Title: Inhibiting the proteasome reduces molecular and biological impacts of the natural product insecticide, spinosad

Running title: Inhibiting the proteasome reduces molecular and biological impacts of spinosad

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Abstract:

BACKGROUND

Insecticide targets are often identified by mutations that confer resistance, but the intricacies of insecticide binding and downstream processes leading to insect death often remain obscure. Mutations in α6-like nicotinic acetylcholine receptor subunit genes have been associated with high levels of resistance to spinosad across many insect species, including *Drosophila melanogaster*. Here, we aimed to expand the understanding of the effects of the natural product insecticide, spinosad on its protein target, the α6 subunit, using genetic tools available in *D. melanogaster*.

RESULTS

Functional, fluorescently tagged Dα6 subunits (Dα6^{YFP}) were developed to allow observation of the protein *in vivo*. Larvae expressing Dα6^{YFP} were exposed to a sub-lethal spinosyn A concentration (0.025ppm) for six days leading to a 64% reduction in fluorescence relative to unexposed larvae. Direct application of high-doses of spinosyn A to dissected larval brains resulted in a visible 38.25% decrease in Dα6^{YFP} occurring within 20 minutes, indicating that degradation of the Dα6 protein was occurring in response to spinosyn A exposure. Chemical inhibition of the proteasome system using the multiple myeloma treatment drug, PS-341 reduced the loss of Dα6^{YFP} in response to spinosyn A at the 20 minute timepoint to 6.35%. In addition, *in vivo* administration of PS-341 prior to spinosad exposure reduced the effect of spinosad on larval activity.

CONCLUSION

Based on these data, we propose that exposure to spinosad leads to degradation of the α6-like target protein, a potentially novel element in the mode of action of spinosyns which may contribute to their toxicity towards insects.

Keywords:

Insecticide resistance

Spinosad

Nicotinic acetylcholine receptor

Proteasome

1. INTRODUCTION

There are rising concerns about the use of chemical insecticides to control insect pests, due to evidence of collateral damage to beneficial insects, including honey bees, through impacts on behavior and fitness [1, 2]. A link to behavior is not surprising as many classes of insecticide act on targets in the central nervous system, causing fast insect knockdown at extremely low doses [3-5]. A more comprehensive understanding of the mode of insecticide action and the downstream effects that are triggered is vital to inform the prediction of resistance mechanisms that may evolve, the design of resistance management strategies, the development of analogues and novel compounds, and the evaluation of the potential impacts on beneficial insects.

The nicotinic acetylcholine receptors (nAChRs) are targeted by multiple classes of insecticides. These pentameric proteins are ligand gated ion channels. The ligand, acetylcholine (ACh), binds at the interface of two adjacent subunits. This causes a conformational shift and opens the central ion-permeable pore, mediating fast synaptic transmission [6-8]. Insecticides can act through the orthosteric ligand binding site and have clear modes of action. One class of insecticides targeting nAChRs, the neonicotinoids (IRAC), act as competitive agonists, activating the receptor [3, 5]. The mode of action of the spinosyn class of insecticides that also target nAChRs is less clear. The spinosyns (IRAC class 5) are fermentation products of the soil actinomycete, *Saccharopolyspora spinosa* [9]. One widely used active ingredient, spinosad (SPIN), is a mixture of two structurally similar macrocyclic lactones, 85% spinosyn A (SPINA) and 15% spinosyn D. Early mode of action studies showed that spinosyn A depolarizes insect neurons, acting on nAChRs in the central nervous system which, in turn, activate motor neurons and cause involuntary muscle contractions leading to paralysis of the insect [10-12]. SPINA prolongs the response of nAChRs to the natural ligand ACh, suggesting that, in contrast to the neonicotinoids, it does not bind to the ligand binding domain and acts allosterically [11, 13]. Later work on resistant insects identified α6-like nAChR subunits in *Drosophila melanogaster* as integral for the mode of action of SPIN with the loss of α6-subunit function conferring high levels of SPIN resistance [10, 14]. Further studies on a wide variety of insect species similarly associated high levels of SPIN resistance with orthologs of α6-like nAChR

subunits [15-20]. Additional evidence supporting the allosteric interactions of SPIN with the Dα6 receptor comes from chimeric studies of Dα6 and Dα7 subunits which implicate the α6 transmembrane domain region with the binding of SPIN [21].

