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

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

Abstract

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

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

1. Introduction

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

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

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

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

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

In mammals and other vertebrates, the VGSCs are known to belong to a superfamily of voltage-gated ion channels where the pore forming subunits (α-subunits) comprise a family of proteins encoded by 10 genes of which 9 have been functionally expressed (Goldin 2001; Catterall *et al* 2005). In contrast insect genomes appear to contain only one gene encoding a VGSC, the *para* gene, first identified in *Drosophila* (Loughney *et al* 1998), which has a high sequence similarity to mammalian VGSCs. *Para* orthologous genes have also been identified in a number of arthropods including several insect, tick and mite species which are economically or medically important (Davies *et al* 2007a, b; Dong 2007). Recent genomic studies have identified heterodimeric VGSC in aphids (Amey *et al* 2015; Zuo *et al* 2016) which appear to have functional similarities to other VGSC, but have a different ion selectivity filter from *para* and VGSC in other taxa. Extensive diversity of physiological function in the VGSC is seen in both vertebrates and arthropods, and whereas part of the variability in vertebrate signalling

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

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

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

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

2. Materials and methods

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

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

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149 *2.2 Short Term Culture of Isolated Neurons:* Adult houseflies were anaesthetised with CO_2 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 Ca^{2+}/Mg^{2+}-free Rinaldini's saline (in mM; 135 NaCl, 2.5 KCl, 0.4 NaH_2PO4, 1.25 $NaHCO_3$,
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 Ca^{2+} / Mg^{2+}-free Rinaldini's saline for 1 hour at room temperature. Ganglia were
156 washed several times with Ca^{2+}/Mg^{2+}-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} streptomyocin). 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 $CaCl_2$, 4 $NaHCO_3$, 1.0 $MgCl_2$, 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 $MgCl_2$, 0.1 $CaCl_2$, 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

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

Sodium currents were isolated by adding channel blockers to the bath solution; K^+ channel blockers were 30 mM tetraethylammonium chloride (TEA) and 1.0 mM 4-aminopyridine (4-AP) (Sigma); the $Ca2^+$ channel blocker was 1.0 mM $CoCl_2$ and the Na^+ channel-blocker was tetrodotoxin (Sigma) 20 – 60 nM).

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

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

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

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

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

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

where G is conductance (Lin and Baines 2015).

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

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

where the parameters are as for Eq 1 and 2.

Tail currents were investigated by a single 50ms pulse to -10mV from a holding potential of -70mV. The length of recording at the holding potential after repolarisation was extended to 100s to allow for visualisation and measurement of tail currents.

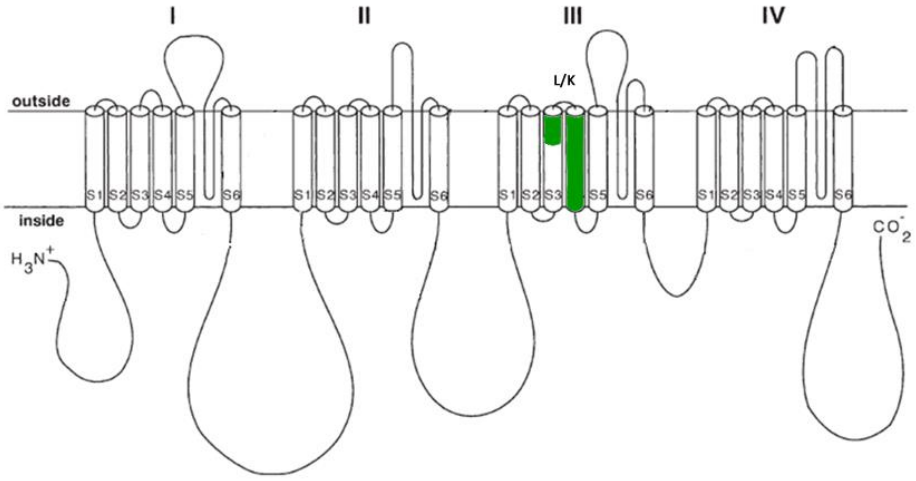
The percentage channel modification was calculated using:

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

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

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

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



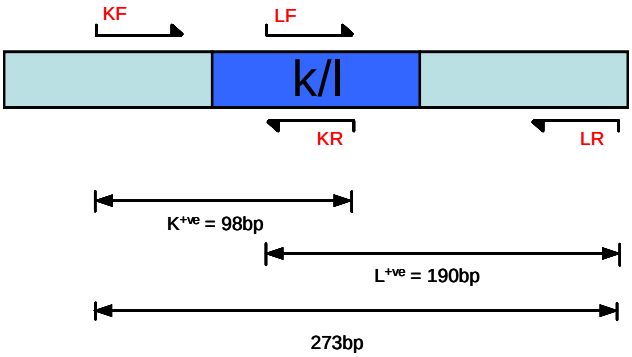


Figure 1. A*: Diagram of the housefly and Drosophila para VGSC with the location of the k/l splice variants indicated****. B****. Two primers in the exons flanking k/l coupled with one k-specific and one l-specific primer were designed such that the sizes of the fragments produced were indicative of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

