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In vivo functional analysis of the *Drosophila melanogaster* nicotinic acetylcholine receptor *Dα6* using the insecticide spinosad

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ABSTRACT

The vinegar fly, *Drosophila melanogaster,* has been used to identify and manipulate insecticide resistance genes. The advancement of genome engineering technology and the increasing availability of pest genome sequences has increased the predictive and diagnostic capacity of the *Drosophila* model. The *Drosophila* model can be extended to investigate the basic biology of the interaction between insecticides and the proteins they target. Recently we have developed an *in vivo* system that permits the expression and study of key insecticide targets, the nicotinic acetylcholine receptors (nAChRs), in controlled genetic backgrounds.

Here this system is used to study the interaction between the insecticide spinosad and a nAChR subunit, Dα6. Reciprocal chimeric subunits were created from Dα6 and Dα7, a subunit that does not respond to spinosad. Using the *in vivo* system, the Dα6/Dα7 chimeric subunits were tested for their capacity to respond to spinosad. Only the subunits containing the C-terminal region of Dα6 were able to respond to spinosad, thus confirming the importance this region for spinosad binding.

A new incompletely dominant, spinosad resistance mechanism that may evolve in pest species is also examined. First generated using chemical mutagenesis, the $D\alpha6^{P146S}$ mutation was recreated using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/*Cas9* system, the first use of this technology to introduce a resistant mutation into a controlled genetic background. Both alleles present with the same incompletely dominant, spinosad resistance phenotype, proving the P146S replacement to be the causal mutation. The proximity of the P146S mutation to the conserved Cys-loop indicates that it may impair the gating of the receptor. The results of this study enhance the understanding of nAChR structure:function relationships.

1. Introduction

Nicotinic acetylcholine receptors (nAChR) mediate fast action potentials at cholinergic synapses upon activation by acetylcholine (ACh). They are members of the Cys-loop ligand-gated ion channel (LGIC) superfamily that also includes 5-HT_3, ɣ-aminobutyric-acid (GABA) and glycine receptors (Grutter et al., 2005; Lester et al., 2004). nAChRs are formed by 5 subunits surrounding a central cation permeable pore that transiently opens when activated by agonists. Each subunit has an extra cellular ligand binding domain coupled to an ion pore domain that consists of four transmembrane segments (TM1 – TM4). The ligand binding domain has 6 important regions for binding ACh (loops A – F), as well as the characteristic Cys-loop motif ($CX_{13}C$) that is crucial in the fast gating of the receptor (Grutter et al., 2005). ACh binding occurs between the interface of two subunits, each of which contributes 3 loops to the binding pocket. The principle face of the binding pocket is always formed by loops A – C of an α subunit, whereas the complementary face is formed by the neighbouring subunit that contributes loops D – F. Loop C contains two vicinal Cysteine residues necessary for ACh binding. Subunits containing this di-Cys motif are referred to as α subunits (Corringer et al., 2000; Karlin, 2002). nAChR subtypes can be homomeric containing only a single type of α subunit, or heteromeric either mixed α-only or α and non-α subunits. The four α-helical transmembrane segments are arranged in an orientation that has TM2 lining the ion pore. Separating the TM3 and TM4 segments is a large cytoplasmic loop, variable in length and

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sequence that is important in the assembly and distribution of receptors within a cell (Kracun et al., 2008; Williams et al., 1998).

Nicotinic receptors are well conserved across animal taxa where they are expressed in both neuronal and non-neuronal cells. In *Drosophila melanogaster*, ACh is the major excitatory neurotransmitter and expression of nAChRs is highly enriched in the central nervous system (Lee and O'Dowd, 1999). Invertebrate nAChR families generally have fewer subunits then vertebrates. *D. melanogaster* has 10 subunits, 7 α subunits (Dα1 − Dα7) and 3 β subunits (Dβ1 − Dβ3) (Sattelle et al., 2005). While *Drosophila* have a limited number of subunit genes, in theory, they could produce a massive number of different receptor subtypes composed of different combinations of receptor subunits. Subunit diversity can also be increased by alternate splicing and/or A-to-I RNA editing (Grauso et al., 2002; Jin et al., 2007). Although the number of subunit genes vary in other insect species, a *Dα6* orthologue is typically present and is highly conserved (Jones et al., 2006, 2010, 2005; Shao et al., 2007).

Spinosad is the first member of the spinosyn class of insecticides. First identified in metabolites from the soil actinomycete *Saccharopolyspora spinosa* (Mertz and Yao, 1990), it is a mixture of two macrocyclic lactones, spinosyn A and spinosyn D. Spinosad is primarily an allosteric agonist of insect nAChRs (Salgado, 1997), but it may also elicit some effect on GABA receptor function (Watson, 2001). Unlike neonicotinoids, which also target nAChRs, spinosad has been shown to activate nAChRs that have been blocked by α-bungarotoxin, indicating that it does not bind to the α-bungarotoxin/ACh binding site. Consistent with this observation, spinosad acts synergistically with ACh, enhancing voltage-clamp inward currents in dissociated cockroach neurons (Salgado and Saar, 2004). The *D. melanogaster* Dα6 subunit was identified as a major target of spinosad in a recessive null mutant identified over a deficiency chromosome in a line that exhibited over 1000-fold resistance (Perry et al., 2007). The viability of the mutant lacking the highly conserved Dα6 protein is striking, as is the lack of obvious fitness costs under laboratory conditions. Several other recessive spinosad resistant *dα6* mutants have also been isolated in *Drosophila* using ethyl methanesulfonate (EMS) mutagenesis. The majority of these mutations are premature truncations of the Dα6 protein (Perry et al., 2015; Watson et al., 2010). It appears that any mutation that causes a loss of *Dα6* function will confer resistance by removing the capacity for spinosad to activate its target, nAChR subtypes that contain Dα6. Importantly major fitness costs associated with Dα6 loss of function mutation have not been reported, reducing the selection pressure on having specific mutations confer the resistance. Since there are a vast array of possible mutations that could cause a loss of function, spinosad resistance is predicted to evolve more readily than if specific amino acid replacement mutations were required. *Dα6* orthologues are well conserved across many insect species in both amino acid identity and genomic structure, with intron/exon boundaries shared as widely as its closest vertebrate orthologue, the vertebrate α7 nAChR subunit (Grauso et al., 2002; Sattelle et al., 2005). It is therefore not surprising that recessive mutations in *Dα6* orthologues in other insect species have been associated with spinosad resistance.

