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Distinct Roles of the DmNa$_v$ and DSC1 Channels in the Action of DDT and Pyrethroids

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Abstract

Voltage-gated sodium channels (Na$_v$ channels) are critical for electrical signaling in the nervous system and are the primary targets of the insecticides DDT and pyrethroids. In *Drosophila melanogaster*, besides the canonical Na$_v$ channel, Para (also called DmNa$_v$), there is a sodium channel-like cation channel called DSC1 (*Drosophila* sodium channel 1). Temperature-sensitive paralytic mutations in *DmNa$_v$* (para^{ts}) confer resistance to DDT and pyrethroids, whereas *DSC1* knockout flies exhibit enhanced sensitivity to pyrethroids. To further define the roles and interaction of DmNa$_v$ and DSC1 channels in DDT and pyrethroid neurotoxicology, we generated a *DmNa$_v$/DSC1* double mutant line by introducing a *para^{ts1}* allele (carrying the I265N mutation) into a *DSC1* knockout line. We confirmed that the I265N mutation reduced the sensitivity to two pyrethroids, permethrin and deltamethrin of a DmNa$_v$ variant expressed in *Xenopus* oocytes. Computer modeling predicts that the I265N mutation confers pyrethroid resistance by allosterically altering the second pyrethroid receptor site on the DmNa$_v$ channel. Furthermore, we found that I265N-mediated pyrethroid resistance in *para^{ts1}* mutant flies was almost completely abolished in *para^{ts1};DSC1$^{-/-}$* double mutant flies. Unexpectedly, however, the *DSC1* knockout flies were less sensitive to DDT, compared to the control flies (*w^{1118A}*), and the *para^{ts1};DSC1$^{-/-}$* double mutant flies were even more resistant to DDT compared to the *DSC1* knockout or *para^{ts1}* mutant. Our findings revealed distinct roles of the DmNa$_v$ and DSC1 channels in the neurotoxicology of DDT vs. pyrethroids and implicate the exciting possibility of using DSC1 channel blockers or modifiers in the management of pyrethroid resistance.

Keywords

DSC1; voltage-gated sodium channel; DDT; pyrethroids insecticide resistance

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Introduction

Insect pests and disease vectors pose tremendous medical, agricultural and economic threats to humans. As such, various control methods, including the use of insecticides, have been developed to mitigate their impact (Perry et al., 1998). DDT and pyrethroids (particularly type I pyrethroids) have a long history of wide usage due to their rapid knockdown and high potency (Narahashi, 2002). Pyrethroids continue to be used extensively in controlling arthropod pests and disease vectors due to their low mammalian toxicity and favorable environmental properties (Narahashi et al., 2007). Although the use of DDT is largely banned in most parts of the world due to its detrimental impact on the ecosystem, it is still one of 12 pesticides recommended by the WHO for indoor residual spray programs in malaria control in African countries (http://www.who.int/mediacentre/news/releases/2006/pr50/en/).

DDT and pyrethroids affect the function of voltage-gated sodium channels (Na_v), which are critical for electrical signaling in excitable cells (Narahashi, 1971). Early studies revealed characteristic repetitive discharge in cockroach nerves treated with DDT or pyrethrins (Eaton and Sternburg, 1964; Lalonde and Brown, 1954). Subsequent studies demonstrated that the insecticide-induced repetitive discharge was the result of prolonged sodium currents (Hille, 1968; Narahashi, 1962; Narahasi and Haas, 1967). Since then, numerous studies have shown that DDT and pyrethroids prolong the opening of Na_v channels by inhibiting channel deactivation and inactivation and stabilizing the open state of sodium channels (Bloomquist, 1996; Bloomquist and Soderlund, 1988; Narahashi, 1992, 2002, 2000; Vijverberg et al., 1982). To date, more than 50 Na_v mutations have been identified that are associated with resistance to pyrethroids in various arthropod pests and disease vectors, and some of them are also responsible for DDT resistance (Rinkevich et al., 2013). Elucidation of mechanisms of pyrethroid resistance led to the identification of two pyrethroid receptor sites, PyR1 and PyR2 (formally Site 1 and Site 2, respectively) on the Na_v channel (Du et al., 2013; O'Reilly et al., 2006), and provided the molecular basis of the action of DDT and pyrethroids on Na_v channels (Dong et al., 2014; Soderlund, 2005).

In *D. melanogaster*, besides the canonical voltage-gated Na_v channel, $DmNa_v$, (also known as Para) (Loughney et al., 1989), there is a Na_v channel-like gene called *DSC1 (Drosophila Sodium Channel 1*, also known as NaCP60E in Flybase). *DSC1* was considered as a putative Na_v channel gene based on its high sequence similarity to Na_v channels in the transmembrane regions (Salkoff et al., 1987). The overall topology of the DSC1 channel is similar to that of the $DmNa_v$ channel, consisting of four homologous domains, each of which has six transmembrane segments (Kulkarni et al., 2002; Zhang et al., 2011). However, functional analysis of the DSC1 channel and a *DSC1* ortholog from the German cockroach, BSC1, in *Xenopus* oocytes revealed that BSC1/DSC1 channels represent a novel family of voltage-gated cation channels with high permeability to Ca^{2+} (Zhang et al., 2011; Zhou et al., 2004). A recent study showed that the *DSC1* knockout line of *D. melanogaster* exhibits enhanced sensitivity to pyrethroids (Zhang et al., 2013), suggesting potential functional interactions between DSC1 channels and Na_v channels in modulating insecticide neurotoxicity. *DSC1* orthologs were isolated from *B. germanica* and *Heliothis virescens* (Liu

et al., 2001; Park et al., 1999) and have now also been found in all insect species with a complete genome sequence (Cui et al., 2012)

To further discern the role of the DSC1 channel in the toxicology of DDT and pyrethroids, we introduced a temperature-sensitive paralytic allele (*para*^{ts1}), carrying an I265N mutation in DmNa$_v$ (Loughney et al., 1989; Pittendrigh et al., 1997) into a *DSC1* knockout line. We then conducted bioassays to compare the susceptibilities of the mutant lines to DDT, permethrin and deltamethrin. In addition, we also evaluated the effects of the I265N mutation on the response of DmNa$_v$ to DDT and pyrethroids. Our analyses revealed surprisingly distinct roles of DmNa$_v$ and DSC1 channels in mediating the toxicological effects of DDT and pyrethroids.

MATERIALS AND METHODS

Site-directed mutagenesis

We introduced the *para*^{ts1} (I265N) mutation into DmNa$_v$22, a pyrethroid-sensitive Na$_v$ channel variant from *D. melanogaster* (Olson et al., 2008). Site-directed mutagenesis was performed by polymerase chain reaction (PCR) using primers (Forward Primer GGCCTGAAGACGATCGTCGGCGCCGTCAACGAATCGGTGAAGAATCTGCGCGA TGTG; Reverse Primer CACATCGCGCAGATTCTTCACCGATTCGTTGACGGCGCCGACGATCGTCTTCAG GCC) and Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). Mutagenesis was verified by DNA sequencing.

