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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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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 10 nM 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} 42 nM) than the k splice variant (EC_{50} 866 nM). We conclude that in addition to the presence of point mutations, target site resistance to pyrethroids may involve the differential expression of splice variants.

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 et al. (1996) and single nucleotide polymorphisms were identified in resistant

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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, 2007; 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, 1997a, 2007; 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 orthologue 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 1989), 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; Schäfer 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., 2005; 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 2007a,b, 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 h light/dark cycle.

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

2.2. Short term culture of isolated neurons

Adult houseflies were anaesthetised with CO_2 and placed on ice. Flies were pinned through the abdomen onto Sylgard (Dow-Corning) coated dishes, decapitated and the thorax dissected along the dorsal midline. Thoracic ganglia were removed and maintained in Ca^{2+}/Mg^{2+}-free Rinaldini's saline (in mM; 135 NaCl, 2.5 KCl, 0.4 NaH_2PO4, 1.25 $NaHCO_3$, 0.5 Glucose, 5.0 HEPES, pH 7.2). Connective tissue was removed from each ganglion and the neural sheath disrupted mechanically prior to treatment with 0.5 mg ml^{-1} collagenase (Sigma) and 2 mg ml^{-1} dispase (Sigma) in Ca^{2+}/Mg^{2+}-free Rinaldini's saline

for 1 h at room temperature. Ganglia were washed several times with Ca^{2+}/Mg^{2+}-free Rinaldini's saline and transferred to modified Schneider's culture medium (85% Schneider's Drosophila medium, 15% Foetal Bovine Serum, plus 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). Ganglia were gently triturated through the flame polished tip of a Pasteur pipette to liberate neurons into the culture media, and the supernatant was plated directly onto 35 mm Petri dishes (Nunc, Roskilde). Dishes were left overnight at 18 °C to allow neurons to settle and stick to the surface of the dish.

2.3. Whole-cell electrophysiology

Dishes plated with housefly neurons were used as static baths and 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 7.2). Currents were recorded using the whole-cell configuration of the patch clamp technique with agarose-bridge earth electrodes. Unpolished patch pipettes (5–10 MΩ) were pulled from borosilicate glass capillaries (World Precision Instruments, UK) using a P-97 Flaming Brown Micropipette Puller (Sutter Instrument Co., USA) and filled with 'housefly pipette' saline (in mM; 70 CsCl, 70 CsF, 1.1 EGTA, 2 $MgCl_2$, 0.1 $CaCl_2$, 5.0 HEPES, pH 7.2).

Experiments used an Axopatch 200 patch-clamp amplifier (Axon Instruments, USA) controlled using WCP software (Dr John Dempster, University of Strathclyde) run on a Windows PC. Whole cell 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 Ca^{2+} 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 min 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 min. 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 −70 mV 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:

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

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 $G = 1/(V_T - V_{rev})$ 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:

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

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 fitted with the Boltzmann equation::

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

where the parameters are as for Eqs (1) and (2).

Tail currents were investigated by a single 50 ms 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:

$$M = ((I_{tail} / (V_{hold} - V_{rev})) / G_{max}) \times 100 \quad (4)$$

where M is percentage modification, I_{tail} is the tail current amplitude measured as the peak value in the 50 ms 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. 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 (Fig. 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.

2.6. Electophysiological properties of drosophila VGSC splice variant

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 min 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^{-1} 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 1 nM–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