While developing heterologous expression systems for the analysis of insect nAChRs has until recently proven difficult [22], pentamers of Dα5/Dα6 (co-expressed with a chaperone Ric-3) have been functionally characterized [10, 23]. Testing of spinosyns showed SPINA can activate the receptor, although Watson and colleagues also noted that there may be other native receptor subtypes that could have different response profiles [10].

This study examines the effects of SPIN on its target receptor, *Dα6,* using fluorescently tagged constructs to conduct both *in vivo* and *ex vivo* analysis in *D. melanogaster.* We characterized impacts of SPIN on $D\alpha6^{YFP}$ levels and used chemical inhibitors to identify a novel mechanism that may contribute to SPIN toxicity. Based on our findings we propose an additional component in the mode of action of spinosyns, whereby in addition to the reported activation of the receptor [11], SPIN binding results in the Dα6 subunit being removed from the membrane and degraded and that this is associated with the toxicity of the insecticide.

2. MATERIALS AND METHODS

2.1. Media, compounds and solvents used in this study

Each litre of fly media used for rearing flies contained 5g/L agar, 12g/L yeast, 53g/L glucose, 27g/L sucrose, 67g/L semolina, 5ml/L propionic acid, 0.5ml/L phosphoric acid and 1.5g/L p-hydroxy benzoic acid methyl ester. 10ml of media was added to individual 25mm x 95mm polystyrene vials (Genesee Scientific). The chemicals used in this study were dissolved in a variety of solvents as follows. Ivermectin (IVR), (Sigma Aldrich) was dissolved in DMSO. The imidacloprid-containing formulation (IMI) was Confidor® 200 SC (Bayer Crop Sciences) which was diluted using water as a solvent. The spinosad-containing formulation (SPINF) was Success® (Yates) and this was diluted using water as a solvent prior to addition to media for rearing assays or it was diluted using 5% sucrose for the larval activity assays. Pure spinosyn A (SPINA) was synthesised by Dow Agrosciences, while technical grade spinosad (SPIN) was purchased from Chem Service Inc and were dissolved in DMSO. PS-341 (Cell signalling

technologies) was dissolved in DMSO when applied to dissected brains and in ethanol when used for the larval activity assays.

2.2. *D. melanogaster* strains used in this study

The *D. melanogaster* strains used in this study are listed in Table 1.

2.2.1. *D. melanogaster* crosses

For all experiments using Dα6>GAL4 to drive expression of the Dα6^{YFP} or Dα7^{YFP} receptor subunits (GAL4>UAS system [24]), virgin females of the driver line were crossed to males from the appropriate tagged subunit line. All crosses for analysis of the tagged receptors were in the genetic background of the hypomorphic *dα6* allele, *dα6^{nx}*. Hence, females were heterozygous for the Dα6>GAL4 construct and both males and females were heterozygous for the 3rd chromosome UAS-*Dα6^{YFP}* or UAS-*Dα7^{YFP}* constructs and homozygous for *dα6^{nx}*.

2.3. Sub-lethal exposure of larvae during development

30 1st instar larvae (1-8 hours post hatching) were counted onto undosed fly media, or onto fly media containing doses of insecticide (0.025ppm SPINF, 0.08ppm IMI or 0.0025ppm IVR). Vials were kept at room temperature, in the dark, for six days. Larvae that were exposed were variously used in the relative movement assay, dissected for imaging or collected for protein quantification.

2.4. Relative movement assay

Larvae, collected after hatching and then reared for 6 days on undosed fly media or on the relevant sub-lethal exposure doses as described above were used in the assay. Individual wells of a NUNC cell culture treated 24 well plate (Thermo Fisher Scientific), containing 200ul of 5% sucrose media had 25 larvae placed into them with a minimum of 4 wells used per treatment. Larvae were then filmed at the 0 minute time point and then 50 ul of insecticide was added into the wells, mixed and then 50ul taken out to return the well volume to 200ul. The final concentrations in assay wells were 14ppm SPINF in 200ul of

5% sucrose. 30 second video recordings were taken at 0, 15, 30, 45, 60 and 90min timepoints after addition of insecticide [25].

