1 **Title:** N318L Blocks the Interaction of Fluralaner but Not Broflanilide or Fipronil

2 with the Insect GABA Receptor *in vivo*

3

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26 **GRAPHICAL ABSTRACT**

27

28

29 **ABSTRACT:** Fluralaner is a novel insecticide targeting the ionotropic GABA receptor
30 (GABAR) subunit, RDL. A recent study revealed that N316L, a substitution of
31 asparagine (N) with leucine (L), in the second transmembrane (M2) -spanning region
32 reduced the antagonist action of fluralaner on housefly *Musca domestica* RDL (*Md*RDL)
33 *in vitro*. To verify the impact of N316L *in vivo*, the corresponding mutation (N318L) in
34 the fruitfly *Drosophila melanogaster* RDL (*Dm*RDL) was constructed using
35 CRISPR/Cas9 genome editing. The homozygous *Dm*RDL^{N318L} mutants showed 9.87-
36 fold resistance to fluralaner compared with w^{1118}, whilst still being highly sensitive to
37 broflanilide and fipronil, which is consistent with those findings observed in the
38 electrophysiology assays of homomeric *Dm*RDL^{WT} or *Dm*RDL^{N318L} channel. Moreover,
39 N318L led to malformed ovaries, stunted eggs and sterility in homozygous females.
40 These results highlighted the N318 as a molecular site for fluralaner *in vivo* and *in vitro*
41 and might elucidate the resistance mechanisms of insect against fluralaner.

42

43 **KEYWORDS:** chloride channel; resistance to dieldrin; isoxazolines; *Drosophila*;
44 CRISPR/Cas9

45

46 **INTRODUCTION**

47 Ionotropic γ-aminobutyric acid (GABA) receptors (GABARs) function as a
48 GABA-gated chloride channel in both insect and mammalian nervous systems [1-2] .
49 Ionotropic GABAR is one member of the cys-loop ligand-gated ion channel
50 superfamily, which shares common structural features, including an extracellular N-
51 terminal region where neurotransmitter binding occurs and four transmembrane
52 domains (M1-4) [3]. Insect GABARs are the molecular targets of insecticides [4-6]. The first
53 generation of GABAergic insecticides, which were developed in the 20th century,
54 mostly consisted of non-competitive antagonist (NCA) type IA (NCA-IA) compounds
55 (e.g., polychlorocycloalkanes, cyclodienes and fiproles) and the macrocyclic lactones
56 [1]. The effectiveness of GABAergic insecticides was greatly reduced with the
57 appearance of the mutation at A2' (alanine at the second residue of M2) in the insect
58 GABAR subunit, RDL (resistant to dieldrin) [5, 7-11]. Therefore, new compound(s) acting
59 on GABAR with novel action are required to solve the resistance problem.

60 Both isoxazolines (e.g., fluralaner) and meta-diamides (e.g., broflanilide)
61 insecticides are GABAergic insecticides designated as NCA-Type II (NCA-II) with
62 completely novel action. To date, fluralaner has released to the market as
63 ectoparasiticide for flea and tick management with little or no toxicity to mammals [12-
64 16]. NCA-II compounds do not show cross-resistance with NCA-IA compounds, even
65 though both of them interact with RDL and inhibit insect GABA-induced neuroactivity
66 [17-20]. Homology modeling of *Drosophila melanogaster* RDL (*Dm*RDL) subunit
67 indicated that the binding sites of NCA-II are located in an interstitial area, which is
68 adjacent to the interface of M1/M3 domains [1, 21]. Previous evidence from *in vitro* assays
69 suggested that isoxazolines and meta-diamides probably act on the same site(s) [6, 22-24].
70 Recently, an essential role of *Dm*RDL G3' (glycine at the third residue of M3) in the

71 action of both isoxazolines and meta-diamides [25-26] has been demonstrated *in vivo*.
72 However, it remains to be determined whether both groups of compounds act on other
73 sites of RDL. A recent study reported that an asparagine (N316) located at the outer end
74 of the channel-lining M2 α-helix also plays an important role in the antagonism of
75 *Md*RDL by fluralaner *in vitro* [27].

76 To determine whether the residue (N316) could play a role in fluranaler
77 interactions *in vivo*, a mutation at the equivalent residue (N318L) in *Dm*RDL was firstly
78 generated using CRISPR/Cas9 genome editing in this study. The sensitivity of these
79 genome-edited flies to fluralaner was then measured. Secondly, electrophysiological
80 assays using *Dm*RDL expressed in the African clawed frog *Xenopus laevis* oocytes were
81 also conducted to determine the effect of N318L on the potency of fluralaner, demethyl-
82 broflanilide (DMBF) or fipronil. Knowledge gained from this study can contribute to
83 the theoretical basis for resistance risk assessment of fluralaner, as well as provide
84 further insights into the mode of action of isoxazolines and meta-diamides insecticides.
85

86 **Materials and Methods**

87 **Ethics statement.** The use of *X. laevis* in the present study strictly followed the
88 ethics of the China (GB/T 35892-2018) and Nanjing Agricultural University guidelines
89 (https://dongwu.njau.edu.cn/info/1003/1192.htm) for the protection of animal welfare.
90 A proof/certificate of approval is available upon request.

