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Role of nicotinic acetylcholine receptor subunits in the mode of action of neonicotinoid, sulfoximine and spinosyn insecticides in *Drosophila melanogaster*

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A B S T R A C T

Insecticides remain valuable tools for the control of insect pests that significantly impact human health and agriculture. A deeper understanding of insecticide targets is important in maintaining this control over pests. Our study systematically investigates the nicotinic acetylcholine receptor (nAChR) gene family, in order to identify the receptor subunits critical to the insect response to insecticides from three distinct chemical classes (neonicotinoids, spinosyns and sulfoximines). Applying the CRISPR/Cas9 gene editing technology in *D. melanogaster*, we were able to generate and maintain homozygous mutants for eight nAChR subunit genes. A ninth gene (*Dβ1*) was investigated using somatic CRISPR in neural cells to overcome the low viability of the homozygous germline knockout mutant. These findings highlight the specificity of the spinosyn class insecticide, spinosad, to receptors containing the Dα6 subunit. By way of contrast, neonicotinoids are likely to target multiple receptor subtypes, beyond those receptor subunit combinations previously identified. Significant differences in the impacts of specific nAChR subunit deletions on the resistance level of flies to neonicotinoids imidacloprid and nitenpyram indicate that the receptor subtypes they target do not completely overlap. While an R81T mutation in β1 subunits has revealed residues co-ordinating binding of sulfoximines and neonicotinoids differ, the resistance profiles of a deletion of *Dβ1* examined here provide new insights into the mode of action of sulfoxaflor (sulfoximine) and identify Dβ1 as a key component of nAChRs targeted by both these insecticide classes. A comparison of resistance phenotypes found in this study to resistance reported in insect pests reveals a strong conservation of subunit targets across many different insect species and that mutations have been identified in most of the receptor subunits that our findings would predict to have the potential to confer resistance.

Abbreviations: Imidacloprid, (IMI); Sulfoxaflor, (SFX); Nitenpyram, (NIT); Spinosad, (SPIN); Nicotinic acetylcholine receptor, (nAChR); Insecticide Resistance Action Committee, (IRAC); Insecticide resistance management, (IRM); Resistance ratio, RR); Clustered Regularly Interspaced Short Palindromic Repeats, (CRISPR); CRISPR Associated protein 9, (Cas9).

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1. Introduction

For more than a century insecticide resistance has reduced the capacity of successive generations of insecticides to control pest insects that damage crops and threaten human and animal health (Ffrench-Constant, 2013; McKenzie and Batterham, 1998; Nauen and Denholm, 2005; Whalon et al., 2008). Insecticides are still pivotal for the control of many pest insects, although a wide range of other practices are routinely implemented. Efforts to prevent or stall the evolution and spread of resistance to current and future insecticidal compounds are facilitated by a detailed understanding of their mode of action (Nauen et al., 2019; Sparks and Nauen, 2015). This knowledge is increasingly valuable in light of losses caused by ineffective pest control and the increasing technological and regulatory costs of bringing new insecticides to market (Sparks and Lorsbach, 2017).

The insect nervous system provides targets for a majority of the most commercially successful insecticides, including acetylcholinesterases (organophosphates and carbamates), voltage gated sodium channels (pyrethroids and DDT) and γ-aminobutyric acid (GABA) and Glutamate-gated chloride channels (cyclodienes, avermectins, and phenylpyrazoles) (Ffrench-Constant et al., 2016; Sparks and Nauen, 2015; Sparks et al., 2019b). The nicotinic acetylcholine receptor family (nAChRs) has, over time, been targeted by a number of insecticides of diverse chemical structures including nicotine, nereistoxin analogs, the neonicotinoids, spinosyns, sulfoximines, butenolids, and most recently, the mesoionics (Sparks and Nauen, 2015; Casida, 2018; Nauen et al., 2019; Matsuda et al., 2020). At present insecticides acting at the nAChR account for approximately 29% of the global insecticide market, rendering the nAChR the most used target site for insect pest control (Sparks et al., 2020).

Insect nAChRs are members of the cys-loop ligand-gated ion-channel superfamily. In the model insect *Drosophila melanogaster* there are 10 receptor subunit genes (seven α-subunits and three β-subunits) (Sattelle et al., 2005). The insect nAChR is a pentameric assembly of these subunits, expressed mainly in the central nervous systems of insects (Sattelle and Breer, 1990). Thus, many possible combinations of these subunits could exist producing an array of nAChR subtypes, each potentially having different physiological properties. The endogenous ligand, acetylcholine (ACh), binds at the interface between two receptor subunits. The principal face of the binding pocket for ACh is formed by loops A-C of an α-subunit, while the complementary face is formed from loops D-F of the adjacent α or β subunit (Corringer et al., 2000). This subunit interface is also believed to be the primary site of binding for the neonicotinoid and sulfoximine insecticide classes (Ihara et al., 2015; Matsuda et al., 2005; Wang et al., 2016). In contrast other nAChR acting insecticides, the spinosyns, appear to act at an allosteric site (Geng et al., 2013; Puinean et al., 2013; Silva et al., 2016; Somers et al., 2015; Watson et al., 2010).

The Insecticide Resistance Action Committee (IRAC) (Nauen et al., 2019; Sparks and Nauen, 2015) classifies insecticides according to their mode of action. Compounds are also allocated to distinct subgroups based on their chemical structure and information demonstrating differential metabolism and reduced resistance from testing of resistant insect strains. Whereas the insecticides imidacloprid (IMI), nitenpyram (NIT) and sulfoxaflor (SFX) are categorized as nAChR competitive modulators; the neonicotinoids (including IMI & NIT) are both in the same subgroup (4A) (Nauen et al., 2019; Sparks and Nauen, 2015), while the sulfoximine, SFX, is a different subgroup (4C) (Sparks and Nauen 2015) due to differences in chemistry and metabolic resistance (Sparks et al., 2013; Watson et al., 2011, 2017). The spinosyns, (including spinosad (SPIN)) are nAChR allosteric modulator – site I compounds and are thus in an entirely different group (Group 5) (Sparks and Nauen, 2015).

Given the contributions of two different subunits to a single ligand binding pocket and that five interfaces are present in a pentameric receptor, it is important to determine which particular nAChR subunits are present and their arrangement. Heterologous expression studies have provided some insights into the interaction of insecticides at specific residues/possible receptor interfaces when insect nAChR subunits are co-expressed with vertebrate β2 or β4 subunits (Lansdell et al., 2008; Lansdell and Millar, 2000a, b; Matsuda et al., 1998; Matsuda et al., 2005) or with an accessory protein from *Caenorhabditis elegans*, RIC3 (Lansdell et al., 2012; Watson et al., 2010). Until recently, difficulties expressing insect nAChRs limited investigation via these methods (Lansdell et al., 2012; Watson et al., 2010); however co-expression of additional cofactors has led to robust functional expression of several combinations of nAChR subunits from *D. melanogaster*, *Apis mellifera* and *Bombus terrestris* in *Xenopus laevis* oocytes (Ihara et al., 2020).

Genetic studies offer another line of investigation into the mode of action of insecticides to improve our understanding of the roles and functions of the different nAChR subunits. For example, in *D. melanogaster,* loss of function mutants were used to show that the Dα1 and Dβ2 subunits are involved in binding of neonicotinoid insecticides including IMI and NIT (Perry et al., 2008). The modest levels of resistance measured for these mutants, singly and in combination, suggested that receptors comprised of additional subunits are targeted by neonicotinoids (Perry et al., 2008). Because the *Dα1* and *Dβ2* mutants were not highly resistant to SFX or SPIN insecticides (Perry et al., 2012), this suggested that the neonicotinoids, sulfoximines and spinosyns target different nAChR subtypes. In contrast to the neonicotinoids, the Dα6 subunit has been established as the main target of the spinosyns in *Drosophila* (Perry et al., 2007; Watson et al., 2010). Mutations in *Dα6* or its orthologues are associated with high levels of resistance in *Drosophila* (Crouse et al., 2018; Perry et al., 2007; Sparks et al., 2019a; Watson et al., 2010) and a wide range of pest insects (Bao and Xu, 2011; Baxter et al., 2010; Geng et al., 2013; Puinean et al., 2013; Silva et al., 2016). One caveat to the findings of previous studies is that only a limited set of receptor mutations known to confer resistance were identified and tested, making it unclear as to whether other subunits play a role, either major or minor. The advent of the capability to specifically manipulate genomes at will provides a new avenue to understand the target site of insecticides and their respective resistance mechanisms (Perry and Batterham, 2018; Somers et al., 2015; Zimmer et al., 2016), and hence options for insecticide resistance management (IRM) programs.