2.4.1.Relative movement assay following pre-incubation with PS-341

Larvae were prepared as per the standard relative movement assay protocol as described above without insecticide exposure and placed into wells. There was a step added to pre-incubate larvae, where 50µl of PS-341 (4000nM) dissolved in EtOH, or a control of EtOH only was added to wells and larvae left for 60 minutes. Following this, 50ul of 10ppm SPIN or SPINF was added to wells as per the original assay above, with a final well concentration in this assay of 2ppm. Larvae were filmed at time point 0 (pre addition of insecticide), 15, 30, 45, 60 and 90 min as described above.

2.5. Western Blot

Replicates of 20-30 larvae from the chronic exposure protocol (control, 0.08ppm IMI, 0.025ppm SPINF and 0.025ppm SPINA) were individually bisected, their anterior half containing the brain were collected, snap frozen and homogenized in 1X Radioimmunoprecipitation assay buffer (10X RIPA buffer, Abcam) with c0mplete protease inhibitor (Roche) to lyse the samples. Samples were centrifuged at 15000g for 15 minutes and the supernatant collected. A total of 40ug of protein, as measured by Qubit protein assay (Thermo Fisher Scientific), was boiled in 6X Laemmli loading buffer and loaded onto two replicate 12.5% polyacrylamide gels and these were run at 150V for 70 minutes and then transferred to a Polyvinylidene difluoride (PVDF) membrane at 30 volts for 1 hour. Subsequently the membranes were blocked with 5% skim milk dissolved in Tris-buffered saline. One blot underwent primary antibody incubation overnight at 4ºC with the Dα6 antibody [10] at a concentration of 1/1000 in Tris-buffered saline. For the other blot, we used the rabbit anti-Beta actin (Cell Signaling Technologies) at a dilution of 1:3000 as a housekeeper to normalize samples. Both blots were probed using anti-rabbit HRP conjugated secondary antibody (Cell Signaling Technologies) at a dilution of 1:10000 as per manufacturer's instructions and protein bands detected by chemiluminescence using Clarity™ Western ECL substrate (Bio-Rad). Blots were imaged using the Chemidoc Chemiluminescence Imager (Bio-Rad) and analysed using 'Image Lab software' to quantify band intensity.

2.6. Quantification of transcript expression levels in larvae developed on sublethal doses of insecticide

Following manufacturer's instructions, TriSURE (Bioline) RNA was extracted from the Armenia[14] larvae that had been exposed to sublethal doses of insecticide treatment as described (0.025 ppm SPINF, control media and 0.08 ppm IMI). 1µg of RNA was used to make cDNA using the GoScript reverse transcriptase system (Promega). Three technical replicates for each of the 3 biological replicates were used as templates for the real time PCR which also included a no template control. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) table is provided (Table S1) and analysis of relative expression was performed using the $2^{-\Delta\Delta Ct}$ method [26]. The sequence and purpose of primers used in the study are provided (Table S2).

2.7. Recombineering yellow fluorescent proteins into dα6 and dα7.

RNA was extracted (using Trizol as per manufacturer's protocol) from whole Armenia[14] 3rd instar larvae The cDNA generated using Superscript III (Invitrogen) was cloned into the pGEM-T-easy plasmid using primers for *dα6* and *dα7* (Table S2). The clones were sequenced (Macrogen, Korea) and clones for *dα6* and *dα7* shuttled into the p[ACMAN] vector [27] using NotI (NEB) digestion and ligation with T4 DNA ligase (Promega). The protocol for recombineering of the three constructs was performed as per [27], using primers as indicated in Table S2 to create the GalK and YFP cassettes for each step. The diagnostic primers were used to determine correct recombineering events. The *Dα6^{YFP}* construct was shuttled to the pUASTattB vector [28] using NotI (NEB) while *Dα7^{YFP}* was shuttled into the pUASTattB vector using XhoI/NotI double digest, due to loss of its 5' NotI site. The Wizard Plasmid miniprep kit (Promega) was used to purify these prior to sequencing to confirm constructs were correct (Macrogen, Korea). The verified *UAS-Dα6^{YFP}* and *UAS-Dα7^{YFP}* plasmids were quantified using a Qubit dsDNA BR Assay kit (Thermo Fisher Scientific) and the concentration adjusted to 250ng/ul. Individual clones were then injected into the *φX-86Fb; dα6^{nx}* background following a standard *D. melanogaster* microinjection protocol [29].

