Pleiotropic Effects of Loss of the *Dα1* Subunit in *Drosophila melanogaster*: Implications for Insecticide Resistance

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ABSTRACT Nicotinic acetylcholine receptors (nAChRs) are a highly conserved gene family that form pentameric receptors involved in fast excitatory synaptic neurotransmission. The specific roles individual nAChR subunits perform in *Drosophila melanogaster* and other insects are relatively uncharacterized. Of the 10 *D. melanogaster* nAChR subunits, only three have described roles in behavioral pathways; *Dα3* and *Dα4* in sleep, and *Dα7* in the escape response. Other subunits have been associated with resistance to several classes of insecticides. In particular, our previous work has demonstrated that an allele of the *Dα1* subunit is associated with resistance to neonicotinoid insecticides. We used ends-out gene targeting to create a knockout of the *Dα1* gene to facilitate phenotypic analysis in a controlled genetic background. To our knowledge, this is the first report of a native function for any nAChR subunits known to be targeted by insecticides. Loss of *Dα1* function was associated with changes in courtship, sleep, longevity, and insecticide resistance. While acetylcholine signaling had previously been linked with mating behavior and reproduction in *D. melanogaster*, no specific nAChR subunit had been directly implicated. The role of *Dα1* in a number of behavioral phenotypes highlights the importance of understanding the biological roles of nAChRs and points to the fitness cost that may be associated with neonicotinoid resistance.

KEYWORDS nicotinic acetylcholine receptors; neonicotinoids; resistance; behavior; sleep

NICOTINIC acetylcholine receptors (nAChRs) belong to the Cys-loop receptor subfamily of ligand-gated ion channels (LGICs) that mediate the transduction of a chemical signal into an electrical signal. Activation in response to acetylcholine (ACh), the major excitatory neurotransmitter of the *Drosophila melanogaster* central nervous system, is on a micro- to submicrosecond timescale (Breer and Sattelle 1987; Gundelfinger and Hess 1992; Lee and O'Dowd 1999). nAChRs are expressed in a wide range of tissues, including but not limited to the nervous system (Chintapalli *et al.* 2007). When expressed presynaptically, nAChRs can enhance neurotransmitter release, while postsynaptic expression mediates excitation (Dani *et al.* 2007). Like all

LGICs, nAChRs are pentameric and can consist of several different subunits forming multiple receptor subtypes that vary in both their sensitivity to particular ligands and their permeability to particular cations. Individual subunits consist of a pore-forming transmembrane domain coupled to a large extracellular N-terminal domain, which forms the endogenous ligand-binding site with adjacent subunits (Corringer *et al.* 2000). Upon ACh binding, a conformational change occurs opening the channel pore to permit the flow of cations.

D. melanogaster has 10 nAChR subunits; however, only three of these have been associated with behavioral phenotypes, with specific roles described for *Dα7* in the escape response (Fayyazuddin *et al.* 2006) and for both *Dα3* and *Dα4* in sleep behavior (Shi *et al.* 2014; Wu *et al.* 2014). Mutations in three *D. melanogaster* nAChR subunits confer resistance to two important classes of insecticides; *Dα1* and *Dβ2* to neonicotinoids (Perry *et al.* 2008), and *Dα6* to spinosyns (Perry *et al.* 2007, 2015; Watson *et al.* 2010; Somers *et al.* 2015). Modification of an insecticide target protein can decrease the efficacy of an insecticide through altered affinity or pharmacological response (Ffrench-Constant *et al.* 1993; Williamson *et al.* 1996; Liu *et al.* 2005). Modifications of this

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doi: 10.1534/genetics.116.195750

Manuscript received September 8, 2016; accepted for publication October 25, 2016; published Early Online November 9, 2016.

Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.195750/-/DC1.