In diamondback moth, *Plutella xylostella,* field resistance to spinosad has been attributed to a mis-spliced transcript of *Pxα6*. The mis-spliced transcript results in a truncated protein that lacks the majority of the intracellular loop and the TM4 domain (Baxter et al., 2010). The Pxα6 truncation is similar to the $d\alpha6^{W337^*}$ allele used in this study as described in Perry et al., 2015. In the *Dα6* orthologue of both the western flower thrips, *Frankliniella occidentalis* and the melon thrips, *Thrips palmi*, the same G275E residue replacement has been associated with spinosad resistance (Bao et al., 2014; Puinean et al., 2013). The G275E replacement occurs 4 residues from the start of TM3 segment. This replacement is in close proximity to an analogous amino acid in the glutamate-gated chloride (GluCl) channel of *Caenorhabditis elegans*, which has been implicated in contributing to the ivermectin binding site (Hibbs and Gouaux, 2011). Like spinosad, ivermectin is a mixture of two macrocyclic lactones. The structural similarities between the two chemicals and their Cys-loop receptor targets may indicate they have similar binding sites. Due to the difficulties of heterologous expression of insect nAChRs, the effect of spinosad on the human $\alpha7_{(5)}$ nAChR was investigated. Spinosad was found to act as a negative allosteric modulator on the human $\alpha7_{(5)}$ nAChR and the introduction of the analogous replacement (A272E) reduces this modulatory effect (Puinean et al., 2013). This result suggests that spinosad binds at an allosteric site on nAChRs at a similar position to that of the ivermectin binding site on GluCl channels.

This study uses transgenic expression of *Dα6* and chimeric subunits in *D. melanogaster* to investigate the interactions between Dα6, spinosad binding and resistance mechanisms. By using response to spinosad as a proxy for insecticide function, the contribution of different subunit isoforms and mutations can be studied *in vivo*. Previous work has shown that expression of wild-type Dα6 is able to rescue the spinosad response while expression of Dα7 does not (Perry et al., 2015). To investigate regions important for spinosad binding two reciprocal chimeric subunits were created, fusing the N-terminal half of Dα6 to the C-terminal half of Dα7 and vice versa. These constructs were then expressed in a spinosad resistant, *Dα6* null background to test their ability to rescue susceptibility to spinosad. EMS mutagenesis and selection experiments revealed an incompletely dominant spinosad resistant allele that was mapped and characterised. Sequencing uncovered a single non-synonymous point mutation in the ligand binding domain of Dα6 located between two functionally important regions of the subunit, loop E and the Cys-loop. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/*Cas9* system was used to confirm the identified mutation as the causative mutation and transgenic expression was used to investigate the mutations consequences on spinosad susceptibility.

2. Materials & methods

2.1. Fly lines

Armenia[14] is an isofemale line derived from the Armenia[60] strain (DGRC stock no. 103394) used in the EMS mutagenesis screen due to its high fecundity. $d\alpha6^{W337^*}$ and $d\alpha6^{nx}$ are recessive *Dα6* alleles that confer high levels of spinosad resistance. The $d\alpha6^{W337^*}$ line has a G -> A transition mutation in exon 9 of *Dα6* resulting in a premature stop codon, that truncates the protein in the large intracellular loop. While the molecular lesion in $d\alpha6^{nx}$ has not been identified in this mutant, very little, if any, *dα6* transcript is produced (Perry et al., 2015). dα6 > GAL4 has a pCaSpeR-Gal4 insertion on the X chromosome containing 1069bp from position +84 to −985 (2L: 9887199..9886130) relative to the transcription start site of *Dα6*. When used to drive expression of a wildtype UAS-*Dα6* cDNA construct in the $d\alpha6^{nx}$ mutant background it restores susceptibility (Perry et al., 2015). elav > GAL4 (DGRC stock no. 458) is an enhancer trap line that expresses GAL4 in neurons from early embryogenesis. ϕ86Fb (DGRC stock no. 24749) is the ϕ31C integrase background line that has an attP insertion site on chromosome arm 3R (Bischof et al., 2007). The double balancer line used has the genotype $w^{[*]};If^{[*]}/CyO;MKRS/TM6b, Tb^{[1]}$. RAL-059 from the Drosophila Genome Reference Panel (http://dgrp.gnets.ncsu.edu/) was chosen as the wildtype spinosad susceptible line for CRISPR experiments.

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