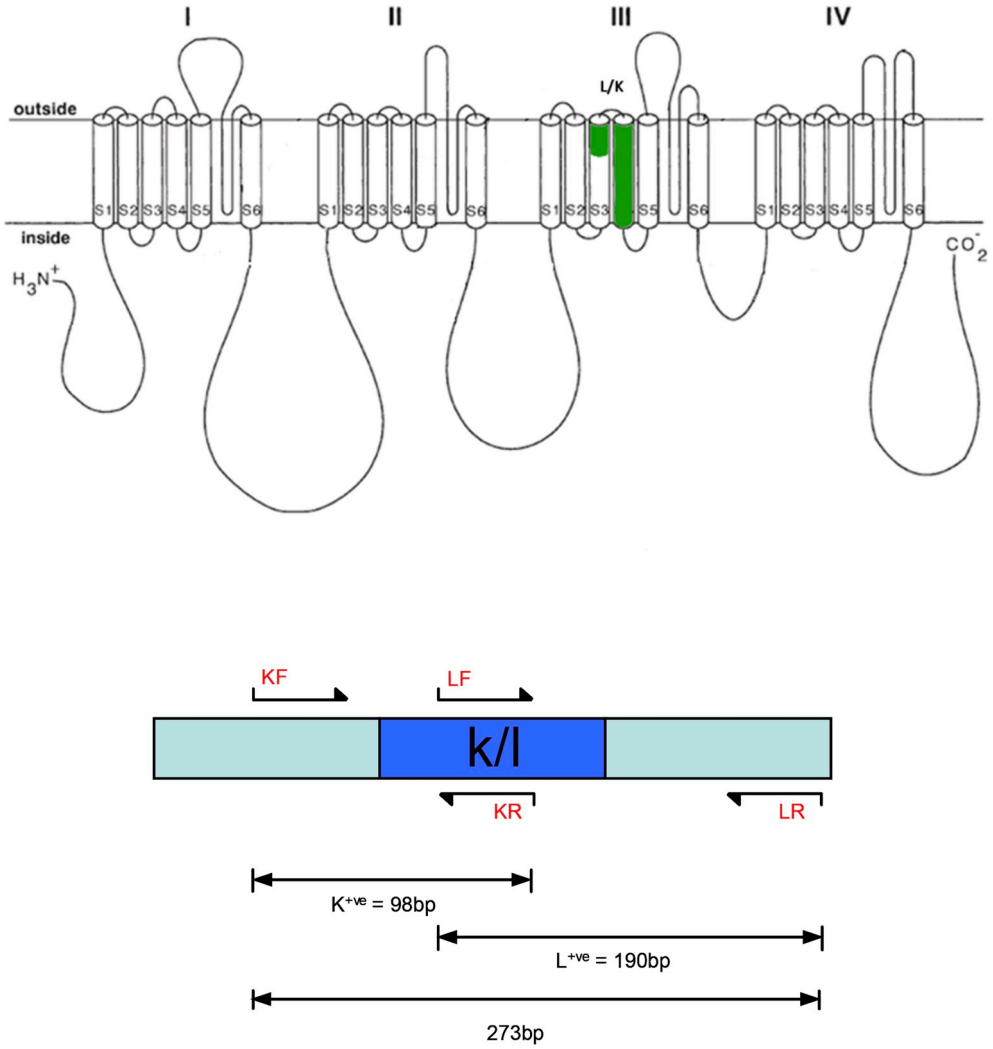

Fig. 1. A: Diagram of the housefly and Drosophila para VGSC with the location of the k/l splice variants indicated. B. Two primers in the exons flanking k/l coupled with one k-specific and one l-specific primer were designed such that the sizes of the fragments produced were indicative of which exon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

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 °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); 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 a,b). 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 5 ms 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 10–30 μm in diameter with a residual axonal stub 5–60 μm in length (Fig. 2).

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 (Fig. 3) and could be blocked by 20 nM TTX (data not shown) further characterising these currents as sodium currents.

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 (Fig. 4A). Half-maximal activation in

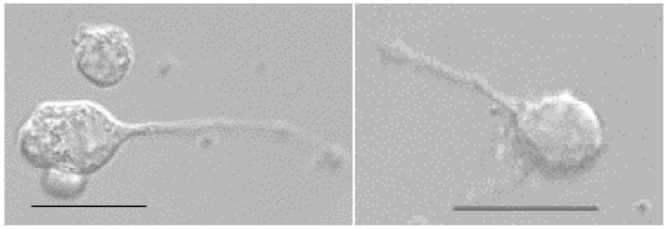

Fig. 2. Phase-contrast micrographs. Individual neuronal cell bodies isolated from the thoracic ganglion of houseflies and attached to glass coverslips. Scale bar 50 μm.

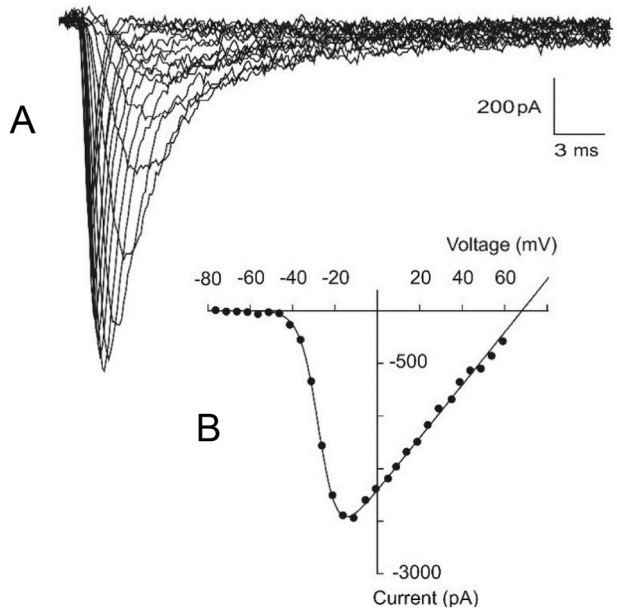

Fig. 3. Voltage-activated currents in neurons isolated from thoracic ganglia of adult wild-type houseflies. A. Family of whole-cell currents recorded in the presence of 30 mM teraethylammonium (TEA), 1 mM 4-aminopyridine (4-AP) and 1 mM $CoCl_2$, following depolarisation of an isolated cell from −80mV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