91 **Insecticides.** Fluralaner (purity ≥ 99%) was purified from Bravecto™ [28]. Fipronil
92 (purity ≥ 96%) was provided by J & K Scientific (China) Ltd. (Beijing, CHN).
93 Broflanilide (purity ≥ 98.67%) was provided by Mitsui Chemicals Agro, Inc. (Tokyo,
94 JPN). DMBF (purity ≥ 96%) was kindly provided by Professor Xusheng Shao (East
95 China University of Science and Technology).

96 ***Drosophila* strains.** *Drosophila melanogaster* strain nos.Cas9 (stock #54591 at
97 Bloomington *Drosophila* Stock Center, Bloomington, IL, USA) was used as the zeroth
98 generation (G_0) for genome editing. The *Drosophila* strain w^{1118} was used as wild type
99 (WT) strain. The *Drosophila* strain w^{1118}; TM2 Ubx^{130}/TM6B Tb^{1}(stock #FWB00002
100 at Fungene Biotech, Qidong city, Jiangsu Province, CHN) was used as the balancer
101 strain. All the flies were cultured on standard fly diet at 25 °C, relative humidity of
102 60%-70% and a photoperiod of 12 h:12 h (L: D) [25, 29].
103 **CRISPR design and plasmid construction.** Potential CRISPR targets in the
104 genomic region of interest were identified using the online platform CRISPR Optimal
105 Target Finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder/), and two target
106 sequences (N318L-gRNA1: 5'-ATTTGTTGACGACATCAAAG-3'; N318L-gRNA2:
107 5'-CAAATCGATTGACGTCTATC-3') without predicted off-target hits were selected
108 to construct the gRNA-expressing plasmid using pCFD4 plasmid backbone
109 (Addgene#49411) as previously described [25].
110 For the construction of donor plasmid, a 2114-bp fragment which contained two
111 homology arms (~1000 bp/arm) flanking the targeted *Dm*RDL genomic region was
112 amplified from the genome of nos.Cas9 strain by PCR using primers *Dm*RDL-donor-
113 F/R (**Table 1**). PCR fragments were purified and cloned into the *pEASY*®-Blunt3
114 cloning vector (TransGen Biotech, Beijing, CHN) as an initial donor plasmid. Then the
115 initial donor plasmid served as a template for introducing N318L and two synonymous
116 mutations on the protospacer adjacent motif (PAM) sequences in the donor DNAs using
117 the Fast Mutagenesis System (TransGen Biotech) with primers N318Ldonor-F1/R1,
118 F2/R2 and F3/R3 (**Table 1**).
119

120 **Table 1** Primers used in the present study

Primers name	Nucleotide sequence (5′- 3′)	Function
*Dm*RDL-F	CCACCATGAGTGATTCAAAAATGG	Cloning of *Dm*RDL
*Dm*RDL-R	CTACTCCTCGCCCAGGA	
*Dm*RDL-*Eco*R I-F	ATTCCCCGGGGATCC***GAATTC***ATGAGTGATTCAAAAATGGACAAACTGG	Generation of pGH19-*Dm*RDL plasmid
*Dm*RDL-*Xba* I-R	TCGGCGATCGGGCCC***TCTAGA***CTACTCCTCGCCCAGGAGCA	
*Dm*RDL-donor-F	CAACGATTATTGCTTCGGTT	Amplification of the homology arms flanking the targeted *Dm*RDL genomic region of nos.Cas9 strain
*Dm*RDL-donor-R	TTCGATTCTAGGTGCGAGGT	
N318Ldonor-F1	CGTGTTGACAATGAC***A***ACTTTGATGT	Generation of mutated donor plasmid
N318Ldonor-R1	***T***GTCATTGTCAACACGGTTGTCACAC	
N318Ldonor-F2	GATTGACGTCTATCT***A***GGAACATGCT	
N318Ldonor-R2	***T***AGATAGACGTCAATCGATTTGACGT	
N318Ldonor-F3	AACTTTGATGTCGTCAACA***CT***TGCAGCGCTG	
N318Ldonor-R3	***AG***TGTTGACGACATCAAAGTTGTCATTGTCA	
pCFD4-gRNA-F	GATATCCGGGTGAACTTCG***ATTTGTTGACGACATCAAAG***GTTTTAGAGCTAGAAATAGCAAG	Generation of DNA fragment with gRNA sequences fused
pCFD4-gRNA-R	CTATTTCTAGCTCTAAAAC***GATAGACGTCAATCGATTTG***CGACGTTAAATTGAAAATAGGTC	
N318Ldet-F	GCAACTATTCGCGTTTAGCC	Amplification of the genomic region of *Drosophila* for sequencing
N318Ldet-R	CTGGCTGTTGATCGACGACT	