2.8. Bioassay to test function of the Dα6^{YFP} protein

Survival of larvae through to adulthood was assayed using *D. melanogaster* media dosed with SPINF as per previous studies [29]. Doses ranged from 0.05ppm through to 0.5ppm SPINF. Vials of fly media, each having 50 1st instar larvae (8-16 hours post hatching) placed in them (5 replicates), were kept in the dark at 25^{o}C until adult eclosion was recorded at 16-18 days. Results were corrected for control mortality (Abbott's correction) and 95% confidence intervals calculated [30].

2.9. Confocal Microscopy and image analysis of larval brains

A Leica TCS SP5 confocal microscope (Biological Optical Microscopy Platform, University of Melbourne), was used for all confocal imaging with the use of the Leica Application Suite. Laser power was adjusted to 20% and the gain settings were adjusted to prevent oversaturation of the image. We used the 514nm wavelength to image Dα6^{YFP} and Dα7^{YFP} while the 488nm wavelength was used to image CD8:GFP tagged protein in larval brains. The 20x objective lens was used in conjunction with a frame rate of 4 and line rate of 2 to image the brains. Image resolution was 512 x 512 pixels, Z stacks were comprised of 2µm slices. Confocal images were processed with Image J software [31] and 3D stacks were converted to maximum intensity Z projections. Thresholding was applied to remove background fluorescence and analysis was limited to these areas. Overall brain fluorescence was measured, and measurements were pooled into their respective treatment groups. Fluorescence changes were measured as a percentage change and samples were then pooled into either control or appropriate insecticide treatment groups. A Student's t-test was performed comparing all treatments with the undosed control brains.

2.10. Standard *ex vivo* exposure assay

Brains were dissected from 3rd instar *D. melanogaster* larvae that had reached the late wandering stage after being reared on fly media. The dissected brains were placed on 8 well cell culture slides (IBIDI) with 8 µl of Vectashield®. After the brain was imaged initially, 2 µl of 120ppm SPINA dissolved in DMSO was dropped onto the brain (24ppm final SPINA concentration). When imaging dissected larval brains during the *ex vivo* exposure assay, we measured maximal intensity Z projection of stacks at time points 0, 10 and 20 minutes, where time 0 was when the drop of insecticide was applied to the larval brain.

Thresholding was applied to the stacks to remove background fluorescence and analysis was limited to these areas. Overall brain fluorescence was measured from each time point and a paired t-test was performed, comparing all the time points for individual brains to time 0, which acted as the control time point. Changes in fluorescence were measured as a percentage change.

2.11. Pre-incubation *ex vivo* exposure assay

Brains were dissected and prepared as per standard *ex vivo* exposure assay protocol above, however larval brains were pre-incubated with 2ul of 20uM PS-341 (or 2ul 100% ethanol) mixed with 6ul of Vectashield® for 30 minutes before being exposed to 2ul of 30ppm SPINA (6ppm final SPINA concentration). The stacks were imaged before the chemical pre-exposure, immediately after the exposure, 10 minutes after the SPINA exposure and 20 minutes after SPINA was added and data analyzed as per the standard *ex vivo* assay.

3. RESULTS AND DISCUSSION

3.1. Effects of SPIN exposure during larval development

3.1.1. Larvae reared on low doses of SPINF are less affected by subsequent high doses of SPINF.

Initially the effects of insecticide exposure during development of wildtype (Armenia[14]) *D. melanogaster* larvae was examined. Following six days of exposure to sub-lethal doses of SPINF or IMI, 3rd instar larvae were collected and tested for their response to SPINF using a standard relative movement assay [25]. Differences in activity levels of these pre-exposed larvae in response to SPINF exposure was compared. After 90 minutes of the assay, control larvae had a 77.3% reduction in movement, while those raised on SPINF were not as affected, with only a 32.6% reduction in movement (Fig. 1A.). Larvae reared on IMI, a neonicotinoid insecticide that also acts on different nAChRs [3] also did not show any difference in movement compared with controls (Fig. 1A.).