Expression of DmNa$_v$22 and mutant channels in *Xenopus* oocytes

The procedures for oocyte preparation and cRNA injection were identical to those described previously (Olson et al., 2008). For robust expression of sodium currents, cRNA was co-injected into oocytes with *Drosophila melanogaster tipE* cRNA (1:1 ratio), which enhances sodium channel expression (Feng, 1995; Warmke, 1997).

Measurement of effects of DDT and pyrethroids on of DmNa$_v$22 and mutant channels expressed in *Xenopus* oocytes

The method for application of DDT and pyrethroids in the recording system was identical to that described previously (Tan et al., 2002b). The effects of pyrethroids were measured 10 min after their application. The pyrethroid-induced tail current was recorded during a 100-pulse train of 5-ms step depolarizations from – 120 to 0 mV with 5-ms interpulse intervals (Vais et al., 2000). The percentage of channels modified by pyrethroids was calculated using the equation $M = \{[I_{tail}/(E_h - E_{Na})]/[I_{Na}/(E_t - E_{Na})]\} \times 100$ (Tatebayashi and Narahashi, 1994), where I_{tail} is the maximal tail current amplitude, E_h is the potential to which the membrane is repolarized, E_{Na} is the reversal potential for sodium current determined from the current-voltage curve, I_{Na} is the amplitude of the peak current during depolarization before pyrethroid exposure, and E_t is the potential of step depolarization.

We incubated oocytes expressing DmNa$_v$22 and I265N channels in DDT solutions overnight in order to detect the inhibitory effect of DDT on fast inactivation. The degree of DDT

inhibition was assayed by measuring the remaining current at the end of 20 ms depolarization to -10 mV from the holding potential of -120 mV and normalized to peak current. Because of an intrinsic non-inactivating current from $DmNa_v22$ channels (Olson et al., 2008) as well as I265N channels, we subtracted it from the normalized non-inactivating current in the presence of DDT to obtain the percentage of DDT inhibition of fast inactivation.

Fly Strains

Four *D. melanogaster* strains were used in this study: w^{1118A}, $DSC1^{-/-}$, $para^{ts1}$ and $para^{ts1}$; $DSC1^{-/-}$. The w^{1118A} line was obtained from Steve Crews' lab in the University of North Carolina, Chapel Hill, and was used in generating the *DSC1* knockout strain, $DSC1^{-/-}$. $DSC1^{-/-}$ is one of two DSC1 knockout founder lines (Zhang et al., 2013). $para^{ts1}$ is a temperature-sensitive paralytic mutant, which has an I265N mutation in the linker connecting transmembrane segments S4 and S5 of domain I of $DmNa_v$ (Pittendrigh et al., 1997).

To generate the $para^{ts1}$;$DSC1^{-/-}$ double mutants, the following crosses were carried out. Virgin females of $para^{ts1}$ were crossed to males from a stock carrying dominant second chromosome marker Bc (Black cell) to generate F1 male $para^{ts1}$ with second chromosome marked by Bc because the *DSC1* gene resides on the second chromosome. They were then back-crossed to $para^{ts1}$ females to obtain F2 $para^{ts1}$ virgin females with second chromosome carrying marker *Bc*, which were further crossed to homozygous $DSC1^{-/-}$ males to generate F3 $para^{ts1}$ males carrying heterozygous DSC1 over *Bc* marker on the second chromosome. These flies were crossed with virgin females from a double balancer stock, with attached-X (X^X) and the second chromosome balancer *CyO* (Curly Oster). The progeny from this final cross yields a balanced stock with $para^{ts1}$ or XX chromosomes over Y, and *DSC1* over CyO. Since the double mutant $para^{ts1}$;$DSC1^{-/-}$ stock was kept over X^X, the females were X^X/Y; $DSC1^{-/-}$ and only males were hemizygous for $para^{ts1}$ and homozygous for $DSC1^{-/-}$, which were used in bioassays.

Bioassays

Contact bioassays with deltamethrin, permethrin and DDT (Chem Service, West Chester PA) were performed as described previously with 1-3 day old male flies (Rinkevich and Scott, 2012). Probit analyses were performed using Minitab (State College, PA). The LC_{50} values were considered to be significantly different if the 95% confidence intervals (CI) did not overlap.

Deltamethrin and DDT Knockdown

Scintillation vials were coated with 0.5 ml of 0.02 mg/ml deltamethrin in acetone (40 μM, approximately 475x the LC_{50} for w^{1118A}) or 2 mg/ml DDT (5 mM, approximately 333x the LC_{50} for w^{1118A}) and allowed acetone to evaporate completely under a fume hood for 1 hr. Ten 1- to 3-day old male flies of each strain were placed in separate vials. The number of flies that were ataxic or unable to stand without exaggerated wobbling was recorded as knocked down at 5 minute intervals for 30 minutes for deltamethrin and 60 minutes for DDT.

Computer modeling

Previous experimental and molecular-modeling studies proposed models of PyR1 and PyR2 in insect sodium channels. Both are homology models based on the X-ray structure of the open voltage-gated potassium channel $K_v1.2$. The model for PyR1 contains residues from transmembrane helices IIS5 and IIIS6, as well as from a linker-helix, IIL45, that connects IIS4 and IIS5 (O'Reilly et al., 2006; Usherwood et al., 2007). The model for PyR2 is formed by residues from helices IL45, IS5, IS6, and IIS6 (Du et al., 2013). Within all these helices, the amino acid sequences of the $DmNa_v$ channel are identical to those in the mosquito $AaNa_v1$-1 channel. Therefore, we used this structural similarity to generate a $K_v1.2$-based homology model of the open $DmNa_v$ channel with deltamethrin (Du et al., 2013) {Du, 2013 #10077; Du, 2013 #10077} and explore possible effects of the I265N mutation on binding of pyrethroids. The energy of the deltamethrin-bound complexes of $DmNa_v$ channel and its mutant was Monte Carlo minimized as described before (Du et al., 2013).