Herein we report our findings from the first systematic investigation of the potential involvement of *Drosophila melanogaster* nAChR subunits in the mode of action of these three insecticide classes (neonicotinoids, sulfoximines, spinosyns). We took advantage of gene editing techniques using Clustered regularly Interspaced Palindromic Repeats (CRISPR) and the CRISPR Associated Protein 9 (Cas9) nuclease to disrupt the function of nine of the ten individual nAChR subunit genes in *D. melanogaster*. Our findings help to refine the list of subunits that are involved in the insecticidal activity of the tested nAChR-acting compounds and the level of resistance conferred from loss of function. They also provide insights into the potential combinations of subunits that may co-assemble into native insect nAChR subtypes, an area highly relevant to the understanding of insecticide mode of action and resistance as well as insect neurophysiology. A detailed understanding of an insecticide's mode of action is an important component for the development of effective IRM strategies (Nauen et al., 2019; Sparks and Nauen, 2015), especially those involving the rotation of groups possessing a different mode of action, in an effort to reduce selection for resistance and cross-resistance (Roush, 1989).

2. Materials and methods

2.1. Compounds

Spinosad (Success®; Dow AgroSciences), imidacloprid 99% (Pestanal®; Sigma-Aldrich), nitenpyram 99% (Pestanal®; Sigma-Aldrich) were purchased commercially. Sulfoxaflor (99%, racemic mixture) was synthesised and provided by Dow AgroSciences (now Corteva

Agriscience).

2.2. Toxicology bioassays

D. melanogaster larval bioassays were performed as described previously (Perry et al., 2012). Briefly, 1st instar larvae were collected and reared on semolina-based fly media containing selected doses of the insecticides. Control media received only the respective solvent. Spinosad and nitenpyram were diluted in distilled water while imidacloprid and sulfoxaflor were diluted in DMSO for media preparation. Flies were kept in the dark at 25 ± 1 °C until adults had eclosed (between 17 and 20 days). A minimum of 750 larvae (750–2800) were screened for calculation of LC_{50} for each strain. Data analyses were performed in R software. R script (Appendix S1) was modified from an original analysis package obtained from Github (https://github.com/shanedenecke/insect.toxicology). Briefly, raw toxicology bioassay data was corrected for control mortality using Abbott's correction (Abbott, 1925). Probit analysis was used to develop regression lines from which the LC_{50}, the concentration lethal to 50% of the tested larvae was then determined (Finney, 1971) (Tables S2-5). Resistance ratio (RR) and its 95% confidence limits between LC_{50} of mutant strain and LC_{50} of respective control strain were calculated as described previously (Robertson and Robertson, 2007) where ratio that had confidence limits not including 1.0 is considered statistically significant ($\alpha = 0.05$) (Tables S6-9).

2.3. Drosophila melanogaster strains

The existing *D. melanogaster* strains used in this study are shown in Table 1 while those generated for this study are listed in Table 2.

2.4. Creation of mutant strains using CRISPR/Cas9

Several alternative or more efficient CRISPR/Cas9 approaches became available during the course of this study and so mutant strains were generated with the increasingly efficient techniques (Gratz et al., 2013, 2014; Port et al., 2014). The specific methods used for each strain are described below while sgRNA targets are provided in Table 3.

2.4.1. Genomic deletion for the Dα1, Dα2 and Dα3 strains

Two "pU6-BbsI-gRNA" plasmids (Gratz et al., 2013) harboring correct sgRNA sequences to target the Cas9 nuclease to the genome (Table 3) were cloned as per the published protocol (http://flycrispr.molbio.wisc.edu/protocols/gRNA) and methods (Gratz et al., 2013). Plasmids were isolated using a Midiprep kit (Qiagen) as per manufacturer's instructions and concentration quantified using a Qubit machine with the dsDNA BR kit (Invitrogen). Plasmids were then microinjected (each plasmid at 200ng/ul in ddH2O) into embryos of the *AC9* fly strain, which had been collected and prepared as previously described (Perry et al., 2015). F1 adults of injected survivors were crossed to virgin females of the *Dbal* fly strain, which has dominant phenotypic markers on the 2nd and 3rd chromosomes and enables the introduction of balancer chromosomes for either the 2nd or 3rd chromosomes. After successful mating these were individually tested for the presence of a deletion (using primers indicated in Table 4) and the deletion positive flies made homozygous through the use of the dominant markers on the balancer chromosomes.

For *Dα3* knockout, the *Dα3*$^{Δ10203'}$ sgRNAs were microinjected and the adult male survivors were initially crossed back to *AC9* and F1 males collected. A wing was clipped from males, DNA extracted and diagnostic PCR for deletion events performed. Flies carrying deletions were individually crossed to virgin female *FM7* balancer strain and these were backcrossed to then remove the *FM7* balancer. The CRISPR/Cas9 microinjection was repeated for *Dα3*$^{Δ10205'}$ sgRNAs (Table 3) to produce the homozygous deletion strain of *Dα3*^{Δ1020} used in this study.

Table 1
D. melanogaster strains obtained for this study.

Name	Description	Source
AC9	Cas9 driven by Actin5c promotor	BDSC 54590
NC9	Cas9 driven during oogenesis by *nanos* regulatory sequences	BDSC 54591
25709	2nd chromosome attP landing site	BDSC 25709
25710	3rd chromosome attP landing site	BDSC 25710
Dbal	Balanced 2nd and 3rd chromosomes	Lab strain *w*;If*/CyO; MKRS/TM6b, Tb*[1]
FM7	X chromosome balancer	FM7c from BDSC 616
elaV > GAL4	enhancer trap line expressing GAL4 in neurons at early embryogenesis	BDSC 458
TM3. GFP, Ser	Third chromosome balancer	*TM3, P{w*$^{+mC=ActGFP}$*} JMR2, Ser*[1]
UAS > Cas9	2nd chromosome Cas9 under control of the UAS promotor	BDSC 54594

BDSC - Bloomington Drosophila Stock Centre.

Table 2
D. melanogaster strains created in this study.

Strain	Description	Background
pCFD4_Dα7	Transgenic sgRNA source	25709
pCFD4_Dα4	Transgenic sgRNA source	25709
pCFD4_Dβ3	Transgenic sgRNA source	25710
pCFD4_Dα6	Transgenic sgRNA source	25709
pCFD4_Dβ1	Transgenic sgRNA source	25709
Dα1^{ΔDH}	*Dα1* deletion strain	wAC9
Dβ2^{KO}	*Dβ2* deletion strain	wAC9
Dβ1^{Δex5}	*Dβ1* deletion strain	Balanced over TM3.GFP,*Ser*
Dβ3$^{ΔB4.2}$	*Dβ3* deletion strain	wAC9
Dα2$^{Δ(3)4E}$	*Dα2* deletion strain	wAC9
Dα3^{Δ1020}	*Dα3* deletion strain	AC9
Dα4^{ΔBA}	*Dα4* deletion strain	wAC9
Dα6^{KO}	*Dα6* deletion strain	wAC9
Dα7^{ΔD6}	*Dα7* deletion strain	AC9
Elav > GAL4; pCFD4_Dβ1	Somatic *Dβ1* deletion strain	*Elav > GAL4;*pCFD4_Dβ1
wAC9	Background strain for autosomal nAChR genes	BDSC 54590 with *w*$^-$ X chromosome substituted

2.4.2. Genomic deletions of Dα4, Dα6, Dα7, Dβ1, Dβ2 and Dβ3

These were created through the use of transgenic flies carrying the pCFD4 plasmid (with sgRNAs as per Table 3) as per Port et al. (2014) with minor variations (Port et al., 2014). Briefly, flies were crossed to *AC9* (or *NC9* for isolating a deletion of the Dβ2 genomic region) and F1 males were then individually crossed to *Dbal* virgin females to allow identification of those individuals heterozygous for a deletion as per 2.4.1. In the case of *Dα6*, larvae from the F1 cross to the *Dbal* strain were screened on 1 ppm SPIN, given the resistance phenotype allowed selection for those individuals carrying a *Dα6* deletion balanced over the *CyO* balancer chromosome (Perry et al., 2007). Sequencing across the edited region was used to confirm the deletion was present.