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nature can provide a significant fitness boost to individuals in a population during insecticide treatment periods and can rapidly increase in frequency leading to control failures (McKenzie and Batterham 1994). However, allele frequencies will also be shaped by fitness costs if resistant mutations negatively impact reproductive output, either by reducing viability or the capacity to mate (McKenzie 1994; Berticat *et al.* 2002; Foster *et al.* 2003). This could be particularly true for alleles that directly modify the neonicotinoid-binding site as neonicotinoids occupy the same binding site as ACh (Liu and Han 2006; Liu *et al.* 2008). Loss-of-function mutants for the *Dα1*, *Dα6*, and *Dβ2* genes are insecticide-resistant and viable (Perry *et al.* 2007, 2008). The viability phenotype suggests a level of functional redundancy among nAChR subunits. Questions remain as to whether individual subunits have other, nonredundant functions in controlling behavior or if the subtle pharmacological and physiological differences from compensating subunits manifests in altered behaviors. Impacts on mating behavior are of particular interest with respect to potential fitness costs.

While invertebrate nAChR pharmacology and biochemistry with respect to insecticide binding has been examined in detail (Bai *et al.* 1991; Lansdell and Millar 2000; Tomizawa *et al.* 2005), little research has been devoted to the endogenous functions of these receptors. *D. melanogaster* is commonly used as a model insect system to study many facets of biology, including insecticide resistance (Perry *et al.* 2012). A large number of well-defined behavioral paradigms exist to investigate the roles of genes in traits, including sleep and cognition through to mating and auditory faculties (Van den Berg 1986; Eberl *et al.* 1997; Hendricks *et al.* 2001; Smith *et al.* 2008).

Here, we report the creation of a *Dα1* knockout mutant and a rescue system through transgene expression. This provided a consistent genetic background to analyze several behavioral paradigms. These reagents were used to identify roles for the *D. melanogaster* *Dα1* subunit in mating, locomotion, and sleep, demonstrating the diverse pleiotropic influences that nAChRs can have on insect behavior. This research has relevance to the consideration of fitness costs that might be associated with resistance conferring mutations in *Dα1* orthologs in pest insects (Liu *et al.* 2005; Liu and Han 2006), and the behavioral impact that exposure to neonicotinoids may have in beneficial species such as the western honey bee, *Apis mellifera* (Gill *et al.* 2012; Henry *et al.* 2012; Williams *et al.* 2015).

Materials and Methods

Fly lines

To create the *Dα1* deletion, ends-out gene targeting was carried out as described in Huang *et al.* (2009). Briefly, *hs-hid/hs-FLP* (BL25680) flies were crossed to *Dα1* donor flies then allowed to lay in vials or bottles for 24 hr. Adults were then transferred to fresh food and the embryos were heat-shocked in a 38° water bath for 60 min (vials) or 90 min (bottles). F1

progeny with mosaic *w⁺* eyes were then crossed to *GAL4^{447w-}* (BL26258) to select for mobilized donor constructs, and F2 progeny with homogenous *w⁺* eyes were crossed to a double-balancer line to map the donor construct to a chromosome. Of the 314 solid *w⁺* eyed F2 progeny, 29 mapped to the target chromosome. PCR was used to verify the replacement of the *Dα1* gene with the donor construct and then the *white* (*w*) marker was removed by crossing the deletion to a line expressing the *Cre* recombinase (BL1092). The deletion was verified with Southern blot analysis. The *w⁻* X chromosome was replaced by the X chromosome from the wild-type isogenic RAL059 line (BL28129).

UAS-*Dα1* flies were created by cloning the *Dα1* ORF into pGEMT-Easy (Promega, Madison, WI), then subcloning it in the *NotI* site of pUAST-attB. The construct was then injected into the attP40 landing site and crossed into the *Dα1^{KO}* background. The *elav::GAL4* (BL458) driver construct was also crossed into the *Dα1^{KO}* background.

Insecticide bioassays

Insecticide bioassays were carried out according to Perry *et al.* (2012). Dosage mortality curves were plotted from dose-response data, and Probit (Sakuma 1998) was used to calculate LD50 values. Resistance ratios with confidence intervals were calculated as in Bioassays with Arthropods (Russell *et al.* 2009). Data from *Dα1* GAL4-UAS rescue experiments were corrected using Abbott's formula (Rosenheim and Hoy 1989).