RESULTS

The *para*^{ts1} (I265N) mutation reduced the sensitivity of $DmNa_v22$ channels expressed in *Xenopus* oocytes to pyrethroids and DDT

$DmNa_v22$ and I265N channels were expressed in *Xenopus* oocytes and examined for channel gating and sensitivity to DDT, permethrin and deltamethrin. The I265N mutation did not alter the voltage-dependence of channel activation or inactivation (Table 1). A 100-pulse train of 5-ms depolarizations from -120 to 0 mV with a 5-ms interpulse interval was used to elicit pyrethroid-induced tail currents from oocytes expressing $DmNa_v22$ and I265N mutant channels (Vais et al., 2000). The resulting pyrethroid-induced tail currents serve as a measure of channel sensitivity to pyrethroids (Tatebayashi and Narahashi, 1994). As shown in Fig. 1, large tail currents were induced by 1 μM of permethrin (Fig. 1A) and 1 μM of deltamethrin (Fig. 1B) in oocytes expressing $DmNa_v22$ channels, indicating that $DmNa_v22$ is highly sensitive to pyrethroids. There was no difference between $DmNa_v22$ and I265N mutant channels in the kinetics of decay of permethrin or deltamethrin-induced tail currents (Fig. 1). However, the amplitude of pyrethroid-induced tail currents was reduced for I265N channels (Fig. 1), indicating that the I265N mutation reduced $DmNa_v22$ channel sensitivity to both permethrin and deltamethrin.

Like pyrethroids, DDT induced a tail current of one $DmNa_v$ channel variant expressed in oocytes (Burton et al., 2011; Usherwood et al., 2005). Apparently this effect was observed only after overnight incubation of oocytes expressing $DmNa_v$ channels in a DDT solution (Ian Mellor, personal communication). However, we found that DDT-induced tail currents from $DmNa_v22$ or I265N channels were still too small for quantitative analysis even after overnight incubation with DDT at high concentrations (~100 μM). Instead, the more drastic effect of DDT on sodium channel gating was the inhibition of fast inactivation. DDT at 100 μM induced a non-inactivating current that is 41% of peak current at the end of 20 ms depolarization of $DmNa_v22$ channels (Fig. 2). However, DDT at the same concentration induced a non-inactivating current of I265N channels that is only 12% of peak current (Fig. 2) indicating that the I265N mutation reduced $DmNa_v22$ channel sensitivity to DDT.

Knockout of *DSC1* antagonizes I265N-mediated pyrethroid resistance

A recent study showed that knockout of *DSC1* caused enhanced sensitivity to pyrethroids in *D. melanogaster* (Zhang et al., 2013). To investigate any potential functional interactions between DSC1 and $DmNa_v$ channels in modulating pyrethroid toxicology, we conducted bioassays to examine the sensitivities of w^{1118A}, $para^{ts1}$, $DSC1^{-/}$, and $para^{ts1}$;$DSC1^{-/-}$ flies to permethrin and deltamethrin.

Compared to w^{1118A}, the $para^{ts1}$ or $DSC1^{-/-}$ strains were 4.6-fold less sensitive or 5-fold more sensitive to permethrin, respectively. However, the $para^{ts1}$;$DSC1^{-/-}$ double mutant was 1.8-fold more sensitive to permethrin compared with w^{1118A} (Table 2). This value is lower than what would be expected if the interactions between the parental $para^{ts1}$ and $DSC1^{-/-}$ were merely additive, as simply adding the Resistance Ratios (RRs) would yield an expected RR of -0.4. Furthermore, averaging the mortality at each dose from $DSC1^{-/-}$ and $para^{ts1}$ yields a RR of 2.4-fold. Recalculating the LC_{50} by Probit analysis on the combined bioassay data produced a RR of 1-fold. The 1.8-fold increase in sensitivity to permethrin observed for $para^{ts1}$;$DSC1^{-/-}$ is significantly lower than all of these expected values. Therefore, these results suggest that the absence of *DSC1* has an epistatic effect on permethrin resistance conferred by the $para^{ts1}$ mutation.

A similar pattern of epistasis was observed with sensitivity of these fly strains to deltamethrin. Compared to w^{1118A}, the $para^{ts1}$ strain was more than 6-fold less sensitive to deltamethrin, but the $DSC1^{-/-}$ strain was more than 2-fold more sensitive. However, the $para^{ts1}$;$DSC1^{-/-}$ double mutant was 1.4-fold less sensitive to deltamethrin compared with w^{1118A} (Table 2). Adding RRs, averaging mortality, and recalculating the LC_{50} using Probit analysis of the combined data from $DSC1^{-/-}$ and $para^{ts1}$ gives RRs of 3.9, 3.4 and 2.4, respectively, for $para^{ts1}$;$DSC1^{-/-}$. The 1.4 RR observed for $para^{ts1}$;$DSC1^{-/-}$ was significantly lower than all of these expected values.

The onset of knockdown due to deltamethrin exposure was more rapid for $DSC1^{-/-}$ mutant flies. The proportion of flies knocked down by deltamethrin was higher for $DSC1^{-/-}$ than all the other strains during the 5^{th} and 10^{th} min (Fig. 3A). The differences in the proportion of $DSC1^{-/-}$ flies knocked down compared to $para^{ts1}$ and $para^{ts1}$;$DSC1^{-/-}$ continued through the 15^{th} minute. There were differences in the speed of knockdown between w^{1118A} and $para^{ts1}$ at 10, and 15 min, and no differences between w^{1118A} and $para^{ts1}$;$DSC1^{-/-}$ or between $para^{ts1}$ and $para^{ts1}$;$DSC1^{-/-}$ at any time. There were no differences in knockdown proportion between any of the strains over the last 10 minutes of the knockdown bioassay. In addition, the time to knock down 50% of population was significantly faster for $DSC1^{-/-}$ than for all other strains. It took significantly longer to knockdown $para^{ts1}$ flies than to knockdown w^{1118A} flies, but no such difference was observed between $para^{ts1}$ and $para^{ts1}$;$DSC1^{-/-}$ or between w^{1118A} and $para^{ts1}$;$DSC1^{-/-}$ (Table 3).

Knockout of DSC1 confers resistance to DDT

Unexpectedly, $DSC1^{-/-}$ flies were 4-fold less sensitive to DDT compared to w^{1118A} (Table 2). The slope of the mortality response curve is significantly lower for $DSC1^{-/-}$ flies compared to w^{1118A} flies demonstrating a significant increase in the resistance ratios at

higher concentrations (i.e. RR @ LC_{95} = 27, Table 2). The lower slope of the mortality response curve also demonstrates a more heterogeneous response for *DSC1*$^{-/-}$ flies, suggesting *DSC1* knockout produces a number of changes in the nervous system to shape the mortality response curve. Consistent with previous results (Pittendrigh et al., 1997), the *para*^{ts1} mutation conferred a 12-fold decrease in DDT insensitivity. The 18-fold increase in the RR of *para*^{ts1};*DSC1*$^{-/-}$ flies was within the range to be considered additive with respect to both individual mutant lines.

Consistent with the mortality results, it took longer for *DSC1*$^{-/-}$ flies to be knocked down by exposure to DDT than *w*^{1118A} starting at minute 25 and continuing through the rest of the duration of the assay (Fig. 3B). The time to knock down 50% of the population was significantly faster for *w*^{1118A} compared to *DSC1*$^{-/-}$ (Table 3). Both *para*^{ts1} and *para*^{ts1};*DSC1*$^{-/-}$ flies were knocked down significantly less than *w*^{1118A} and *DSC1*$^{-/-}$ at 25 and 35 minutes, respectively, and these differences continued throughout the rest of the experiment. Both *para*^{ts1} and *para*^{ts1};*DSC1*$^{-/-}$ flies were not affected by DDT during the time course of this experiment, and these results are consistent with bioassay results demonstrating high levels of DDT insensitivity in those strains.

