

1 **The effects of knock-down resistance mutations and alternative splicing on voltage-gated**
2 **sodium channels in *Musca domestica* and *Drosophila melanogaster***

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19 Short Title: Alternative splicing of insect voltage-gated sodium channels affects pyrethroid sensitivity

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21

22 **Abstract**

23 Voltage-gated sodium channels (VGSCs) are a major target site for the action of pyrethroid insecticides
24 and resistance to pyrethroids has been ascribed to mutations in the VGSC gene. VGSCs in insects are
25 encoded by only one gene and their structural and functional diversity results from posttranscriptional
26 modification, particularly, alternative splicing. Using whole cell patch clamping of neurons from
27 pyrethroid susceptible (*wild-type*) and resistant strains (*s-kdr*) of housefly, *Musca domestica*, we have
28 shown that the V_{50} for activation and steady state inactivation of sodium currents (I_{Na+}) is significantly
29 depolarised in *s-kdr* neurons compared with *wild-type* and that 10nM deltamethrin significantly
30 hyperpolarised both of these parameters in the neurons from susceptible but not *s-kdr* houseflies.
31 Similarly, tail currents were more sensitive to deltamethrin in *wild-type* neurons (EC_{15} 14.5 nM) than
32 *s-kdr* (EC_{15} 133 nM). We also found that in both strains I_{Na+} are of two types: a strongly inactivating (to
33 6.8% of peak) current, and a more persistent (to 17.1 % of peak) current. Analysis of tail currents
34 showed that the persistent current in both strains (*wild-type* EC_{15} 5.84 nM) was more sensitive to
35 deltamethrin than was the inactivating type (*wild-type* EC_{15} 35.1 nM). It has been shown previously,
36 that the presence of exon I in the *Drosophila melanogaster* VGSC gives rise to a more persistent I_{Na+}
37 than does the alternative splice variant containing exon k and we used PCR with housefly head cDNA
38 to confirm the presence of the housefly orthologues of splice variants k and I. Their effect on
39 deltamethrin sensitivity was determined by examining I_{Na+} in *Xenopus* oocytes expressing either the k
40 or I variants of the *Drosophila para* VGSC. Analysis of tail currents, in the presence of various
41 concentrations of deltamethrin, showed that the I splice variant was significantly more sensitive (EC_{50}
42 42nM) than the k splice variant (EC_{50} 866nM). We conclude that in addition to the presence of point
43 mutations, target site resistance to pyrethroids may involve the differential expression of splice
44 variants.

45 **Keywords** Voltage-gated sodium channel; Pyrethroid; Splice variant; Insecticide resistance; *Xenopus*
46 oocyte

47

48 **1. Introduction**

49 Voltage-gated sodium channels (VGSC) have been shown to be a major target site for the action of
50 pyrethroid insecticides where they bind to the ion channel and modify its operation leading to
51 disruption of neural signalling, incapacity and death of the insect (Davies *et al* 2007a, 2007b). The
52 response of the insect has been described as “knock-down”. An important mechanism of resistance
53 to pyrethroids results from a reduced sensitivity of the nervous system (Sawicki 1978), a phenomenon
54 termed “knockdown resistance or *kdr*” that had been found previously in houseflies, *Musca*
55 *domestica*, resistant to DDT (Busvine 1951, Milani 1954). Later, ‘*super-kdr* (*s-kdr*)’ was also identified
56 as an allelic form of *kdr* which can provide up to 500-fold resistance to Type-II pyrethroids such as
57 deltamethrin (Sawicki 1978).

58 A number of studies associated the *kdr* and *s-kdr* phenotype in houseflies (Williamson *et al* 1993;
59 Knipple *et al* 1994) and similar resistance mechanisms in other insect species (Taylor *et al* 1993; Dong
60 and Scott 1994) with the *para*-type VGSC. The *para*-type sodium channel in housefly was fully
61 sequenced by Williamson and colleagues (1996) and single nucleotide polymorphisms were identified
62 in resistant insects. In *kdr* flies this led to an amino acid substitution of phenylalanine for leucine at
63 position 1014 (L1014F) whereas for *s-kdr* there was an additional substitution at position 918 (M918T);
64 findings which were confirmed by studies in other labs (Ingles *et al* 1996; Miyazaki *et al* 1996; Smith
65 *et al* 1997).

66 Subsequently, mutations in the *para*-orthologous genes of many other arthropod species have been
67 identified and *kdr* resistant types have been associated with L1014F and other amino acid
68 substitutions at the same site, some with the additional M918T or other Domain II S4-S6 changes in
69 amino acid composition related to a *s-kdr* phenotype (Davies *et al* 2007a, b; Dong 2007a, b; Soderlund
70 2008; Rinkevich *et al* 2013). Additionally, there have now been a range of mutations in other regions

71 of arthropod VGSCs which have been associated with resistance to a range of pyrethroids (Du *et al*
72 2016; Smith *et al* 2016; Wu *et al* 2017; Chen *et al* 2017).

73 Analysis of putative pyrethroid resistance mutations has benefitted greatly from functional expression
74 of the insect sodium channels in *Xenopus* oocytes. Such studies usually involve the injection of oocytes
75 with cRNA encoding either the *wild-type* channel or one in which site-directed mutagenesis has been
76 used to make precise mutations. Subsequent expression allows the properties and responses to
77 pyrethroid of the *wild-type* or modified channel to be compared using electrophysiological recordings
78 (Ingles *et al* 1996; Warmke *et al* 1997; Vais *et al* 1997, 2000a, 2000b; Lee *et al* 1999; Dong 1997, 2007b;
79 Wang *et al* 2003; Tan *et al* 2002; 2005). Robust functional expression of insect sodium channels in
80 *Xenopus* oocytes has usually required co-expression of another transmembrane protein TipE from
81 *Drosophila* (Feng *et al* 1995). A housefly ortholog of TipE acts in a similar way (Lee *et al* 2000) and
82 homologs of TipE (TEH 1-4) have been shown to modulate the function of insect sodium channels
83 (Wang *et al* 2013, 2015), leading to the hypothesis that these proteins act as auxiliary subunits to the
84 insect channel, analogous to the function of the β subunits of mammalian sodium channels.

85 In mammals and other vertebrates, the VGSCs are known to belong to a superfamily of voltage-gated
86 ion channels where the pore forming subunits (α -subunits) comprise a family of proteins encoded by
87 10 genes of which 9 have been functionally expressed (Goldin 2001; Catterall *et al* 2005). In contrast
88 insect genomes appear to contain only one gene encoding a VGSC, the *para* gene, first identified in
89 *Drosophila* (Loughney *et al* 1998), which has a high sequence similarity to mammalian VGSCs. *Para*
90 orthologous genes have also been identified in a number of arthropods including several insect, tick
91 and mite species which are economically or medically important (Davies *et al* 2007a, b; Dong 2007).

92 **Recent genomic studies have identified heterodimeric VGSC in aphids (Amey *et al* 2015; Zuo *et al***
93 **2016) which appear to have functional similarities to other VGSC, but have a different ion selectivity**
94 **filter from *para* and VGSC in other taxa.** Extensive diversity of physiological function in the VGSC is
95 seen in both vertebrates and arthropods, and whereas part of the variability in vertebrate signalling

96 can be explained by the differential expression of members of the family of sodium channels,
97 arthropods achieve similar diversity through post-transcriptional modification of *para*. In particular,
98 alternative splicing and RNA editing appear to be important in VGSC function in insects (Tan *et al* 2002;
99 Song *et al* 2004; Olson *et al* 2008; Lin *et al* 2009, Lin and Baines 2015, Sun *et al* 2019). Nine splice sites
100 have been identified in the *para* gene in *Drosophila* with 7 optional splice sites (a, b, i, j, e, f, and h) as
101 well as two sites (c/d and l/k) where the exons are mutually exclusive and code for amino acids in the
102 transmembrane spanning regions of the channel. The currents gated by VGSCs with the splice variants
103 have been compared by expressing the individual clones in *Xenopus* oocytes (Lin *et al* 2009). This has
104 shown that the presence of exons f, j, e, and h affect the voltage sensitivity of the channel whereas
105 the presence of exon k, rather than l, results in a significant reduction in the persistence of the sodium
106 current. Lin *et al* (2009) have shown that the RNA-binding protein Pasilla (Ps) regulates the alternate
107 splicing, with the k isoform increasing, at the expense of l, in the absence of Ps. A similar pattern of
108 developmentally regulated alternative exon usage was seen in the housefly *para* orthologue (Vssc1)
109 (Lee *et al* 2002), however the functional significance of alternative splicing on sodium channel function
110 in houseflies has not yet been determined. The cockroach *Blattella germanica* VGSC (BgNa_v) has also
111 been characterised showing that at a position corresponding to the k/l site in *Drosophila* there are
112 three mutually exclusive exons G1, G2 and G3. Functional expression of clones with G1 or G2 revealed
113 that these exons confer different electrophysiological properties and sensitivity to deltamethrin on
114 the VGSC (Tan *et al* 2002). Likewise, VGSCs from the brown plant hopper, *Nilaparvata lugens*, have
115 been shown to express variants based on the expression of nine alternative exons including the
116 mutually exclusive k/l variants and which again exhibit distinct electrophysiological properties and
117 sensitivities to pyrethroids (Sun *et al* 2019).

118

119 Electrophysiological recordings of sodium currents in neurons from several species of insects have
120 been shown to have heterogeneous properties (Byerly and Leung 1988; Saito and Wu 1991, 1993;
121 Schafer *et al* 1994; O'Dowd *et al* 1995; Le Corrionc *et al* 1999; Lapied *et al* 1999; Grolleau and Lapied

122 2000; Wicher *et al* 2001; Zhao *et al* 2004; Defaix and Lapied 2005) and it is widely assumed that this is
123 due to the differential expression of splice variants. The differences in VGSC expression on neuronal
124 excitability have been considered (Lin and Baines 2015) but there has been less consideration of the
125 implications for pyrethroid toxicity. Evidence from work on VGSCs with insecticide-resistance
126 mutations, expressed in *Xenopus* oocytes has provided insights into the molecular interactions of
127 pyrethroids with the target ion channel (Davies *et al* 2007, Usherwood *et al* 2007, Dong *et al* 2014,
128 Field *et al* 2017). It is apparent that pyrethroid action is facilitated by the activation of the VGSC, and
129 mutations which shift the voltage sensitivity of the channel, or promote closed-state inactivation, can
130 reduce pyrethroid sensitivity.

131

132 We report here an electrophysiological investigation of housefly VGSCs in neurons from *wild-type* and
133 *s-kdr* insects, which aims to correlate the properties of sodium channels, in their native cellular
134 environment, with published data from VGSCs expressed in *Xenopus* oocytes, and to investigate the
135 effects of resistance mutations and splice variants on the response of the VGSC to the pyrethroid
136 deltamethrin.

137

138 **2. Materials and methods**

139 *2.1 Isolation, culture and recording from housefly neurons: wild-type and s-kdr (530sel) (Farnham et al*
140 *1987) strains of M. domestica (with a resistance factor to deltamethrin of s-kdr/wild-type of 497 fold*
141 *(Foster et al 2003))* were obtained from Rothamsted Research (Harpenden U.K.) and the full life-cycle
142 maintained using standard rearing techniques (Foster *et al* 2003) in an insectary at 25°C and a 12 hour
143 light/dark cycle.

144

145 The pyrethroid resistance status of the two populations was checked regularly by a discriminating
146 dose bioassay (using 0.1µg deltamethrin in 1µl acetone applied to the thorax (Foster *et al* 2003)) and
147 DNA sequencing of PCR fragments amplified from total genomic DNA, extracted from adults.