Mating assays

Flies were raised at constant temperature with a 12-hr light/dark (L:D) cycle. Experiments were always performed within 3 hr of perceived dawn. Virgin adult males and females were collected under light CO₂ anesthesia, males were stored individually, and females in groups of 5–10. A single 3–5-day-old male was gently aspirated into a mating chamber (12 mm diameter × 4 mm height) and allowed to acclimatize for 2 min. A single female was then introduced and video recording started. Mating pairs were recorded for 10 min or until copulation was initiated. Videos were then analyzed manually noting the times of courtship initiation and copulation initiation. Due to the low number of frames/sec of recordings, unilateral wing extension behavior was used to mark the initiation of courtship. Statistical significance was determined using an unpaired two-tailed *t*-test.

Sleep assays

For sleep measurements, 4–5-day-old males were monitored individually using the DAM2 system (Trikinetics). Activity counts were collected in 1-min bins for both entrainment period (2 days of L:D) and experimental period (4–5 days of L:D). Sleep parameters were computed using pySolo (Gilestro and Cirelli 2009).

Longevity assays

For longevity assay, 1-day-old males were collected in vials of 20 flies. They were then changed into fresh food vials and the

Table 1 *Dα1^{KO}* LC50 and resistance ratios

Line	Imidacloprid LC50 ^a	Imidacloprid RR ^b	Nitenpyram LC50 ^a	Nitenpyram RR ^b
<i>w¹¹¹⁸</i>	0.12 (0.10–0.13)	1 (0.88–1.14)	0.55 (0.47–0.62)	1 (0.82–1.22)
<i>Dα1^{KO}</i>	2.88 (2.40–3.32)	24.86 (20.63–29.96)	25.72 (18.06–33.84)	47.02 (33.55–65.89)

RR, resistance ratio.

^a Calculated insecticide dose (part per million) required for 50% mortality (95% Confidence Interval).^b Calculated resistance ratio (95% Confidence interval).

number of dead flies recorded every 2 days until no survivors were left. A Kaplan–Meier survival curve was constructed and the *P*-value was calculated using a custom R script.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All fly strains are available upon request.

Results and Discussion

To generate a *Dα1* null mutant, a modified ends-out targeting scheme (Huang *et al.* 2009) was used. A precise 57-kb deletion of the *Dα1* genomic region was created, then validated by Southern blot and sequencing (Supplemental Material, Figure S1). This mutant is referred to as *Dα1^{KO}*. Given that previously created *Dα1* alleles are resistant to neonicotinoids (Perry *et al.* 2008), *Dα1^{KO}* was screened on two different neonicotinoids (imidacloprid and nitenpyram) and was found to be highly resistant to both (Figure S2 and Table 1). Calculated LC50 values and resistance ratios were consistent with those measured for *Dα1* mutants studied previously (Perry *et al.* 2008). *Dα1* and orthologs in pest species are well-established targets for several different neonicotinoid insecticides (Liu *et al.* 2005; Perry *et al.* 2008, 2012). Heterologous expression and affinity chromatography studies have also implicated *Dα1* in directly binding imidacloprid at a site that overlaps that normally occupied by the ligand, ACh (Tomizawa and Yamamoto 1992; Lansdell and Millar 2000). Therefore, these resistance data matched our prediction that a mutant with a genomic deletion of *Dα1* would be resistant to neonicotinoid insecticides.

The GAL4-UAS system was employed to create a phenotypic rescue in which a *Dα1* cDNA clone was expressed in the *Dα1^{KO}* background. Expression of the *Dα1* clone using the pan-neuronal *elav*::GAL4 driver rescued sensitivity to both imidacloprid and nitenpyram (Figure S3). The reversion of the resistance phenotype indicates that the subunit expressed from the *Dα1* transgene is assembling into functional nAChRs that bind these insecticides.