2.4.3. Somatic CRISPR/Cas9 of Dβ1

Virgin female *elaV > Gal4;Dβ1PCFD4* flies express GAL4 pan-neuronally, while simultaneously expressing two different sgRNAs against *Dβ1* from U6 promotors. These were crossed to *UAS > Cas9* male flies resulting in neuronal expression of Cas9 with the genotype *elav > GAL4/+ (or y);pCFD4_Dβ1/UAS > Cas9*. The background used for comparison was the 25710 fly strain crossed to UAS > Cas9.

2.5. Production of transcriptomes and analysis of nAChR subunit expression

Flies were allowed to lay eggs on a fresh juice plate over a 3-h laying period. Eggs were left at 25 °C overnight and hatched larvae were cleared the next morning. Freshly hatched larvae over a 3-h hatching period were collected into new plates to synchronize the larval

Table 3
sgRNA sequences used to target Cas9 to specific sites in the genome.

Mutant	sgRNA sequence 1	sgRNA sequence 2	sgRNA type
$D\alpha2^{\Delta(3)4E}$	CGGAGTCCAGACCACTTTGC	GCGGCGGATGCAAGGTCGTG	T7
$D\alpha1^{\Delta DH}$	GATGGAAAATATGTTAGATT	GAAACATGTCTGCTCGTCGA	pU6
$D\beta2^{KO}$	GTTTAATACAACCTCACGGT	GAAGCATTGTTAACTCAGAC	pU6
$D\alpha7^{\Delta D6}$	GCGCACGAACAGTAGTAGAC	GTAAGCGAAGGAGCGATGCT	pC
$D\alpha4^{\Delta BA}$	GGAAAGTATCCAAGCTCTGA	GGAAAAGGCTTAATCGCATA	pC
$D\beta3^{\Delta B4.2}$	GCGCCTTTGTTTCGGATGTA	GTTCAGAGTGCGCACTGGCT	pC
$D\alpha3^{10205'}$	GTGTGTGTTCTTCAATTTGG	GTCGACGGGCTTTGTTTGAA	pU6
$D\alpha3^{10203'}$	GTCGCCGCAAACGCACACGA	GCCCATCGATATAAAGCTAT	pU6
$D\alpha6^{KO}$	TTTCGGTGTCTCTTGGTCAG	AGGACAGTTGAGGGATTGAG	pC
$D\beta1^{\Delta ex5}$ and $D\beta1^{Somatic}$	GAGCGCAGGCGCGAAAAATT	AGGGCGCTTCATGAATAGGA	pC

Transcribed cRNA (T7), pU6-BbsI-gRNA plasmid (pU6), pCFD4 dual sgRNA plasmid (pC).

Table 4
Diagnostic Primers to detect CRISPR/Cas9 events.

Mutant	Forward Diagnostic primer 1 (5'-3')	Reverse Diagnostic primer 2 (5'-3')
$D\alpha2^{\Delta(3)4E}$	CCTTTCCTCTAGTGCCGATG	TACCCCCAAAACTCGTTAGC
$D\alpha1^{\Delta DH}$	GTTCGGCACTTTTAGTCGATTTCG	CGAGTCAAGGCAACGTTTG
$D\beta2^{KO}$	CCACCAGCTCGAAGTTTTGC	CATGCTGATGAATGCGAGGC
$D\alpha7^{\Delta D6}$	AACGAAAAGCGTGGGATTAG	AGCTTAGATTCGGGCTGTTG
$D\alpha4^{\Delta BA}$	CCTTTCCTCTAGTGCCGATG	TGCCTCAATAGCCTTTGCTC
$D\beta3^{\Delta B4.2}$	ATGTCGGCTGCTGCTTTATC	GCAGAGCAATACTCGGAAAC
$D\alpha3^{10205'}$	TCGCGGAATAACGTGGAACA	ATCGGTGGATGGTGGTTTCC
$D\alpha3^{10203'}$	GTGTGACTGTATTGGTGCTG	CACACACAGTCTGATGGAGC
$D\alpha6^{KO}$	ACAATTGGCAGGACTCCGTT	AGTGTTGGTCCACGTGATCC
$D\beta1^{\Delta ex5}$ and $D\beta1^{Somatic}$	ACAGCTGATAGGGCCAACTG	CTGATATGCTTGGGCAGCTC

development stage and after 5 h, 100 first instar larvae (5–8 h old) were flash-frozen in liquid nitrogen. Samples were homogenized in TRIsure (Bioline) for at least 5 min. RNA was extracted using 0.2 vol of chloroform and then precipitated using 1 volume of isopropanol, following manufacturer's instructions. The RNA pellet was washed with 75% ethanol before being resuspended in RNase-free water (Sigma). To remove DNA traces, RNA was treated with RQ1 RNase-free DNase (Promega) and the reaction was stopped using RQ1 Stop solution (Promega). Sample quality was tested by running a 1% agarose gel electrophoresis. RNA purity was evaluated using a Nanodrop spectrophotometer. RNA concentration was quantified using a Qubit 2.0 fluorometer (Thermo Fisher). Library preparation (250-300bp cDNA insert) and Illumina sequencing (150bp paired end) were carried out by Novogene Bioinformatics Technology in Singapore (raw sequencing or BAM file output has been archived in the NCBI Sequence Read Archive under the accession BioProject PRJNA656174; SRR12474696-SRR12474728 and SRR13125765-SRR13125767). For analysis, reads were aligned to the *D. melanogaster* genome and exons using Tophat2 (Kim et al., 2013). Coverage plots of the aligned reads along the nAChR genes were created using pyGenomeTracks (Lopez-Delisle et al., 2020). Reads representing gene transcripts were counted using HTSeq (Anders et al., 2015). Differential expression analysis was performed using DESeq2 (Anders and Huber, 2010) and for significant DEGs, adjusted p-value was set to $p < 0.05$. Only the CRISPR/Cas9 germline mutants were examined. For *Dβ1*, homozygous *Dβ1* knockout larvae were identified and collected, taking advantage of the green fluorescence associated with the *TM3.GFP,Ser* balancer they were maintained over to exclude heterozygotes.

2.6. Cladogram for nAChR subunits

Protein FASTA files were compiled for nAChR subunits (Table S11) using *D. melanogaster* ligand-gated ion channel Rdl as an outgroup. These were aligned using ClustalW (BLOSUM:Gap penalty 10, Gap extend 0.1) (Thompson et al., 1994) and a Neighbor-Joining consensus tree calculated (Jukes-Cantor with resampling (1000 Bootstraps) using Geneious Prime software (BioMatters Ltd).

3. Results

3.1. Creation of a set of nAChR mutants for analysis

3.1.1. nAChR subunit deletions created using CRISPR/Cas9

Deletions of varying lengths were isolated for nine nAChR subunit genes using a variety of CRISPR/Cas9 methods (Fig. 1). Flies homozygous for a deletion of *Dα2, Dα3* or *Dβ3* were viable. Consistent with published data, the *Dα1, Dβ2, Dα4, Dα6 and Dα7* mutants were also viable (Fayyazuddin et al., 2006; Perry et al., 2007, 2008; Shi et al., 2014). With the exception of Dβ1, the mean 1st instar to adult viability of these mutants on untreated media ranged from 84% to 112.8% when compared to the appropriate genetic background control strains, *AC9* and *wAC9* (Table S1). We were not able to generate a mutant for *Dα5* despite multiple attempts. A strain carrying CRISPR/Cas9 mediated events deleting regions of *Dβ1* was successfully created in this study (Fig. 1). A large deletion of *Dβ1* ($D\beta1^{\Delta ex5}$) was used to produce the *Dβ1* loss of function transcriptome (section 3.3). However, poor larval to adult viability and adult survival meant that the *Dβ1* mutant could not be maintained as a homozygous strain and, thus, were not suitable for use in our insecticide assays.