148

149 *2.2 Short Term Culture of Isolated Neurons:* Adult houseflies were anaesthetised with CO₂ and placed
150 on ice. Flies were pinned through the abdomen onto Sylgard (Dow-Corning) coated dishes,
151 decapitated and the thorax dissected along the dorsal midline. Thoracic ganglia were removed and
152 maintained in Ca²⁺/Mg²⁺-free Rinaldini's saline (in mM; 135 NaCl, 2.5 KCl, 0.4 NaH₂PO₄, 1.25 NaHCO₃,
153 0.5 Glucose, 5.0 HEPES, pH 7.2). Connective tissue was removed from each ganglion and the neural
154 sheath disrupted mechanically prior to treatment with 0.5 mg ml⁻¹ collagenase (Sigma) and 2 mg ml⁻¹
155 dispase (Sigma) in Ca²⁺ / Mg²⁺-free Rinaldini's saline for 1 hour at room temperature. Ganglia were
156 washed several times with Ca²⁺/Mg²⁺-free Rinaldini's saline and transferred to modified Schneider's
157 culture medium (85% Schneider's *Drosophila* medium, 15% Foetal Bovine Serum, plus 100 units ml⁻¹
158 penicillin and 100 µg ml⁻¹ streptomycin). Ganglia were gently triturated through the flame polished
159 tip of a Pasteur pipette to liberate neurons into the culture media, and the supernatant was plated
160 directly onto 35 mm Petri dishes (Nunc, Roskilde). Dishes were left overnight at 18°C to allow neurons
161 to settle and stick to the surface of the dish.

162

163 *2.3 Whole-cell Electrophysiology:* Dishes plated with housefly neurons were used as static baths and
164 filled with 'housefly' saline (in mM; 140 NaCl, 5.0 KCl, 0.75 CaCl₂, 4 NaHCO₃, 1.0 MgCl₂, 5.0 HEPES, pH
165 7.2). Currents were recorded using the whole-cell configuration of the patch clamp technique with
166 agarose-bridge earth electrodes. Unpolished patch pipettes (5-10 MΩ) were pulled from borosilicate
167 glass capillaries (World Precision Instruments, UK) using a P-97 Flaming Brown Micropipette Puller
168 (Sutter Instrument Co., USA) and filled with 'housefly pipette' saline (in mM; 70 CsCl, 70 CsF, 1.1 EGTA,
169 2 MgCl₂, 0.1 CaCl₂, 5.0 HEPES, pH 7.2).

170

171 Experiments used an Axopatch 200 patch-clamp amplifier (Axon Instruments, USA) controlled using
172 WCP software (Dr John Dempster, University of Strathclyde) run on a Windows PC. Whole cell
173 capacitance compensation was done using the Axopatch 200 and leak current subtraction performed

174 using WCP software. The filtering rate was 5 KHz in all experiments. The sampling rate was 50 KHz
175 except in the experiments where pyrethroid-induced tail currents were measured, where it was
176 reduced to 3 KHz.

177

178 Sodium currents were isolated by adding channel blockers to the bath solution; K⁺ channel blockers
179 were 30 mM tetraethylammonium chloride (TEA) and 1.0 mM 4-aminopyridine (4-AP) (Sigma); the
180 Ca²⁺ channel blocker was 1.0 mM CoCl₂ and the Na⁺ channel-blocker was tetrodotoxin (Sigma) 20 – 60
181 nM).

182

183 Deltamethrin was dissolved in dimethylsulphoxide (DMSO) to create stock solutions that were diluted
184 1000-fold by addition to the single-use bath to give the required final concentrations (1 nM – 300 nM).
185 Unless otherwise stated, cells were allowed to equilibrate for 10 minutes after entering the whole-cell
186 patch clamp recording configuration. Current voltage relationships were recorded in triplicate
187 immediately before addition of deltamethrin and again after 5 minutes. Control experiments
188 demonstrated that concentrations of up to 0.1 % DMSO had no effect on Na⁺ currents in housefly
189 neurons.

190

191 *2.4 Physiology of isolated housefly neurons.* Current-voltage relationships were measured by applying
192 30 ms depolarising steps between –70mV and +60 mV. From a holding potential of –100 mV, steps
193 were delivered in 5 mV increments at a frequency of 0.5 Hz. The peak current amplitude at each step
194 was plotted against the corresponding test potential and fitted by applying an iterative non-linear
195 regression protocol to the modified Boltzmann function:

196

$$197 \quad I_{peak} = G_{max}(V_T - V_{rev}) / (1 + \exp((V_T - V_{0.5})/k)) \quad \text{Eq 1}$$

198

199 where I_{peak} is the peak current elicited by the voltage pulse, G_{max} is the maximum conductance for the
200 series of voltage pulses, V_T is the test potential, V_{rev} is the reversal potential, $V_{0.5}$ is the voltage that
201 elicits a half-maximal response and k is the slope factor in mV.

202

203 Voltage-dependence of activation was measured using the same methods. Currents were converted
204 to conductance using $G = I / (V_T - V_{rev})$ and normalised by dividing by G_{max} . The mean \pm SEM was plotted
205 against the corresponding test potential and was fitted with a Boltzmann equation to fit conductance:

206

$$207 \quad G/G_{max} = 1 / (1 + \exp(V_{50} - V_T / k)) \quad \text{Eq 2}$$

208

209 where G is conductance (Lin and Baines 2015).

210

211 Voltage-dependence of inactivation was measured using holding potentials ranging from -120 mV to
212 $+40$ mV, immediately followed by a test pulse to a potential that elicited the maximum peak current
213 for the cell tested. A pre-pulse duration of 30 ms was used to induce steady-state inactivation. Peak
214 current amplitudes were normalised to the maximum peak current for the cell tested and plotted
215 against the corresponding test potential and fit with the Boltzmann equation:

216

$$217 \quad I_{peak} = I_{max} / (1 + \exp(-(V_T - V_{0.5})/k)) \quad \text{Eq 3}$$

218

219 where the parameters are as for Eq 1 and 2.

220

221 Tail currents were investigated by a single 50ms pulse to -10 mV from a holding potential of -70 mV.

222 The length of recording at the holding potential after repolarisation was extended to 100s to allow for

223 visualisation and measurement of tail currents.

224

225 The percentage channel modification was calculated using:

$$226 \quad M = ((I_{tail} / (V_{hold} - V_{rev})) / G_{max}) * 100 \quad \text{Eq4}$$

227

228 where M is percentage modification, I_{tail} is the tail current amplitude measured as the peak value in
229 the 50ms immediately following repolarisation, V_{hold} is the holding potential, V_{rev} is the reversal
230 potential, and G_{max} is the conductance transformation of the peak current elicited by the depolarising
231 pulse under control conditions. This was obtained for different deltamethrin concentrations.

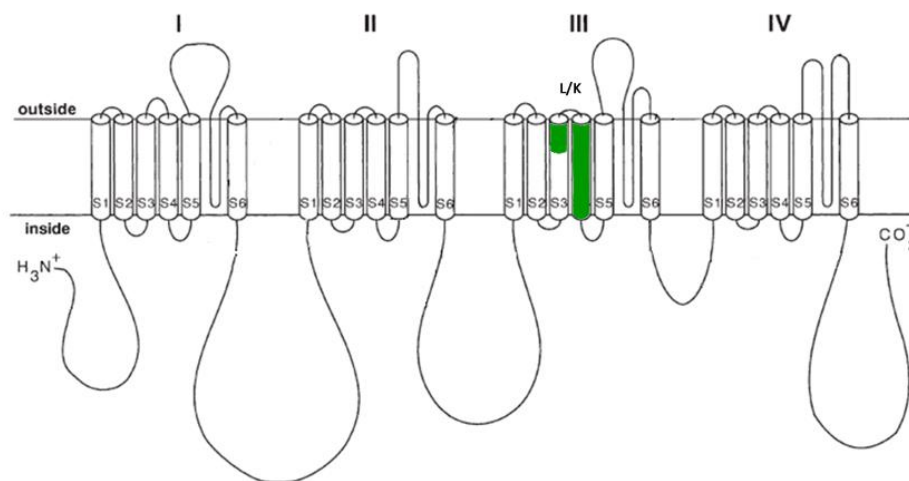
232

233 M was plotted against deltamethrin concentration and fitted with a concentration-response function
234 with a 100% modification upper plateau restriction, to give a concentration-response relationship. All
235 curve fitting and statistical analyses used GraphPad Prism 8 software.

236

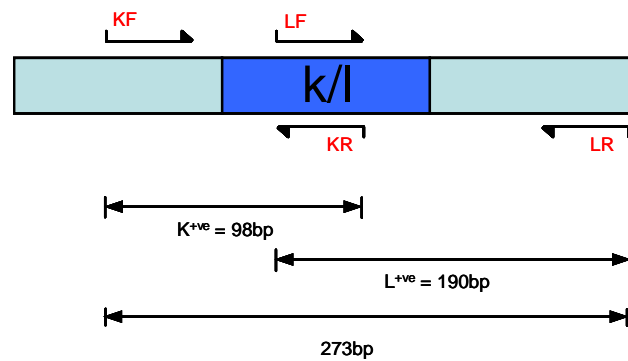
237 *2.5 Identification of mutually exclusive k and I exons in housefly heads:* Total cDNA was extracted from
238 housefly heads and PCRs performed using Primers designed to amplify fragments spanning the
239 mutually-exclusive exons k and/or I (Figure 1). A multiplex approach was used, whereby a pair of
240 primers in the exons flanking k/l were coupled with one k-specific and one l-specific primer, with the
241 size of the fragments produced being diagnostic for which exon sequence is present.

242



243

244



245

246

247 **Figure 1. A:** Diagram of the housefly and *Drosophila para* VGSC with the location of the *k/l* splice
248 variants indicated. **B.** Two primers in the exons flanking *k/l* coupled with one *k*-specific and one *l*-
249 specific primer were designed such that the sizes of the fragments produced were indicative of which
250 exon was expressed.

251

252 **2.6 Electrophysiological properties of *Drosophila* VGSC splice variants:** DNA constructs of *Drosophila*

253 VGSC clone *DmNav10/pGH19 (para13-5)* (Warmke *et al* 1997) were expressed as mutually exclusive *k*
254 (*13-5k*) and *l* (*13-5L*) exons, as described in Lin *et al* (2009).

255

256 *Xenopus laevis* ovarian tissue was obtained from the Biomolecular Science Unit of the University of
257 Portsmouth and dissociated in 0.2 mg ml⁻¹ type 1A collagenase enzyme (Sigma, UK) in Ca²⁺-free Barth's
258 solution: (mM) 96 NaCl, 2 KCl, 1 MgCl₂, 5.0 HEPES, 2.5 pyruvic acid and 100 IU ml⁻¹ / 100 µg ml⁻¹
259 streptomycin/penicillin for 60 minutes followed by six washes in Ca²⁺-free Barth's solution. It was then
260 transferred to Barth's GTP solution: (mM) 96 NaCl, 2.0 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 5.0 HEPES, 2.5 pyruvic
261 acid, 0.5 theophylline and 0.05 mg/ml of gentamycin pH 7.5 and incubated at 18°C. Stage IV and V
262 oocytes were defolliculated and co-injected with 10 ng of *TipE* and 10 ng of either *para 13-5k* or
263 *para13-5L* cRNA. Oocytes were incubated for 2 - 4 days at 18 °C in Barth's GTP solution.

264

265 Oocytes were moved to disposable 35 mm dishes containing 2 ml of recording solution (Barth's
266 solution without sodium pyruvate, theophylline and gentamycin). Solutions of deltamethrin in DMSO
267 were added directly to the recording solution to obtain a 1nM – 30 µM final concentration of

268 deltamethrin. The bath DMSO concentration did not exceed 1 % (v / v) which had no effect on the
269 *para* VGSC response (data not shown).