The loss of *Dα1* results in significant levels of resistance to neonicotinoids. However, the level of resistance is not of the same magnitude observed in the *Dα6* knockout mutant, which is over 1000-fold resistant to spinosad (Perry *et al.* 2007). The *Dα1^{KO}* mutant is still susceptible when exposed to a high enough dose, which may be due to expression of other neonicotinoid-sensitive nAChR subtypes. Mutations in

the orthologs of *Dα3* and *Dβ1* have been identified in neonicotinoid-resistant strains of *Nilaparvata lugens* (Liu *et al.* 2005) and *Myzus persicae* (Bass *et al.* 2011), respectively. While only one imidacloprid-binding site has been reported in adult *D. melanogaster* and other Dipteran and Lepidopteran species (Tomizawa and Casida 1997; Lind *et al.* 1998), multiple binding sites have been reported in several Hemipteran species (Lind *et al.* 1998; Xu *et al.* 2010; Bass *et al.* 2011). Unlike Hemipterans, Dipterans and Lepidopterans are holometabolous insects that undergo complete metamorphosis from larva to adult. It is possible that as yet undescribed imidacloprid-sensitive nAChR subtypes are expressed in the larval life stages. Another possibility is that a novel subtype is formed as a consequence of the loss of the *Dα1* subunit that alters the mutant's sensitivity to neonicotinoids.

Dα1 courtship and copulation phenotypes

RNAi knockdown of the *Dα1* subunit resulted in defects in courtship and copulation behavior (Table S1). Previous microarray studies (Chintapalli *et al.* 2007), confirmed by RT-PCR (Figure S4), highlighted expression of *Dα1* in both neuronal and reproductive tissues. Taken together, these lines of evidence suggested the potential for *Dα1* to function in mating behavior.

Mating behavior can be influenced by genetic background, for example expression of *w* is important for visual cues and misexpression of this gene can trigger male–male courtship (Zhang and Odenwald 1995). As the *Dα1^{KO}* line was generated in the *w¹¹¹⁸* background, both the mutant and the control line have functionally null copies of *w*. Therefore, the *w⁺* X chromosome from another isogenic line, RAL059 (BL28129), was used to replace the *w¹¹¹⁸* X chromosome present in both the *Dα1^{KO}* mutant line and the *w¹¹¹⁸* background line. These lines will be referred to as the mutant and wild-type lines respectively.

Courtship behavior was measured in terms of the latency of courtship initiation by males after female introduction into the mating chamber (Figure 1). Flies that failed to initiate courtship within the allowed 10-min period were given a maximum value. Wild-type males initiated courtship in every trial, regardless of the genotype of the female partner. Mutant males only initiated courtship 65% of the time with wild-type females and 79% of the time with mutant females. Mutant males also took significantly longer to initiate courtship than wild-type males when paired with either wild-type or mutant females.

Figure 1 Courtship initiation and copulation initiation of wild-type and $D\alpha 1^{KO}$ mutant males measured when paired with either wild-type or mutant females. Mutant males consistently took longer to initiate both courtship and copulation compared to wild-type males, regardless of the genotype of the female partner. Wild-type males initiated courtship with both wild-type and mutant females with equal vigor; however, copulation initiation took significantly longer with mutant females compared to wild-type females. Error bars display 95% confidence intervals (** $P < 0.01$, Student's t -test).