3.1.2. Development and validation of a tagged Dα6 protein to visualize protein levels *in vivo*

Given the link between spinosyn insecticides and the Dα6 subunit, [10, 14], we examined the transcript levels of *dα6* and no change was detected for those larvae reared on either SPINF or IMI (Fig. 1B). We then attempted to quantify Dα6 protein levels in wildtype larvae (Fig. S1). We used a polyclonal Dα6 antibody which detected a band of the previously published protein size (approximately ~53kD) and intensity of this band was analysed (Fig S1A, D). Analysis showed a decreased intensity of this protein band for larvae that had developed for six days on SPINA (16% decrease) or SPINF (29% decrease) dosed fly media (Fig. S1D). Interestingly, Dα6 protein levels increased 27% when reared on IMI (Fig. S1D), but this does not result in an altered response to SPINF (Fig. 1A). Strong higher and lower molecular weight bands were observed both in wildtype and in the $d\alpha6^{nx}$ mutant (Fig. S1C) indicating there was non-specific protein recognition by the antibody. This could impact accurate measurement of changes in protein levels, particularly if Dα6 aggregates were present and masked by these higher molecular weight bands, so an alternative method of examining Dα6 was developed to overcome this.

In order to more accurately assess the impact of spinosad on the Dα6 protein *in vivo*, a transgenic line expressing a YFP tagged Dα6 ($D\alpha6^{YFP}$) was created (Fig. 2A). We expressed $D\alpha6^{YFP}$ in the $d\alpha6^{nx}$ genetic background and observed a fluorescence pattern in the dissected brains of 3rd instar larvae (Fig. 2B.) that is similar to the published expression pattern of the driver [29]. The $d\alpha6^{nx}$ mutant is highly resistant to SPINF [29] and was unaffected at all doses tested (Fig. 2C). The wild type *Dα6* subunit was able to complement the null mutation and restore sensitivity to SPINF at all doses (Fig. 2C). The *Dα6-YFP* subunit partially complemented the null mutation at 0.005ppm SPINF and fully complemented it at 0.1ppm SPINF and above, indicating the fusion protein was functional and that it can respond to SPINF in a similar manner to wildtype Dα6 (Fig. 2C). Larvae were again reared from hatching on a sub-lethal dose of 0.025ppm SPINA and 0.025ppm SPINF for six days. Imaging revealed a 64% decrease in fluorescence for larvae reared on SPINA relative to control larvae (Fig. 3A-C) and a 62.8% decrease in levels of fluorescence in brains of larvae reared on SPINF (Fig. 3C). No decrease was observed in flies expressing a membrane localized GFP with the *dα6*>GAL4 driver when reared on SPINF (Fig. S2A-C).

3.1.3. Testing effects of SPINF on different subunits and of different insecticides on Dα6 levels indicates a specific rather than general response.

In order to test whether the impact of SPINF on Dα6^{YFP} was nAChR subunit specific, a closely related nAChR subunit Dα7 (53% identity/63% similarity to Dα6) [32], not previously associated with SPIN resistance [29], was tested. We tagged Dα7 at an analogous position to that of Dα6 in the TM3-TM4 loop, Dα7^{YFP}. The fluorescence levels for Dα7^{YFP} in unexposed 3rd instar larval brains were lower than for Dα6^{YFP}. This was likely to be due, in part, to native expression of untagged Dα7, but also potentially due to post-transcriptional regulation of Dα7^{YFP} subunit protein levels. Rearing of larvae on sub-lethal doses of SPINF did not alter levels of Dα7^{YFP} fluorescence observed (Fig. S3 A-C).

To examine the specificity of the reduced levels of Dα6^{YFP} in response to rearing on SPINA (Fig. 3), larvae were also subjected to sub-lethal exposure to IMI and IVR during development. The binding sites for imidacloprid overlap the ACh binding site [3, 5]. The macrocyclic lactone, IVR, targets the insect GluCl1α receptor [33, 34], possibly at a site analogous to the binding site of SPIN on nAChRs [18]. IMI exposure increased Dα6^{YFP} fluorescence (28.73%, $p<0.00001$ Student's t-test) (Fig. 3C). IVR exposure did not significantly impact the Dα6^{YFP} levels ($p=0.114$, Student's t-test), Fig 3C). Of the insecticide treatments examined in this study, only SPINF or SPINA decreased the expression levels of Dα6^{YFP}.

3.2. Direct observation of decreasing Dα6^{YFP} levels in larval brains using an *ex vivo* assay.

An *ex vivo* exposure assay was developed to allow a high-dose of SPINA to be directly applied to larval brains to mitigate factors such as uptake, metabolism and transport, allowing us to assess the speed with which Dα6 levels are impacted. Using this *ex vivo* exposure assay, fluorescence was again measured and significant changes were observed from 10 minutes, with a 38.25% reduction in fluorescence observed at 20 minutes (Fig. 4D-G). There was no observed change in fluorescence for brains exposed to DMSO only as a control ($p=0.136$) (Fig. 4A-C & G).