3.1.2. Somatic CRISPR/Cas9 bioassays used for Dβ1 subunit knockout

The crossing scheme outlined in 2.4.4 led to viable F1 larvae and adults. The F1 flies from this cross will be henceforth referred to as the $D\beta1^{Somatic}$ genotype. The adults had reduced locomotion, similar to that observed in the deletion homozygotes (not shown).

The 1st instar to adult viability of $D\beta1^{Somatic}$ (74.5%) was comparable to that of its control background (75.4%) (Table S1). This genotype was used in subsequent insecticide bioassays.

3.2. Insecticide bioassay results

3.2.1. Response of genetic backgrounds to insecticides

The viability of germline knockout mutants and $D\beta1^{Somatic}$ was sufficient to conduct insecticide bioassays. All of the germline mutants were isolated in the *AC9* or *NC9* genetic backgrounds which only differ due to

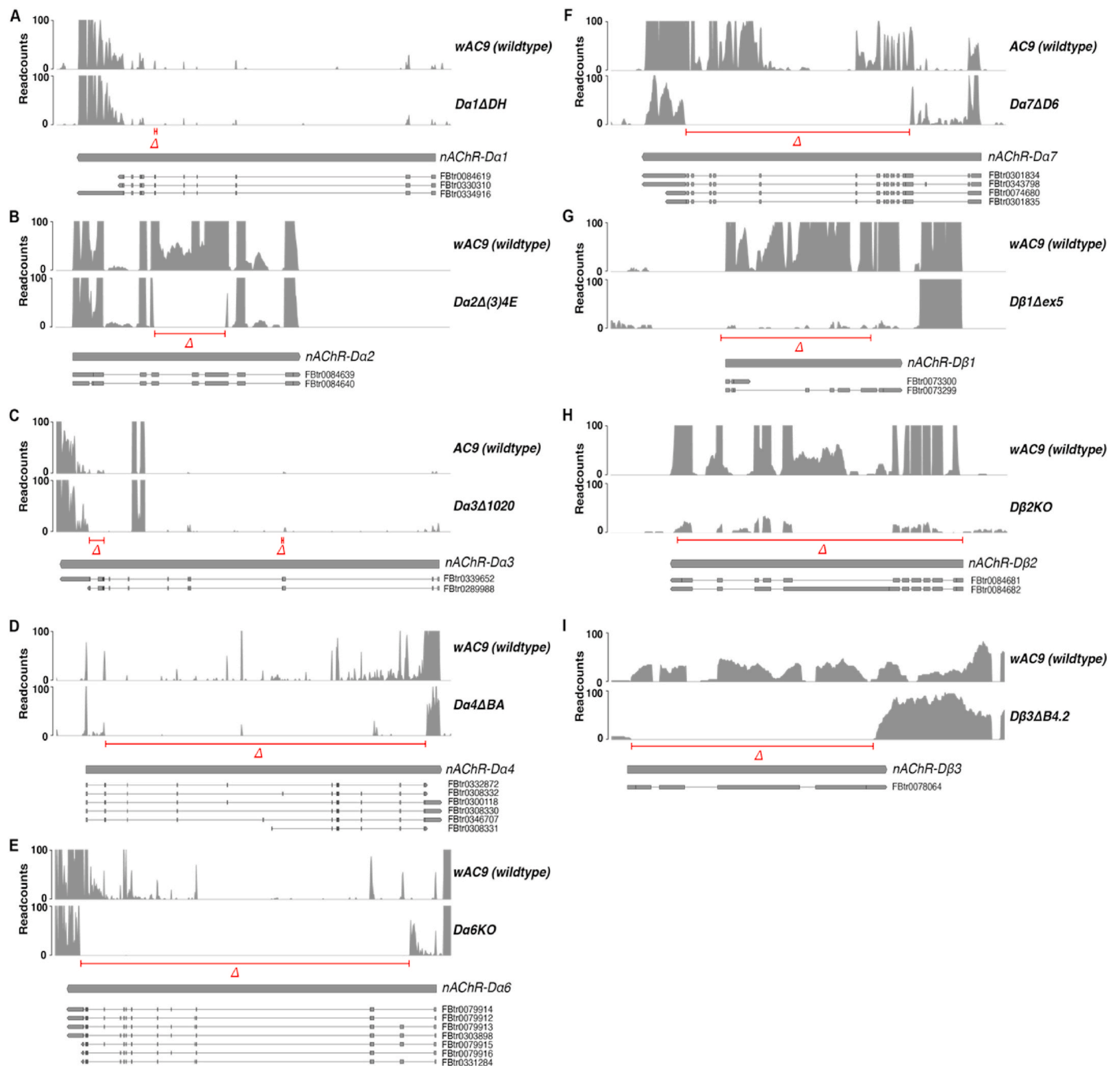

Fig. 1. Genomic deletions and RNA expression for each nAChR mutant created using CRISPR/Cas9. Coverage tracks of mapped RNAseq reads were plotted across nAChR subunit genomic loci together with the annotated gene transcripts for the wildtype strain (*wAC9* or *AC9* as appropriate) and each of the nAChR mutant strains. The number of mapped reads displayed was capped at 100 (y-axis). Panels correspond to genomic regions for **A.** *Dα1*, **B.** *Dα2*, **C.** *Dα3*, **D.** *Dα4*, **E.** *Dα6*, **F.** *Dα7*, **G.** *Dβ1*, **H.** *Dβ2*, **I.** *Dβ3*. Red bars indicate the region that has been deleted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the insertion of the different Cas9 expression plasmid constructs. However, for the autosomal genes (*Dα1, Dα2, Dα4, Dα6, Dβ2, Dβ3*) the X chromosome was replaced for with the X chromosome from w^{1118} (*wAC9* strain). Thus, the respective backgrounds are *AC9* for *Dα3* and *Dα7*, and *wAC9* for all other mutants. For SPIN, NIT and SFX there is no significant difference between LC_{50} values for *wAC9* and *AC9* strains. The differences between the IMI LC_{50} 95% fiducial limits for *wAC9* (0.169–0.192 ppm) and *AC9* (0.196–0.231 ppm) were significant, but small compared to differences observed between some mutants and either of these strains (Tables S2-5). We also note that the LC_{50} for $25710^{Somatic}$ is significantly higher than that for both *AC9* and *wAC9* backgrounds for all four insecticides tested. In determining the RR for mutants (Fig. 2, Tables S6-9), all comparisons were made using data from the appropriate background strain.