121 Note: For primers *Dm*RDL-*Eco*R I-F/R, sequences of the pGH19 backbone are underlined and the
122 restriction enzyme *Eco*R I and *Xba* I sites are in bold italic; for primers pCFD4-gRNA-F/R, sequences
123 of the pCFD4 backbone are underlined and of the target sequences are in bold italic; for primers
124 N318Ldonor-F1/R1 and N318Ldonor-F2/R2, two single-base pair synonymous mutations are in bold
125 italic; for primers N318Ldonor-F3/R3, the 2-base pair mutation of N318L was in bold italic.
126

127 **Generation and identification of genome-edited *Drosophila* strain.** To generate
128 *Dm*RDL^{N318L} in *Drosophila*, a mixture of gRNA-expressing plasmid (75 ng/μL) and
129 donor plasmid (100 ng/μL) was injected into 300 embryos of nos.Cas9 strain. The G_0
130 adult flies were individually crossed with nos.Cas9 strain. The G_1 progeny was screened
131 for *Dm*RDL^{N318L} alleles. Screening was performed by isolating genomic DNA from
132 sets of at least 15 G_1 adult flies per vial and by using the genomic DNA as template for
133 amplification with primers N318Ldet-F/R (**Table 1**), then sequence-verified. Once the
134 *Dm*RDL^{N318L} alleles were detected in G_1 flies, other male flies from the same vial were
135 individually crossed with females of the balancer strain. As G_2 progeny emerged, male
136 flies with a TM2 *Ubx*^{130} allele were identified and individually crossed with the females
137 of balancer strain, and the lines arising from *Dm*RDL^{N318L} G_2 males were retained. By
138 this step, all nos.Cas9 in the X chromosome were replaced with WT strain background.
139 In the G_3 progeny, adult flies with both *Dm*RDL^{N318L} and TM2 *Ubx*^{130} alleles were self-
140 crossed to generate the heterozygous *Dm*RDL^{N318L} strain (G_4), which was verified by
141 sequencing. This strain was subsequently maintained by crossing between siblings
142 bearing both *Dm*RDL^{N318L} and TM2 *Ubx*^{130} alleles.

143 **Bioassays.** For the bioassays, homozygous *Dm*RDL^{N318L} flies were selected
144 against the marker phenotype (*Ubx*^{130}) for the relevant balancer [30], and heterozygous
145 *Dm*RDL^{N318L} flies were selected from the progeny of homozygous *Dm*RDL^{N318L} males
146 mated to the WT females. The bioassay method for *D. melanogaster* adults was based
147 on ffrench-Constant and Roush (1991) [31] with minor modifications. Insecticide was
148 coated internally on the inside of glass vials (diameter × height, 20 mm × 80 mm) by
149 applying 150 μL of acetone containing various concentrations of each insecticide, and
150 rolling the vials until the acetone evaporated. Ten female flies (1-3 days post-eclosion)
151 were transferred into the vial as one replicate, and each vial was plugged with absorbent

152 cotton (~0.5 g) soaked with 5% sucrose (4.0 mL). The mortality was recorded at 24 h
153 after treatment. For these assays, each concentration was replicated five times and
154 acetone treatment was used as the control.

155 **Electrophysiological assay.** The coding sequence of WT or N318L mutant
156 *Dm*RDL subunit was cloned into the pGH19 plasmid backbone from WT or
157 homozygous *Dm*RDL^{N318L} flies respectively, for expression in *X. laevis* oocytes.
158 Procedures for oocyte preparation, cRNA injection into oocytes and two-electrode
159 voltage-clamp electrophysiology were carried out as previously described [32]. The
160 concentration (e.g., 3 μM) of insecticides was used according to Asahi et al. (2023)
161 report [27].

162 **Detection of *Drosophila* female sterility.** Ten single female flies (3 days post-
163 eclosion) were individually mated to two WT male flies [33] as one replication and the
164 proportion of fertile individuals were scored. Afterwards, ovaries were dissected from
165 all the tested females in phosphate-buffered saline (PBS) by pulling on the stalk region
166 of older egg chambers. Five replicates of each genotype were tested using WT female
167 as control.

168 **Statistical analysis.** For the bioassays, the median lethal concentration (LC_{50})
169 values were calculated by probit analysis using SPSS 17.0 software (SPSS Inc.,
170 Chicago, IL), non-linear dose–response curves were generated in GraphPad Prism 9.5.0
171 (GraphPad Software Inc., La Jolla, CA). For the electrophysiological assay, the values
172 of median effective concentration (EC_{50}), median inhibitory concentration (IC_{50}) and
173 the scatter plot were determined from the mean of 3-9 replicates using non-linear
174 regression analysis in GraphPad Prism 9.5.0. Two values of EC_{50} or IC_{50} were
175 considered as significantly different if their 95% confidence intervals (CIs) did not
176 overlap. Inhibition of GABA-induced currents by fluralaner, DMBF or fipronil at 3 μM

177 in WT and N318L mutants were analyzed with Student's *t*-test using SPSS 17.0

178 software. Significant difference was determined by *P* values: ns indicates $P \geqslant 0.05$,

179 **** indicates $P < 0.0001$. For the detection of *Drosophila* female sterility, proportions

180 were analyzed with Student's *t*-test using SPSS 17.0 software. Significant difference

181 was determined by *P* values: **** indicates $P < 0.0001$.