270

271 Voltage activated sodium currents were recorded by two-electrode voltage clamp using a Dagan CA-
272 1B high performance oocyte clamp amplifier (Dagan Instr., Minneapolis, MN, USA). Microelectrodes
273 were made from thin walled borosilicate glass capillaries (TW150F-4, World Precision Instruments,
274 UK) using a micropipette puller (model P-97, Sutter Instrument Company, USA), with a resistance of 1
275 -2 M Ω when filled with 0.7 M KCl and 1.7 M K⁺ citrate. Signals were recorded using Pulse and PulseFit
276 software running on a Windows PC coupled to a HEKA ITC-16 interface (Digitimer Ltd. Welwyn Garden
277 City UK) with a sampling frequency of 50 kHz. Leak and capacitance currents were subtracted using a
278 P / 5 protocol. All experiments were carried out at 21 - 23°C.

279

280 From a holding potential of -70 mV, voltage-dependence of activation was measured using 35 ms step
281 depolarisations, to the test potential from -65 mV to +45 mV in 5 mV increments at 1 s intervals. Peak
282 current (I_{peak}) was plotted against the test voltage and fitted with a modified Boltzmann equation. Data
283 were then converted to conductance and fitted with a modified Boltzmann equation (Eq. 1; Eq. 2;
284 Usherwood *et al* 2007).

285

286 Type II pyrethroids preferentially target the open channel thus modification by these pyrethroids can
287 be enhanced by application of conditioning pulses which open and close the channel (Vais *et al.*, 2000).
288 Tail currents which are an observable effect of pyrethroids, slowing channel inactivation and
289 deactivation processes (Vais *et al.*, 2001) were elicited using a standard protocol designed to visualise
290 modification of channel activity by pyrethroids. A train of 100 5ms conditioning pulses to 0 mV from
291 the holding potential of -70 mV with 10 ms intervals (sufficient time for recovery from open state
292 inactivation) was followed by a single 12 s repolarisation pulse to -110 mV. The amplitude of tail

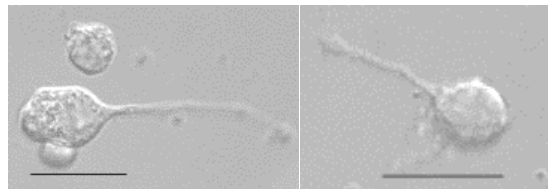
293 currents was used to establish the percentage of modified channels (M) using Eq 4, according to
294 Tatebayashi and Narahashi (1994).

295

296 3. Results

297

298 *3.1 Properties of isolated housefly neurons:* Housefly neurons were isolated from thoracic ganglia and
299 maintained in culture overnight. Cells were heterogeneous but those selected for whole cell patch-
300 clamp were typically 10 to 30 μm in diameter with a residual axonal stub 5 - 60 μm in length (Figure
301 2).



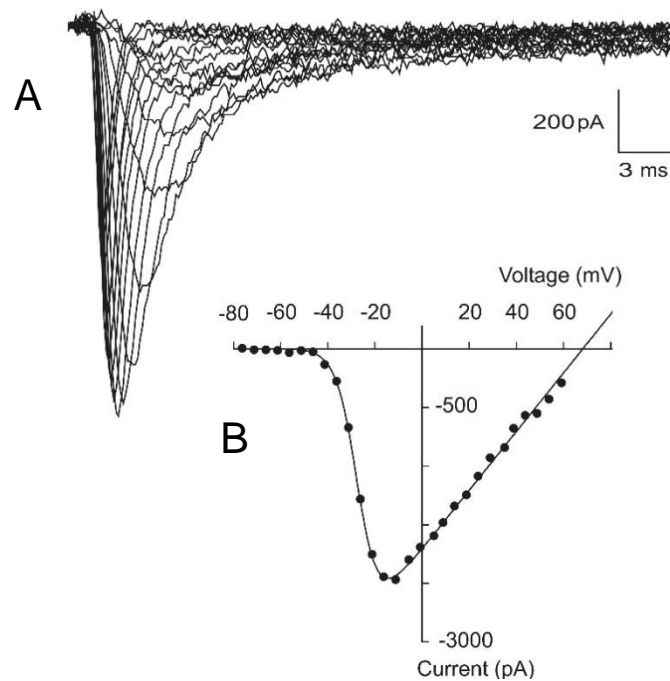
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304 **Figure 2. Phase-contrast micrographs.** Individual neuronal cell bodies isolated from the thoracic
305 ganglion of houseflies and attached to glass coverslips. Scale bar 50 μm .

306 Patch-clamped cells produced a variety of currents in response to depolarisation (from a holding
307 potential of -80mV) including outward currents with profiles typical of those carried by potassium
308 channels and inward currents with characteristics of calcium and sodium channels. In the presence
309 of TEA, 4-AP and CoCl_2 , isolated voltage-gated sodium currents were recorded (Figure 3) and could

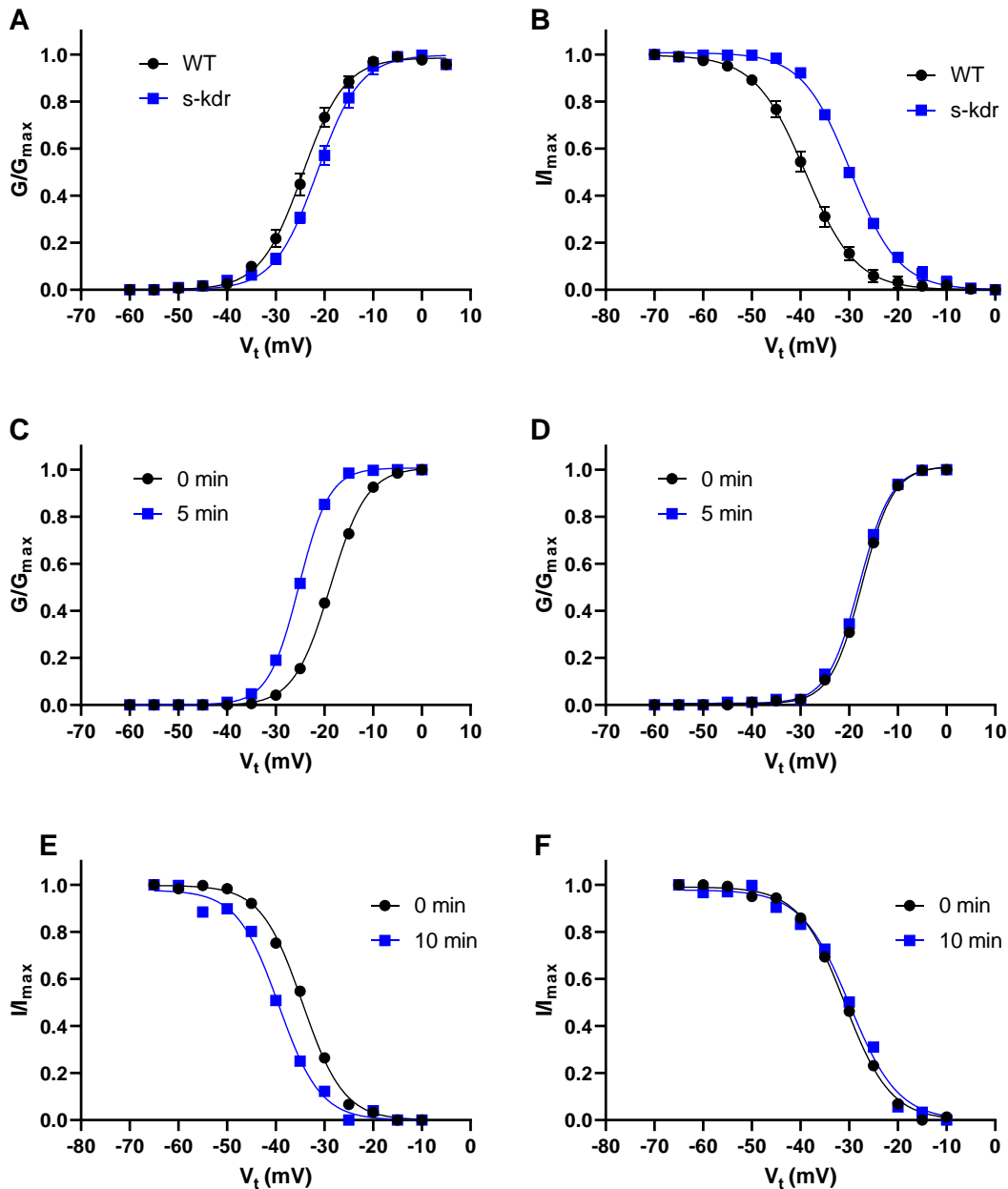
310 be blocked by 20nM TTX (data not shown) further characterising these currents as sodium currents.



311

312 **Figure 3. Voltage-activated currents in neurons isolated from thoracic ganglia of adult wild-type**
313 **houseflies.** A. Family of whole-cell currents recorded in the presence of 30mM tetraethylammonium
314 (TEA), 1mM 4-aminopyridine (4-AP) and 1mM CoCl₂, following depolarisation of an isolated cell from -
315 80mV to a range of potentials. B. Current/voltage relationship of the same neuron.

316 Comparative studies of voltage-gated sodium currents in neurons from *wild-type* and *s-kdr* houseflies
317 showed a significant depolarising shift of the activation curves (Figure 4A). Half-maximal activation in
318 neurons from *s-kdr* insects (-21.4 ± 0.2 mV, $n = 15$) showed a significant depolarising shift of 2.76 mV
319 when compared to the susceptible *wild-type* strain (-24.1 ± 0.2 mV, $n = 17$). Slope factors for the two
320 curves were indistinguishable ($k = 4.6 \pm 0.2$ mV, *wild-type*; 4.5 ± 0.2 mV, *s-kdr*). The voltage-
321 dependence of inactivation also differed between the strains (Figure 4B). Inactivation curves were
322 described by Eq3, giving $V_{0.5}$ values of -39.0 ± 0.1 mV (*wild-type*, $n = 17$), and -29.7 ± 0.2 mV (*s-kdr*, n
323 $= 15$), revealing that the inactivation curves were significantly depolarised in the resistant insects. The
324 slope factors of *wild-type* ($k = 5.2 \pm 0.1$ mV) and *s-kdr* ($k = 4.9 \pm 0.2$ mV) inactivation curves were
325 similar.