3.2.2. Response of nAChR mutant strains to selected insecticides

We analysed the effect of the nine subunit mutants against the four different nAChR acting insecticides (Tables S2-5). Four mutants have shifts in LC_{50} values to all insecticides ($D\alpha1^{\Delta DH}$, $D\alpha2^{\Delta(3)4E}$, $D\alpha7^{\Delta D6}$ and $D\beta1^{Somatic}$) with only one, $D\alpha7^{\Delta D6}$, changing in a consistent direction for each compound (higher LC_{50} c.f. background). Assays using SPIN identified $D\alpha1^{\Delta DH}$ and $D\beta1^{Somatic}$ mutants as hypersensitive, while

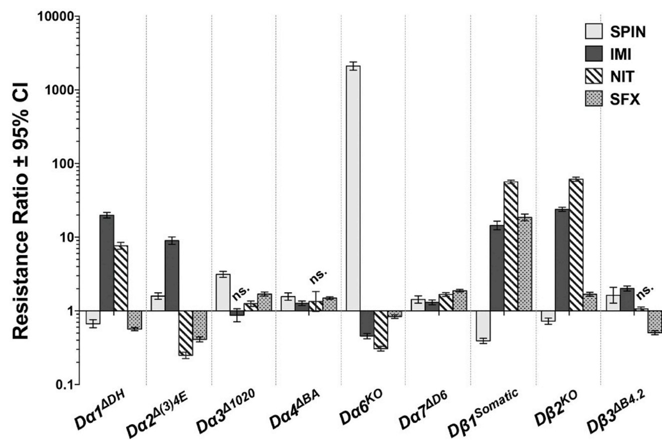

Fig. 2. Resistance ratio of each nAChR mutant on SPIN, IMI, NIT and SFX compounds with 95% confidence limits shown. Confidence intervals non-overlapping a value of 1 (x-axis) are statistically significant (α = 0.05) with non-significant RRs marked (ns.).

Dα2$^{Δ(3)4E}$, *Dα3*^{Δ1020}, *Dα4*^{ΔBA}, and *Dα7*^{ΔD6} showed small (2–3 fold), but statistically significant, increases in RR for SPIN (Table S6). In contrast, *Dα6*^{KO} exhibited a high RR (>2100 fold) to SPIN, in agreement with prior studies (Table S6). Bioassays with SFX resulted in only one mutant strain *Dβ1*^{Somatic} exhibiting a substantial change in RR (20-fold) (Table S7). All of the other mutant strains showed either a modest increase in their RR to SFX (*Dα3*^{Δ1020}, *Dα4*^{ΔBA}, *Dα7*^{ΔD6}, *Dβ2*^{KO}) or small increase in sensitivity to SFX (*Dα1*^{ΔDH}, *Dα2*$^{Δ(3)4E}$, *Dβ3*$^{ΔB4.2}$) (Table S7). The *Dα1*^{ΔDH}, *Dα2*$^{Δ(3)4E}$, *Dβ1*^{Somatic} and *Dβ2*^{KO} mutants exhibited substantially increased RRs when tested against IMI. Whereas, the *Dα4*^{ΔBA} and *Dβ3*$^{ΔB4.2}$ mutants showed small (2–3 fold increases) in RR for IMI, the *Dα6* ^{KO} mutant was hypersensitive (Table S8). *Dα1*^{ΔDH}, *Dβ1*^{Somatic} and *Dβ2*^{KO} exhibited substantial increases in RR to the neonicotinoid, NIT (Table S9) whilst the *Dα3*^{Δ1020} and *Dα7*^{ΔD6} mutants showed only small RR increases (Fig. 2, Table S9). Both *Dα2*$^{Δ(3)4E}$ and *Dα6*^{KO} mutants were hyper-sensitive to NIT (Table S9). The bioassay data for the *Dβ1*^{Somatic} genotype clearly show high levels of resistance to IMI, NIT and SFX, indicating the efficiency of somatic CRISPR.

3.3. Expression profile of the nAChR subunits in the mutant backgrounds

For most deletion mutants we observed the expected loss of expression in deleted regions (Fig. 1A–F, H,I). The coding region knockouts, *Dα4*^{ΔBA}, *Dα6*^{KO}, *Dα7*^{ΔD6} and *Dβ3*^{ΔB4} have no or very few reads, as was to be expected. We observed that the partial deletions *Dα1*^{ΔDH}, *Dα2*$^{Δ(3)4E}$ and *Dα3*^{Δ1020} while having reads map to the gene transcripts, did not have any reads mapping to their deleted regions (Fig. 1). A significant >16-fold reduction in transcript expression of *Dβ2* ($log_2FC < -4$, Fig. 3) was also observed in the *Dβ2*^{KO} mutant relative to *wAC9* (Fig. 1H), this was despite some potential contamination, as low read counts were observed in the three RNA samples that were analysed (Figure S1). Expression of *Dβ1* was detected in the *Dβ1*^{Δex5} mutants (Fig. 1G) which may be from maternally inherited transcripts. Contamination, with the sequenced sample containing heterozygotes, again cannot be completely ruled out as a possibility, but is unlikely as all three samples collected had very similar, low levels of read counts for *Dβ1* (Figure S2). All mutant strains have at least one subunit with a significantly changed expression level (Fig. 3).

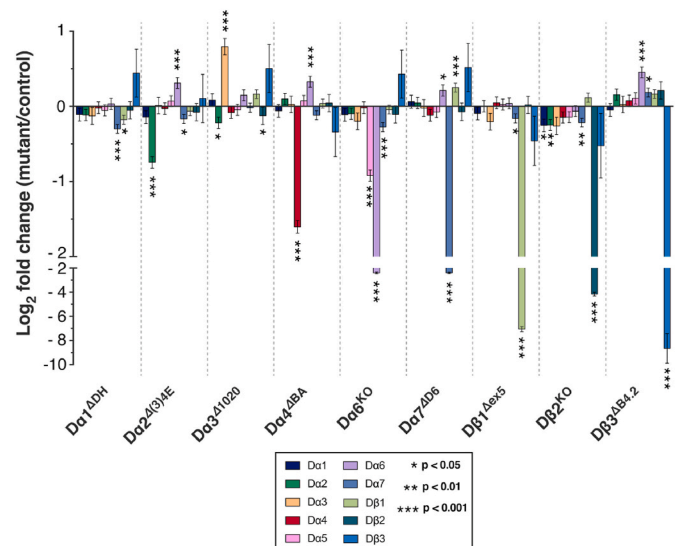

Fig. 3. Differential expression analysis of the nAChR genes in nAChR mutants. RNAseq analyses were performed using whole 1st instar *D. melanogaster* larvae. Differential expression analysis using DESeq2 reveals relative transcript expression levels of 10 nAChR subunit genes in each of the CRISPR-generated mutants strains in comparison to their respective AC9 or wAC9 control backgrounds. Bar graph shows log2 fold change of relative transcript expression levels ±standard error. Statistical significance is from the Benjamini-Hochberg adjusted p-value from the DESeq2 analysis. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

4. Discussion

4.1. Germline and somatic CRISPR/Cas9 gene editing can be used to examine insecticide targets

The capacity of CRISPR/Cas9 methods to specifically target and manipulate genes to validate their role in insecticide resistance generated in the lab (Somers et al., 2015), or identified in the field (Zimmer et al., 2016), is clear. Here, we used CRISPR/Cas9 to systematically create mutations in nAChR subunit genes to examine the contribution of individual gene family members to resistance phenotypes. Microinjection of the CRISPR/Cas9 components in combination with transgenic approaches (Gratz et al., 2013, 2014; Port et al., 2014) were used to generate nine deletions in nAChR subunit genes. The lower fitness of the *Dβ1* knockout strain precluded the maintenance of it as a homozygous line, however, a close to wildtype level of larval to adult eclosion survival rate was observed for the eight other nAChR mutant strains (Table S1).

Despite several attempts using these different CRISPR/Cas9 approaches, we were unable to isolate a heterozygous fly carrying a deletion in *Dα5*. The reasons for this are not clear given we were able to isolate heterozygotes carrying a deletion for *Dβ1*. Present data suggest that deletion of the nAChR *Dα5* is a lethal event, highlighting its likely importance in the overall function of the nAChR in *D. melanogaster* (D. Christesen, unpublished information). The *Dα5* subunit has an atypical N-terminal region prior to the nAChR domains, the function of which is currently unknown (Grauso et al., 2002).

The *Dβ1* knockout strain we generated was found to be homozygous infertile, with adults that are highly uncoordinated upon emergence and unable to either mate or produce offspring. This observation is in agreement to a recent study reporting poor fecundity and egg viability for *D. melanogaster* carrying an R81T mutation in the *Dβ1* gene (Homem et al., 2020). The nAChR mutations associated with resistance to the insecticides examined in the present study have displayed predominately recessive phenotypes (Bass et al., 2011; Perry et al., 2011) meaning that larval to adult viability bioassays on balanced

heterozygotes would not be informative. Hence, we employed a somatic CRISPR strategy for characterising the resistance profile of the Dβ1 subunit using a crossing scheme to produce flies with pan-neuronal somatic CRISPR knockout of the *Dβ1* gene for our assays (2.4.4). The toxicology results from our screening suggest that a high efficiency of the CRISPR/Cas9 cutting is occurring, in particular, it yielded the highest level of resistance towards SFX for any knockout strain (Fig. 2, Table S7).