2.6 Electophysiological properties of Drosophila VGSC splice variants: DNA constructs of *Drosophila* VGSC clone *DmNav10*/pGH19 (*para13-5*) (Warmke *et al* 1997) were expressed as mutually exclusive k (13-5k) and l (13-5L) exons, as described in Lin *et al* (2009).

Xenopus laevis ovarian tissue was obtained from the Biomolecular Science Unit of the University of Portsmouth and dissociated in 0.2 mg ml^{-1} type 1A collagenase enzyme (Sigma, UK) in Ca^{2+}-free Barth's solution: (mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 5.0 HEPES, 2.5 pyruvic acid and 100 IU ml^{-1} / 100 µg ml^{-1} streptomycin/penicillin for 60 minutes followed by six washes in Ca^{2+}-free Barth's solution. It was then transferred to Barth's GTP solution: (mM) 96 NaCl, 2.0 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.0 HEPES, 2.5 pyruvic acid, 0.5 theophylline and 0.05 mg/ml of gentamycin pH 7.5 and incubated at 18˚C. Stage IV and V oocytes were defolliculated and co-injected with 10 ng of *TipE* and 10 ng of either *para 13-5k* or *para13-5L* cRNA. Oocytes were incubated for 2 - 4 days at 18 °C in Barth's GTP solution.

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

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

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

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

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

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

3. Results

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

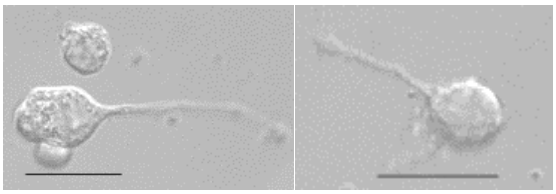


Figure 2. Phase-contrast micrographs. *Individual neuronal cell bodies isolated from the thoracic ganglion of houseflies and attached to glass coverslips. Scale bar 50µm.*