326

327 **Figure 4. Properties of susceptible (wild-type), and super-kdr VGSC currents in isolated thoracic**
 328 **neurons from houseflies. A-B:** Voltage dependence of activation (A) is expressed as normalised
 329 conductance following a 30ms depolarising step from -100mV to a range of potentials from -70 to
 330 +30mV. The V_{50} is significantly ($p < 0.0001$; extra sum of squares F-test) depolarised in the s-kdr strain.
 331 Steady-state inactivation (B) is expressed as the mean normalised peak current plotted against a range
 332 of pre-pulses from -120mV to +40mV, followed by a test depolarisation to the potential giving
 333 maximum peak sodium current for that cell. $V_{50inact}$ was significantly ($p < 0.0001$; extra sum of squares
 334 F-test) depolarised in the s-kdr strain. Wild-type $n = 17$ neurons; s-kdr, $n = 15$ neurons. C-F: 10nM
 335 deltamethrin shifted activation and steady-state inactivation of sodium currents in the hyperpolarising
 336 direction for susceptible (C and E) but much less so for s-kdr (D and F) sodium channels in isolated
 337 neurons. Currents were elicited as in A and B. Conductance/current values were plotted against the

338 *test/pre-pulse potential, n=3 neurons for susceptible and n=4 for pyrethroid resistant neurons. All plots*
339 *are fitted with a Boltzmann equation.*

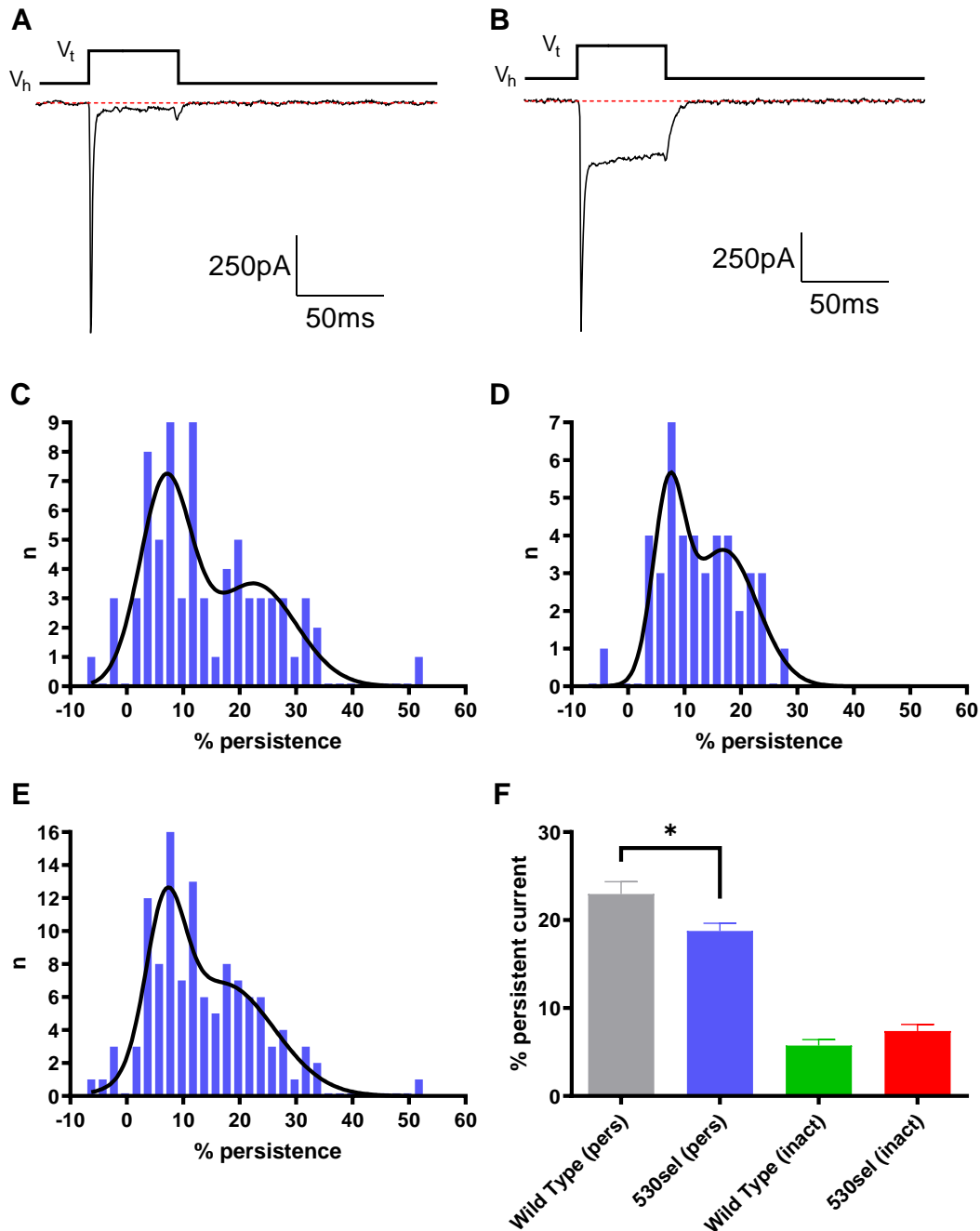
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341 VGSC currents observed in neurons of both housefly strains demonstrated diversity in terms of
342 amplitude, rate of onset and decay and the extent to which they inactivate during a 50-ms depolarising
343 pulse.

344

345 *3.2 Effect of deltamethrin on sodium currents in isolated housefly neurons:* The effects of deltamethrin
346 on sodium currents were examined in isolated neurons from susceptible and pyrethroid resistant (*s-*
347 *kdr*) housefly strains. Sodium currents from susceptible insects displayed a significant hyperpolarising
348 shift ($p < 0.0001$; extra sum of squares F-test) of 6.47 mV in V_{50act} in the presence of 10 nM deltamethrin
349 (Figure 4C) whereas a hyperpolarising shift of only 0.53 mV ($p = 0.0067$; extra sum of squares F-test)
350 in the current voltage relationship was seen in sodium currents from *s-kdr* flies (Figure 4D). The same
351 neurons were subsequently retested for the effect of deltamethrin on steady state inactivation
352 (recorded after 10 minutes of exposure) and again a significant ($p < 0.0001$; extra sum of squares F-
353 test) hyperpolarising shift of 4.86 mV in the voltage dependence of inactivation ($V_{50inact}$) was seen in
354 the susceptible but not the *s-kdr* flies (Figure 4E-F).

355



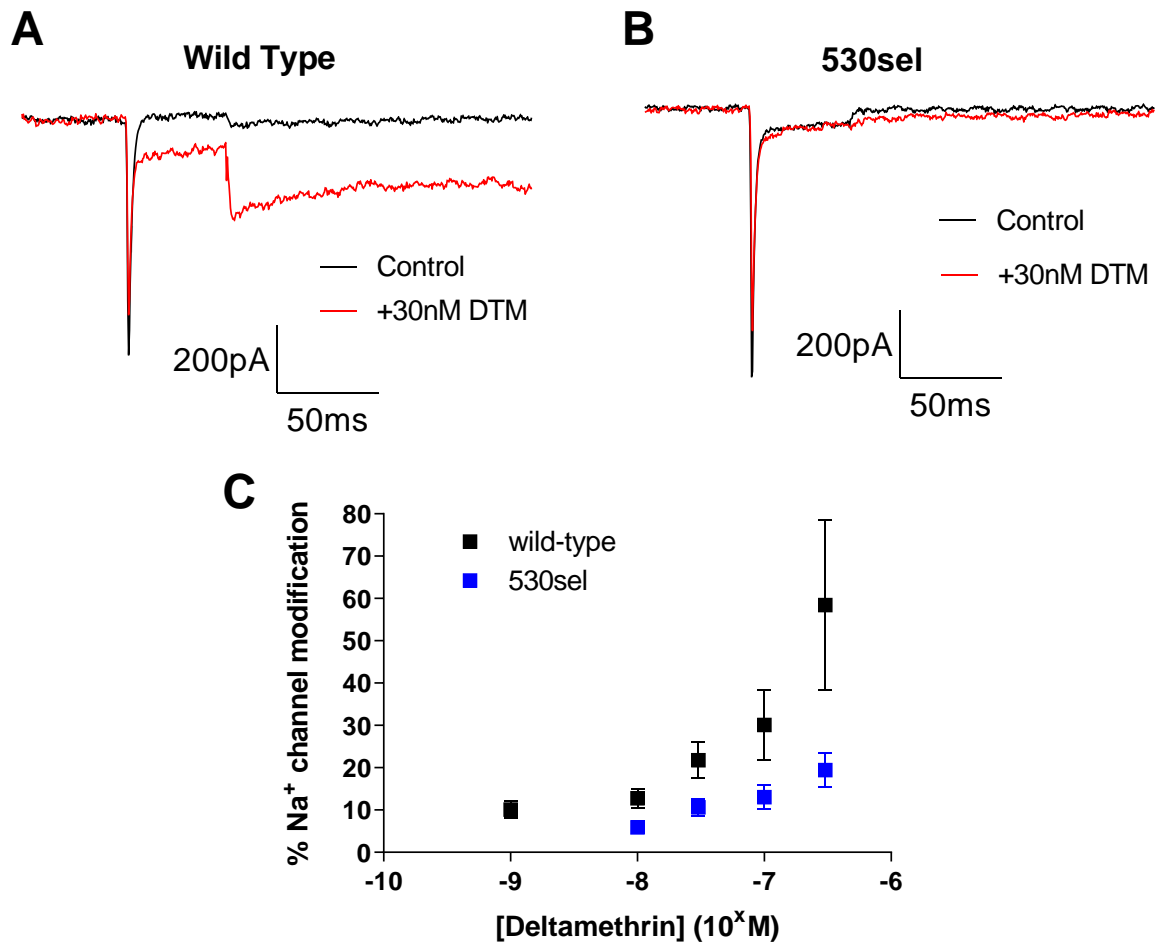
356

357 **Figure 5. Rapidly inactivating and persistent sodium currents in housefly thoracic neurons.** In a sub-
 358 population of isolated neurons a single 50ms depolarization to a V_t of -10mV from a V_h of -70mV evokes
 359 Na^+ currents which activate and inactivate rapidly (A) whereas in other cells the same depolarisation
 360 results in Na^+ currents which activate rapidly but do not fully inactivate resulting in a persistent current
 361 (B). Frequency distributions of the percentage persistence of Na^+ currents in housefly neurons for the
 362 wild-type strain (C), the 530sel strain (D) and the pooled data (E). The data were best fitted with a sum
 363 of two Gaussian distributions confirming the presence of two distinct current populations. Mean
 364 percentage persistence (F) showed that persistent currents in the wild-type strain had significantly
 365 greater percentage persistence than did their counterparts in the resistant (530sel) strain (* $p < 0.05$,
 366 unpaired t-test) whereas the inactivating currents were not significantly different.

367

368 During a 50ms depolarising pulse, Na⁺ currents in some cells activated and inactivated rapidly (Figure
369 5A); whereas, other cells produced a Na⁺ current that activated rapidly but did not fully inactivate
370 (Figure 5B) and as a result exhibited a persistent current component. Both types were observed in
371 neurons isolated from thoracic and head ganglia but data in fig 5 were from thoracic ganglia only. To
372 facilitate analysis of VGSC channel diversity in housefly neurons, Na⁺ currents were divided into
373 separate classes by calculating the amplitude of the inactivating current at the end of the step
374 depolarisation, relative to the peak current and expressing this as ‘% persistence’. Frequency
375 histograms of this, in both strains, were best fitted with a sum of two Gaussian distributions ($p < 0.05$,
376 F-test to compare fits of a single vs sum of two Gaussian distributions), confirming the existence of
377 two distinct current populations with mean persistence (\pm SD) of $6.8 \pm 3.4\%$ and $17.1 \pm 9.0\%$ (Figure
378 5C-E). The inflection point (12 % persistence) between the two fits was taken as the threshold for the
379 two populations and showed that the strongly inactivating currents predominated. Both types of
380 current were blocked by 20 nM TTX. It is also apparent that the “persistent” current in *wild-type*
381 neurons had significantly greater persistence than did that from *s-kdr* houseflies (Figure 5F). Peak
382 currents for neurons from the two strains of insect had almost identical mean amplitudes (mean \pm
383 SEM : *wild-type* 764.0 ± 42.2 nA, n= 76; *s-kdr* 763.4 ± 67.2 nA, n=43; $p=0.99$).

384 Na⁺ currents in neurons of the *wild-type* strain of the housefly were more sensitive to deltamethrin
385 than those in neurons of the resistant (*s-kdr*) strain as demonstrated by the larger tail currents seen
386 in *wild-type* channels when exposed to the same deltamethrin concentrations (Figure 6).