When analysing the RNAseq data we observed that sequence reads are not mapped to regions of the gene that were deleted as would be expected (Fig. 1) with the exception of *Dβ1* and *Dβ2* as discussed earlier. Interestingly for $D\alpha3^{\Delta1020}$, the level of transcription of the majority of the *Dα3* gene was increased, however the deleted regions were not detected (Fig. 1C). These data suggest that there is a compensatory transcriptional response to this mutation.

Taking advantage of the specific genome editing afforded by CRISPR, in both germline and somatic cells, we were able to assess deletions of six previously untested *D. melanogaster* nAChR subunits (*Dα2*, *Dα3*, *Dα4*, *Dα7*, *Dβ1* and *Dβ3*) with insecticide response, as well as provide further information on the resistance profiles for three other nAChR subunits (*Dα1*, *Dα6* and *Dβ2)* (Perry et al., 2012).

4.2. Effects of mutations on toxicity

4.2.1. Spinosyns

In agreement with previous studies (Crouse et al., 2018; Perry et al., 2007, 2015; Sparks et al., 2019a; Watson et al., 2010), the $D\alpha6^{KO}$ larvae were found to survive on very high doses of SPIN (LC_{50}, 30.5 ppm, Table S2). The RR of 2106-fold was the highest RR observed in this study (Fig. 2, Table S6) and is of a similar magnitude to that reported for the $D\alpha6^{nx}$ allele (estimated at >1176 fold) (Perry et al., 2015). Importantly, the level of SPIN resistance observed for the $D\alpha6^{KO}$ (2106-fold) is far higher than that observed for any of the other mutant strains (RR = 0.7–3.1-fold) (Fig. 2, Table S6–S9). There is the possibility that these minor increases in RR for mutations in *Dα2, Dα3*, *Dα4,* and Dα7 subunits (Fig. 2) actually represents a reduction in the number of targets that bind SPIN. Interestingly, in the $D\alpha2^{\Delta(3)4E}$, $D\alpha4^{\Delta BA}$, and $D\alpha7^{\Delta D6}$ mutants, there is increased transcription of the Dα6 gene (Fig. 3), however whether this alters Dα6 subunit protein levels and how this would result in increased resistance to SPIN is not clear. It is also possible that these small shifts in RR are caused by as yet unidentified changes occurring at synapses when specific receptor subtypes are absent. Regardless, the resistance levels are extremely minor in comparison to the resistance conferred by mutations in α6-like receptors (Crouse et al., 2018; Perry et al., 2007; Sparks et al., 2019a; Watson et al., 2010); (Bao and Xu, 2011; Baxter et al., 2010; Geng et al., 2013; Puinean et al., 2013; Silva et al., 2016; Wang et al., 2020) and thus likely do not play a role in the mode of action of the spinosyns.

4.2.2. Neonicotinoids

The NIT and IMI data for *Dα1* and *Dβ2* concur with previous studies (Perry et al., 2008, 2012; Somers et al., 2017). In the present study, resistance was also observed with, mutants for these two subunits, as well as two others $D\alpha2^{\Delta(3)4E}$ and $D\beta1^{Somatic}$ (Fig. 2). These four mutants ($D\alpha1^{\Delta DH}$, $D\alpha2^{\Delta(3)4E}$, $D\beta2^{KO}$ and $D\beta1^{Somatic}$) were shown to exhibit IMI resistance levels (8.9–23.8 fold) that were statistically significant (Fig. 2, Table S8). Furthermore, three of these subunits, $D\alpha1^{\Delta DH}$, $D\beta2^{KO}$ and $D\beta1^{Somatic}$ were also resistant to NIT (Fig. 2, Table S9). Resistance to IMI (32.6-fold) has been recently reported for a *D. melanogaster* strain which had a $D\beta1^{R81T}$ mutation created using CRISPR/Cas9, supporting the importance of Dβ1 containing receptors as insecticide targets (Homem et al., 2020). Altogether, the available data indicate that these four subunits are all components of IMI targeted native nAChR subtypes. This conclusion is also supported by the recent functional expression studies showing that nAChRs Dα1, Dα2, Dβ1 and Dβ2 are IMI responsive as are some receptors containing subsets of these subunits and that both agonist and antagonistic actions of neonicotinoids are reduced when the *Dβ1* R81T mutation is co-expressed with *Dα1, Dβ2 and Dα2* (Ihara et al., 2020). Importantly, the Dα1 and Dβ1 subunits were required in all combinations in which functional nAChR expression led to a response to IMI (Ihara et al., 2020). Taken together these data may explain why the levels of resistance observed in the *Dα1*, *Dα2* and *Dβ2* mutants are two orders of magnitude lower than the resistance observed for SPIN (Fig. 2, Tables S6, S8), with the highest levels of resistance to IMI in the present study observed for the $D\beta2^{KO}$ strain (23.8-fold). In the absence of any one of these subunits there may remain a population of nAChR subtypes that are targeted by IMI. We cannot draw firm conclusions on the importance of *Dβ1* because the levels of resistance observed in $D\beta1^{Somatic}$ may be an underestimate of the resistance levels that would be observed in a homozygous loss of function *Dβ1* mutant.

4.2.3. Sulfoxaflor

The only nAChR subunit knockout associated with significant, high levels of resistance to SFX (c.a. 20-fold) was $D\beta1^{Somatic}$ (Fig. 2, Table S7). nAChR β subunits lack the loop C “YXCC” motif critical for ACh binding and thus need to assemble with α subunits in order to form ACh responsive receptors (Kao and Karlin, 1986), As such, it is therefore noteworthy that none of the α subunit mutations tested conferred resistance to SFX, a response profile distinguishing it from the multiple receptor mutants conferring resistance to IMI and NIT. The previously mentioned Homem et al. (2020) study on the $D\beta1^{R81T}$ *D. melanogaster* strain also observed that the presence of the $D\beta1^{R81T}$ point mutation was associated with a modest level of resistance to SFX (5.1-fold).

A 2.8-fold RR to SFX was identified in a *D. melanogaster* EMS induced allele of *Dβ2* (Perry et al., 2012), but here the $D\beta2^{KO}$ strain presented with a smaller SFX RR of 1.7-fold (Fig. 2, Table S7). This result, together with other low-level RRs identified in $D\alpha3^{\Delta1020}$, $D\alpha4^{\Delta BA}$ and $D\alpha7^{\Delta D6}$ are discussed later (Section 4.2.4).

SFX sensitive nAChR subtypes in *D. melanogaster* are likely to contain the Dβ1 subunit, however, given the lack of resistance conferred by other individual subunit knockouts, our data also suggest that other nAChR subunits which co-assemble with Dβ1 may have only minor impacts on the level of response to SFX. Therefore, it is possible that SFX is able to bind to all Dβ1 containing nAChRs in neurons, which could explain why resistance was not observed in the other receptor knockouts. Examining double knockouts, if they are viable, may shed further light on the role of nAChR subunits aside from Dβ1 in the action of SFX. Since the role of *Dα5* could not be explored in the present study, its impact on SFX efficacy, or lack thereof, must await further investigation.

A *Dβ1* native driver was not available to drive expression of the CRISPR/Cas9 and gene editing is not 100% efficient, so some neurons which natively express *Dβ1* may not have deletions of the *Dβ1* gene. It is, therefore, possible that the 18-fold SFX resistance observed in the present study is an underestimate of the importance of Dβ1 binding to the SFX response. Nonetheless these data attest to the usefulness of somatic CRISPR in examining the resistance phenotype associated with recessive alleles that impact viability.