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

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

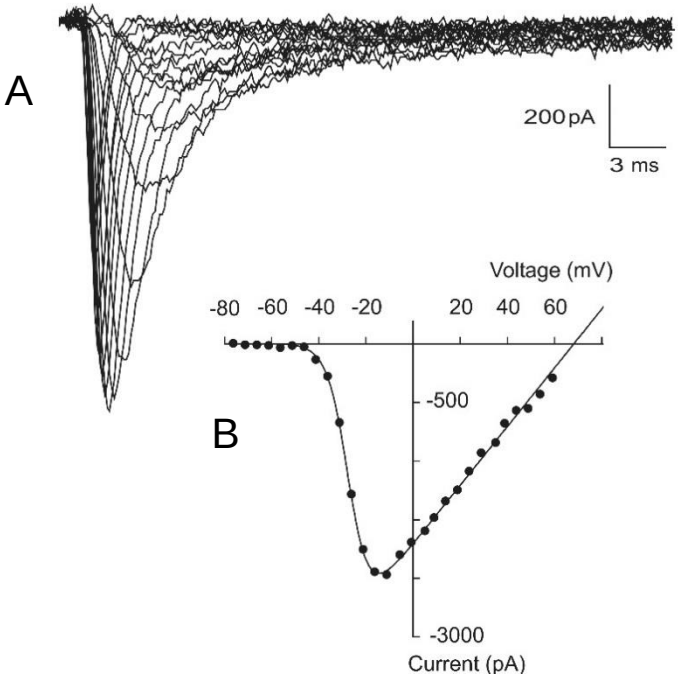
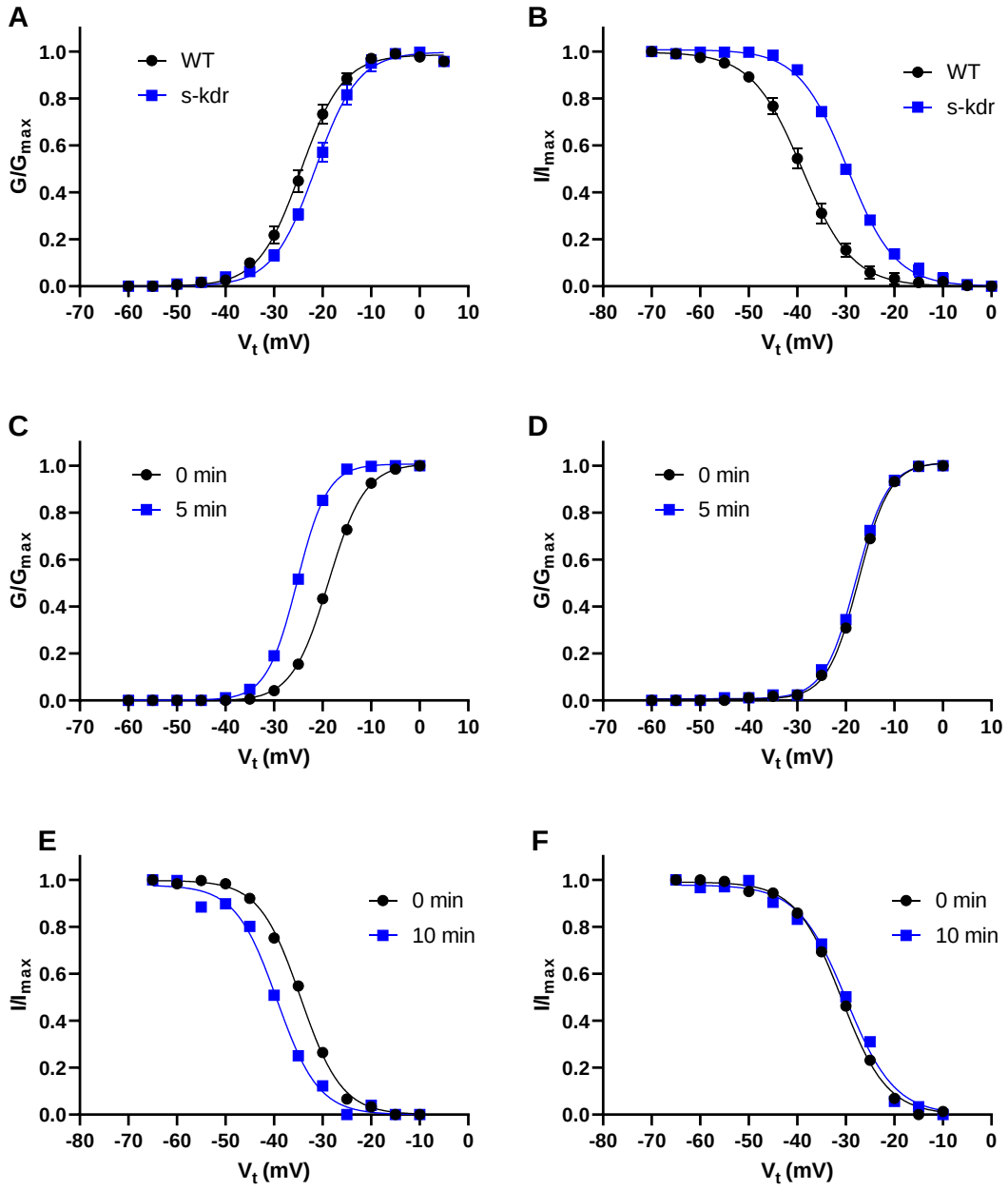


Figure 3. Voltage-activated currents in neurons isolated from thoracic ganglia of adult wild-type houseflies. *A. Family of whole-cell currents recorded in the presence of 30mM teraethylammonium (TEA), 1mM 4-aminopyridine (4-AP) and 1mM $CoCl_2$, following depolarisation of an isolated cell from -80mV to a range of potentials. B. Current/voltage relationship of the same neuron.*

Comparative studies of voltage-gated sodium currents in neurons from *wild-type* and *s-kdr* houseflies showed a significant depolarising shift of the activation curves (Figure 4A). Half-maximal activation in neurons from *s-kdr* insects (−21.4 ± 0.2 mV, *n* = 15) showed a significant depolarising shift of 2.76 mV when compared to the susceptible *wild-type* strain (−24.1 ± 0.2 mV, n = 17). Slope factors for the two curves were indistinguishable (*k* = 4.6 ± 0.2 mV, *wild-type*; 4.5 ± 0.2 mV, *s-kdr*). The voltage-dependence of inactivation also differed between the strains (Figure 4B). Inactivation curves were 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* = 15), revealing that the inactivation curves were significantly depolarised in the resistant insects. The slope factors of *wild-type* (*k* = 5.2 ± 0.1 mV) and *s-kdr* (*k* = 4.9 ± 0.2 mV) inactivation curves were similar.



