle, 2000) (Summarised in Figure 3).

### **Conclusions**

The cloned RDL receptor is not only a convenient model of insect ionotropic GABARs but is also a convenient homomeric anionic ligand-gated ion channel accessible to combined computational studies and site-directed mutagenesis approaches to understanding the structural basis of anion channel function, as well as insecticide resistance. The experimental work to date indicates that RDL is widely distributed throughout the CNS of insects of different orders (*Lepidoptera*, *Diptera*, *Coleoptera* and *Dictyoptera*), is located at synaptic sites and on neuronal cell bodies, and that RDL homomers mimic the characteristic agonist, barbiturate and pregnane steroid pharmacology of native bicuculline-insensitive insect GABARs. In all these respects, RDL very closely resembles *in situ* insect GABARs. The close similarity between RDL homomers and native insect GABARs suggests that RDL-like subunits, which are widely distributed in the *Drosophila* nervous system, may underlie several aspects of the distinct pharmacology of bicuculline-insensitive insect GABARs.

The RDL subunit offers potential for generating insect-vertebrate chimeric GABARs, which will help identify the binding sites of modulators that differ in their actions on vertebrate and insect GABARs. Its ability to form homomeric receptors that resemble *in situ* receptors also facilitates computational studies, just as the vertebrate  $\alpha 7$  receptor enabled molecular modelling of cationic ligand-gated ion channels. Expression studies on the fourth, as yet uncharacterised splice variant will complete the description of all splice variants for this receptor. Finally, expression studies of edited RDL receptors will provide the first functional characterisation of the consequences of pre-mRNA A-to-I editing in any anionic ligand-gated ion channel.

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## Legends for figures

Figure 1. Structure and post-transcriptional modifications of RDL. (A) Exon structure of the *Rdl* gene; grey boxes represent transmembrane (TM) regions 1-3 coded by exon 7 and the TM 4 region encoded by exon 9. Alternative splicing occurs in exons 3 and 6. The two versions of exon 3 (**a** and **b**) differ by 2 amino acid residues. The two versions of exon 6 (**c** and **d**) differ by 10 amino acid residues (Adapted from Hosie, et al. (1997) *TiNS* 20: 578-583, with permission). (B) Model of the extracellular domain (ECD) of RDL<sub>ac</sub>, based on the ACh binding protein (RSCBiol:119B), with red and yellow spheres denoting alternatively spliced residues of exons 3 and 6 respectively and orange spheres showing the site of RNA-editing on the N-terminus (R122G). (C) Alternative splicing of RDL exon 6 produces variations in the agonist dose-response curves for GABA and its analogues isoguvacine (ISOG), isonipicotic acid (ISON) and 3-aminopropanesulphonic acid (3-APS). All 4 agonists were more potent on RDL<sub>ac</sub> homomeric receptors, compared to homomeric RDL<sub>ad</sub> receptors (points represent mean  $\pm$ S.E.M. from at least 3 oocytes). Reprinted from *Neuroscience*, 102, Hosie AM, Buckingham SD, Presnail JK, Sattelle DB., Alternative splicing of a *Drosophila* GABA receptor subunit gene identifies determinants of agonist potency, 709-714 copyright (2001), with permission from Elsevier.

Figure 2 Pre-mRNA A-I editing of RDL. (A) Schematic representation of the RDL subunit, showing how potential A-I editing of mRNA for RDL results in changes to the amino acid sequence at residues 122, 283, 294 and 360. (C-D) Comparative model illustrating residues changed by alternative splicing and RNA-editing. Images in the left-hand panels are side-views and the right hand panels show pentamers viewed from above. (B) Model of the TM domain of RDL<sub>ac</sub>, based on coordinates of Torpedo nAChR (1OED.pdb), with orange and red spheres denoting sites

of RNA-editing in the TM1 region and the TM1-TM2 loop respectively. Yellow spheres denotes site of A to S/G mutation inferring resistance to insecticides.

Figure 3. Physiological characteristics of RDL receptors. (A) Chemical structures for convulsant compounds BIDN, Dieldrin and Fipronil. (B) Actions of selected convulsant antagonists on the currents evoked by 10  $\mu$ M GABA at wild-type RDL receptors expressed in *Xenopus* oocytes.

Traces for GABA alone, followed by GABA and 10  $\mu$ M picrotoxinin (PTX), fipronil, *t*-butylbicyclophosphorothionate (TBPS) and 4-*n*-propyl-4'-ethynylbicycloorthobenzoate (EBOB) respectively (Adapted from Buckingham et al. (1994) *Neurosci. Letts.* 181: 137-140, with permission). (C) Effects of the convulsant BIDN on GABA-evoked currents at the RDL receptor.