Copulation latency was also measured for the same flies. Mutant males rarely copulated within the 10-min period, only 15% of trials when paired with a wild-type female and only 3% when paired with a mutant female. Wild-type males were more successful, initiating copulation in 90% of the trials when paired with a wild-type female and in 56% of the trials when paired with a mutant female. No significant difference was observed in copulation latency of mutant males when paired with either wild-type or mutant females. In contrast,

Figure 2 Rescue of courtship and copulation initiation by $D\alpha 1$ expression using the *elav::GAL4* driver. Left panels are pairs consisting of wild-type male flies crossed to control or rescue female flies, right panels are pairs consisting of wild-type female flies crossed to control or rescue male flies. No significant differences in courtship initiation of wild-type males were observed regardless of the female's genotype. Wild-type females were courted significantly faster by rescue male flies compared to control male. Copulation initiation was significantly faster for both rescue crosses compared to the appropriate controls. Error bars display 95% confidence intervals (** $P < 0.01$, Student's t -test).

wild-type males did initiate copulation significantly faster with wild-type females compared to mutant females. This suggests that, unlike the latency of courtship initiation that is primarily influenced by the genotype of the male, both sexes contribute to the latency of copulation initiation.

The rescue system was again employed to see if expression of a $D\alpha 1$ transgene could rescue the courtship and copulation phenotypes observed for the $D\alpha 1^{KO}$ mutant (Figure 2). Appropriate driver-only and UAS-only control flies were used for comparison to rescue flies. No discernible differences in courtship initiation were observed when wild-type males were crossed to control or rescue female flies. However, when crossed to wild-type female flies, male rescue flies were more successful in initiating courtship than male control flies (Table S2). Male rescue flies also showed a significant decrease in courtship initiation when crossed to female wild-type compared to male control flies. Significant rescue of copulation

Figure 3 Analysis of $D\alpha 1^{KO}$ sleeping behavior. (A) No significant difference in total mean sleep is observed between wild-type and mutant flies; however, mutant flies sleep significantly less at night and significantly more during the day. (B and C) Mutant flies experience significantly less and shorter sleep episodes during the night and significantly more episodes during the day. Error bars display 95% confidence intervals (* $P < 0.05$, ** $P < 0.01$, Student's t -test).

initiation was also observed in both female and male rescue flies. Rescue flies were both more successful and faster at initiating copulation than the appropriate controls.

It is clear from our data that $D\alpha 1$ plays a role in *Drosophila* mating behavior; however, the underlying mechanism remains unknown. Defects in ACh signaling have previously been associated with abnormal mating behavior (Greenspan 1980). Analysis of mosaic mutants, defective for cholinergic signaling, identified a neuropile in the mushroom body calyx critical for normal male courtship behavior (Greenspan *et al.* 1980). Male-specific, cholinergic neurons have also been identified in the abdominal ganglion, the disruption of which significantly decreased male fertility, potentially due to their innervation of the male reproductive system (Acebes *et al.* 2004). The disruption of these neurons has been hypothesized to result in the uncoordinated or altered release of sperm, seminal fluid, and accessory proteins. The study performed by Acebes *et al.* (2004) used the presynaptic choline acetyltransferase marker to identify these neurons; however, the receptors receiving this signal were not identified. The data from our mating experiments and expression analysis suggests that $D\alpha 1$ may be one of the specific nAChR subunits expressed in this pathway. Another possibility to consider is a higher processing role for $D\alpha 1$ in mating behavior. The phenotypes observed in the $D\alpha 1^{KO}$ mutant are consistent with a defect in one or more sensory modalities. While there are specialized receptors responsible for detecting sensory stimuli, cholinergic signaling has been identified in connecting sensory circuitry to processing centers in the brain (Wong *et al.* 2002). $D\alpha 1$ expression has previously been observed in the

mushroom body calyx and lateral protocerebrum, which includes the lateral horn (Schuster *et al.* 1993). Recently, the role of the lateral horn in locusts has been proposed to serve as a site for multimodal sensory integration (Gupta and Stopfer 2012). This may suggest that $D\alpha 1$ plays a role in integrating multimodal courtship circuitry to higher sensory processing centers. Challenging $D\alpha 1^{KO}$ mutants with sensory-specific behavioral paradigms and neuron-specific rescue could test this hypothesis.