Computer modeling of the $DmNa_v$ 22 channel carrying the I265N mutation

We designate the I265 residue as I^{1k12} using the nomenclature that is universal for sodium channels and other P-loop ion channels (Du et al., 2013; Zhorov and Tikhonov, 2004). This designation reflects the location of the I^{1k12}N mutation: domain D**1**, lin**k**er-helix IL45 (k), and relative position **12** within the linker. The linker helix contains two recently identified pyrethroid-sensing residues I^{1k7} and V^{1k11} (Du et al., 2013). In our model of PyR2 in the mosquito sodium channel, the side chains of both I^{1k7} and V^{1k11} extend towards helix IIS6 and interact directly with the halogen-containing end of the pyrethroid molecule, which binds in the domain I/II interface, between helices IL45 and IIS6. In contrast the side chain of I^{1k12} faces lipids and does not form contacts with the bound pyrethroid molecule (Du et al., 2013).

In our model of the $DmNa_v$ I^{1k12}N channel with deltamethrin bound to PyR2 (Fig. 4), the N^{1k12} side chain also faces lipids and occurs too far from the pyrethroid molecule to noticeably interact with it. In high-resolution X-ray structures, 26.4% of asparagine residues participate in side chain-backbone hydrogen bonds. In the three most populated motifs the asparagine side chain accepts an H-bond from the backbone NH group two or three residues downstream or donates an H-bond to the backbone carbonyl four residues upstream (Vasudev et al., 2012). We used distance constraints to impose such H-bonds for N^{1k12} and Monte Carlo-minimized respective channel-deltamethrin complexes. The N^{1k12} H-bonds with the NH groups of two other pyrethroid sensing residues in IS5 S^{1o2} (Wang et al., 2014) and V^{1o3} (Du et al., 2013) caused noticeable backbone deformation in IL45 (Fig. 4). However, in all the structures the N^{1k12} side chain remained far from deltamethrin. Thus, the effect of I^{1k12}N mutation on pyrethroid sensitivity is likely allosteric.

DISCUSSION

It is well established that both DDT and pyrethroids act on Na_v channels and modify the gating of Na_v channels (Bloomquist and Soderlund, 1988; Narahashi, 2002; Soderlund, 2010). Identification of naturally occurring mutations in insect Na_v channels that confer cross-resistance to DDT and pyrethroids further confirms that DDT and pyrethroids have a rather common mode of action. Although the DSC1 protein shares a high sequence homology with the $DmNa_v$ channel, the role of the DSC1 channel in the molecular action of DDT and pyrethroids remain unclear. In this study, we first examined the effects of DDT and pyrethroids on $DmNa_v$ channels carrying the *para*^{ts1} mutation I265N ($I^{1k12}N$) in *Xenopus* oocytes and then evaluated the toxicity of DDT and pyrethroids against *DSC1* knockout *D. melanogaster* flies as well as *DSC1* knockout flies carrying the *para*^{ts1} mutation (I265N). Our findings revealed striking differences in the activities of DDT and pyrethroids on flies lacking expression of DSC1 channels, providing the first molecular evidence that DSC1 channels play distinct roles in the neurotoxicity of DDT and pyrethroids *in vivo*.

Our results from both bioassays and functional analysis corroborate the fact that the *para*^{ts1} mutation (I265N) causes reduced sensitivity to DDT and pyrethroids (permethrin and deltamethrin). Our finding is therefore different from the finding from an early study, which argued for a hypersensitivity of *para*^{ts1} flies to deltamethrin (Pedra et al., 2004). It is likely that Pedra and colleagues compared the sensitivity of the *para*^{ts1} mutant to that of another susceptible strain Canton-S, which had an LC_{50} of 0.343 μg deltamethrin/vial, whereas the *w*^{1118A} strain used in this study had a much lower LC_{50} value of 0.011 (95% CI 0.009-.012) μg deltamethrin/vial. The LC_{50} of deltamethrin for *para*^{ts1} flies in our experiments was 0.067 (95% CI 0.058-0.078) μg/vial (Table 2), which is in agreement with the LC_{50} of deltamethrin (0.049 μg /vial; 95% CI 0.019-0.091) previously reported (Pedra et al., 2004). Furthermore, the *para*^{ts2} strain which carries the same I265N mutation is also more resistant to deltamethrin (Pittendrigh et al., 1997). Taken together, these results demonstrate that the I265N mutation is responsible for resistance of *para*^{ts1} flies to DDT and pyrethroids.

The mechanism through which the I265N mutation confers DDT and pyrethroid resistance appears to be a unique mechanism that is not currently known because the mutation does not affect channel gating properties and, based on computer modeling, the I265N mutation does not appear to be located within either of the known pyrethroid-binding sites. In contrast, many other Na_v channel mutations that reduce pyrethroid sensitivity occur at residues that either are located within the two pyrethroid-binding sites or that are involved in regulating channel kinetics and voltage-dependent gating (Du et al., 2013; Soderlund and Knipple, 2003; Usherwood et al., 2007). Nevertheless, the I265N ($I^{1k12}N$) mutation appeared to allosterically deform pyrethroid receptor PyR2 resulting in reduced pyrethroid binding. Isoleucine has long been known to stabilize α-helices, whereas asparagine is a prominent α-helix breaker (Chou and Fasman, 1974). Therefore, the I265N mutation could destabilize the α-helical structure of the IL45 linker. Notably, there is another helix-breaking residue, G^{1k9}, three positions upstream of I265N ($I^{1k12}N$). The combined effects of two helix-destabilizing residues, G^{1k9} and N265 (N^{1k12}), may be likely sufficient to deform the linker helix. This deformation then may change the mutual disposition of the pyrethroid-sensing residues, I^{1k7}

and V^{1k11}, on the one hand, and helix IIS6, on the other hand, thus distorting the geometry of PyR2.

Unlike the *para*^{ts1} mutation, *DSC1* knockout increased sensitivity of flies to pyrethroids (this study; Zhang et al., 2013), suggesting that the DSC1 channel is not a direct target of pyrethroid insecticides, but has epistatic effects on pyrethroid action on the Na_v channels. In addition, *DSC1* knockout did not alter the expression of Na_v channel transcripts in these flies (Zhang et al., 2013). Our previous study showed that loss of the DSC1 channel results in membrane hyperexcitability and the DSC1 channel stabilizes the nervous system likely through regulating synaptic transmission (Zhang et al., 2013). The membrane hyperexcitability in the *DSC1* knockout is expected to potentiate the activating effects of pyrethroids on sodium channels/the nervous system, which could explain why *DSC1* knockout flies are more sensitive to pyrethroids. Consistent with this expectation, we found that the permethrin and deltamethrin resistance caused by *para*^{ts1} is significantly antagonized by the *DSC1* knockout mutation. The antagonism of permethrin and deltamethrin resistance between the *para*^{ts1} and *DSC1* mutations is a unique example in which two homologous genes have a negative epistatic interaction to modulate insecticide toxicity by exerting opposing effects (inhibitory by DSC1 channels and excitatory by Na_v channels) on membrane excitability.