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 ($k = 4.6 \pm 0.2$ mV, *wild-type*; 4.5 ± 0.2 mV, *s-kdr*). The voltage-dependence of inactivation also differed between the strains (Fig. 4B). Inactivation curves were described by Eq (3), 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* ($k = 5.2 \pm 0.1$ mV) and *s-kdr* ($k = 4.9 \pm 0.2$ mV) inactivation curves were similar.

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 (Fig. 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 (Fig. 4D). The same neurons were subsequently retested for the effect of deltamethrin on steady state inactivation (recorded after 10 min 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 (Fig. 4E–F).

During a 50 ms depolarising pulse, Na^+ currents in some cells activated and inactivated rapidly (Fig. 5A); whereas, other cells produced a Na^+ current that activated rapidly but did not fully inactivate (Fig. 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% (Fig. 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 (Fig. 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 (Fig. 6).

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 Tatebayashi and Narahashi (1994).

The limited aqueous solubility of deltamethrin results in problems in recording and analysing currents at concentrations in excess of 1 μM, 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 132.9 nM, a 9.2-fold increase in the value for the *wild-type* of 14.5 nM (Fig. 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 (Fig. 7). For example, 1 nM 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 (Fig. 7A). The greater susceptibility of persistent type currents was seen in both the *wild-type* (Fig. 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 (Fig. 7C).