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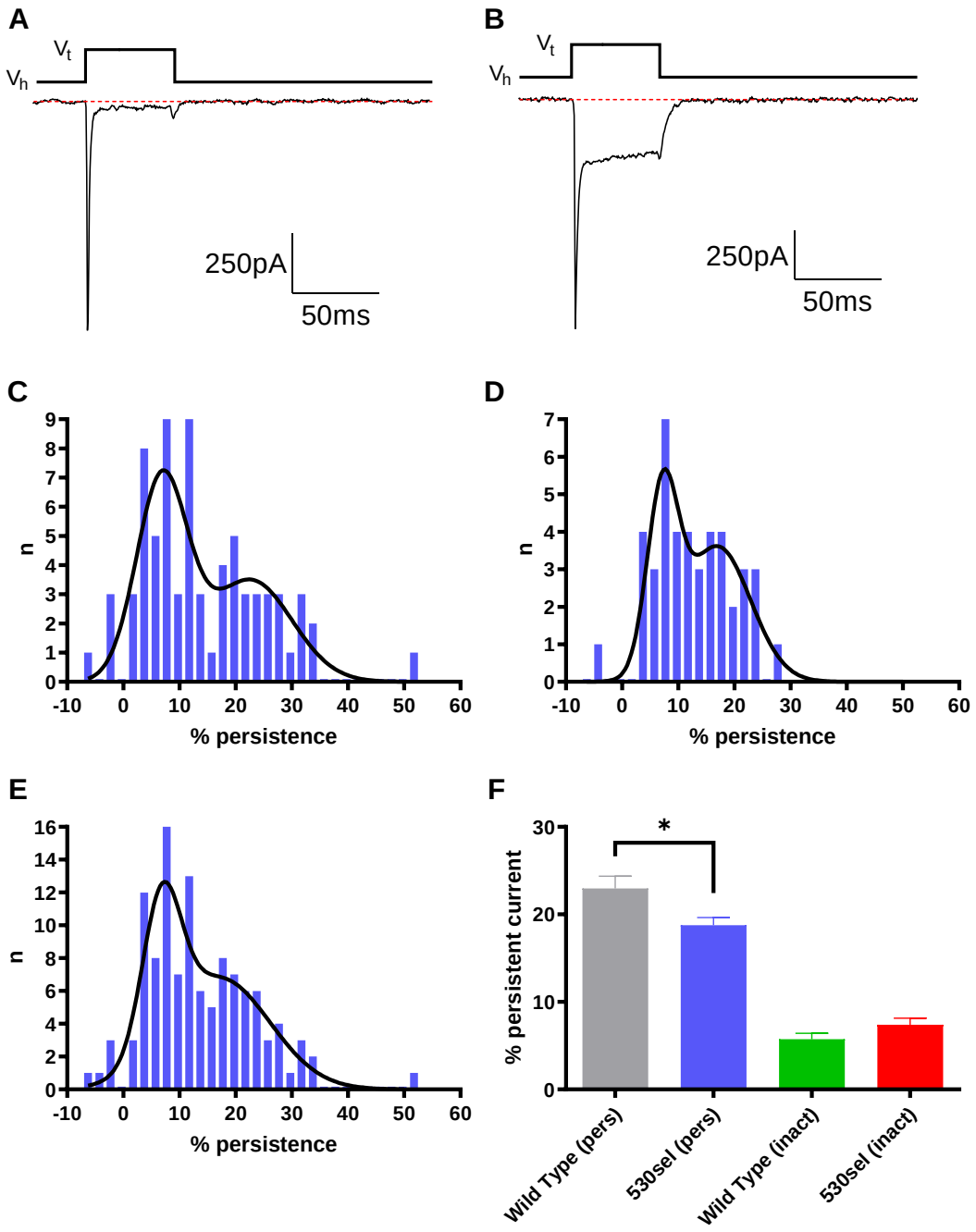
327 ***Figure 4. Properties of susceptible (wild-type), and super-kdr VGSC currents in isolated thoracic***
328 ***neurons from houseflies. A-B****: Voltage dependence of activation (A) is expressed as normalised*
329 *conductance following a 30ms depolarising step from -100mV to a range of potentials 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*

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

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

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



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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.*