The epistatic effects that *DSC1* knockout has on pyrethroid resistance may have significant practical implications. For example, identifying compounds that affect the activity of DSC1 channels could theoretically be used to enhance the sensitivity of pyrethroid-resistant insects to pyrethroids. Importantly, the DSC1 family of genes appears to be restricted to only insects and several invertebrate phyla; they are not found in mammals and other vertebrates (Cui et al., 2012). In this regard, the DSC1/BSC1 family of ion channels represents an attractive target for future development of new and safe chemicals against insect pests.

Because DDT and pyrethroids exert rather similar effects on Na_v channels, we had expected that knockout of the DSC1 channel would also potentiate the action of DDT. Surprisingly, however, *DSC1* knockout flies were less sensitive to DDT compared to *w*^{1118A} flies in both toxicity and knockdown bioassays. Furthermore, the *para*^{ts1};*DSC1*$^{-/-}$ double mutant showed an additive effect of these two gene mutations, which is in contrast to the pyrethroid antagonism described above. These results raise the intriguing possibility that the DSC1 channel could be a major target of DDT activity. This is an important finding because although the Na_v channel has been the focus for understanding the mechanism of DDT resistance, DDT-resistance has been linked to a mutation in the DSC1 channel (Amichot et al., 1992). As such, a significant implication of this study is that future investigations of the DDT mode of action and resistance mechanisms to DDT in mosquitoes should include characterization of the *DSC1* gene, in addition to the Na_v gene.

The involvement of the DSC1 channel in the action of DDT, but not of pyrethroids, could also explain some puzzling findings in earlier studies. For example, injection of a sublethal dose of the sodium channel blocker tetrodotoxin (TTX) protects cockroach peripheral nervous system (PNS) from the effects of allethrin, a pyrethroid insecticide (Gammon, 1978). However, TTX does not protect cockroach PNS from the effect of DDT,

demonstrating that DDT acts on a target aside from a Na_v channel (Gammon, 1977). We predict that the DSC1 channel might be the non-sodium channel target because TTX does not block the DSC1 channel (Zhang et al., 2011).

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Literature Cited

Amichot M, Castella C, Cuany A, Berge JB, Pauron D. Target modification as a molecular mechanism of pyrethroid resistance in *Drosophila melanogaster*. Pestic. Biochem. Physiol. 1992; 44:183–190.

Beeman RW. Recent advances in mode of action of insecticides. Annu. Rev. Entomol. 1982; 27:253–281. [PubMed: 7044265]

Bloomquist, JR.; Mittler, TE.; Radovsky, FJ.; Resh, VH. Ann. Rev. Entomol. Annual Reviews, Inc.; Palo Alto: 1996. Ion Channels as Targets for Insecticides; p. 163-190.

Bloomquist JR, Soderlund DM. Pyrethroid insecticides and DDT modify alkaloid-dependent sodium channel activation and its enhancement by sea anemone toxin. Molec. Pharmacol. 1988; 33:543–550. [PubMed: 2452970]

Burton MJ, Mellor IR, Duce IR, Davies TGE, Field LM, Williamson MS. Differential resistance of insect sodium channels with kdr mutations to deltamethrin, permethrin and DDT. Insect Biochem. Mol. Biol. 2011; 41:723–732. [PubMed: 21640822]

Chou PY, Fasman GD. Conformational parameters for amino acids in helical, Î²-sheet, and random coil regions calculated from proteins. Biochemistry. 1974; 13:211–222. [PubMed: 4358939]

Cui Y-J, Yu L-L, Xu H-J, Dong K, Zhang C-X. Molecular characterization of *DSC1* orthologs in invertebrate species. Insect Biochem. Molec. Biol. 2012; 42:353–359. [PubMed: 22321571]

Dong K, Du Y, Rinkevich F, Nomura Y, Xu P, Wang L, Silver K, Zhorov BS. Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem. Molec. Biol. 2014; 50:1–17. [PubMed: 24704279]

Du Y, Nomura Y, Satar G, Hu Z, Nauen R, He SY, Zhorov B, Dong K. Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. PNAS. 2013; 110:11785–11790. [PubMed: 23821746]

Eaton JL, Sternburg J. Temperature and the mode of action of DDT on the nervous system of *Periplaneta americana*. J. Ins. Physiol. 1964; 10:471–485.

Farley JM, Narahashi T, Holan G. The mechanism of action of a DDT analog on the crayfishneuromuscular junction. Neurotoxic. 1979; 1:191–207.

Feng G, Deák P, Chopra M, Hall LM. Cloning and functional analysis of TipE, a novel membrane protein that enhances Drosophila para sodium channel function. Cell. 1995; 82:1001–1011. [PubMed: 7553842]

Gammon, DW. Neural correlates of insecticide poisoning in the cockroach. University of Cambridge; 1977.

Gammon DW. Neural effects of allethrin on the free walking cockroach, *Periplaneta americana*: an investigation using defined doses at 15 and 32°C. Pestic. Sci. 1978; 9:79–91.

Hille B. Pharmacological modifications of the sodium channels of frog nerve. J. Gen. Physiol. 1968; 51:199–219. [PubMed: 5641635]

Kulkarni NH, Yamamoto AH, Robinson KO, Mackay TFC, Anholt RRH. The DSC1 channel, encoded by the *smi60E* locus, contributes to odor-guided behavior in *Drosophila melanogaster*. Genetics. 2002; 161:1507–1516. [PubMed: 12196396]

Lalonde DIV, Brown AWA. The effects of insecticides on teh action potentials of insect nerve. Canadian J. Zool. 1954; 32:74–81.

Narahashi T. Effect of the insecticide allethrin on membrane potentials of cockroach giant axons. J. Cellular Comp. Physiol. 1962; 59:61–65. [PubMed: 14478362]

Narahashi, T. Mode of Action of Pyrethroids. Bulletin of the World Health Organization; International Conference on Alternative Insecticides for Vector Control; Atlanta. World Health Organization; 1971.

Narahashi T. Nerve Membrane Na+ Channels as Targets of Insecticides. TiPS Rev. 1992; 13:236–241.

Narahashi T. Nerve membrane ion channels as the target site of insecticides. Mini Rev. Medic. Chem. 2002; 2:419–432.