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

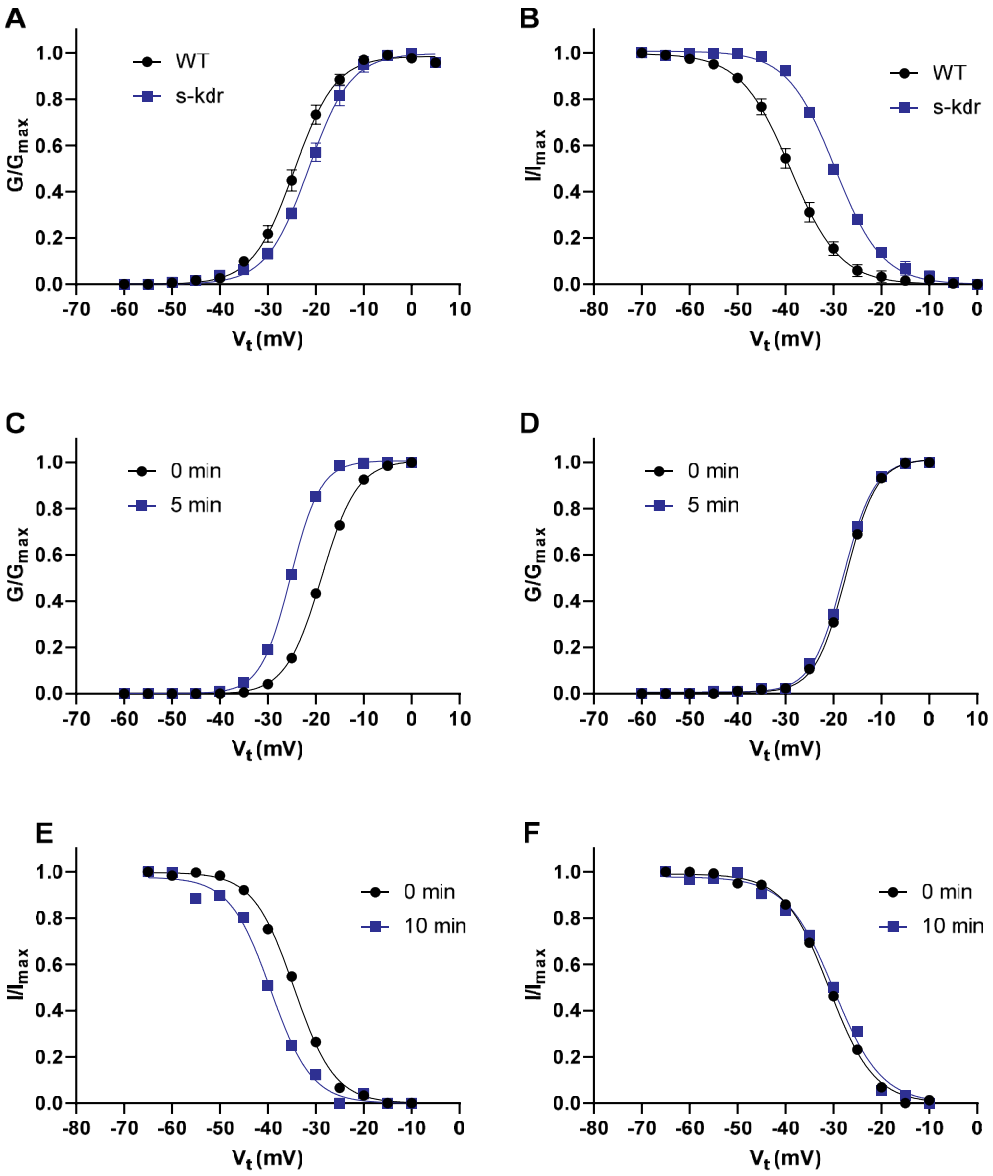

Fig. 4. Properties of susceptible (wild-type), and super-kdr VGSC currents in isolated thoracic neurons from houseflies. A-B: Voltage dependence of activation (A) is expressed as normalised conductance following a 30 ms depolarising step from −100mV to a range of potentials from −70 to +30 mV. The V_{50}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

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., 2002). In this study we have investigated the k/l mutually exclusive exons (Fig. 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). Fig. 8 shows the presence of both exons.

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 (Fig. 9A).

The effect of a 5 min application of deltamethrin on activation channel kinetics was investigated. This showed that in the l splice