3.2.1. Chemical disruption of the ubiquitin proteasome system inhibits SPINA induced loss of Dα6^{YFP} and reduces effects of SPIN and SPINF on larval activity.

The speed of the response we observed suggested a mechanism involving protein degradation rather than reduced protein production. To further investigate this, we modified our *ex vivo* exposure assay to include pre-treatment with a chemical inhibitor of the Ubiquitin Proteasome system (UPS), PS-341. PS-

341 binds to the catalytic site of the 26S subunit of the proteasome and has been shown to inhibit the function of the proteasome in both vertebrates [35, 36] and in *D. melanogaster* [37]. The pre-treatment with PS-341 limited the reduction in fluorescence to 6.35% over a 20 minute SPINA exposure ($p<0.0001$, Paired t-test) (Fig 5. D-G). No change in fluorescence was observed when pre-treating the brains with PS-341 (Fig. 5G).

Based on the earlier observation that sub-lethal pre-exposure to SPINF or SPINA during development reduced both the level of $D\alpha6^{YFP}$ (Fig 3C) and in the case of developmental exposure to SPINF, the impact of subsequent SPINF exposure on larval movement (Fig. 1A) we wanted to test whether blocking the degradation of Dα6 using PS-341 would also change the response of larvae to SPIN. The activity levels of the PS-341 pre-treated wildtype larvae were significantly higher in our movement assay than those of the ethanol-only control at both 60 and 90 minutes after SPIN exposure (Fig. 5H). At the 90 minute endpoint the final relative movement ratio for PS-341 pre-treated larvae was 0.71 and for solvent only pre-treated larvae it was 0.42 ($p<0.0001$, Student's t-test) (Fig. 5H). Additional controls showed that activity levels of PS-341 pre-treated wildtype larvae were similar to those which had not been exposed to PS-341 (Fig. S4). This result appears counter-intuitive, given that loss of function *Dα6* mutants are highly resistant [25]. PS-341 appeared to prevent the degradation of the insecticide target, Dα6, so we had expected an increase in the level of sensitivity. Proteasomal inhibitors, and specifically PS-341, are known to lead to a number of detrimental impacts on cells, particularly neurons, including oxidative stress and a build-up of misfolded proteins [38] so the reduced effect of SPIN in the presence of PS-341, albeit in an acute assay, was somewhat surprising. The doses applied in our *ex vivo* model system are relatively high in comparison to those known to elicit a response in electrophysiological assays, however given the congruence between the receptor degradation observed in the chronic assays and the ability of the PS-341 to both reduce the loss of the fluorescently tagged receptor and reduce the effects of the dose on wildtype larval activity we believe it provides a valuable tool for examining the interaction of spinosyns and the Dα6 receptor.

4. CONCLUSIONS

4.1. The level of the Dα6 subunit responds dynamically to insecticide exposure.

It has long been known that ligands can influence nAChR levels. This is particularly true of nicotine and its influence in increasing cell surface expression of α4/β2 receptors and other subtypes in the brains of smokers, in cultured cells and animal models [39-41]. Chronic exposure to nicotine has also been shown to down-regulate human α6 containing nAChRs in rat brains (note human α6 is not an ortholog to Dα6) [42, 43]. IMI binds at the orthosteric nAChR acetylcholine binding site (as does nicotine), [44] and IMI increased the level of Dα6^{YFP} (Fig. 3C). Spinosyn's likely bind at an allosteric site on the nAChR [13], and here we demonstrated that exposure clearly results in a decreased level of Dα6^{YFP} (Fig. 3C, 4D-G.). Such changes in response to insecticide exposure reflects a dynamism in their nAChR targets not previously reported and strongly suggests exposure to both high and low concentrations of insecticides may impact the proportion of different nAChR subtypes present at synapses.