Narahashi T. Neuroreceptors and ion channels as the basis for drug action: past, present, and future. J Pharmacol Exp Ther. 2000; 294:1–26. [PubMed: 10871290]

Narahashi T, Zhao X, Ikeda T, Nagata K, Yeh J. Differential actions of insecticides on target sites: basis for selective toxicity. Human & Experimental Toxicology. 2007; 26:361–366. [PubMed: 17615118]

Narahasi T, Haas HG. DDT: Interaction with nerve membrane conductance changes. Science. 1967; 157:1438–1440. [PubMed: 6037859]

Olson R, Liu Z, Nomura Y, Song W, Dong K. Molecular and functional characterization of voltage-gated sodium channel variants from *Drosophila melanogaster*. Insect Biochem. Molec. Biol. 2008; 38:604–610. [PubMed: 18405837]

O'Reilly AO, Khambay BPS, Williamson MS, Field LA, Wallace BA, Davies TGE. Modelling insecticide-binding sites in the voltage-gated sodium channel. Biochem. J. 2006; 396:255–263. [PubMed: 16475981]

Pedra JHF, Hostetler A, Gaffney PJ, Reenan RA, Pittendrigh B. Hyper-susceptibility to deltamethrin in *parats-1* DDT resistance *Drosophila melanogaster*. Pestic. Biochem. Physiol. 2004; 78:58–66.

Perry, AS.; Yamamoto, I.; Ishaaya, I.; Perry, RY. Insecticides in agriculture and environment; Retrospects and prospects. Springer Publishing Co.; New York, NY: 1998.

Pittendrigh B, Reenan R, ffrench-Constant RH, Ganetzky B. Point mutations in the *Drosophila* sodium channel gene *para* associated with resistance to DDT and pyrethroid insecticides. Mol. Gen. Genet. 1997; 256:602–610. [PubMed: 9435785]

Rinkevich FD, Du Y, Dong K. Diversity and convergence of sodium channel mutations involved in resistance to pyrethroids Pestic. Biochem. Physiol. 2013; 106:93–100.

Rinkevich FD, Scott JG. Reduction of *dADAR* activity affects the sensitivity of *Drosophila melanogaster* to spinosad and imidacloprid. Pesticide Biochemistry and Physiology. 2012; 104:163–169.

Salkoff L, Butler A, Wei A, Scavarda N, Giffen K, Ifune C, Goodman R, Mandel G. Genomic organization and deduced amino acid sequence of a putative sodium channel gene in *Drosophila*. Science. 1987; 237:744–749. [PubMed: 2441469]

Shafer TJ, Meyer DA. Effects of pyrethroids on voltage-sensitive calcium channels: a critical evaluation of strengths, weaknesses, data needs, and relationship to assessment of cumulative neurotoxicity. Tox. Appl. Pharmacol. 2004; 196:303–318.

Soderlund, DM.; Gilbert, LI.; Iatrou, K.; Gill, SS. Comprehensive molecular insect science. Elsevier; Amsterdam: 2005. Sodium channels; p. 1-24.

Soderlund DM. State-dependent modification of voltage-gated sodium channels by pyrethroids. Pesticide Biochemistry and Physiology. 2010; 97:78–86. [PubMed: 20652092]

Soderlund DM, Knipple DC. The molecular biology of knockdown resistance to pyrethroid insecticides. Insect Biochem. Molec. Biol. 2003; 33:563–577. [PubMed: 12770575]

Tatebayashi H, Narahashi T. Differential mechanism of action of the pyrethroid tetramethrin on tetradotoxin-sensitive and tetradotoxin-resistant sodium channels. J. Pharmacol. Exper. Therap. 1994; 270:595–603. [PubMed: 8071852]

Usherwood PNR, Davies TGE, Mellor IR, O'Reilly AO, Peng F, Vais H, Khambay BPS, Field LM, Williamson MS. Mutations in DIIS5 and the DIIS4-S5 linker of *Drosophila melanoaster* sodium channel define binding domains for pyrethroids and DDT. FEBS Letters. 2007; 581:5485–5492. [PubMed: 17991435]

Usherwood PNR, Vais H, Khambay BPS, Davies TGE, Williamson MS. Sensitivity of the Drosophila *para* sodium channel to DDT is not lowered by the *super-kdr* mutation M918T on the IIS4-S5 linker that profoundly reduces sensitivity to permethrin and deltamethrin. FEBS Lett. 2005; 579:6317–6325. [PubMed: 16263118]

Vais H, Williamson MS, Goodson SJ, Devonshire AL, Warmke JW, Usherwood PN, Cohen CJ. Activation of Drosophila sodium channels promotes modification by deltamethrin. Reductions in affinity caused by knock-down resistance mutations. J Gen Physiol. 2000; 115:305–318. [PubMed: 10694259]

Vasudev PG, Banerjee M, Ramakrishnan C, Balaram P. Asparagine and glutamine differ in their propensities to form specific side chain-backbone hydrogen bonded motifs in proteins. Proteins: Structure, Function, and Bioinformatics. 2012; 80:991–1002.

Vijverberg HPM, vanden Zalm JM, vanden Bercken J. Similar mode of action of pyrethroids and DDT on sodiumchannel gating in myelinated nerves. Nature. 1982; 295:601–603. [PubMed: 6276777]

Warmke J, Reenan RA, Wang P, Qian S, Arena JP, Wang J, Wunderler D, Liu K, Kaczorowski GJ, Van der Ploeg LH, Ganetzky B, Cohen CJ. Functional expression of Drosophila para sodium channels. Modulation by the membrane protein TipE and toxin pharmacology. J Gen Physiol. 1997; 110:119–133. [PubMed: 9236205]

Zhang T, Liu Z, Song W, Du Y, Dong K. Molecular characterization and functional expression of the DSC1 channel. Insect Biochem. Molecular Bio. 2011; 41:451–458.

Zhang T, Wang Z, Wang L, Luo N, Jiang L, Liu Z, Wu CF, Dong K. Role of the DSC1 channel in regulating neuronal excitability in *Drosophila melanogaster*: Extending nervous system stability under stress. PLoS Genetics. 2013; 9:e1003327. [PubMed: 23505382]

Zhorov BS, Tikhonov DB. Potassium, sodium, calcium, and glutamate-gated channels: Pore architechture and ligand action. J. Neurochem. 2004; 88:782–799. [PubMed: 14756799]

Zhou W, Chung I, Goldin A, Dong K. A voltage-gated calcium-selective channel encoded by a sodium channel-like gene. Neuron. 2004; 42:101–112. Z., L. [PubMed: 15066268]