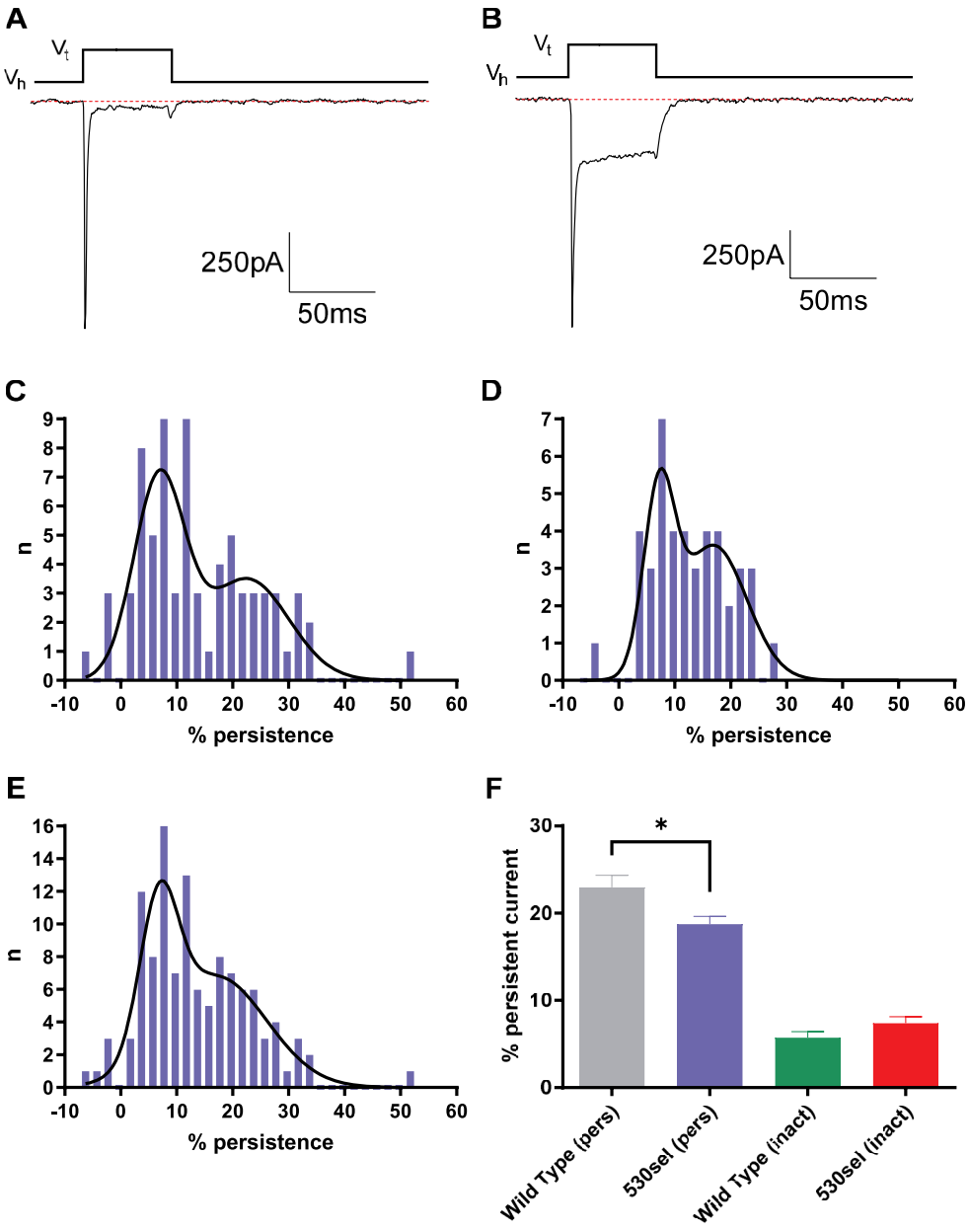

Fig. 5. Rapidly inactivating and persistent sodium currents in housefly thoracic neurons. In a sub-population of isolated neurons a single 50 ms depolarisation to a V_t of −10mV from a V_h of −70mV evokes Na^+ currents which activate and inactivate rapidly (A) whereas in other cells the same depolarisation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

variant, the $V_{50.act}$ was shifted by 7 mV in the hyperpolarising direction to −24.22 mV in the presence of 30 nM deltamethrin ($P < 0.001$; *t*-test) (Fig. 9B) and for the k splice variant there was an approximate 5 mV shift in $V_{50.act}$ to −19.10 mV (Fig. 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

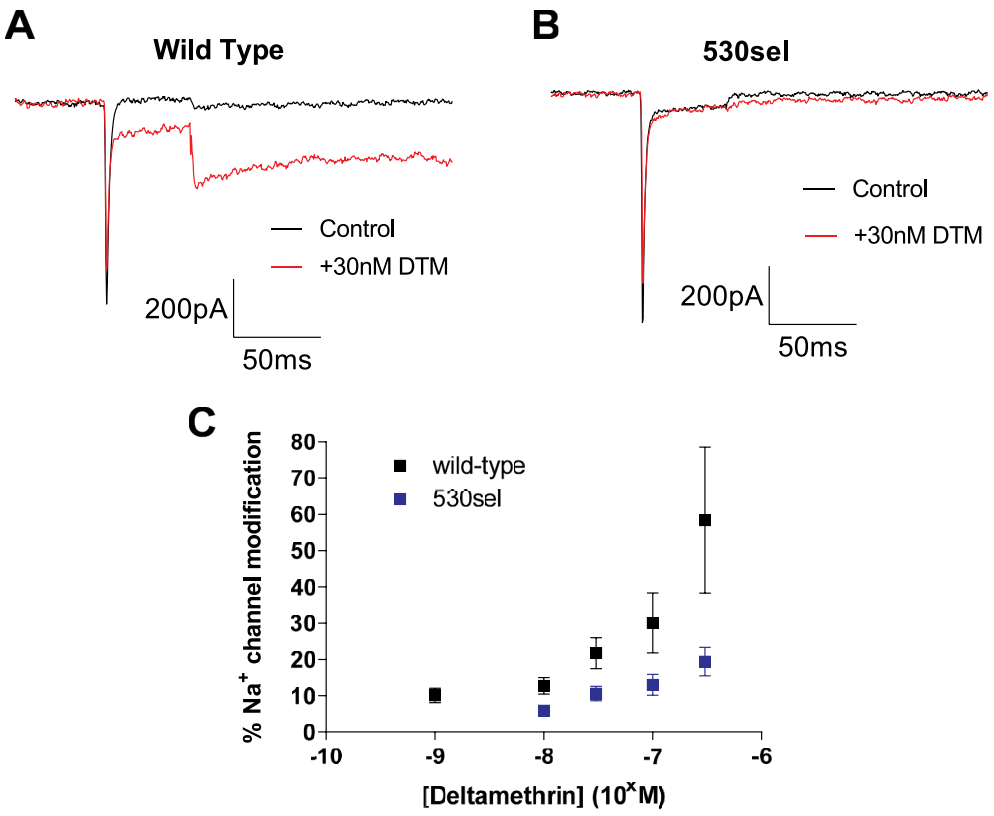

Fig. 6. Tail currents in isolated housefly thoracic neurons. Inactivating Na^+ currents in neurons of the wild-type strain (A) are more sensitive to deltamethrin than Na^+ currents in the s-kdr (530sel) strain (B). In response to 30 nM deltamethrin tail current amplitude is greater in the wild-type than the resistant strain (relative to control current). Whole-cell currents were generated in response to a single 50 ms depolarisation to a Vt of −10 mV from a Vh of −70 mV (voltage protocol 4). Currents from single neurons are shown following application of deltamethrin for 5 min. **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.