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

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

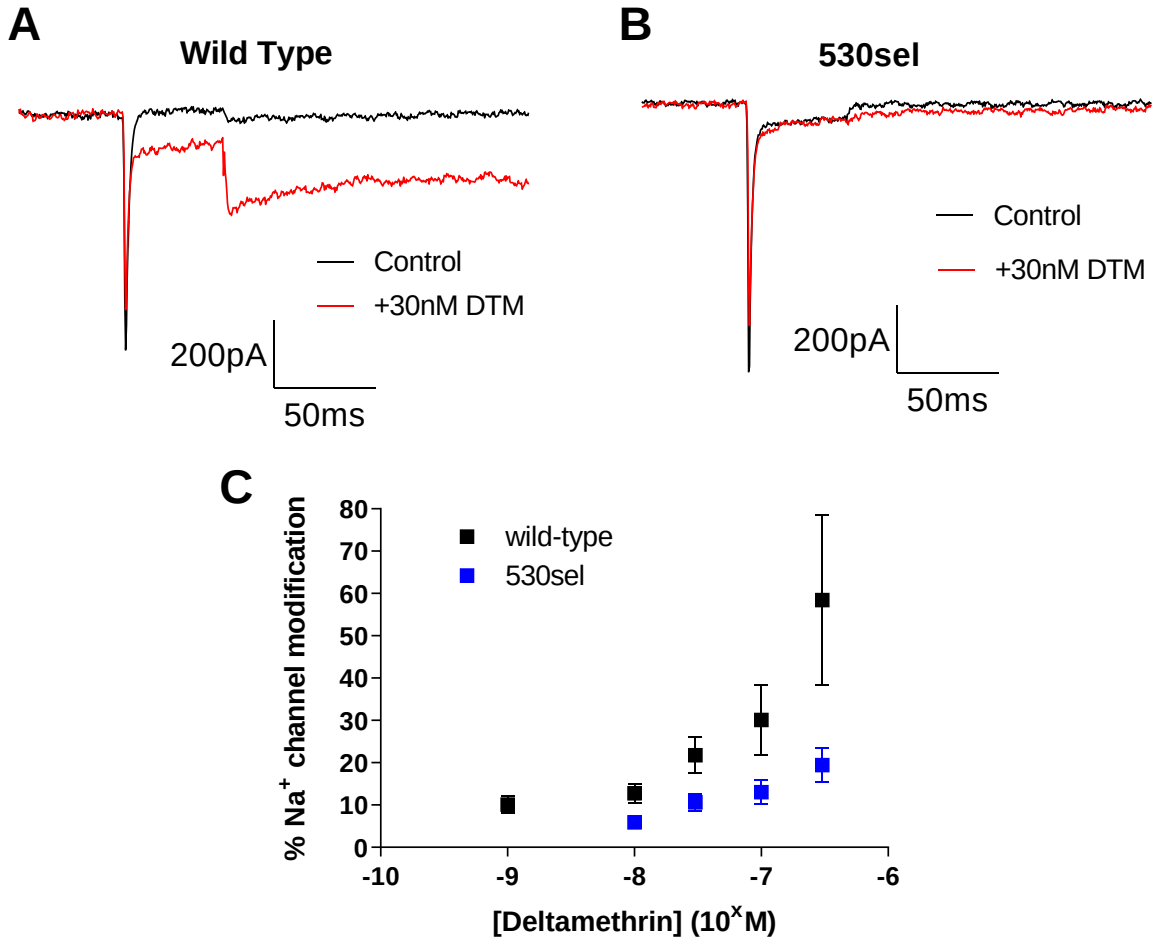


Figure 6. Tail currents in isolated housefly thoracic neurons. *Inactivating Na^+ currents in neurons of the wild-type strain (A) are more sensitive to deltamethrin than Na^+ currents in the s-kdr (530sel) strain (B). In response to 30nM deltamethrin tail current amplitude is greater in the wild-type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