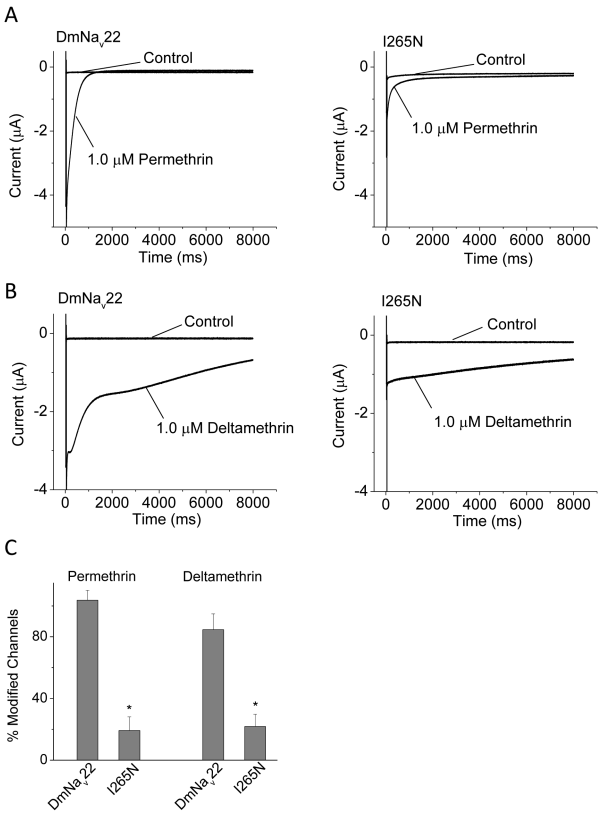

Figure 1.

The para^{ts1} (I265N) channels are less sensitive to the pyrethroids permethrin and deltamethrin than DmNa$_v$22. A and B. Tail-current induced by permethrin (A) or deltamethrin (B) from DmNa$_v$22 or I265N channels. The I265N mutation is located in the linker connecting S4 and S5 of domain I. C. Percent channel modification by pyrethroids. The number of oocytes for each channel was >5. The protocols and quantitative analysis of pyrethroid-induced tails are described in the Materials and Methods. Data are the average ± SEM. * indicates significantly different from DmNa$_v$22 channels (Student's t-test, $P < 0.05$).

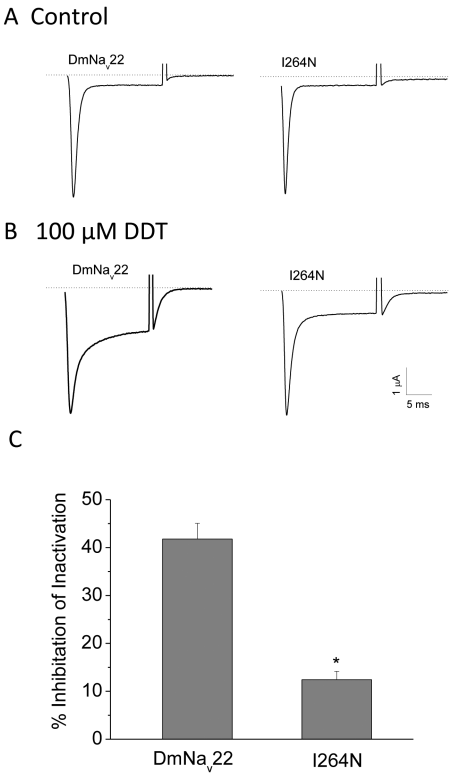

Figure 2.

The para^{ts1} (I265N) channels are less sensitive to DDT than DmNa$_v$22 channels. A. Representative sodium current traces from oocytes expressing DmNa$_v$22 and I265N channels. B. Representative sodium current traces from DmNa$_v$22 and I265N channels after overnight incubation in 100 µM DDT. C. Inhibition of fast inactivation by DDT. The number of oocytes for each channel was >15. The protocol and quantitative analysis of DDT inhibition are described in the Materials and Methods. Data are the average ± SEM. * indicates significantly different from DmNa$_v$22 channels (Student's t-test, $P < 0.05$).

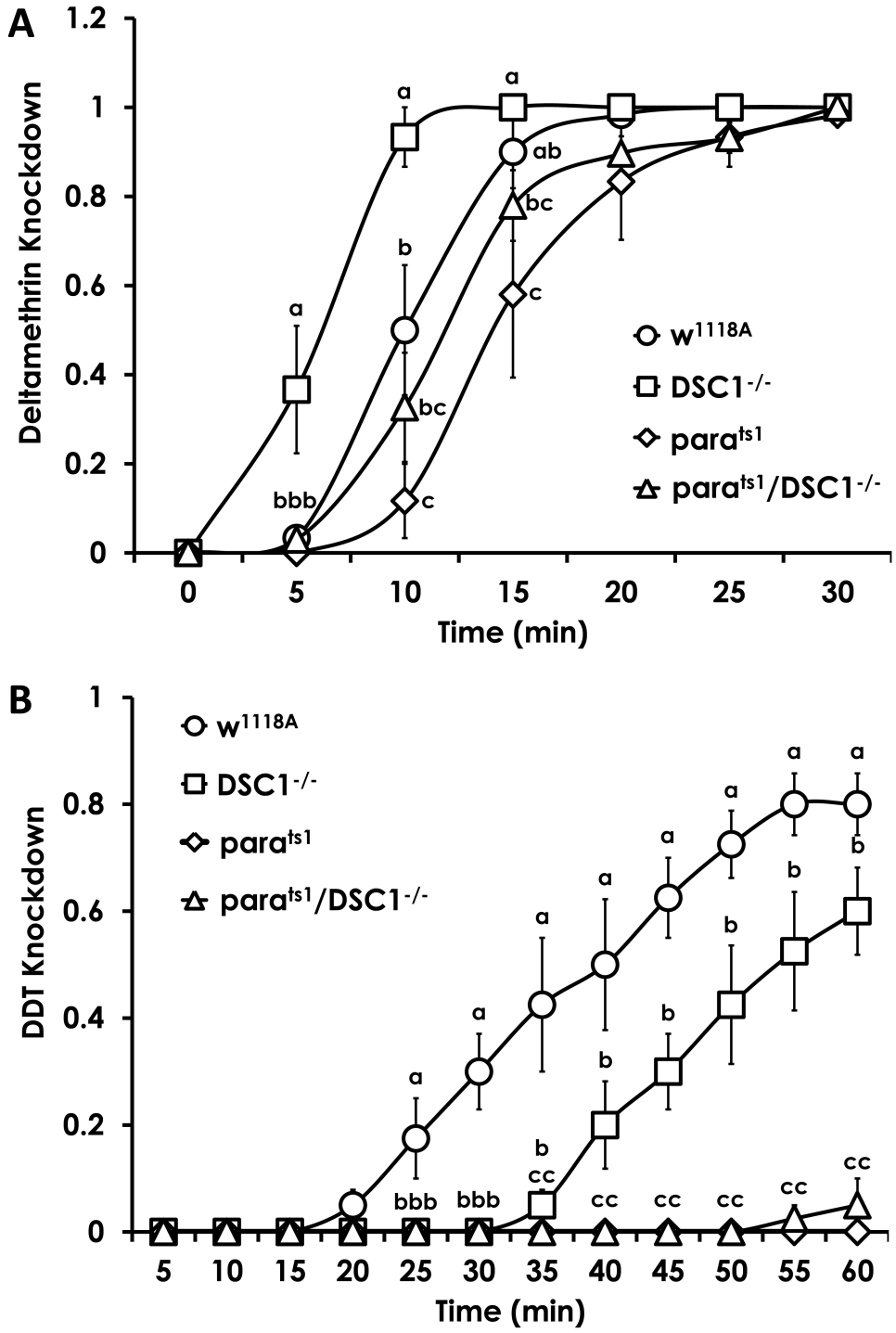

Figure 3.