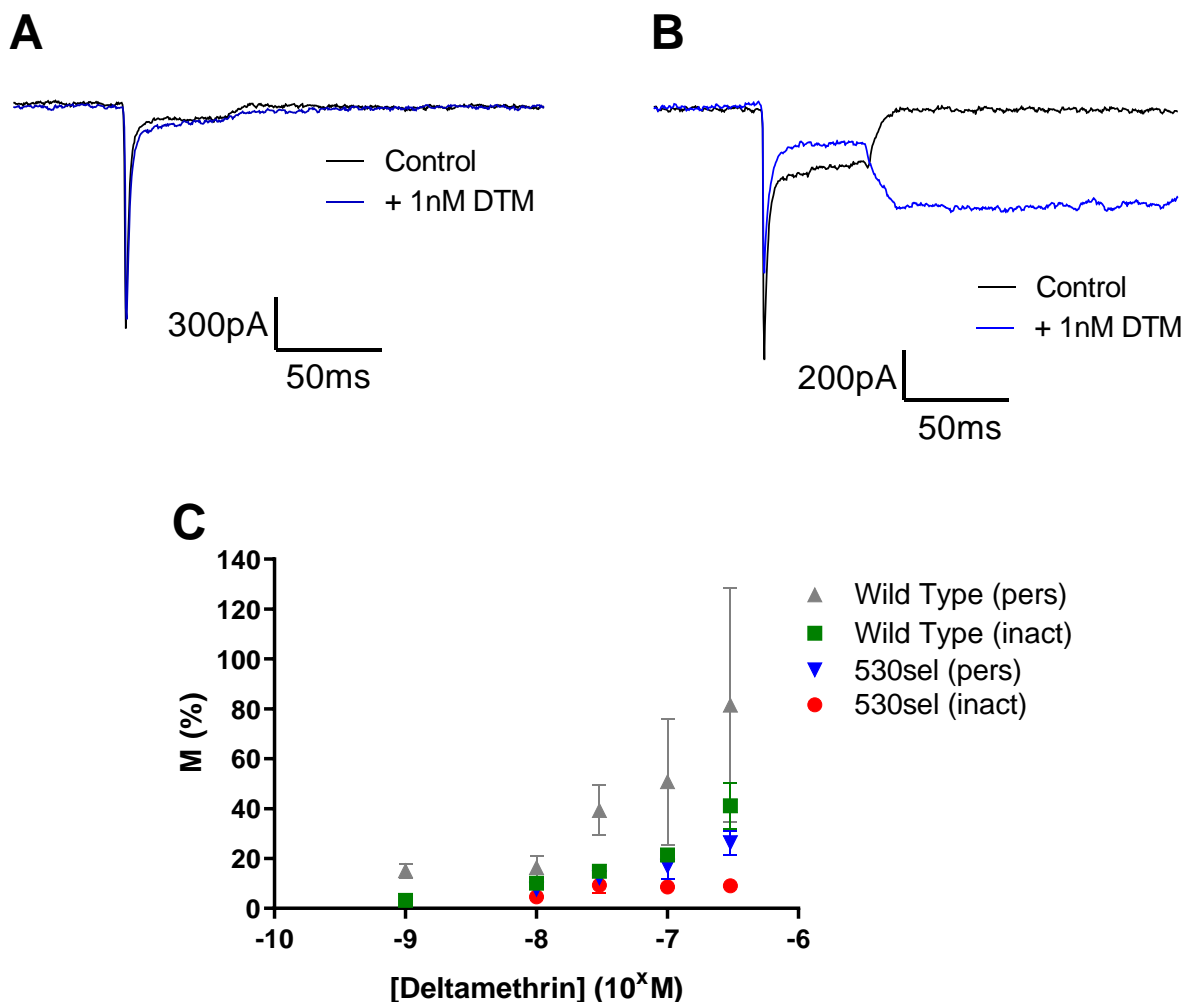
387
 388 **Figure 6. Tail currents in isolated housefly thoracic neurons. Inactivating Na⁺ currents in neurons of**
 389 **the wild-type strain (A) are more sensitive to deltamethrin than Na⁺ currents in the *s-kdr* (530sel) strain**
 390 **(B). In response to 30nM deltamethrin tail current amplitude is greater in the wild-type than the**
 391 **resistant strain (relative to control current). Whole-cell currents were generated in response to a single**
 392 **50ms depolarization to a V_t of -10mV from a V_h of -70mV (voltage protocol 4). Currents from single**
 393 **neurons are shown following application of deltamethrin for 5 minutes. C: Concentration-response**
 394 **relationship for deltamethrin-induced tail currents in central neurons from wild-type and *s-kdr* (530sel)**
 395 **housefly strains (includes both inactivating and persistent currents). Wild-type currents are modified**
 396 **more than resistant-type currents. M% was calculated according to Equation 4 and plotted against**
 397 **deltamethrin concentration.**

398
 399 Peak Na⁺ current and tail current amplitudes were used to calculate the percentage channel
 400 modification by various concentrations of deltamethrin (Equation 4) using the method of Tatebyashi
 401 and Narahashi (1994).

402 The limited aqueous solubility of deltamethrin results in problems in recording and analysing currents
 403 at concentrations in excess of 1μM, thus the concentration response curves do not reach an upper

404 plateau. It is therefore more meaningful to consider lower percentage modification e.g. 15% (EC_{15}). At
 405 this percentage modification, *s-kdr* houseflies have a lower sensitivity to deltamethrin with an EC_{15} of
 406 132.9nM, a 9.2-fold increase in the value for the *wild-type* of 14.5nM (figure 6C).

407 **3.3 Persistent and Inactivating sodium currents in housefly neurons have different sensitivities to**
 408 **deltamethrin:** Closer inspection of tail current data revealed that the persistent type Na^+ current is
 409 much more sensitive to deltamethrin than is the inactivating type current (Figure 7). For example,
 410 1nM deltamethrin has little or no effect on an inactivating type Na^+ current, but has a considerable
 411 impact on persistent type Na^+ currents in *wild-type* flies (Figure 7A). The greater susceptibility of
 412 persistent type currents was seen in both the *wild-type* (Figure 7B) and *s-kdr* strains, with both of the
 413 *wild-type* Na^+ currents showing greater susceptibility than either of the currents in the *s-kdr* strain
 414 (Figure 7C).
 415



416

417 **Figure 7. Persistent-type currents are modified by deltamethrin more than are inactivating-type**
 418 **currents.** **A-B:** Data from isolated housefly *thoracic* neurons of the *wild-type* strain. In response to 1nM
 419 deltamethrin tail current amplitude is greater for persistent currents (B) compared to inactivating
 420 currents (A), relative to control current. Whole-cell currents were generated in response to a single

421 50ms depolarization to a V_t of -10mV from a V_h of -70mV (voltage protocol 4). Currents from single
422 neurons are shown following application of deltamethrin for 5 minutes. **C**: Concentration-response
423 relationship for deltamethrin-induced tail currents in inactivating and persistent type currents in
424 central neurons from wild-type and *s-kdr* (530sel) housefly strains. Persistent type currents are
425 modified more than are inactivating type currents. $M\%$ was calculated according to Equation 4 and
426 plotted against deltamethrin concentration.

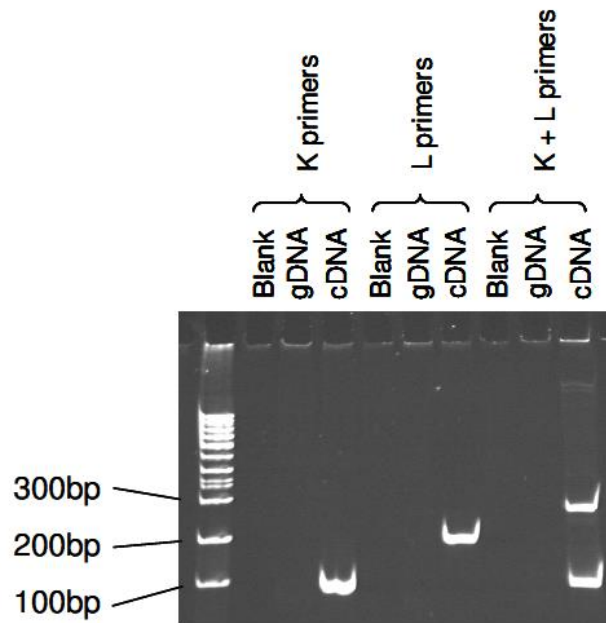
427

428 3.4 Housefly neurons contain splice variants that affect persistence of sodium currents in *Drosophila*

429 *para* sodium channels: In view of the importance of Na^+ current persistence on the sensitivity of
430 housefly neurons to pyrethroid insecticides the question arises as to what is the basis of the diversity
431 of sodium currents recorded in different neurons. One likely possibility is the variable expression of
432 different splice variants of the VGSC gene in different cells. In particular, it is known that both housefly
433 *Vssc1* and *Drosophila (para)* have a number of splice variants including two pairs of mutually exclusive
434 exons *c/d* and *k/l*. No functional significance has been attributed to the *c* exon in *Drosophila* (Lin *et al*
435 2009) and in houseflies it may give rise to a non-functional channel (Lee *et al* 2009). In this study we
436 have investigated the *k/l* mutually exclusive exons (Figure 1) which give rise to channels with
437 differences in current properties, in particular, the persistence of the current they gate, with the *k*
438 exon producing persistent currents of smaller amplitude (Lin *et al* 2009)

439

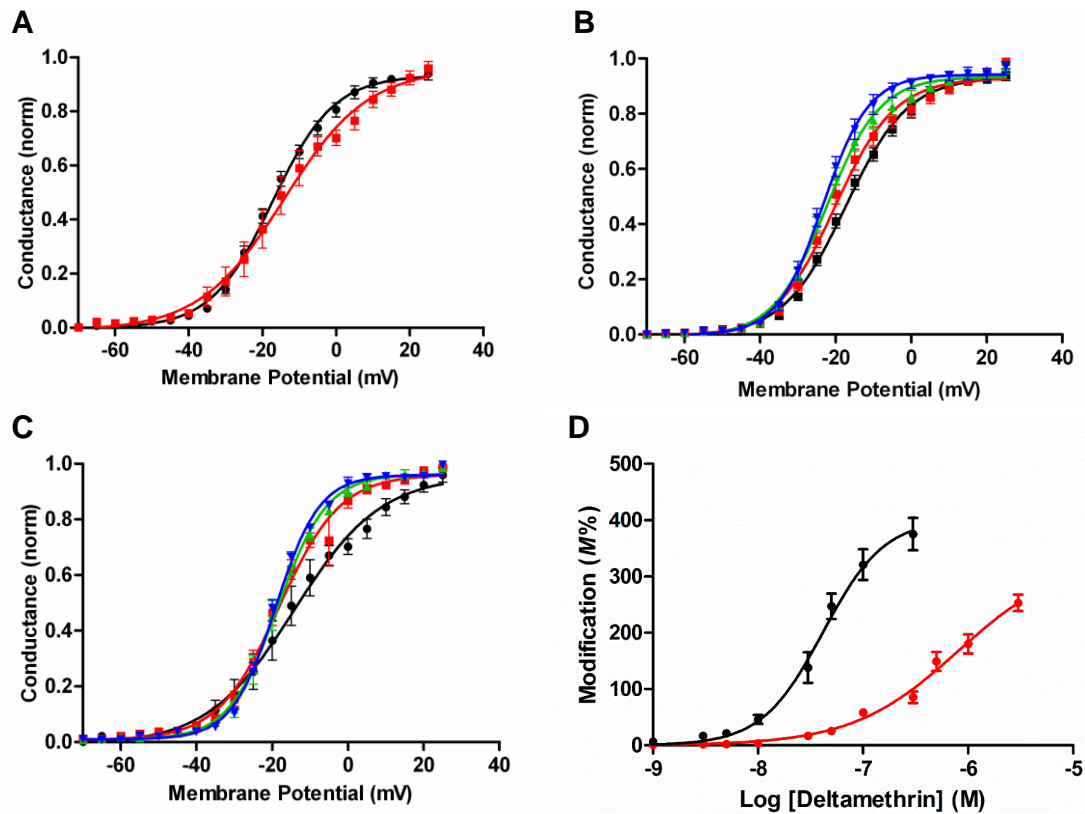
440 A multiplex PCR approach was adopted to investigate expression levels of transcripts containing either
441 of the mutually exclusive exons *k* and *l* in housefly heads where neurons were found to exhibit both
442 inactivating and persistent sodium currents (data not shown). Figure 8 shows the presence of both
443 exons.