182

183 **Results**

184 **Generation of genome-edited *D. melanogaster* bearing the N318L in *Dm*RDL.**

185 The N318L was introduced into *Dm*RDL using CRISPR/Cas9 genome editing (**Figure**

186 **1**). Embryos of the nos.Cas9 strain were injected with specific gRNAs/donor plasmid

187 mixture, and were defined as the G_0. In G_0, 86 larvae (28.67%) hatched from 300

188 injected embryos and 38 adults finally emerged. Then, 38 G_0 adults were individually

189 crossed with the nos.Cas9 flies, and the resulting G_1 progeny were screened for the

190 *Dm*RDL^{N318L} allele by sequencing after emerging. The *Dm*RDL^{N318L} allele was

191 detected in 1 out of 36 lines that gave G_1 progeny. Afterwards, 22 G_1 male flies from

192 this positive line were individually crossed with the balancer strain and screened to

193 identify positive heterozygotes (**Figure S1**). The *Dm*RDL^{N318L} alleles were found in 4

194 of 21 G_1 flies (19.05%) that gave G_2 progeny. Although the sibling mating using

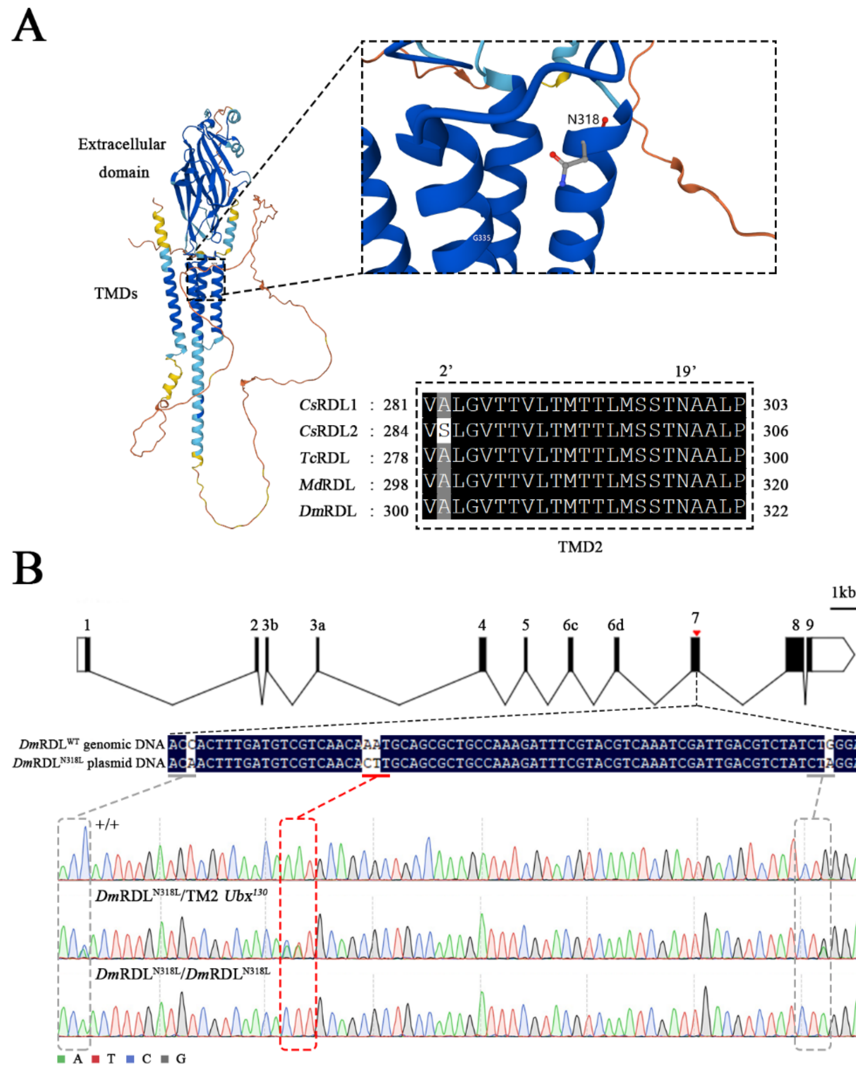
195 positive G_2 flies, which bear the *Dm*RDL^{N318L} allele opposite to the TM2 *Ubx*^{130}

196 balancer chromosomes, produced homozygous mutant G_3 progeny (**Figure 1B**), all

197 female homozygotes were sterile. Therefore, a heterozygous *Dm*RDL^{N318L} strain was

198 generated and subsequently maintained by crossing between siblings bearing both

199 *Dm*RDL^{N318L} and TM2 *Ubx*^{130} alleles (**Figure 1B**).