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

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

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

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

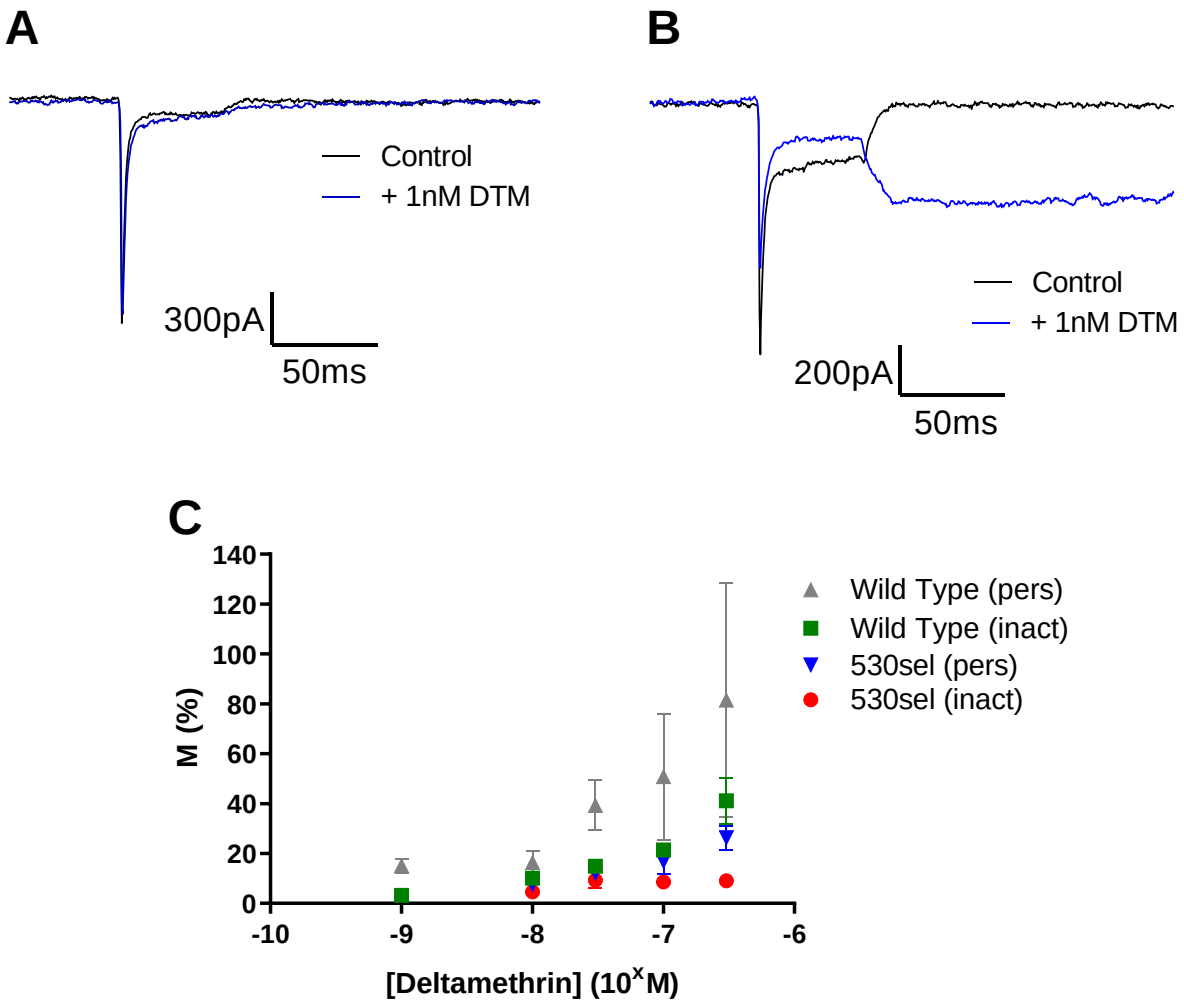


Figure 7. Persistent-type currents are modified by deltamethrin more than are inactivating-type currents. A-B*: Data from isolated housefly thoracic neurons of the wild-type strain. In response to 1nM deltamethrin tail current amplitude is greater for persistent currents (B) compared to inactivating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