444 **Figure 8. PCR amplification of Na⁺ channel-specific fragments from (wild-type) housefly cDNA**
 445 **confirms that transcripts containing both exon k and exon l are expressed.** The presence of an exon
 446 **k band but no exon l band in the multiplex cDNA lane (far right) indicates that expression levels are**
 447 **higher for Na⁺ channels containing exon k than those containing exon l.**
 448

449 *3.5 k and l exons confer different sensitivity to deltamethrin in expressed Drosophila para VGSCs:*
 450 *Drosophila para* channels expressed in *Xenopus* oocytes have been used to interpret resistance
 451 mutations identified in the field. However, in these studies the “wild-type *para* channel” has been the
 452 l exon variant. We tested the effects of deltamethrin on *Drosophila para* sodium channels using
 453 *Xenopus* oocytes injected with cRNA encoding either k (13-5K) or l (13-5L) exon (no other changes,
 454 including point mutations, are present between these two clones, see Lin et al., 2009). Activation
 455 kinetics of the two splice variants were compared and showed there was a significant 3 mV (P< 0.01;
 456 *t* test) depolarising shift of the V₅₀ for activation in the k splice variant (Figure 9A).

457



458

459 **Figure 9. Properties of 13-5L and 13-5K para splice variant VGSCs expressed in *Xenopus oocytes*.** **A:**
 460 Plots of normalized conductance against test depolarization for 13-5L and 13-5K. The voltage
 461 dependence of activation ($V_{50.act}$) for each was: ● 13-5L: $V_{50} = -17.32 \pm 0.4$ mV, $k = 8.4 \pm 0.4$, $n = 16$
 462 and ■ 13-5K: $V_{50} = -14.23 \pm 1.3$ mV, $k = 11.5 \pm 1.3$, $n = 7$. A two-tailed t -test comparison of the $V_{50.act}$
 463 showed a significant difference $P < 0.01$. **B-C:** Conductance-Voltage relationships for the 13-5L (B) and
 464 13-5K (C) para splice variants in the absence and presence of increasing concentrations of
 465 deltamethrin. **13-5L:** ■ No deltamethrin ($V_{50} = -17.32 \pm 0.4$ mV, $k = 8.3 \pm 0.4$, $n = 16$); ■ 1 nM
 466 deltamethrin ($V_{50} = -20.31 \pm 0.5$ mV, $k = 8.0 \pm 0.4$, $n = 11$); ▲ 5 nM deltamethrin ($V_{50} = -22.42 \pm 0.4$
 467 mV, $k = 7.1 \pm 0.4$, $n = 10$); ▼ 30 nM deltamethrin $V_{50} = -24.22 \pm 0.4$ mV, $k = 5.6 \pm 0.3$, $n = 7$. **13-5K:** ●
 468 No deltamethrin ($V_{50} = -14.23 \pm 1.3$ mV, $k = 11.5 \pm 1.3$, $n = 7$); ■ 1 nM deltamethrin ($V_{50} = -18.23 \pm 0.7$
 469 mV, $k = 8.1 \pm 0.6$, $n = 9$); ▲ 5 nM deltamethrin ($V_{50} = -18.51 \pm 0.4$ mV, $k = 6.5 \pm 0.4$, $n = 7$); ▼ 30 nM
 470 deltamethrin ($V_{50} = -19.10 \pm 0.3$ mV, $k = 5.8 \pm 0.2$, $n = 7$). **D.** Log [deltamethrin](M) – response curves
 471 were fitted by a four-parameter logistic equation establishing an EC_{50} value of 42 nM and a maximum
 472 modification value of 402 %, $n = 10$ for 13-5L, and an EC_{50} value of 866 nM and a maximum modification
 473 of 346 %, $n = 11$ for 13-5K.

474

475 The effect of a 5 minute application of deltamethrin on activation channel kinetics was investigated.

476 This showed that in the l splice variant, the $V_{50.act}$ was shifted by 7 mV in the hyperpolarising direction

477 to -24.22 mV in the presence of 30 nM deltamethrin ($P < 0.001$; t test) (Figure 9B) and for the k splice

478 variant there was an approximate 5 mV shift in $V_{50.act}$ to -19.10 mV (Figure 9C)

479 The sensitivity to deltamethrin was also investigated by determining the percentage of channel
480 modification by analysing the tail currents at various deltamethrin concentrations. This gave an EC₅₀
481 value of 42 nM for the 13-5L variant and a 20-fold larger (866 nM) value for the 13-5K splice variant
482 The upper plateau in the relationship for the k variant could not be fully established owing to the
483 insolubility of deltamethrin at higher concentrations.

484 These data are consistent with the data we have obtained from isolated housefly neurons where
485 pyrethroid sensitivity is closely associated with the biophysical properties and time course of the
486 whole cell sodium current in particular the amplitude of the persistent current component.

487 **4. Discussion**

488 We show here that neurons isolated from *wild-type* and *s-kdr* houseflies exhibit voltage-gated sodium
489 currents that may have a rapidly inactivating or more persistent time-course. We further show that
490 both in isolated housefly neurons and in *Xenopus* oocytes expressing *Drosophila melanogaster para*
491 VGSC splice variants, sodium currents with greater persistence are much more sensitive to the
492 pyrethroid deltamethrin. We present evidence that difference in voltage sensitivity of VGSC and
493 persistence of the sodium current may be factors in differences in pyrethroid sensitivity between
494 housefly strains. Isolated housefly neurons where VGSC are expressed in their native environment
495 provide a useful model for studying resistance mutations and can extend our knowledge of the
496 physiological effects of resistance mutations. For example sodium currents recorded from housefly
497 neurons showed no difference in peak amplitude between the *wild-type* and *s-kdr* strains, this is in
498 contrast to the results obtained for housefly channels expressed in *Xenopus* oocytes where the
499 channel carrying the *s-kdr* double mutant had significantly smaller peak currents (Lee S. *et al* 1999).

500 In the present study, neurons from *s-kdr* houseflies showed positive shifts in the voltage dependence
501 of activation and steady-state inactivation. Similar results have been reported previously for *Heliothis*
502 *virescens* neurons *in vitro* where a pyrethroid resistant strain exhibited sodium currents with

503 activation properties with positive voltage shifts (Lee, D. *et al* 1999). It is also consistent with previous
504 studies of the housefly VGSC (Vssc1) containing the L104F *kdr* mutation (Smith *et al* 1997) or the
505 L1014F and M918T *s-kdr* double mutation (Lee, S. *et al* 1999) expressed in *Xenopus* oocytes, although
506 Vais *et al* (2000b) showed a depolarising shift compared with *wild-type* with the L1014F (*kdr*)
507 mutation, but no significant change when the M918T (*s-kdr*) was also present. Usherwood *et al* (2007)
508 found that the M918T mutation, expressed in isolation in the *para* channel of *Drosophila*, also
509 produced a small but significant depolarisation of the mid-point activation voltage but had no
510 significant effect on steady-state inactivation.

511 It has been known for many years that pyrethroids have effects on activation and steady-state
512 inactivation of insect VGSCs, increasing excitability by shifting the voltage dependence in the
513 hyperpolarising direction (Narahashi 1996). Our data confirm that deltamethrin (10nM) shifts the
514 voltage dependence of activation and steady-state inactivation of housefly *wild-type* neuronal sodium
515 channels in a negative direction, whereas VGSCs from *s-kdr* flies are unaffected by the pyrethroid.
516 These findings are again consistent with the electrophysiological properties of housefly Vssc1 (Lee, S.
517 *et al* 1999) and *Drosophila para* (Burton *et al* 2011) expressed in oocytes. The data presented here
518 also show that the effects of deltamethrin on the voltage dependence of the housefly neuronal sodium
519 channels are abolished in neurons from *s-kdr* insects.

520 Pyrethroids have been shown to slow inactivation and deactivation of VGSCs leading to the
521 appearance of insecticide-induced tail-currents (Vijverberg *et al* 1982; Tatebayashi and Narahashi
522 1994) which serve as a measure of channel modification (Narahashi 1996; Vais *et al* 2000b). We have
523 demonstrated that tail currents can be recorded from isolated housefly neurons in the presence of
524 deltamethrin, which are comparable with the tail currents induced by pyrethroids in neurons from *H.*
525 *virescens* and also in *Drosophila para* channels (Vais *et al* 2000 a, b; Usherwood *et al* 2005), housefly
526 Vssc1 (Smith *et al* 1997) and *Blatella germanica* (Tan *et al* 2002), expressed in *Xenopus* oocytes.

527 Here, 1nM deltamethrin produced some modification of *wild-type* neuronal housefly sodium channels
528 which was similar to the sensitivity of *Drosophila para* channels, although it was necessary to use ATX
529 (which prevents sodium channel inactivation) to record tail currents in the latter study (Vais *et al*
530 2000b). *Wild-type* housefly channels expressed in oocytes were also modified by cismethrin, but this
531 was only seen at concentrations above 20nM (Smith *et al* 1998), which is similar to the threshold of
532 10nM for modification of sodium channels by permethrin seen in *H. virescens* neurons from a
533 susceptible strain (Lee D. *et al* 1999).

534 It is difficult to compare these studies quantitatively due to slight differences in methodology and the
535 pyrethroid being used, however they each provide a basis for comparing the *wild-type* channel with
536 that of a pyrethroid-resistant strain tested alongside it. In the present study there was a significant 9.2
537 -fold increase in the EC₁₅ value for channel modification from 14.5nM for the *wild-type* to 132.9 nM
538 deltamethrin for the sodium channels from *s-kdr* houseflies. This is smaller than the difference seen
539 in heterologously expressed sodium channels, where the *s-kdr* double mutation completely abolished
540 the pyrethroid sensitivity of the housefly channel, whilst for *Drosophila para* channels in the presence
541 of ATX the *s-kdr* mutant channel was 100 -fold less sensitive. The reduction in sensitivity in this study
542 is more reminiscent of the study by Lee D. *et al* (1999) on *H. virescens* channels where over a similar
543 range of pyrethroid modification (5% to 20%), neurons from the resistant strain showed a 21-fold
544 reduction in sensitivity to permethrin.

545 Further insight was gained into the relatively small impact of the *s-kdr* mutations on pyrethroid
546 resistance seen in the present study compared with others, by considering the heterogeneity of the
547 sodium currents recorded from different neurons. Sodium currents from both *wild-type* and *s-kdr*
548 neurons were characterised as “inactivating” or “persistent” and it is apparent that the persistent
549 current was much more sensitive to deltamethrin than the rapidly inactivating current (Figure 8). In
550 view of the correlation between the degree of persistence and the sensitivity to deltamethrin it is
551 worth noting that the degree of persistence varies between the two strains, with the resistant *s-kdr*

552 strain showing significantly reduced persistent current amplitude and raising the possibility that
553 reduced persistent currents may be one component of the resistance mechanism. **Alternatively the**
554 **mutation may itself be responsible for changing the kinetics of the channel from a persistent to an**
555 **inactivating mode.** The concentration of deltamethrin required to produce 15% modification in
556 inactivating and persistent channels of both strains can be compared with the values for the whole
557 populations. For the persistent currents, the EC₁₅ value is 6.6nM and 33.4nM (resistance factor 5.06 -
558 fold) whereas for the inactivating currents the EC₁₅ values are 35.8nM and 20µM (resistance factor
559 558 -fold) for the *wild-type* and *s-kdr* insects respectively.

560 The identification of two Na⁺ current types, inactivating and persistent, allows for more detailed
561 analysis of Na⁺ current properties between strains. Inactivating type currents are like the Na⁺ currents
562 seen in oocyte expression studies, with rapid activation followed by rapid and near complete
563 inactivation. By contrast, inactivating type Na⁺ currents in the resistant strain are highly resistant to
564 tail current generation which is in agreement with previous studies of the L1014F and M918T double
565 mutations in housefly Na⁺ channels (Lee S. *et al.* 1999) and other species. However, the non-
566 inactivating type currents characterised here have a major impact on the action of pyrethroids and
567 are therefore likely to strongly influence the sensitivity of the overall population of neurons and
568 therefore the sensitivity of the nervous system of susceptible and resistant insects to pyrethroids *in*
569 *vivo*.