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

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

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

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

It is difficult to compare these studies quantitatively due to slight differences in methodology and the pyrethroid being used, however they each provide a basis for comparing the *wild-type* channel with that of a pyrethroid-resistant strain tested alongside it. In the present study there was a significant 9.2 -fold increase in the EC_{15} value for channel modification from 14.5 nM for the *wild-type* to 132.9 nM deltamethrin for the sodium channels from *s-kdr* houseflies. This is smaller than the difference seen in heterologously expressed sodium channels, where the *s-kdr* double mutation completely abolished the pyrethroid sensitivity

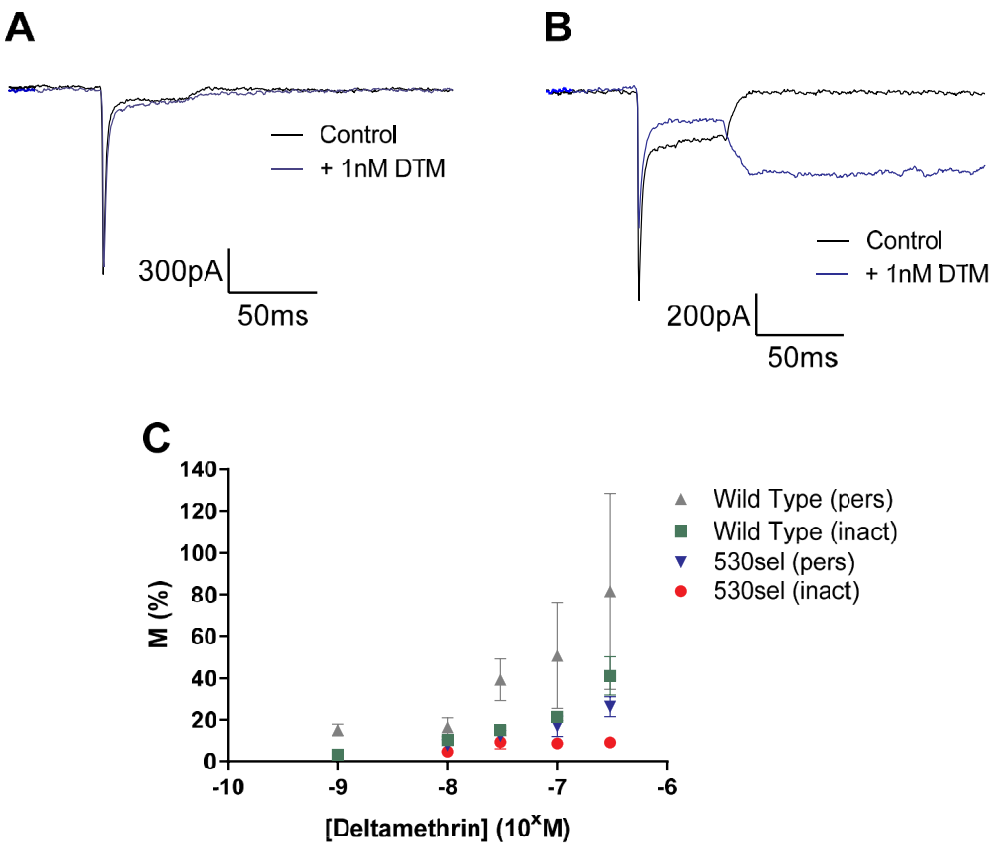

Fig. 7. Persistent-type currents are modified by deltamethrin more than are inactivating-type currents. A-B: Data from isolated housefly thoracic neurons of the wild-type strain. In response to 1 nM deltamethrin tail current amplitude is greater for persistent currents (B) compared to inactivating currents (A), relative to control current. Whole-cell currents were generated in response to a single 50 ms depolarisation to a Vt of −10 mV from a Vh of −70 mV (voltage protocol 4). Currents from single neurons are shown following application of deltamethrin for 5 min. **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.