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

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

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

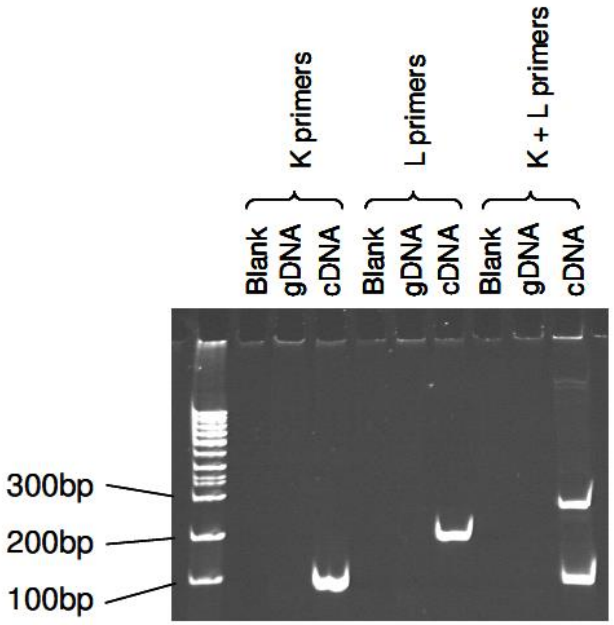
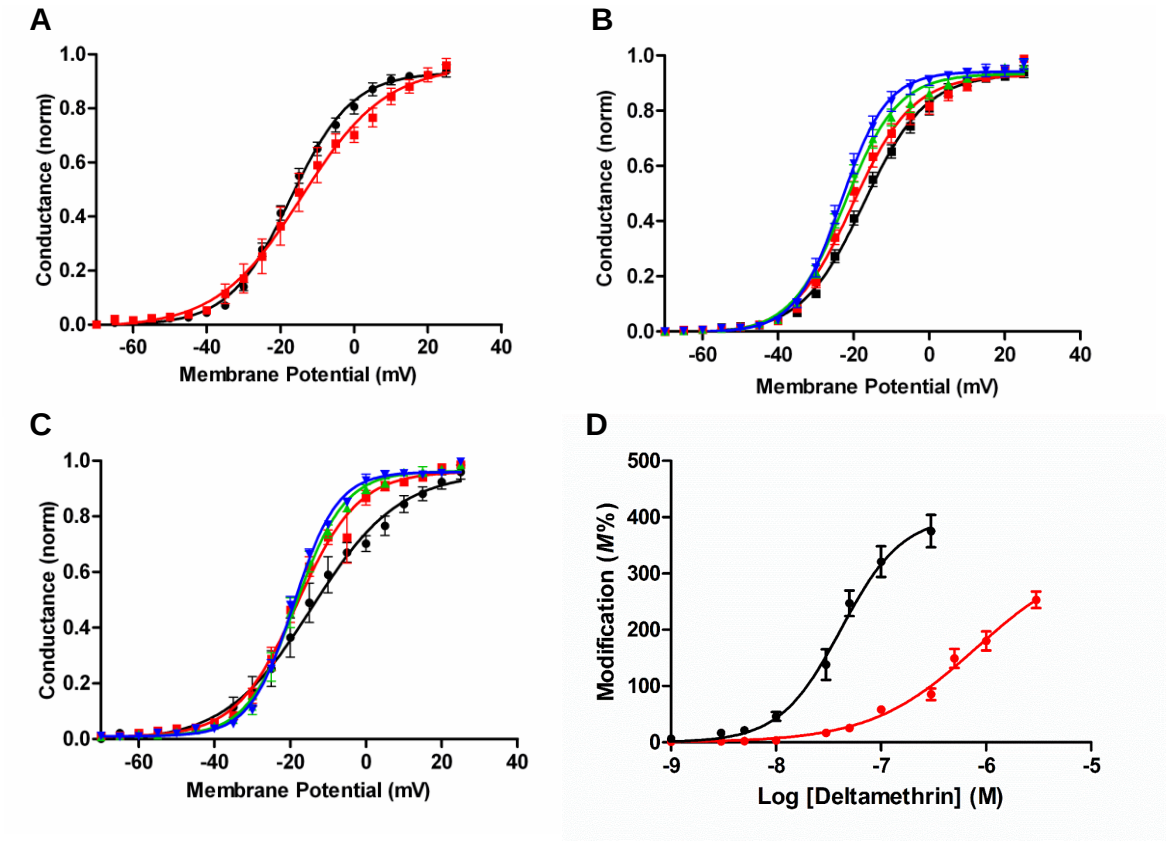


Figure 8. PCR amplification of Na^+ channel-specific fragments from (wild-type) housefly cDNA confirms that transcripts containing both exon k and exon l are expressed. *The presence of an exon k band but no exon l band in the multiplex cDNA lane (far right) indicates that expression levels are higher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

3.5 k and l exons confer different sensitivity to deltamethrin in expressed Drosophila para VGSCs: *Drosophila para* channels expressed in *Xenopus* oocytes have been used to interpret resistance mutations identified in the field. However, in these studies the "*wild-type para* channel" has been the l exon variant. We tested the effects of deltamethrin on *Drosophila para* sodium channels using *Xenopus* oocytes injected with cRNA encoding either k (13-5K) or l (13-5L) exon (no other changes, including point mutations, are present between these two clones, see Lin et al., 2009). Activation kinetics of the two splice variants were compared and showed there was a significant 3 mV ($P< 0.01$; *t* test) depolarising shift of the V_{50} for activation in the k splice variant (Figure 9A).



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 ± 0.4 mV, k = 8.4 ± 0.4, n = 16*
462 *and ■ 13-5K: V_{50} = −14.23 ± 1.3 mV, k = 11.5 ± 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 ± 0.4 mV, k = 8.3 ± 0.4, n = 16); ■ 1 nM*
466 *deltamethrin (V_{50} = −20.31 ± 0.5 mV, k = 8.0 ± 0.4, n = 11); ▲ 5 nM deltamethrin (V_{50} = −22.42 ± 0.4*
467 *mV, k = 7.1 ± 0.4, n = 10); ▼ 30 nM deltamethrin V_{50} = −24.22 ± 0.4 mV, k = 5.6 ± 0.3, n = 7.* ***13-5K****: ●*
468 *No deltamethrin (V_{50} = −14.23 ± 1.3 mV, k = 11.5 ± 1.3, n = 7); ■ 1 nM deltamethrin (V_{50} = −18.23 ± 0.7*
469 *mV, k = 8.1 ± 0.6, n = 9); ▲ 5 nM deltamethrin (V_{50} = −18.51 ± 0.4 mV, k = 6.5 ± 0.4, n = 7); ▼ 30 nM*
470 *deltamethrin (V_{50} = −19.10 ± 0.3 mV, k = 5.8 ± 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)

The sensitivity to deltamethrin was also investigated by determining the percentage of channel modification by analysing the tail currents at various deltamethrin concentrations. This gave an EC_{50} value of 42 nM for the 13-5L variant and a 20-fold larger (866 nM) value for the 13-5K splice variant The upper plateau in the relationship for the k variant could not be fully established owing to the insolubility of deltamethrin at higher concentrations.