570

571 The molecular basis for the finding of both inactivating and persistent sodium currents in housefly
572 neurons is unknown, however we have shown that both the k and I isoforms are present in housefly
573 head cDNA. It is known from studies expressing k and I isoforms of *Drosophila para* cRNA in *Xenopus*
574 oocytes that the k isoform leads to a reduced persistent current relative to the I isoform (Lin and
575 Baines 2015). It is also apparent that most of the studies investigating the effects of pyrethroids on
576 *para* expressed in oocytes used the I splice variant. This is in contrast to the heterologous expression

577 of the housefly cRNA in *Xenopus* oocytes where the published Vssc1 sequence (Lee S. *et al* 1999)
578 identifies the k isoform (Davies *et al* 2007b). This may help to explain the relatively low sensitivity of
579 the channel to pyrethroids and the complete abolition of the effect of pyrethroid when the *s-kdr*
580 (M918T) mutation was present in the study of Lee S. *et al* 1999. This interpretation is also consistent
581 with observations on expression of two isoforms of the VGSC from *B. germanica* (Tan *et al* 2002) where
582 G1 (BgNa_v1-1) has a splice variant which is equivalent to the I isoform of *para* and is 100x more
583 sensitive to deltamethrin than is the G2 isoform (BgNa_v2-1) which is the k variant (Davies *et al* 2007b).
584 A recent study has shown a similar difference in pyrethroid sensitivity in k/I splice variants of
585 *Nilaparvata lugens*, where the VGSCs containing the k variant were less sensitive to etofenprox,
586 permethrin and deltamethrin than the I isoform (Sun *et al* 2019). It can be speculated that pyrethroid
587 sensitivity of neurons significantly depends on which splice variant is predominantly expressed.

588 This hypothesis was further evaluated by assessing the effects of deltamethrin in *Xenopus* oocytes
589 expressing either the k or I isoform of *para*. It was apparent that the k splice variant was inherently
590 less excitable than the I isoform, with V₅₀ for both activation and steady state inactivation being shifted
591 to a significantly more positive value, as recently described by Sun *et al* (2019) for *N. lugens*, for two
592 out of three of the k variants tested. A depolarising shift of the activation voltage was also found for
593 BG Na_v1-1 compared with BG Na_v2-1 (Tan *et al* 2002) whereas V₅₀ for steady-state inactivation was
594 hyperpolarised. Tail currents as a direct indicator of pyrethroid sensitivity also differed substantially,
595 with the *para* channel containing the I exon being much more sensitive to deltamethrin than was the
596 k variant, which is consistent with the pattern observed for cockroach channels BG Na_v1-1 and BG
597 Na_v2-1 (Tan *et al* 2002) and *N. lugens*, (Sun *et al* 2019) expressed in *Xenopus* oocytes.

598 The results presented here show that there is considerable diversity in VGSC function between
599 individual housefly neurons in both *wild-type* and pyrethroid resistant insects and this can produce
600 major differences in neuronal sensitivity to pyrethroids. An important molecular basis for this diversity
601 is the expression of the mutually exclusive k and I exons through alternative splicing of the *para*
602 orthologous genes of insects (Dong 2007, Davies 2007a, b). In view of the parallels between the

603 greater sensitivity of the persistent neuronal currents to pyrethroids and greater sensitivity of the I
604 splice variant of *para* when heterologously expressed, it is tempting to identify the k/l site as crucial
605 for pyrethroid action. However, it is important to bear in mind that this is one of 29 possible splice
606 variants of *para*, many of which have been shown to have different physiological properties (Olson *et*
607 *al* 2008) and the pyrethroid sensitivity of the whole nervous system of an insect is likely to reflect a
608 combination of splice-variant channels.

609 Modelling studies of insect VGSCs (O'Reilly *et al* 2006; Usherwood *et al* 2007; Davies *et al* 2008; Du *et*
610 *al* 2013) have identified two putative binding sites for pyrethroids and DDT, which have been termed
611 PyrR1 and PyrR2 and are thought to involve the interfaces between domains II and III and between
612 domains I and II respectively (Dong *et al* 2014; Zhorov and Dong 2017). The k/l site is located in the S3
613 and S4 segments of Domain III of the VGSC but to date there have been no modelling studies of the
614 direct effect of k/l splice variants on pyrethroid binding. It is possible that there may be allosteric
615 effects such as those reported for the effect of a mutation (N1575Y) on the topology of the PyrR2 site
616 (Wang *et al*, 2015). Molecular modelling and functional studies have established that S3 and S4
617 segments are critical for coupling depolarisation to channel activation and inactivation (Catterall,
618 2010; Shen *et al* 2017) which provides an explanation for the changes in voltage sensitivity and
619 persistence of sodium current between the k/l splice variants discussed above.

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627

628 **5. References**

- 629 Amey, J.S., O'Reilly, A. O., Burton, M. J., Puinean, A. M., Mellor, I.R., Duce, I. R., Field, L. M., Wallace,
630 B. A., Williamson, M. S., Davies, T. G.E. 2015. An evolutionarily-unique heterodimeric voltage-gated
631 cation channel found in aphids *FEBS let.* 589, 598-607.
- 632 Burton, M. J., Mellor, I. R., Duce, I. R., Davies, T. G. E., Field, L. M., Williamson, M. S. 2011. Differential
633 resistance of insect sodium channels with *kdr* mutations to deltamethrin, permethrin and DDT. *Insect*
634 *Biochem. Mol. Biol.* 41, 723-732.
- 635 Busvine, J. R., 1951. Mechanism of resistance to insecticide in houseflies. *Nature.* 168,193–195.
- 636 Byerly, L., Leung, H.T. 1988. Ionic currents of *Drosophila* neurons in embryonic cultures. *J. Neurosci.*
637 8, 4379-4393.
- 638 Catterall, W.A., Goldin, A.L., Waxman, S.G. 2005. International Union of Pharmacology. XLVII.
639 Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol.*
640 *Rev.* 57, 397–409.
- 641 Chen, M., Du Y., Nomura, Y., Zhu, G., Zhorov, B.S., Dong, K. 2017. Mutations of two acidic residues at
642 the cytoplasmic end of segment III S6 of an insect sodium channel have distinct effects on pyrethroid
643 resistance. *Insect Biochem. Mol. Biol.* 82, 1-10.
- 644 Davies, T. G. E., Field, L. M., Usherwood, P. N. R., Williamson, M. S. 2007a. DDT, pyrethrins, pyrethroids
645 and insect sodium channels. *Life* 59, 151 – 162.
- 646 Davies, T. G. E., Field, L. M., Usherwood, P. N. R., Williamson, M.S. 2007b. A comparative study of
647 voltage-gated sodium channels in the Insecta: implications for pyrethroid resistance in Anopheline
648 and other Neopteran species. *Insect Mol. Biol.* 16, 361–375.
- 649 Davies, T. G. E., O'Reilly, A. O., Field, L. M., BA Wallace, B. A., Williamson, M.S. 2008. Knockdown
650 resistance to DDT and pyrethroids: from target-site mutations to molecular modelling. *Pest Manag.*
651 *Sci.* 64, 1126–1130.

652 Defaix, A., Lapied, B. 2005. Role of a novel maintained low-voltage-activated inward current
653 permeable to sodium and calcium in pacemaking of insect neurosecretory neurons. *Invert. Neurosci.*
654 5, 135-46.

655 Dong, K. 1997a. A single amino acid change in the para sodium channel protein is associated with
656 knockdown-resistance (kdr) to pyrethroid insecticides in German cockroach. *Insect Biochem. Mol.*
657 *Biol.* 27, 93-100.

658 Dong, K. 2007. Insect sodium channels and insecticide resistance. *Invert. Neurosci.* 7, 17–30.

659 Dong, K., Scott, J. G. 1994 Linkage of kdr-type resistance and the para-homologous sodium-channel
660 gene in German cockroaches (*Blattella germanica*). *Insect Biochem. Mol. Biol.* 24, 647 – 654.

661 Dong, K., Du, Y., Rinkevich, F., Nomura, Y., Xu, P., Wang, L., Silver, K., Zhorov, B.S. 2014. Molecular
662 biology of insect sodium channels and pyrethroid resistance. *Insect Biochem. Mol. Biol.* 50, 1-17.

663 Du, Y., Liu, Z., Nomura, Y., Khambay, B., Dong, K., 2006 An alanine in segment 3 of domain III (IIIS3) of
664 the cockroach sodium channel contributes to the low pyrethroid sensitivity of an alternative splice
665 variant. *Insect Biochem. Mol. Biol.* 36, 161-168.

666 Du, Y., Nomura, Y., Boris S. Zhorov, B.S., Dong, K. 2016. Sodium channel mutations and pyrethroid
667 resistance in *Aedes aegypti*. *Insects.* 60, 2-11.

668 Feng, G., Deak, P., Chopra, M., Hall, L. M. 1995. Cloning and functional analysis of TipE, a novel
669 membrane protein that enhances *Drosophila* para sodium channel function. *Cell.* 82, 1001-11.

670 Field, L.M., Davies, T. G. E., O'Reilly, A. O., Williamson, M.S., Wallace, B. A. 2017. Voltage-gated sodium
671 channels as targets for pyrethroid insecticides. *Eur. Biophys. J.* 46, 675-679.

672 Foster, S.P., Young, S., Williamson, M.S., Duce, I., Denholm, I., Devine, G. J. 2003. Analogous pleiotropic
673 effects of insecticide resistance genotypes in peach-potato aphids and houseflies. *Heredity* 91, 98-
674 106.

675 Goldin, A. L 2001. Resurgence of sodium channel research. *Annu. Rev. Physiol.* 63, 871–94

676 Grolleau, F., Lapied, B. 2000 Dorsal unpaired median neurones in the insect central nervous system:
677 towards a better understanding of the ionic mechanisms underlying spontaneous electrical activity. J.
678 Exp. Biol. 203, 1633-1648.

679 Ingles, P. J., Adams, P. M., Knipple, D. C., Soderlund, D. M. 1996. Characterization of voltage-sensitive
680 sodium channel gene coding sequences from insecticide-susceptible and knockdown resistant house
681 fly strains. Insect Biochem. Mol. Biol. 26, 319–326.

682 Knipple, D. C., Doyle, K. E., Marsella-Herrick, P. A., Soderlund, D. M., 1994. Tight genetic linkage
683 between the kdr insecticide resistance trait and a voltage-sensitive sodium channel gene in the house
684 fly. Proc. Natl. Acad. Sci. USA 91, 2483–2487.

685 Lapied, B., Stankiewicz, M., Grolleau, F., Rochat, H., Zlotkin, E., Pelhate, M. 1999 Biophysical properties
686 of scorpion alpha-toxin-sensitive background sodium channel contributing to the pacemaker activity
687 in insect neurosecretory cells (DUM neurons). Eur. J. Neurosci. 11, 1449-1460.

688 Le Corronc, H., Hue, B., Pitman, R. M. 1999. Ionic mechanisms underlying depolarizing responses of an
689 identified insect motor neuron to short periods of hypoxia. J. Neurophysiol. 81, 307-318.

690 Lee, D., Park, Y., Brown, T.M., Adams, M.E. 1999. Altered properties of neuronal sodium channels
691 associated with genetic resistance to pyrethroids. Mol. Pharmacol. 55, 584–593.