200

201 **Figure 1.** Generation of the *Dm*RDL^{N318L} by CRISPR/Cas9 genome editing. (A) Structure of

202 *Dm*RDL and sequence alignment of the M2 from several insect species. *Cs*: *Chilo suppressalis*, *Tc*:

203 *Tribolium castaneum*, *Md*: *Musca domestica*, *Dm*: *Drosophila melanogaster*. N318 in *D.*

204 *melanogaster* corresponds to residue 19’ in M2. (B) Genotypes of the heterozygous and

205 homozygous *Dm*RDL^{N318L} flies were confirmed by sequencing genomic DNA. Two synonymous

206 mutations indicated by grey boxes were designed to prevent repeated editing. The corresponding

207 codon of the N318L residue is indicated by a red box.

208

209 **Homozygous *Dm*RDL^{N318L} *Drosophila* were resistant to fluralaner while still**

210 **being sensitive to broflanilide or fipronil.** The LC_{50} values for fluralaner, broflanilide,

211 and fipronil against the WT flies were 0.39, 0.16 and 0.11 μg/vial, respectively.

212 heterozygous *Dm*RDL^{N318L} mutants showed sensitivity to these insecticides similar to

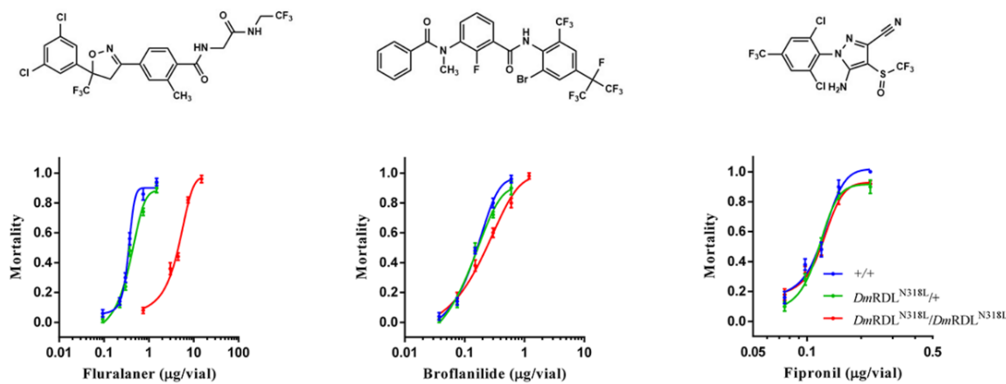
213 those of the WT (**Figure 2** and **Table S1**). However, homozygous *Dm*RDL^{N318L}

214 *Drosophila* mutants exhibited 9.87-fold resistance to fluralaner compared with the WT

215 flies (**Figure 2** and **Table S1**), and were still sensitivity to broflanilide or fipronil

216 (**Figure 2** and **Table S1**).

217



218

219 **Figure 2.** Non-linear dose-response curves for insecticidal effects of fluralaner, broflanilide and

220 fipronil against the homozygous and heterozygous *Dm*RDL^{N318L} *Drosophila* mutants. Data

221 represent means ± SE (n=5).

222

223 **N318L altered the sensitivity of *Dm*RDL homomer to fluralaner but not to**

224 **broflanilide or fipronil *in vitro*.** *Dm*RDL^{WT} and *Dm*RDL^{N318L} were expressed in *X.*

225 *laevis* oocytes, respectively. N318L significantly decreased the potency of GABA to

226 homomeric *Dm*RDL channel with its EC_{50} increasing up to 8.69-fold (**Figures 3A-C**

227 and **Table S2**). Fluralaner showed concentration-dependent antagonistic action on the

228 GABA (EC_{50})-induced currents (**Figures 3D-F**) with an IC_{50} of 39.06 nM in the

229 homomeric *Dm*RDL^{WT} channel (**Table S2**). However, the N318L decreased the potency