(Upper panel) Compared to control, pre-incubation of oocytes with 100  $\mu$ M BIDN shifts the dose response curve for GABA to the right and reduces the maximal currents evoked, suggesting the antagonist actions of BIDN are neither strictly competitive nor non-competitive. (Lower panel)

Dose inhibition curves using 100  $\mu$ M GABA show that compared to wild-type RDL ( $\square$ ), the A302S mutation ( $\circ$ ) decreases the inhibitory effects of the BIDN at the RDL receptor (points represent mean  $\pm$ S.E.M. from at least 3 oocytes). (Adapted from *Brain Research*, 693, Hosie AM, Shirai Y, Buckingham SD, Rauh JJ, Roush RT, Baylis HA, Sattelle DB, Blocking actions of BIDN, a bicyclic dinitrile convulsant compound, on wild-type and dieldrin-resistant GABA receptor homooligomers of *Drosophila melanogaster* expressed in *Xenopus* oocytes, 257-260, copyright (1995), with permission from Elsevier).

Figure 4. Analysis of the actions of BIDN and fipronil using single channel recordings from S2 cells expressing *Rdl*. (A) Examples of currents recorded from an outside-out patch from an S2-RDL cell exposed to 50  $\mu$ M GABA (c = closed, o = open). (B) Current amplitude histograms for GABA and GABA with the convulsant antagonist BIDN show the presence of BIDN leads to a reduction

in the size of channel conductance. (C) The convulsant antagonist fipronil reduces the probability of channel openings ( $P_o$ ) in the S2-RDL cells, with addition of BIDN further reducing the  $P_o$ . (D) Plotting the increasing concentration of BIDN, or 300 nM BIDN with increasing concentrations of fipronil, against the reduction in  $P_o$  reveals that the effects of these two antagonists do not seem to be via the same site on the RDL receptor. (Adapted by permission from British Journal of Pharmacology, 130: 1833-1842 copyright 2000 Macmillan Publishers Ltd.).

Figure 5. Alignments of RDL. (A) Alignments of the *Drosophila* RDL sequence (from above) with other *Drosophila* GABA-gated channels and the human  $\alpha 7$  nicotinic acetylcholine receptor subunit (a cation channel) showing variations in residues of TM2 reported to define ion-selectivity of the channel. Sequences: *Drosophila melanogaster* LCCH3 (Accession number AAL28208), GRD (CAA55144) and Human  $\alpha 7$  nAChR (NP\_000737). Alignments created using ClustalX and GeneDoc programs (B) Alignments of the TM1 and TM2 regions of the RDL sequence in *Drosophila* and other insect species reveals conserved sequence of amino acid residues. Sequences: *Drosophila melanogaster* (fruitfly, Accession number NP\_729462), *Musca domestica* (housefly, BAD16658), *Anopheles gambiae* (malaria mosquito, XP\_316072), *Apis mellifera* (honeybee, AAC63381) and *Heliothis virescens* (tobacco budworm, AAB62575). Region of alignment contains the alanine residue, where mutation to a serine or glycine has been reported to confer resistance to a number of compounds including insecticides.



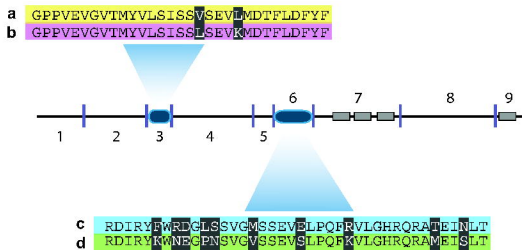
**Tables**

<b>Subunit combination</b>	<b>Heterologous expression</b>	<b>Pharmacology</b>	<b>Reference</b>
RDL homomer	Oocytes, S2 cells (Anion channel)	Picrotoxinin sensitive Bicuculline insensitive	(Hosie and Sattelle, 1996a)
RDL + LCCH3	Sf-9	Picrotoxinin insensitive Bicuculline sensitive	(Zhang et al., 1995)
RDL + GRD	Not reported	Not reported	N/A
GRD + LCCH3	oocytes (Cation channel)	Picrotoxinin sensitive Bicuculline insensitive	(Gisselmann et al., 2004)

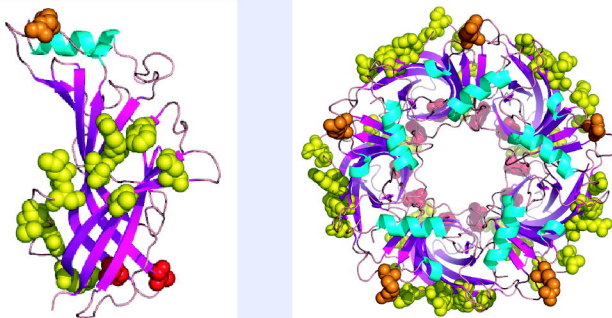
*Table 1 Heterologous expression of RDL alone or with other putative insect GABAR subunits results in receptors with distinct pharmacologies*