4.2.4. Low levels of cross-resistance were observed for other nAChR subunits

Small (3-fold or lower) but significantly increased RRs towards IMI ($D\alpha4^{\Delta BA}$, $D\beta3^{\Delta B4.2}$, $D\alpha7^{\Delta D6}$), NIT ($D\alpha4^{\Delta BA}$, $D\alpha3^{\Delta1020}$, $D\alpha7^{\Delta D6}$), SPIN ($D\alpha2^{\Delta3(4)E}$, $D\alpha3^{\Delta1020}$) and SFX ($\underline{D\alpha4}^{\Delta BA}$, $D\alpha3^{\Delta1020}$, $D\alpha7^{\Delta D6}$, $D\beta2^{KO}$) were observed (Fig. 2, Tables S6-9). As discussed earlier (section 4.2.1), it is possible that these particular receptor subunits are targeted, but may have low binding affinity, are few in number, or are replaced by other subunits that bind insecticide. There have been no reports implicating mutations in receptor subunits from any of the α3, α4, α7 and divergent (β3) clades (Fig. 4) with insecticide resistance in pest insects. This supports the idea that these are not major targets for insecticides acting through the nAChR.

Previous heterologous expression studies have found that Dα1, Dα2,

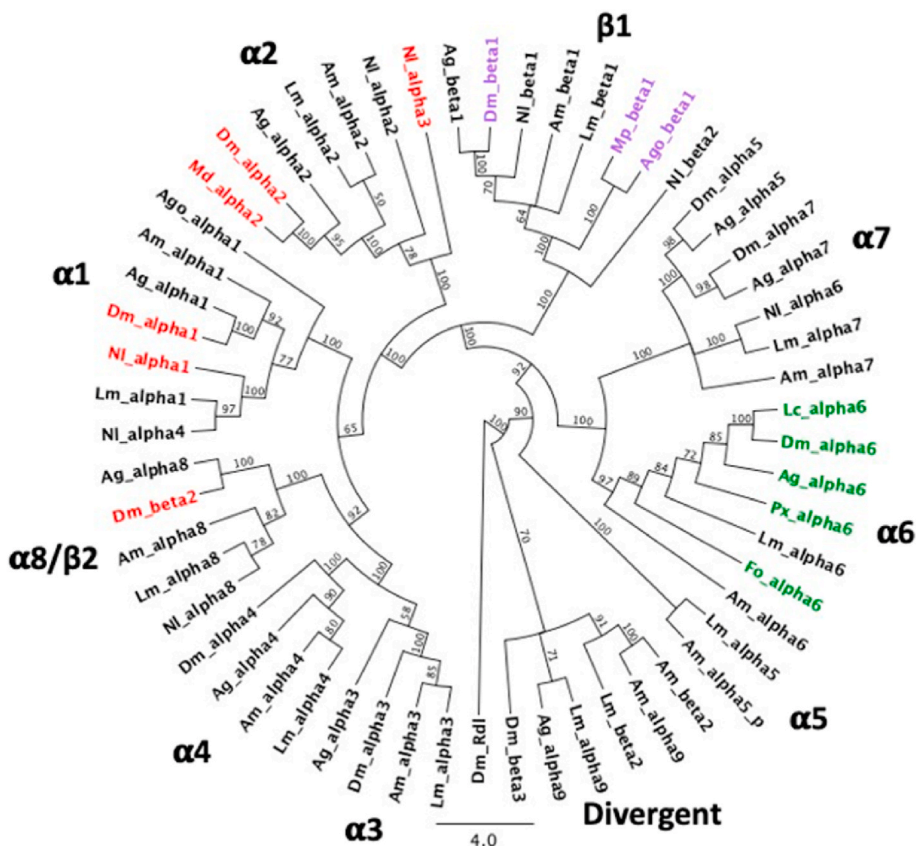

Fig. 4. Receptor subunits targeted by specific classes of insecticides appear to be conserved. The rooted cladogram of nAChR subunits from a range of insects highlights subunits associated with neonicotinoid (red and purple), sulfoximine (purple) and the spinosyn (green) classes of insecticides have orthologues in *D. melanogaster* that are associated with resistance. Abbreviations:Ago - *Aphis gossypii*, Ag - *Anopheles gambiae*, Mp – *Myzus persicae*, Dm – *Drosophila melanogaster*, Lm – *Locusta migratoria*, Md – *Musca domestica*, Fo - *Frankliniella occidentalis*, Nl - *Nilaparvata lugens*, Px - *Plutella xylostella.* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Dα3 can all respond to IMI, when co-expressed with vertebrate β-type subunits (Lansdell and Millar, 2000b; Schulz et al., 1998). Given the level of IMI resistance for $D\alpha3^{\Delta1020}$ was relatively low (2.19-fold), Dα3 may not be a significant *in vivo* target of IMI in *D. melanogaster* larvae. Potentially, the subtype combinations it assembles into are few in number or are not responsive, and/or that excitation of synapses that Dα3-containing nAChRs are localised to do not cause symptoms or lead to insect death. The *Dα3* genomic locus was recently associated with IMI resistance in adult flies. Screening of a *Dα3* knockout strain showed that adult *Dα3* knockout flies were hypersensitive to IMI (Fournier-Level et al., 2019). This observation highlights an additional layer of complexity when identifying targets. Depending on the life stage of an insect, resistance phenotypes to the same insecticide might vary depending on stage specific levels of nAChR subtypes and stage specific subunit combinations.

In multiple receptor mutants we detected a modest (2–4 fold) but significant hypersensitivity to SPIN ($D\beta1^{Somatic}$), IMI ($D\alpha6^{KO}$), NIT ($D\alpha2^{\Delta(3)4E}$, $D\alpha6^{KO}$) and SFX ($D\alpha2^{\Delta(3)4E}$). Based on our transcriptomic data (Fig. 3), this hyper-sensitivity is not due to changes in transcription of the receptor subunits that are targeted by these compounds. There was no increased expression of the spinosad target, *Dα6*, in the $D\beta1^{Somatic}$ mutant, no increase in *Dα1*, *Dα2*, *Dβ2* or *Dβ1* expression in the $D\alpha6^{KO}$ mutant and no change in *Dβ1* expression in the $D\alpha2^{\Delta(3)4E}$ mutant. Hence, the changes in insecticide responses (Fig. 2) are not due to changes in transcription of receptor subunits (Fig. 3). Our working hypothesis is that sensitivity is an effect of perturbations occurring at the synapse to accommodate the loss of the receptor subtype. While these altered RRs could be due to changes unrelated to the nAChRs, it is also possible there are post-transcriptional changes to levels of protein for other receptor subunits. To address how insects are compensating for the loss of the specific nAChRs, approaches using sophisticated next generation sequencing techniques, such as the recent single cell transcriptomic analysis of the adult *D. melanogaster* brain (Croset et al., 2018) together with genomic manipulations such as gene tagging (Kondo et al., 2020) could help to define patterns and levels of subunit co-expression.

4.3. Implications of results for nAChR targets of insecticides

4.3.1. Dα6 again confirmed as a major spinosad target, Dα5 remains a potential target

With the systematic knockout analysis of different receptor subunits in this study, the role of Dα6 as the critical nAChR subunit targeted by SPIN is evident, a conclusion consistent with other studies in *D. melanogaster* (Crouse et al., 2018; Perry et al., 2007; Sparks et al., 2019a; Watson et al., 2010) and other insect species (Bao and Xu, 2011; Baxter et al., 2010; Geng et al., 2013; Puinean et al., 2013; Silva et al., 2016; Wang et al., 2020). However, given we could not create *Dα5* loss-of-function mutants by CRISPR/Cas9, the involvement of this subunit in resistance to SPIN and other insecticides is yet to be tested. Prior studies (Lansdell et al., 2012; Watson et al., 2010) suggest that a Dα5-Dα6 nAChR subtype combination is functional and remains the only published nAChR combination from which a response to spinosyn A has been elicited. A prerequisite for co-assembly is co-expression in the same cells. Recent single cell transcriptomic analysis of gene expression in the *D. melanogaster* adult brain suggests that 63% of *Dα6* expressing cells also express *Dα5* (Croset et al., 2018). While it is likely that the *Dα5* and *Dα6* genes would also be co-expressed in the larval brain, this is yet to be demonstrated. It is interesting to note that our expression analysis of the *Dα6* knockout mutant identified a significant decrease (~50%) in the level of expression of the *Dα5* gene (Fig. 3). This observation could suggest that in some neurons, the expression of these two subunits is co-ordinated, a result that might be expected if they sometimes assemble as part of the same receptor complex. We also observed a small decrease in expression of *Dα7* in the *Dα6* knockout strain but, *Dα7* has not previously been associated with resistance to SPIN (Somers et al., 2015). In the present study, testing the *Dα7* knockout did not reveal any resistance

to SPIN and, hence, Dα7 does not appear to be a critical component of receptor subtypes that bind SPIN (Fig. 2).