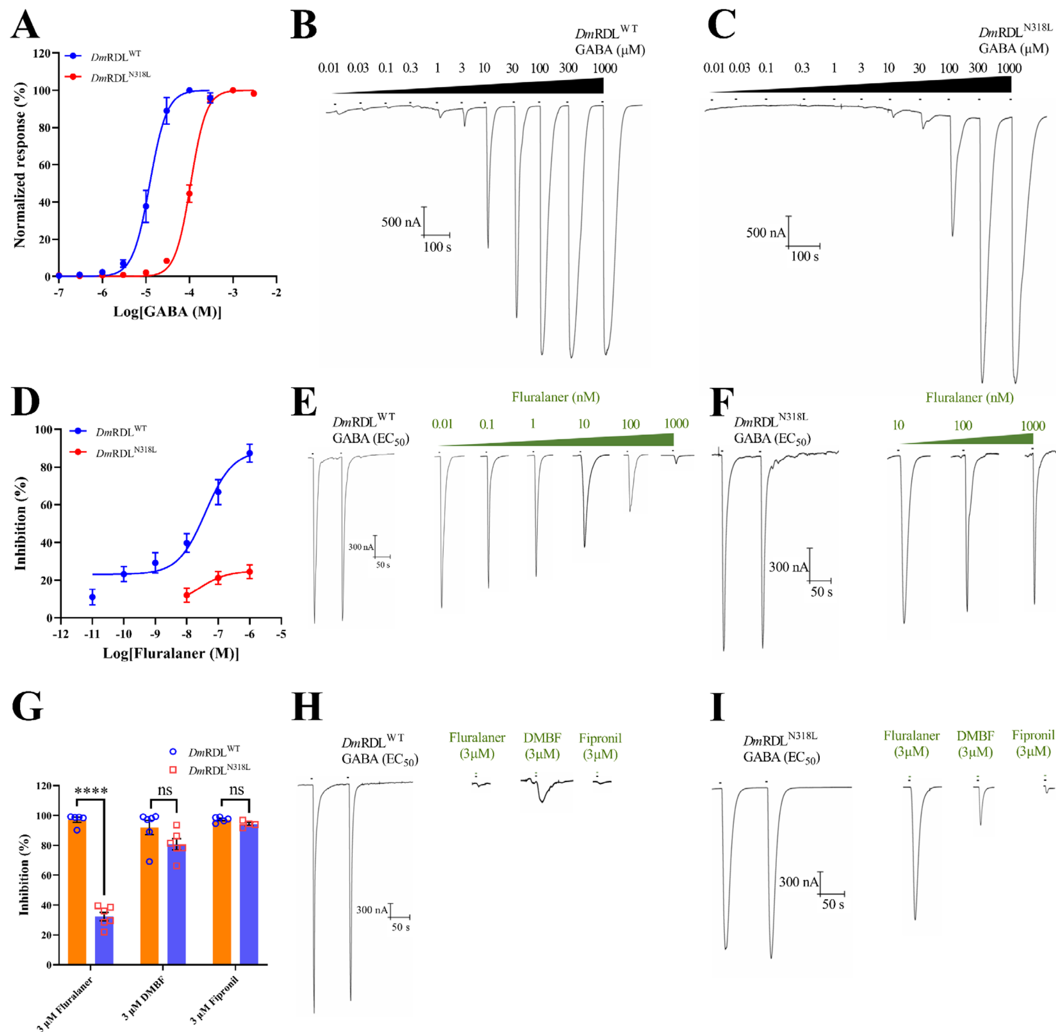
230 of fluralaner by a significantly increased IC_{50} (> 1000 nM) (**Table S2**).

231 Fluralaner, DMBF, and fipronil at 3 μM inhibited the GABA-induced currents by

232 97.07%, 91.46%, and 97.23%, respectively in homomeric *Dm*RDL^{WT} channel, however,

233 by 32.36%, 80.69%, and 94.25% in homomeric *Dm*RDL^{N318L} channel (**Figures 3G-I**

234 and **Table S3**).



235

236 **Figure 3.** Responses to GABA and insecticides of *X. laevis* oocytes expressing homomeric

237 *Dm*RDL^{WT} or *Dm*RDL^{N318L} channel. (A-C) Concentration-response curves of GABA and

238 representative current traces of *Dm*RDL^{WT} or *Dm*RDL^{N318L} channel. Data represent means ± SE

239 (n=6). (D-F) Inhibition of GABA-induced currents and representative current traces by fluralaner

240 in *Dm*RDL^{WT} or *Dm*RDL^{N318L} channel. Data represent means ± SE (n=3−9). (G-I) Inhibition of

241 GABA-induced currents and representative current traces by fluralaner, DMBF or fipronil at 3 μM

242 in *Dm*RDL^{WT} or *Dm*RDL^{N318L} channel . Data represent means ± SE (n =4-6). Significant difference

243 was determined by Student's *t*-test (ns, not significant; ****, $P < 0.0001$).