692 Lee, S. H. Smith, T. J., Knipple, D. C., Soderlund, D. M. 1999. Mutations in the house fly Vssc1 sodium
693 channel gene associated with super-kdr resistance abolish the pyrethroid sensitivity of Vssc1/tipE
694 sodium channels expressed in *Xenopus* oocytes. Insect Biochem. Mol. Biol. 29, 185-194

695 Lee, S. H., Smith, T. J., Ingles, P. J., Soderlund, D. M. 2000. Cloning and functional characterization of a
696 putative sodium channel auxiliary subunit gene from the house fly (*Musca domestica*). Insect Biochem.
697 Mol. Biol. 30, 479-487.

698 Lee, S. H., Smith, T. J., Ingles, P. J., Knipple, D. C., Soderlund, D. M. 2002. Developmental regulation of
699 alternative exon usage in the house fly Vssc1 sodium channel gene. Invert. Neurosci. 4, 125-133.

700 Lin, W. H., Wright, D. E., Muraro, N. I., Baines, R. A. 2009. Alternative splicing in the voltage-gated
701 sodium channel DmNav regulates activation, inactivation, and persistent current. *J. Neurophysiol.* 102,
702 1994-2006.

703 Lin, W. H., Baines, R.A. 2015. Regulation of membrane excitability: a convergence on voltage-gated
704 sodium conductance. *Mol. Neurobiol.* 51, 57–67.

705 Loughney, K., Kreber, R., Ganetzky, B. 1989. Molecular analysis of the para locus, a sodium channel
706 gene in *Drosophila*. *Cell.* 58, 1143-1154.

707 Milani R. 1954. Comportamento mendeliano della resistenza alla azione abbattante del DDT:
708 correlazione tra abbattimento e mortalità in *Musca domestica* L. *Riv. Parassitol.* 15, 513–542.

709 Miyazaki, M., Ohyama, K., Dunlap, D. Y., Matsumura, F. 1996. Cloning and sequencing of the para-type
710 sodium channel gene from susceptible and kdr-resistant German cockroaches (*Blattella germanica*)
711 and house fly (*Musca domestica*). *Mol. Gen. Genet.* 252, 61–68.

712 Narahashi, T. 1996. Neuronal ion channels as the target sites of insecticides. *Pharmacol. Toxicol.* 18, 1-
713 14.

714 O'Dowd, D.K., Gee, J. R., Smith, M. A. 1995. Sodium current density correlates with expression of
715 specific alternatively spliced sodium channel mRNAs in single neurons. *J. Neurosci.* 15, 4005-4012.

716 Olson, R. O., Liu, Z., Nomura, Y., Song, W., Dong, K. 2008. Molecular and functional characterization of
717 voltage-gated sodium channel variants from *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 38,
718 604-10.

719 Rinkevich, F.D., Du, Y., Dong, K. 2013. Diversity and convergence of sodium channel mutations
720 involved in resistance to pyrethroids. *Pestic. Biochem. Physiol.* 106, 93-100

721 Saito, M., Wu, C. F. 1991. Expression of ion channels and mutational effects in giant *Drosophila*
722 neurons differentiated from cell division-arrested embryonic neuroblasts. *J. Neurosci.* 11,
723 2135-2150.

724 Saito, M., Wu, C. F. 1993. Ionic channels in cultured *Drosophila* neurons. *EXS.* 63, 366-389.

725 Sawicki, R. M. 1978. Unusual response of DDT-resistant houseflies to carbinol analogues of DDT.
726 *Nature.* 275, 443-444.

727 Schäfer, S., Rosenboom, H., Menzel, R. 1994. Ionic currents of Kenyon cells from the mushroom body
728 of the honeybee. *J. Neurosci.* 14, 4600-4612

729 Shen, H., Zhou, Q., Pan, X., Li, Z., Wu, J., Yan, N. 2017. Structure of a eukaryotic voltage-gated sodium
730 channel at near-atomic resolution. *Science.* 355 (6328), eaal4326.

731 Smith, L. B., Kasai, S., Jeffrey G. Scott, J.G. 2016. Pyrethroid resistance in *Aedes aegypti* and *Aedes*
732 *albopictus*: Important mosquito vectors of human diseases. *Pesticide Biochem. Physiol.* 133, 1–12.

733 Smith, T. J., Lee, S. H., Ingles, P. J., Knipple, D. C., Soderlund, D. M. 1997. The L1014F point mutation in
734 the house fly *Vssc1* sodium channel confers knockdown resistance to pyrethroids. *Insect Biochem.*
735 *Mol. Biol.* 27, 807–812.

736 Smith, T.J., Ingles, P.J., Soderlund, D. M. 1998. Actions of the pyrethroid insecticides cismethrin and
737 cypermethrin on house fly *Vssc1* sodium channels expressed in *Xenopus* oocytes. *Arch. Insect*
738 *Biochem. Physiol.* 38(3), 126-136.

739 Soderlund, D. M. 2008. Pyrethroids, knockdown resistance and sodium channels. *Pest Manag. Sci.* 64,
740 610–616.

741 Song, W., Liu, Z., Tan, J., Nomura, Y., Dong, K. 2004. RNA editing generates tissue-specific sodium
742 channels with distinct gating properties. *J. Biol. Chem.* 279, 32554-32561.

743 Sun, H., Du, Y., Liu, Z., Dong, K. 2019 Distinct functional properties of sodium channel variants are
744 associated with usage of alternative exons in *Nilaparvata lugens*. *Insect Biochem. Mol. Biol.* 118,
745 103292.

746 Tan, J., Liu, Z., Nomura, Y., Goldin, A. L., Dong, K. 2002. Alternative splicing of an insect sodium channel
747 gene generates pharmacologically distinct sodium channels. *J. Neurosci.* 22, 5300-5309.

748 Tan, J., Liu, Z., Wang, R., Huang, Z. Y., Chen, A. C., Gurevitz, M., Dong, K. 2005. Identification of amino
749 acid residues in the insect sodium channel critical for pyrethroid binding. *Mol. Pharmacol.* 67, 513-
750 522.

751 Tatebayashi, H., Narahashi, T. 1994. Differential mechanism of action of the pyrethroid tetramethrin
752 on tetrodotoxin-sensitive and tetrodotoxin resistant sodium channels. *J Pharmacol Exp Ther*, 270, 595-
753 603.

754 Taylor, M. F. J., Heckel, D. G., Brown, T. M., Kreitman, M. E., Black, B. 1993 Linkage of pyrethroid
755 insecticide resistance to a sodium-channel locus in the tobacco budworm. *Insect Biochem. Mol. Biol.*
756 23, 763 – 775.

757 Usherwood, P.N., Vais, H., Khambay, B. P., Davies, T. G., Williamson, M. S. 2005. Sensitivity of the
758 *Drosophila para* sodium channel to DDT is not lowered by the super-kdr mutation M918T on the IIS4-
759 S5 linker that profoundly reduces sensitivity to permethrin and deltamethrin. *FEBS Lett.* 579, 6317-
760 6325.

761 Usherwood, P. N., Davies, T. G., Mellor, I. R., O'Reilly, A. O., Peng, F., Vais, H., Khambay, B. P., Field, L.
762 M., Williamson, M. S. 2007. Mutations in DIIS5 and the DIIS4-S5 linker of *Drosophila melanogaster*
763 sodium channel define binding domains for pyrethroids and DDT. *FEBS Lett.* 581, 5485-5492.

764 Vais, H., Williamson, M. S., Hick, C. A., Eldursi, N., Devonshire, A. L., Usherwood, P. N. 1997. Functional
765 analysis of a rat sodium channel carrying a mutation for insect knock-down resistance (kdr) to
766 pyrethroids. *FEBS Lett.* 413, 327-332.

767 Vais, H., Atkinson, S., Eldursi, N., Devonshire, A. L., Williamson, M. S., Usherwood, P.N 2000a. A single
768 amino acid change makes a rat neuronal sodium channel highly sensitive to pyrethroid insecticides.
769 *FEBS Lett.* 470, 135-138.

770 Vais, H., Williamson, M. S., Goodson, S. J., Devonshire, A. L., Warmke, J. W., Usherwood, P. N., Cohen,
771 C. J. 2000b Activation of *Drosophila* sodium channels promotes modification by deltamethrin.
772 Reductions in affinity caused by knock-down resistance mutations. *J. Gen. Physiol.* 115, 305-318.

773 Vais, H., Williamson, M. S., Devonshire, A. L., Usherwood, P. N. 2001 The molecular interactions of
774 pyrethroid insecticides with insect and mammalian sodium channels. *Pest Manag. Sci.* 57, 877-888.

775 Vijverberg, H. P., van der Zalm, J. M., van der Bercken, J. 1982. Similar mode of action of pyrethroids
776 and DDT on sodium channel gating in myelinated nerves. *Nature.* 295(5850), 601-603.

777 Wang, R., Huang, Z. Y., Dong, K. 2003. Molecular characterization of an arachnid sodium channel gene
778 from the varroa mite (*Varroa destructor*). *Insect Biochem. Mol. Biol.* 33, 733-739.

779 Wang, L., Nomura, Y., Du, Y., Dong, K. 2013. Differential effects of TipE and a TipE-homologous protein
780 on modulation of gating properties of sodium channels from *Drosophila melanogaster*. *PLoS One.* 8,
781 1-11.

782 Wang, L., Du, Y., Nomura, Y., Dong, K. 2015a. Distinct modulating effects of TipE-homologs 2-4 on
783 *Drosophila* sodium channel splice variants. *Insect Biochem. Mol. Biol.* 60, 24-32.

784 Wang, L., Nomura, Y., Du, Y., Liu, N., Zhorov, B.S., Dong, K. 2015b. A mutation in the intracellular loop
785 III/IV of mosquito sodium channel synergizes the effect of mutations in helix IIS6 on pyrethroid
786 resistance. *Mol. Pharmacol.* 87, 421-429.

787 Warmke, J. W., Reenan, R. A., Wang, P., Qian, S., Arena, J. P., Wang, J., Wunderler, D., Liu, K.,
788 Kaczorowski, G. J., Van Der Ploeg, L. H., Ganetzky, B., Cohen, C. J. 1997. Functional expression of
789 *Drosophila* para sodium channels. Modulation by the membrane protein TipE and toxin pharmacology.
790 *J. Gen. Physiol.* 110, 119-133.

791 Wicher, D., Walther, C., Wicher, C. 2001. Non-synaptic ion channels in insects: basic properties of
792 currents and their modulation in neurons and skeletal muscles. *Prog. Neurobiol.* 64, 431-525.

793 Williamson, M. S., Denholm, I., Bell, C. A., Devonshire, A. L. 1993. Knockdown resistance (kdr) to DDT
794 and pyrethroid insecticides maps to a sodium channel gene locus in the housefly (*Musca domestica*).
795 Mol. Gen. Genet. 2401, 17-22.

796 Williamson, M. S., Martinez-Torres, D., Hick, C. A., Devonshire, A. L. 1996. Identification of mutations
797 in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to
798 pyrethroid insecticides. Mol. Gen. Genet. 252, 51 – 60.

799 Wu, S., Nomura, Y., Du, Y., Zhorov, B. S., Dong, K. 2017. Molecular basis of selective resistance of the
800 bumblebee BiNav1 sodium channel to tau-fluvalinate. Proc. Natl. Acad. Sci. U.S.A. 114 (491), 12922–
801 12927.

802 Zhao, X., Ikeda, T., Salgado, V.L., Yeh, J.Z., Narahashi, T. 2005. Block of two subtypes of sodium
803 channels in cockroach neurons by Indoxacarb insecticides. Neurotoxicol. 26, 455–465.

804 Zhorov, B. S., Dong, K. 2017. Elucidation of pyrethroid and DDT receptor sites in the voltage-gated
805 sodium channel. Neurotoxicol. 60, 171–177.

806 Zuo, Y., Peng, X., Wang, K., Lin, F., Li, Y., Chen, M. 2016. Expression patterns, mutation detection and
807 RNA interference of *Rhopalosiphum padi* voltage-gated sodium channel genes. Sci. Rep. 6, 30166.