Activity and sleep

We explored the possibility that impaired locomotion may be contributing to the mating phenotype observed in the $D\alpha 1^{KO}$ mutant (Figure S5). Over a 24-hr period, the $D\alpha 1^{KO}$ mutant exhibited hyperactivity; however, it moved at a slower average speed. When average speed was binned in 3-hr intervals, the difference was significant during the middle two 3-hr bins of the day and the last three 3-hr bins of the night. Most importantly, there was no significant difference in average speed during the first 3-hr bin of the day, when courtship assays were performed, indicating that general locomotion deficits did not impact the measurement of mating behavior.

The $D\alpha 1^{KO}$ mutant also has an unusual pattern of sleep (Figure 3). Although there were no significant differences observed in total amount of sleep, mutant flies slept significantly less during the night, experiencing less sleep episodes of significantly shorter duration than wild-type controls. The total time of day sleep experienced by the mutant was significantly longer with a higher number of sleep episodes; however, there was no observable difference in episode length. Expression of the $D\alpha 1$

Figure 4 Modulation of $D\alpha 1^{KO}$ sleeping behavior by $D\alpha 1$ expression using the *elav::GAL4* driver. (A) Total mean sleep is significantly increased, as is mean sleep during the day and night, for rescue flies compared to both control genotypes. (B) Sleep episode lengths are significantly increased in rescue flies compared to control genotypes, particularly at night. (C) No significant difference is observed in the number of day sleep episodes rescue flies experience and a reduced number of night episodes was observed. Error bars display 95% confidence intervals (* $P < 0.05$, ** $P < 0.01$, Student's *t*-test).

transgene in the mutant background increased amount of sleep, both during the day and night (Figure 4). This increase was a result of longer sleep episodes, which may explain why both mutant and rescue flies both show less night sleep episodes. Rescue flies have less night sleep episodes due to the length of these episodes, whereas mutant flies may have trouble with sleep initiation and maintenance. The exceptional increase in episode length observed in rescue flies is likely due to nonnative expression of the transgene; however, it is clear $D\alpha 1$ influences sleep.

While activation of all cholinergic neurons inhibits sleep in flies (Seidner *et al.* 2015), different neuronal groups can be wake-promoting (Yi *et al.* 2013) or sleep-promoting (Wu *et al.* 2014). Individual nAChR subunits also appear to have different roles in sleep regulation (Yi *et al.* 2013; Shi *et al.* 2014; Wu *et al.* 2014). From this study, $D\alpha 1$ seems to have net sleep-promoting effects, especially with sleep maintenance. The increase in daytime sleep observed in mutant flies may be a compensation mechanism to cope with the reduced amount of nighttime sleep (Hendricks *et al.* 2000). Further experiments are needed to determine whether the $D\alpha 1^{KO}$ mutant has intact sleep homeostatic regulation, and whether loss of sleep affects sleep quality. Sleep deprivation has been linked to various detrimental effects, namely reduced life span (Tomita *et al.* 2015) and learning deficits (Seugnet *et al.* 2011).

Longevity

We also measured longevity of $D\alpha 1^{KO}$ mutants, revealing a much shorter life span in the mutant compared to the wild-

type (Figure 5). While longevity is not a direct measure of fitness, the mutants' reduced life span highlights the importance of the $D\alpha 1$ subunit in *D. melanogaster* physiology. It is not clear if this effect is due to a single physiological role of $D\alpha 1$, such as the subunits involvement in sleep, or cumulative effects of several impaired physiological roles that impact the mutant's longevity. In either case, it suggests that a resistance allele in the $D\alpha 1$ subunit is likely to impact the fitness levels of the insect. It also supports the notion that sublethal exposures to insecticides that target receptor subunits orthologous to $D\alpha 1$ encountered by beneficial insects, such as honey bees, are likely to affect their behavior in ways that may also impact their fitness.

Conclusions

The $D\alpha 1^{KO}$ mutant generated in this study provides a useful tool to study the role of $D\alpha 1$ in insecticide resistance but, more significantly, to explore the endogenous roles of this gene.