244

245 ***Dm*RDL^{N318L} caused homozygous female sterility.** We found that homozygous

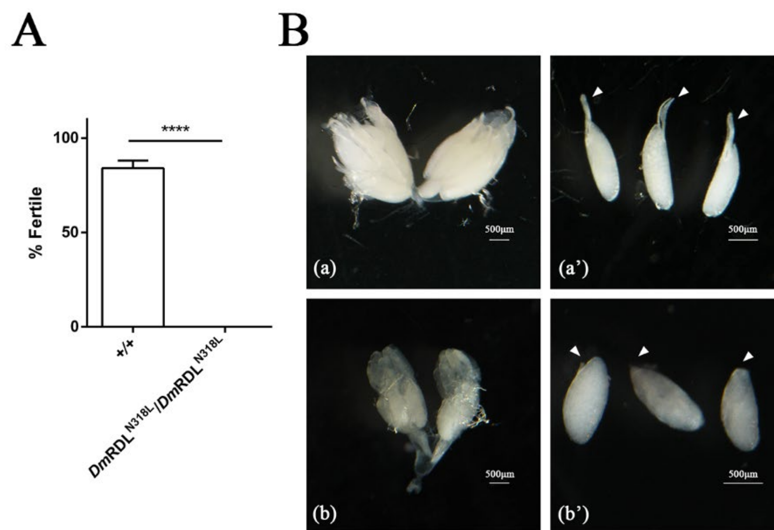
246 *Dm*RDL^{N318L} females were sterile and cannot lay eggs after mating (**Figure 4A**).

247 Therefore, their ovaries were dissected to determine if oogenesis was affected. Ovaries

248 of homozygous *Dm*RDL^{N318L} females were malformed compared with that of the WT

249 flies. Meanwhile, the eggs in the ovaries have stunted dorsal appendages that failed to

250 extend to their normal length (**Figure 4B**).



251

252 **Figure 4.** Detection of sterility in homozygous *Dm*RDL^{N318L} females. (A) Fertility of the WT (*w^{1118}*)

253 and homozygous *Dm*RDL^{N318L} females. Data represent means ± SE (n=5). Significant difference

254 was determined by Student's *t*-test (****, $P < 0.0001$). (B) Photographs of whole ovaries or eggs of

255 the WT (*w^{1118}*) and homozygous *Dm*RDL^{N318L} females. Ovaries of the homozygous *Dm*RDL^{N318L}

256 females were malformed (b) compared with that of *w^{1118}* (a), with stunted dorsal appendages (b') of

257 eggs that failed to extend to their normal length (a'), which were indicated by white arrows.

258

259 **Discussion**

260 RDL is first identified in *D. melanogaster* by genetic screening for mutations

261 associated with dieldrin resistance and is the most studied insect GABAR subunit until

262 now [7, 31, 34]. To date, mutations at the A2' residue (e.g., A2'S, A2'N) in M2 have been

263 reported to confer NCA-I (e.g., cyclodiene and phenylpyrazoles) resistance in several
264 insect species [9-10, 20, 35-40]. However, as the newly emerging NCA-II GABAergic
265 insecticides, isoxazolines and meta-diamides, showed distinct binding site(s) from
266 NCA-IA compounds [17-19, 23].

267 A specific overlap of isoxazoline and meta-diamide binding sites was shown in
268 homology models of *Dm*RDL, indicating that the NCA-II site is situated close to the
269 interface between M1 and M3 [1, 6]. Amino acid substitutions of G3' in M3 substantially
270 reduced the sensitivity of RDL to either DMBF in *Drosophila* mel-2 cells, or to
271 fluralaner in *X. laevis* oocytes [6, 24-25]. Consistent with this, homozygous G3'M or G3'A
272 mutations led to significant resistance to NCA-II insecticides in genome-edited *D.*
273 *melanogaster* [25-26]. Thus, these evidences suggest that G3' is a common and critical
274 target for NCA-II insecticides. Recently, the Asn residue at the extracellular end of the
275 channel-lining M2 α-helix was also found to be involved in the actions of fluralaner on
276 homomeric *Md*RDL channel [27]. Therefore, the function of Asn residue at M2 of
277 *Dm*RDL was verified *in vivo* by CRISPR/Cas9 genome editing in this study.
278 Homozygous *Dm*RDL^{N318L} mutants showed 9.87-fold resistance to fluralaner,
279 suggesting that the Asn residue played a role in the interaction of fluralaner with
280 GABARs *in vivo*. It is worth noting that homozygous *Dm*RDL^{N318L} mutants were still
281 sensitive to fipronil and broflanilide. Therefore, the electrophysiological assay was
282 performed as well.

283 In consistent with the previous study [27], the IC_{50} value of fluralaner against
284 homomeric *Dm*RDL^{N318L} channel significantly increased to more than 1000nM (**Table**
285 **S2**). Even at a higher concentration (3μM), fluralaner did not effectively inhibit
286 homomeric *Dm*RDL^{N318L} channel compared with *Dm*RDL^{WT} (**Figures 3G-I** and **Table**
287 **S3**). Fipronil at 3μM still could effectively inhibit the homomeric *Dm*RDL^{N318L} channel,

288 which is consistent with Asahi et al. (2023) report [27]. Interestingly, DMBF at 3 μM
289 showed much stronger inhibition of *Dm*RDL^{N318L} channel compared with fluralaner at
290 3 μM (**Figures 3G-I** and **Table S3**), which was not examined in Asahi et al. (2023)
291 report [27]. These *in vitro* and *in vivo* findings indicated that the N318 residue might play
292 a role in differential effects of fluralaner and broflanilide.