The time course of knockdown of *D. melanogaster* exposed to deltamethrin (A) and DDT (B). *DSC1*$^{-/-}$ exhibited increased knockdown by deltamethrin, but demonstrated delayed knockdown by DDT. There was no significant knockdown of *para*^{ts1} or *para*^{ts1};*DSC1*$^{-/-}$ by DDT. Data are the average ± SEM. Letters above each data point at every 5 minute intervals

indicate significant differences at each time point (One-Way ANOVA, Fishers LSD, $P<0.05$).

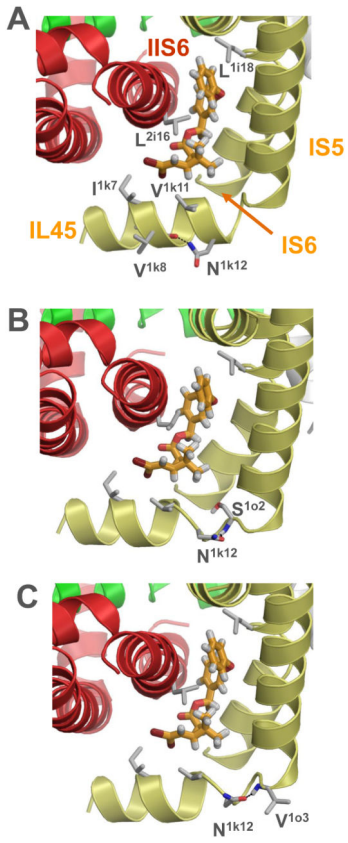

Figure 4.

$K_v1.2$-based model of the open $DmNa_v$ channel phenotype N^{1k12} (N265) with a deltamethrin molecule docked to PyR2 as described before (Du et al., 2013). Helices in domains I, II, III and IV are shown, respectively, by yellow, red, green, and white ribbons. Pyrethroid-sensing residues in the I/II domain interface, as well as N^{1k12} and its possible H-bonding partners V^{1k8} (*A*), S^{1o2} (*B*) and V^{1o3} (*C*) are shown as sticks. Asparagine N^{1k12} is far from deltamethrin and does not contribute to the pyrethroid binding site. However, an H-bond between the N^{1k12} side chain and a backbone atom can deform the backbone and thus affect the mutual disposition of deltamethrin and pyrethroid-sensing residues in PyR2.

Table 1

Effects of the *para*^{ts1} mutation I265N (I^{1k12}N) on the voltage-dependence of sodium channel activation and inactivation

	Activation		Inactivation		
Na^+ Channel Type	**$V_{1/2}$ (mV)**	**k (mV)**	**$V_{1/2}$ (mV)**	**k (mV)**	**n**
$DmNa_v22$	−25.98 ± 0.47	5.46 ± 0.23	−46.06 ± 0.28	5.40 ± 0.13	8
I265N	−23.90 ± 1.40	7.46 ± 0.60	−45.86 ± 0.90	5.85 ± 0.19	10

Table 2

Toxicity of permethrin and deltamethrin against w^{1118A}, *para*^{ts1}, *DSC1*$^{-/-}$ and *para*^{ts1}/*DSC1*$^{-/-}$

	Permethrin			
Strain	**n**	**LC_{50}**	**Slope**	**RR**
w^{1118A}	1140	2.0 (1.9-2.1)	3.8 (±0.2)	1.0[a]
DSC1$^{-/-}$	720	0.4 (0.3-0.5)	1.8(±0.1)	−5.0[b]
para^{ts1}	760	9.2 (8.0-10.5)	2.1 (±0.1)	4.6[c]
para^{ts1}/*DSC1*$^{-/-}$	903	1.1 (1.0-1.2)	2.7 (±0.2)	−1.8[d]
	Deltamethrin			
	n	**LC_{50}**	**Slope**	**RR**
w^{1118A}	780	0.011(0.009-0.012)	2.6 (±0.2)	1.0[a]
DSC1$^{-/-}$	400	0.004 (0.004-0.005)	3.4 (±0.3)	−2.4[b]
para^{ts1}	899	0.067 (0.058-0.078)	1.4 (±0.1)	6.3[c]
para^{ts1}/*DSC1*$^{-/-}$	948	0.015 (0.014-0.016)	3.8 (±0.3)	1.4[d]
	DDT			
	n	**LC_{50}**	**Slope**	**RR**
w^{1118A}	760	0.6 (0.6-0.7)	3.4 (±0.3)	1.0[a]
DSC1$^{-/-}$	980	2.5 (2.1-2.9)	1.2 (±0.1)	4.0[b]
para^{ts1}	820	7.7 (6.2-9.3)	1.1 (±0.1)	12.1[c]
para^{ts1}/*DSC1*$^{-/-}$	960	11.6 (7.9-15.5)	0.7 (±0.1)	18.4[c]

The *DSC1* knockout significantly antagonizes permethrin and deltamethrin resistance conferred by the *para*^{ts1} mutation, but enhances DDT resistance in an additive manner. The LC_{50} values are in units of μg/vial with the 95% CI in parenthesis. The values in parenthesis next to the slope value are the ± SE of the slope. The Resistance Ratios (RR) are calculated relative to the LC_{50} of w^{1118A}. Letters in the RR column represent significant differences between strains within each insecticide treatment.

Table 3

Time (min) to knockdown (KD_{50}) for 50% of flies exposed to 0.02 mg/ml deltamethrin or 2 mg/ml DDT

	KD_{50}	
Strain	**Deltamethrin**	**DDT**
w^{1118A}	8.8 (±0.6)[a]	41.4 (±3.4)[a]
DSC1$^{-/-}$	5.1 (±0.7)[b]	59.1 (±3.5)[b]
para^{ts1}	15.0 (±2.2)[c]	NA
para^{ts1}/*DSC1*$^{-/-}$	11.0 (±1.2)[ac]	NA

KD_{50} values are shown ± SE. We were unable to calculate KD_{50} values for DDT against *para*^{ts1} and *para*^{ts1}/*DSC1*$^{-/-}$ since there was no significant knockdown. Different letters within each column indicate significance of differences (See Fig. 3)