4.3.2. nAChR targets for neonicotinoids and sulfoximines

4.3.2.1. Dβ1 is a shared target for neonicotinoids and sulfoximines. We targeted almost the entire Dβ1 subunit for deletion in the nervous system. The resistance observed for the $D\beta 1^{Somatic}$ mutant provides some clarification regarding the involvement of the Dβ1 subunit in the binding and mode of action of SFX in *D. melanogaster*. Our results are in agreement with studies showing that the Dβ1 subunit is important for the responses to the neonicotinoids (IMI, NIT) and sulfoximines (SFX) in *D. melanogaster* (Ihara et al., 2020; Homem et al., 2020). However, we observed differences in the degree of resistance and cross resistance to these three compounds previously associated with R81T variants in β1-like subunits. The FRC strain of *Myzus persicae* (green peach aphid) that carry the $Mp\beta 1^{R81T}$ mutation are highly resistant to IMI, and to a far lesser degree, NIT and SFX (Bass et al., 2011; Cutler et al., 2013). Likewise, *Aphis gossypii* (cotton aphid) with the Agβ1 R81T mutation also exhibited substantial resistance to IMI and NIT (216, 237-fold, respectively) and, again to a far lesser degree, SFX (23-fold) (Hirata et al., 2015, 2017). This is mirrored in the *D. melanogaster* $D\beta 1^{R81T}$ strain, with resistance to IMI being higher than for SFX (Homem et al., 2020). In contrast to the above reports, in the present study a different rank-order was observed for $D\beta 1^{Somatic}$ mutation with the RR higher for NIT (RR 56.4) than SFX (RR 20.4) or IMI (RR 12.7) (Fig. 2). There are two potential explanations for the observed differences, with the most likely being the effect of deleting the whole subunit vs the specific R81T substitution. Based on molecular modelling analysis, it has been suggested that the higher resistance to IMI in FRC-P is due to the mutation not impacting the binding of SFX as significantly as binding of IMI (Wang et al., 2016), a conclusion supported by other modelling studies (Beck et al., 2015). Some studies have found that not all IMI resistant strains carrying the R81T mutation exhibit the same degree of cross resistance to SFX, or even any cross-resistance at all (Bass et al., 2015; Hirata et al., 2015, 2017; Koo et al., 2014; Mezei et al., 2020) which leaves some uncertainty as to the overall impact of the R81T mutation in the β1 subunit on SFX resistance. These inconsistences in cross resistance patterns observed in various species could be explained if there were contributions from other genes or other amino acid sequence variations in β1 itself. A second possibility, which is not mutually exclusive is that there are differences between species in the insecticide responsive subtypes that are assembled. Co-expression of *D. melanogaster* nAChR subunits Dα1/Dα2/Dβ1 and Dα1/Dβ1 led to formation of functional receptors, however responses to 100uM ACh from co-expression of the orthologs from *Apis mellifera* and *Bombus terrestris* was not found for these combinations (Ihara et al., 2020), suggesting that this may be the case. Teasing apart the evolutionary differences between insect nAChRs is an area that warrants further study with *in vivo* and *in vitro* approaches.

4.3.2.2. Key differences are found between receptor targets of IMI, NIT and SFX. As noted above, the ~nine-fold IMI resistance associated with $D\alpha 2^{\Delta(3)4E}$ indicated that Dα2 is likely to assemble into IMI binding nAChR subtype(s), results consistent with recent heterologous expression data (Ihara et al., 2020). But, in contrast to IMI, the $D\alpha 2^{\Delta(3)4E}$ knockout is hyper-sensitive to NIT (RR 0.25-fold) and to SFX (RR 0.43-fold). The distinct resistance profiles for IMI, NIT and SFX for different nAChR subunit knockouts demonstrates that there is a clear distinction in the way these insecticides target nAChR subtypes (Fig. 2). This includes the specificity for different nAChR subtypes for two insecticides of the IRAC 4A subgroup (IMI and NIT). The sensitivity of the $D\alpha 2^{\Delta(3)4E}$ knockout to SFX supports the idea that there are also important differences between the sets of receptor subtypes that are bound by IMI and those bound by SFX. This distinction highlights the value of analysing the impact of the loss of receptor subunits using an insect model system, something now possible due to the advent of CRISPR/-Cas9. Unlike the R81T point mutation in the *Dβ1,* the resistance observed to IMI, NIT and SFX in the present study results from the loss of the whole $D\beta 1^{Somatic}$ nAChR subunit. As such this data suggests that rotation of neonicotinoids and sulfoximines could lead to an increased risk of resistance to these compounds if this particular mutation was present. The extent to which these insecticides share targets will require a greater understanding of the SFX and neonicotinoid binding sites on the various targeted receptor subtypes. Further assessment of the fitness costs associated with mutations of *Dβ1* should also be considered, given that the capacity for resistance to rise to a frequency that threatens pest control is shaped by the extent of fitness costs. As such, from an insecticide resistance management perspective, and as per IRAC guidelines (Sparks and Nauen, 2015), it is important that the rotation of neonicotinoids and SFX for the control of sap-feeding insect pests be avoided wherever possible (Mezei et al., 2020; Sparks et al., 2013).

4.4. Conclusions

D. melanogaster has long been used as a model for studying the mode of action of insecticides (Crow, 1957; Ffrench-Constant et al., 2004; Perry et al., 2011). The use of CRISPR to selectively knockout specific subunits of the nAChR has provided valuable insights into the function of the nAChR for a selected group current insecticides. Five nAChR subunits stand out as key targets for the four insecticides (IMI, NIT, SFX SPIN) tested in this study: Dα1, Dβ1 and Dβ2 for the neonicotinoid insecticides (IMI, NIT), with an additional nAChR subunit (Dα2) also involved for IMI; Dβ1 for the sulfoximine insecticide (SFX); and Dα6 for the spinosyn insecticide (SPIN). The involvement of different and/or multiple nAChR subunits in the action of these different nAChR acting insecticides underscores the complexity and importance of the nAChR as an insecticide target. That four of these nAChR subunits, (Dα1, Dα2, Dα6, Dβ1) belong to nAChR subunit clades that are reported to be associated with resistance in other insect species is a testament to the conserved function of individual nAChR subunits (Fig. 4). The approach used in this study and the strains developed here could also provide insights into targets of other nAChR-acting insecticides including other neonicotinoids (Group 4A), the butenolides (Group 4D, the mesoionics (Group 4E), nereistoxin analogs (nAChR channel blockers, Group 14) and the new nAChR peptide insecticides (allosteric modulators – site II, Group 32) (Sparks et al., 2020). Further studies employing multi-subunit knockout combinations could help to determine which native subunit combinations are present and the importance of specific combinations to the efficacy of nAChR acting insecticides, a stepping-stone towards improving our understanding of biological role of these complex receptors in insecticide mode of action and resistance.

Declarations of competing interest

T. Perry and P. Batterham received the sulfoxaflor compound and funding assistance towards sulfoxaflor bioassays from Dow AgroSciences. T. C. Sparks currently acts as a consultant for the agrochemical industry including Corteva Agriscience.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2021.103547.

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