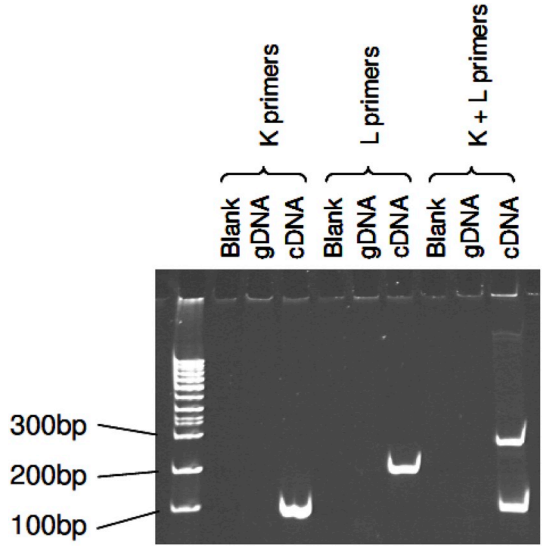

Fig. 8. PCR amplification of Na^+ channel-specific fragments from (wild-type) housefly cDNA confirms that transcripts containing both exon k and exon l are expressed. The presence of an exon k band but no exon l band in the multiplex cDNA lane (far right) indicates that expression levels are higher for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

of the housefly channel, whilst for *Drosophila para* channels in the presence of ATX the *s-kdr* mutant channel was 100 -fold less sensitive. The reduction in sensitivity in this study is more reminiscent of the study by Lee D. et al. (1999) on *H. virescens* channels where over a similar range of pyrethroid modification (5%–20%), neurons from the resistant strain showed a 21-fold reduction in sensitivity to permethrin.

Further insight was gained into the relatively small impact of the *s-kdr* mutations on pyrethroid resistance seen in the present study compared with others, by considering the heterogeneity of the sodium currents recorded from different neurons. Sodium currents from both *wild-type* and *s-kdr* neurons were characterised as “inactivating” or “persistent” and it is apparent that the persistent current was much more sensitive to deltamethrin than the rapidly inactivating current (Fig. 8). In view of the correlation between the degree of persistence and the sensitivity to deltamethrin it is worth noting that the degree of persistence varies between the two strains, with the resistant *s-kdr* strain showing significantly reduced persistent current amplitude and raising the possibility that reduced persistent currents may be one component of the resistance mechanism. Alternatively the mutation may itself be responsible for changing the kinetics of the channel from a persistent to an inactivating mode. The concentration of deltamethrin required to produce 15% modification in inactivating and persistent channels of both strains can be compared with the values for the whole populations. For the persistent currents, the EC_{15} value is 6.6 nM and 33.4 nM (resistance factor 5.06 -fold) whereas for the inactivating currents the EC_{15} values are 35.8 nM and 20 μM (resistance factor 558 -fold) for the *wild-type* and *s-kdr* insects respectively.

The identification of two Na^+ current types, inactivating and persistent, allows for more detailed analysis of Na^+ current properties between strains. Inactivating type currents are like the Na^+ currents seen in oocyte expression studies, with rapid activation followed by rapid and near complete inactivation. By contrast, inactivating type Na^+ currents in the resistant strain are highly resistant to tail current generation which is in agreement with previous studies of the L1014F