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

4. Discussion

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

In the present study, neurons from *s-kdr* houseflies showed positive shifts in the voltage dependence of activation and steady-state inactivation. Similar results have been reported previously for *Heliothis 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 EC_{15} 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 EC_{15} value is 6.6nM and 33.4nM (resistance factor 5.06 -
558 fold) whereas for the inactivating currents the EC_{15} 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 l isoforms are present in housefly
573 head cDNA. It is known from studies expressing k and l isoforms of *Drosophila para* cRNA in *Xenopus*
574 oocytes that the k isoform leads to a reduced persistent current relative to the l 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 l splice variant. This is in contrast to the heterologous expression

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

This hypothesis was further evaluated by assessing the effects of deltamethrin in *Xenopus* oocytes expressing either the k or l isoform of *para*. It was apparent that the k splice variant was inherently less excitable than the l isoform, with V_{50} for both activation and steady state inactivation being shifted to a significantly more positive value, as recently described by Sun *et al* (2019) for *N. lugens*, for two out of three of the k variants tested. A depolarising shift of the activation voltage was also found for BG Na_v1-1 compared with BG Na_v2-1 (Tan *et al* 2002) whereas V_{50} for steady-state inactivation was hyperpolarised. Tail currents as a direct indicator of pyrethroid sensitivity also differed substantially, with the *para* channel containing the l exon being much more sensitive to deltamethrin than was the k variant, which is consistent with the pattern observed for cockroach channels BG Na_v1-1 and BG Na_v2-1 (Tan *et al* 2002) and *N. lugens*, (Sun *et al* 2019) expressed in *Xenopus* oocytes.

The results presented here show that there is considerable diversity in VGSC function between individual housefly neurons in both *wild-type* and pyrethroid resistant insects and this can produce major differences in neuronal sensitivity to pyrethroids. An important molecular basis for this diversity is the expression of the mutually exclusive k and l exons through alternative splicing of the *para* 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 l
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 PyR2 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 PyR2 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

5. References

Amey, J.S., O'Reilly, A. O., Burton, M. J., Puinean, A. M., Mellor, I.R., Duce, I. R., Field, L. M., Wallace, B. A., Williamson, M. S., Davies, T. G.E. 2015. An evolutionarily-unique heterodimeric voltage-gated cation channel found in aphids FEBS let. 589, 598-607.

Burton, M. J., Mellor, I. R., Duce, I. R., Davies, T. G. E., Field, L. M., Williamson, M. S. 2011. Differential resistance of insect sodium channels with kdr mutations to deltamethrin, permethrin and DDT. Insect Biochem. Mol. Biol. 41, 723-732.

Busvine, J. R., 1951. Mechanism of resistance to insecticide in houseflies. Nature. 168,193–195.

Byerly, L., Leung, H.T. 1988. Ionic currents of Drosophila neurons in embryonic cultures. J. Neurosci. 8, 4379-4393.

Catterall, W.A., Goldin, A.L., Waxman, S.G. 2005. International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol. Rev. 57, 397–409.

Chen, M., Du Y., Nomura, Y., Zhu, G., Zhorov, B.S., Dong, K. 2017. Mutations of two acidic residues at the cytoplasmic end of segment IIIS6 of an insect sodium channel have distinct effects on pyrethroid resistance. Insect Biochem. Mol. Biol. 82, 1-10.

Davies, T. G. E., Field, L. M., Usherwood, P. N. R., Williamson, M. S. 2007a. DDT, pyrethrins, pyrethroids and insect sodium channels. Life 59, 151 – 162.

Davies, T. G. E., Field, L. M., Usherwood, P. N. R., Williamson, M.S. 2007b. A comparative study of voltage-gated sodium channels in the Insecta: implications for pyrethroid resistance in Anopheline and other Neopteran species. Insect Mol. Biol. 16, 361–375.

Davies, T. G. E., O'Reilly, A. O., Field, L. M., BA Wallace, B. A., Williamson, M.S. 2008. Knockdown resistance to DDT and pyrethroids: from target-site mutations to molecular modelling. Pest Manag. 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 tran abbattimento e mortalita 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.