Based on prior evidence, it was expected that the $D\alpha 1^{KO}$ mutant would be resistant to neonicotinoid insecticides (Perry *et al.* 2008). However, the data presented here allow a fresh evaluation of the value of $D\alpha 1$ and orthologs in other species as insecticide targets. The ability of the $D\alpha 1$ knockout flies to survive at all presents a potential issue for resistance evolution to compounds that target this receptor. Given that a wide range of mutations would lead to a total loss-of-function phenotype, such mutations will arise frequently, conferring insecticide resistance. The data presented here indicate that,

Figure 5 Kaplan–Meier survival plot of $D\alpha 1^{KO}$. The $D\alpha 1^{KO}$ mutant had significantly shorter median survivorship of 34 days compared to that of the control with a median survivorship of 52 days. ($P < 0.01$, Mantel–Cox test).

looking beyond viability, there is a significant fitness cost associated with the total loss of $D\alpha 1$ function, most obvious in terms of severe mating behavior defects, but possibly contributed to by the sleep and longevity phenotypes. Under optimal environmental conditions in the laboratory, large phenotypic differences between $D\alpha 1^{KO}$ and control flies were observed. It is possible that the fitness cost associated with a null allele of this gene would be even greater under less ideal conditions experienced by natural populations. Therefore, while a nonsense mutation resulting in a resistance allele may be viable, it would likely be associated with fitness costs that would prevent it from persisting in the field. In contrast, mutations that may be null alleles have been found in $D\alpha 6$ orthologs in spinosad-resistant insects in a number of species (Baxter *et al.* 2010; Hsu *et al.* 2012). Therefore, it is possible that the spectrum of mutations in $D\alpha 1$ orthologs that can increase in frequency to confer insecticide resistance in a pest species are constrained by fitness costs.

The potential impact of neonicotinoid insecticides on the behavior of beneficial insects, such as *A. mellifera*, has been intensively researched (Gill *et al.* 2012; Henry *et al.* 2012; Williams *et al.* 2015). Eleven nAChR subunits have been identified in *A. mellifera*, including a 1:1 ortholog of $D\alpha 1$ (Jones *et al.* 2006). While it cannot be assumed that the functional roles of the honey bee ortholog are identical to those of $D\alpha 1$, it is likely to influence a range of behaviors that may be perturbed upon exposure to sufficient concentrations of neonicotinoid insecticides.

The genetic resources that can be developed to study gene function in *D. melanogaster*, such as those described here, are extremely powerful. Research on nonmodel insects, both beneficial and pest species, is more challenging. Some func-

tional analysis of $D\alpha 6$ orthologs from pest species has been possible following the appropriate expression of these genes in a *D. melanogaster* $D\alpha 6$ null mutant (Perry *et al.* 2015; Somers *et al.* 2015). A similar approach may be useful in the functional characterization of the orthologs of $D\alpha 1$.

Insecticides can be powerful probes in neuroscience. To exert toxic effects, these chemicals bind to receptors that have crucial roles in neurotransmission. Research using pyrethroids and cyclodiene insecticides has been productive in elucidating the function of sodium channels and ligand-gated chloride channels, respectively (Dong *et al.* 2014; Hosie *et al.* 1997). Similarly, the use of insecticides that target nAChRs has stimulated research on their function. Our data demonstrates that such research will make a vital contribution in providing a detailed knowledge of the role neurotransmission in a wide range of behaviors. The variety of behavioral traits impacted by $D\alpha 1$ loss-of-function indicates a high level of involvement of $D\alpha 1$ in several behavioral neural circuits, which will require further investigation. Further analysis of $D\alpha 1$ and the remaining members of the nAChR gene family with a range of paradigms is likely to reveal function in a wide range of insect behaviors.

Acknowledgments

The authors thank Bruno van Swinderen for valuable discussions and the loan of DAM2 equipment. This research was funded through an Australian Research Council Discovery Project (DP130102415) awarded to PB.