293 Besides this, the N318L also affected the response of homomeric *Dm*RDL channel
294 to GABA (**Figures 3A-C**). As shown in **Figure 1A**, the Asn also locates at the 19th
295 residue of M2 (hereafter named as N19') in RDL subunits of other insect species. In the
296 heteromeric human $GABA_A$R, the interaction between the 19th residue (R19') of M2
297 and the M2-M3 loops affects conformational changes of the M2 extracellular end,
298 which subsequently modulates the GABAergic signal transduction [41-46]. In the
299 homomeric *Dm*RDL channel, N19'K increased its conductance and homomeric
300 *Dm*RDL$^{N19'K}$ formed a permeable channel to cations, which was supposed to be caused
301 by a structural change around the M2 segment [47]. Similarly, N19'R [48] and N19'L
302 (N318L) (**Figure 3** and **Table S2**) decreased the potency of GABA to homomeric
303 *Dm*RDL channel. Therefore, we speculated that the 19th residue at M2 plays an
304 important role in the activation of GABARs.

305 Except for the change of resistance of homozygous *Dm*RDL^{N318L} mutants against
306 fluralaner, it is surprising that the homozygous *Dm*RDL^{N318L} females were infertile with
307 obviously shortened dorsal appendages. As a neuroreceptor subunit widely expressed
308 in the insect central nervous system [49], RDL is essential for early development of insect
309 [25-26, 31, 50-52]. For example, loss-of-function mutations of *Dm*RDL caused embryonic
310 lethality [31, 51-52]. Varying degrees of physiological defects were found in genome-edited
311 flies with *Rdl* mutations created by CRISPR/Cas9 genome editing, e.g., I276C (M1),
312 A2'N (M2), T305I (M2), or G3'M/S/Q (M3) in *Dm*RDL led to lethality before

homozygotes reaching adult stage, respectively [25-26, 50]. Whilst homozygous *Dm*RDL^{L280C} and *Dm*RDL$^{G3'A}$ flies were able to survive to the adult stage, they were still sterile [26]. In this study, we speculated that such obstruction of oogenesis might be the reason for the failure of the homozygous *Dm*RDL^{N318L} females to lay eggs and thus become sterile. However, further research should be carried out to reveal how RDL participate in the dorsal appendage morphogenesis of oogenesis.

In conclusion, the effect of the RDL^{N318L} *in vivo* on sensitivity to fluralaner was assessed using genome-edited *D. melanogaster*. Homozygous *Dm*RDL^{N318L} mutants were resistant to fluralaner while still being sensitive to broflanilide and fipronil. Meanwhile, electrophysiological assays also showed that N318L did not affect the antagonism of DMBF to homomeric *Dm*RDL channel *in vitro*. It is also worth noting that homozygous *Dm*RDL^{N318L} females were sterile with malformed eggs indicating fitness costs associated with the *Dm*RDL^{N318L} mutation. These results highlight N318 in M2 as potentially being a target site for fluralaner action and could help understand possible mechanisms underlying insect resistance to isoxazoline and meta-diamide insecticides.

ASSOCIATED CONTENT

Supporting Information

Crossing procedures for the generation of the homozygous *Dm*RDL^{N318L} *Drosophila* mutants (**Figure S1**); Toxicity of three insecticides to the heterozygous or homozygous *Dm*RDL^{N318L} *Drosophila* mutants (**Table S1**); Potencies of GABA and fluralaner on the homomeric *Dm*RDL^{WT} or *Dm*RDL^{N318L} channel expressed in *X. laevis* oocytes (**Table S2**); Inhibition of 3 μM insecticide on the homomeric *Dm*RDL^{WT} or *Dm*RDL^{N318L} channel expressed in *X. laevis* oocytes (**Table S3**). Nucleic acid sequence and amino

338 acid sequence of the *Dm*RDL^{WT} and *Dm*RDL^{N318L} (**Text S1**).

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Tao Tang and Andrew K. Jones: Writing – review & editing.
Chunqing Zhao: Conceptualization, funding acquisition, supervision and writing – original draft.
All authors read and approved the final manuscript.

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Notes

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ABBREVIATIONS

CI, confidence interval; **CRISPR/Cas9**, clustered regularly interspaced short palindromic repeats and CRISPR-associated protein; **cRNA**, complementary RNA; **DMBF**, demethyl-broflanilide; **EC_{50}**, median effective concentration; **GABAR**, γ-aminobutyric acid receptors; **gRNA**, guide RNA; **IC_{50}**, median inhibitory concentration; **LC_{50}**, median lethal concentration; **M1**, first transmembrane domain; **M2,** second transmembrane domain; **M3,** third transmembrane domain; **NCA**, non-competitive antagonist; **PAM**, protospacer adjacent motif; **PBS**, phosphate-buffered saline; **PCR**,

414 polymerase chain reaction; **SE**, standard error; **WT**, wild type;

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