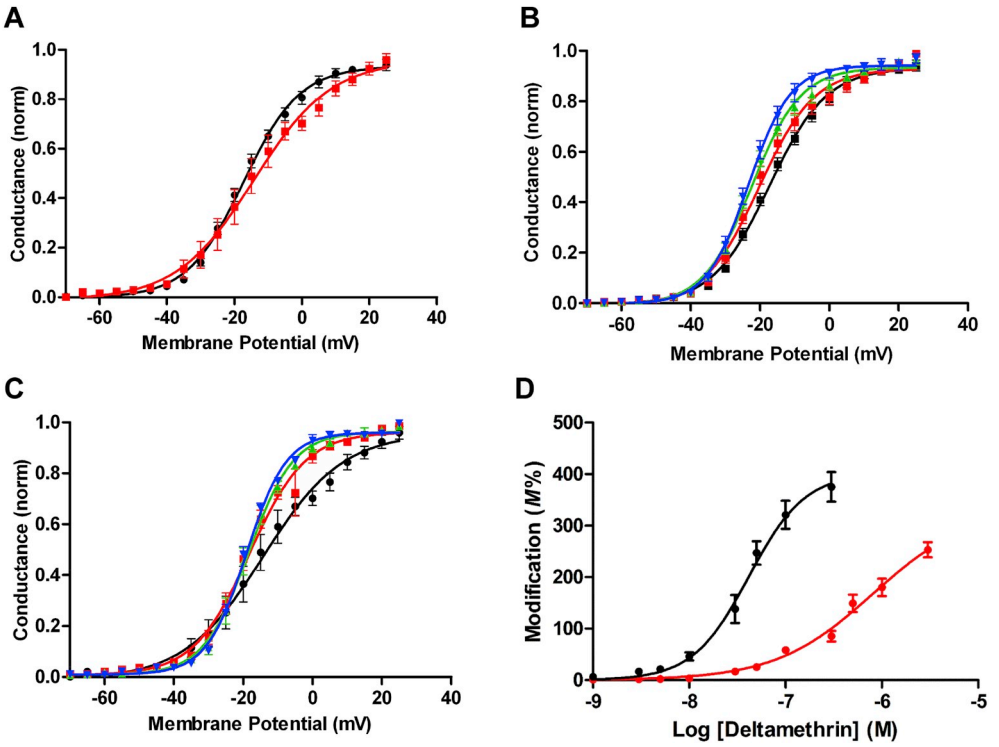

Fig. 9. Properties of 13-5L and 13-5K para splice variant VGSCs expressed in Xenopus oocytes. A: Plots of normalised conductance against test depolarisation for 13-5L and 13-5K. The voltage dependence of activation ($V_{50.act}$) for each was: ● 13-5L: V_{50} = −17.32 ± 0.4 mV, k = 8.4 ± 0.4, n = 16 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 $V_{50.act}$ showed a significant difference $P < 0.01$. B-C: Conductance-Voltage relationships for the 13-5L (B) and 13-5K (C) para splice variants in the absence and presence of increasing concentrations of deltamethrin. 13-5L: ■ No deltamethrin (V_{50} = −17.32 ± 0.4 mV, k = 8.3 ± 0.4, n = 16); ■ 1 nM deltamethrin (V_{50} = −20.31 ± 0.5 mV, k = 8.0 ± 0.4, n = 11); ▲ 5 nM deltamethrin (V_{50} = −22.42 ± 0.4 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: ● 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 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 deltamethrin (V_{50} = −19.10 ± 0.3 mV, k = 5.8 ± 0.2, n = 7). D. Log [deltamethrin](M) – response curves were fitted by a four-parameter logistic equation establishing an EC_{50} value of 42 nM and a maximum modification value of 402%, n = 10 for 13-5L, and an EC_{50} value of 866 nM and a maximum modification of 346%, n = 11 for 13-5K.

and M918T double mutations in housefly Na^+ channels (Lee S. et al., 1999) and other species. However, the non-inactivating type currents characterised here have a major impact on the action of pyrethroids and are therefore likely to strongly influence the sensitivity of the overall population of neurons and therefore the sensitivity of the nervous system of susceptible and resistant insects to pyrethroids *in vivo*.

The molecular basis for the finding of both inactivating and persistent sodium currents in housefly neurons is unknown, however we have shown that both the k and l isoforms are present in housefly head cDNA. It is known from studies expressing k and l isoforms of *Drosophila para* cRNA in *Xenopus* oocytes that the k isoform leads to a reduced persistent current relative to the l isoform (Lin and Baines, 2015). It is also apparent that most of the studies investigating the effects of pyrethroids on *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, Du et al., 2006) 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 et al., 2007a, b). In view of the parallels between the greater sensitivity of the persistent neuronal currents to pyrethroids and greater sensitivity of the l splice variant of *para* when heterologously expressed, it is tempting to identify the k/l site as crucial for pyrethroid action. However, it is important to bear in mind that this is one of 29 possible splice variants of *para*, many of which have been shown to have different physiological properties (Olson et al., 2008) and the pyrethroid sensitivity of the whole nervous system of an insect is likely to reflect a combination of splice-variant channels.

Modelling studies of insect VGSCs (O'Reilly et al., 2006; Usherwood et al., 2007; Davies et al., 2008; Du et al., 2016) have identified two putative binding sites for pyrethroids and DDT, which have been termed PyrR1 and PyR2 and are thought to involve the interfaces between domains II and III and between domains I and II respectively (Dong et al., 2014; Zhorov and Dong, 2017). The k/l site is located in the S3 and S4 segments of Domain III of the VGSC but to date there

have been no modelling studies of the direct effect of k/l splice variants on pyrethroid binding. It is possible that there may be allosteric effects such as those reported for the effect of a mutation (N1575Y) on the topology of the PyR2 site (Wang et al, 2015). Molecular modelling and functional studies have established that S3 and S4 segments are critical for coupling depolarisation to channel activation and inactivation (Catterall, 2005; Shen et al., 2017) which provides an explanation for the changes in voltage sensitivity and persistence of sodium current between the k/l splice variants discussed above.

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