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Communicating editor: G. Bosco

Figure S1. Verification of *DαI* genomic deletion. A) Restriction digest maps showing the expected band patterns from the three restriction enzymes used for each genotype. (A = *Afl*II, B = *Bss*SI and D = *Dra*III). B) Southern blot verifying the deletion of *DαI*, initially the 5' probe was used then the blot was stripped and then probed with the 3' probe. The banding pattern observed was as expected for each genotype with a few unexpected faint bands that was most likely due to incomplete digestion or non-specific binding. C) Sequencing across the *DαI* genomic region in the *DαI*^{KO} mutant showing the replacement of the *DαI* with the attP and loxP sites.

*w*¹¹¹⁸ *Dα1*^{KO}
*w*¹¹¹⁸ 95% C.I. *Dα1*^{KO} 95% C.I.

Figure S2. Imidacloprid and nitenpyram dosage mortality curves for $D\alpha I^{KO}$. A) Imidacloprid dosage mortality curves of w^{1118} and $D\alpha I^{KO}$. B) Nitenpyram dosage mortality curves of w^{1118} and $D\alpha I^{KO}$. $D\alpha I^{KO}$ exhibits the higher survival on both insecticides noted by the rightward shift of the plotted regression line. Probit mortality was calculated using PriProbit (Sakuma 1998) and used to plot a regression line against the log dose of each insecticide. Dashed lines indicate 95% confidence intervals. LC_{50} values (lethal concentration for 50% of the population) are determined at the point where the regression line intersects the X axis.

Figure S3. Larval insecticide bioassays on the $D\alpha I^{KO}$ line expressing the UAS- $D\alpha I$ construct driven by *elav::GAL4*. Significant rescue was observed when UAS- $D\alpha I$ is driven by *elav>GAL4* at A) 1ppm imidacloprid and B) 4ppm nitenpyram. Data were corrected for control mortality using Abbott's formula and plotted with 95% confidence intervals.

Figure S4. D α 1 expression analysis. A) mRNA signal levels of *D. melanogaster* nAChR subunits in neuronal tissues demonstrating that *D α 1* is the lowliest expressed subunits in these tissues. B) Enrichment of *D α 1* mRNA in various tissues compared to whole adult flies. Data for mRNA signal and enrichment are from *Chintapalli et al.* 2007 (www.flyatlas.org). C) To confirm expression of D α 1 in these tissues RT-PCR was performed using cDNA made from different adult tissues. *D α 1* expression was observed in female and male head, testes, accessory glands and the ejaculatory duct.

A)

B)

C)

Figure S5. Activity analysis of the *DαI^{KO}* mutant. A) The *DαI^{KO}* mutant had a higher overall activity index (i.e. spent more time moving) than the wildtype control. This increase in activity could be rescued by *DαI* expression using the *elav::GAL4* driver. B) Over a 24-hour period the *DαI^{KO}* mutant moved at an average slower speed than the wildtype control. C) However, this difference in average speed was dependant on the time of day and was not observed over the first three hours of day time, when mating experiments were performed. (* $p < 0.05$, ** $P < 0.01$)

Table S1 *Dα1* RNAi knockdown

Female	Male	n	n(court)	%(court)	n(cop)	%(cop)
WT	Driver-only	38	32	84	22	57.89
WT	<i>Dα1</i> -RNAi	44	20	45	2	4.55

Table S2. Mating behaviour success

Female	Male	n	n(court)	%(court)	n(cop)	%(cop)
WT	WT	40	40	100	36	90
WT	Da1 ^{KO}	52	34	65.38	8	15.38
Da1 ^{KO}	WT	34	34	100	19	55.88
Da1 ^{KO}	Da1 ^{KO}	29	23	79.31	1	3.45
Driver-only	WT	44	44	100	9	20.45
UAS-only	WT	44	44	100	15	34.09
Rescue	WT	42	42	100	34	80.95
WT	Driver-only	54	18	33.33	38	29.63
WT	UAS-only	36	18	50	10	27.78
WT	Rescue	57	51	89.47	43	75.44