figure 1

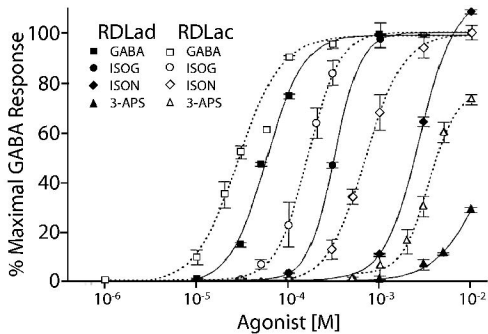
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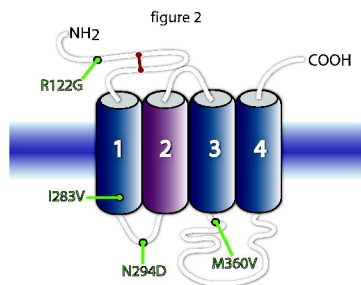
B



C



A



B

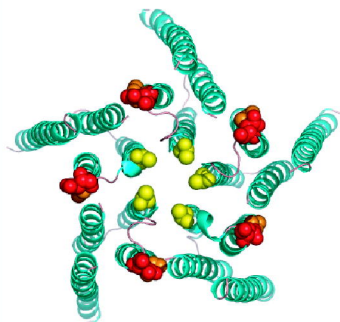
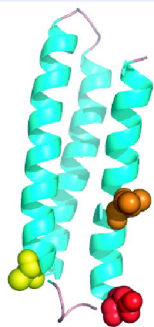


figure 3

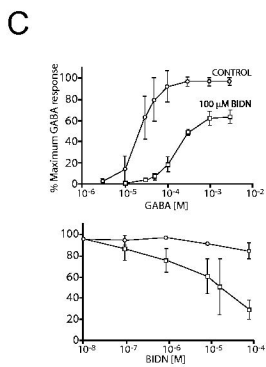
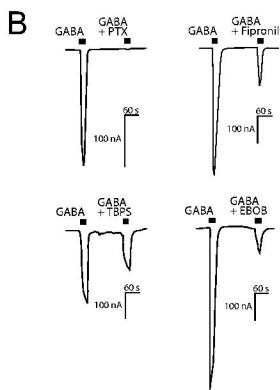
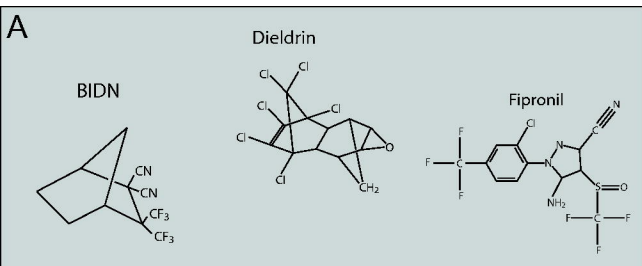
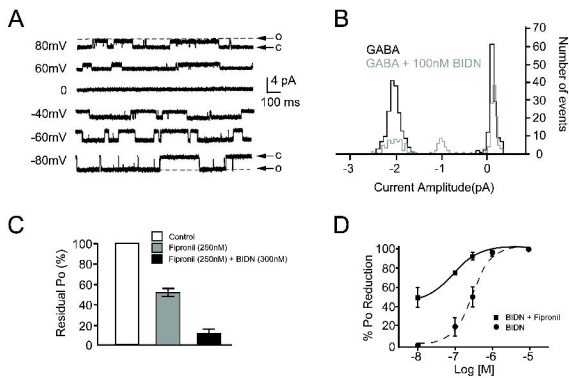


figure 4



**A**

figure 5

TM2

RDL : LIVIISWVSFWLNRNATPARVALGVTTVLTMTTMSSTN  
 LCCH3 : IVMLSWVSFWINHEATSARVALGITTVLTMTTISTGVR  
 GRD : LLVVLSWVSFWLNREATADRVS LGITTVLTMTFI GLEAR  
 α7nAChR : LISALALVLELLPADS-GEKISLGITVLLSLTVFMLLVA

Residues that play a major role in  
 the ion selectivity of the channel  
 blue indicates -vely charged residues

**B**

← TM1

TM2

Drosophila : LIVIISWVSFWLNRNATPARVALGVTTVLTMTTMSSTN  
 Musca : LIVVISWVSFWLNRNATPARVALGVTTVLTMTTMSSTN  
 Anopheles : LIVIISWVSFWLNRNATPARVALGVTTVLTMTTMSSTN  
 Apis : LIVIISWVSFWLNRNATPARVALGVTTVLTMTTMSSTN  
 Heleothis : LIVIISWVSFWLNRNATPARVALGVTTVLTMTTMSSTN  
 LIV6ISWVSFWLNRNATPARVALGVTTVLTMTTMSSTN

A→S/G point mutation  
 conferring insecticide resistance