4.2. The degradation of Dα6 by the UPS contributes to SPIN's mode of action

Previous studies on SPIN revealed spinosyn-activated currents in dissected ganglia, so the death of exposed insects is thought to occur via exhaustion of the nervous system [11]. We have shown that the effects of SPIN are also associated with degradation of the Dα6 target and that there is a reduced effect when proteasomal degradation is impeded. Only one protein degradation pathway was examined here and so it could be interesting to examine additional pathways in future studies. Based on our data we hypothesize that an additional mechanism initiated by SPIN binding to Dα6 may occur. We propose that the receptor or the insecticide-receptor complex might enter the proteasome degradation pathway. While the fate of the SPIN molecule is unknown, if it enters the cell still bound to the receptor, it could be liberated to act on further intracellular targets. For example, SPIN has been reported to cause cellular damage via mitochondrial dysfunction, oxidative stress and cell death [45, 46]. Alternatively, the degradation of Dα6 could overload proteasome function, also known to cause oxidative stress (for review see [47]). Either of these alternatives could potentially account for the resistance observed in Dα6 loss of function mutants as the absence of the SPIN target means there is no receptor degradation, an effect that is phenocopied by PS-341 treatment. Hence, in the mutants, SPIN would not be ferried into cells to interact with other targets and there would be no stress imposed on the degradation system. This could help to explain why we observed a decrease in the sensitivity of larvae to SPIN when PS-341 was used to inhibit

the proteasome and which, based on our hypothesis, would block the loss of Dα6 protein, potentially leaving more target available for SPIN to bind to, something that might otherwise be expected to result in a higher sensitivity to SPIN. Overall, our findings suggest there may be an additional or alternative mode of action for SPIN, beyond the influx of ions into neurons, with the toxicity involving the degradation of the Dα6 receptor target. This suggests a novel mode of action because, to date, the interactions between other ligand gated ion channels and insecticides have been observed to occur at the membrane impacting ion flux. While these studies have been conducted in *D. melanogaster*, the α6 subunit is highly conserved among insects [29], so this mode of action may apply to a wide range of pest and beneficial insects.

In general, our understanding of what transpires downstream of the interaction between an insecticide and its target is fragmented; SPIN is not an isolated case. There is accumulating evidence of low-dose insecticide exposures causing significant perturbations of metabolism and energy production [2, 48]. Given current concerns about the potential for sub-lethal doses of insecticides to threaten the viability of non-pest insect populations [49-51], there is a need to fill the gap in our understanding of what transpires between the insecticide:target interaction and the behavioral and fitness defects that are being so intensively investigated. Unravelling the complete mode of action of insecticides, from binding through to molecular events that lead to the eventual death of the insect for both acute and for chronic exposure levels, is an important step towards this. These insights may offer opportunities to develop interventions which can mitigate any collateral impacts or they might assist in the discovery of new, more pest-selective insecticides that are safer to non-target organisms.

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Conflict of interest declaration

The authors have no conflict of interest to declare.

References

1. Blacquiere T, Smagghe G, van Gestel CA and Mommaerts V, Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21**: 973-992 (2012).
2. Christen V, Krebs J, Bunter I and Fent K, Biopesticide spinosad induces transcriptional alterations in genes associated with energy production in honey bees (*Apis mellifera*) at sublethal concentrations. *J Hazard Mater* **378**: 120736 (2019).
3. Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M and Sattelle DB, Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol Sci* **22**: 573-580 (2001).
4. Sparks TC and Nauen R, IRAC: Mode of action classification and insecticide resistance management. *Pestic Biochem Physiol* **121**: 122-128 (2015).
5. Tomizawa M, Lee DL and Casida JE, Neonicotinoid insecticides: molecular features conferring selectivity for insect versus mammalian nicotinic receptors. *J Agric Food Chem* **48**: 6016-6024 (2000).
6. Changeux JP and Edelstein SJ, Allosteric mechanisms of signal transduction. *Science* **308**: 1424-1428 (2005).
7. Corringer PJ, Le Novere N and Changeux JP, Nicotinic receptors at the amino acid level. *Annual review of pharmacology and toxicology* **40**: 431-458 (2000).
8. Hucho F, The nicotinic acetylcholine receptor and its ion channel. *European Journal of Biochemistry* **158**: 211-225 (1986).
9. Mertz FP and Yao RC, *Saccharopolyspora spinosa sp. nov*. Isolated from Soil Collected in a Sugar Mill Rum Still. *International Journal of Systematic Bacteriology* **40**: 34-39 (1990).
10. Watson GB, Chouinard SW, Cook KR, Geng C, Gifford JM, Gustafson GD, et al., A spinosyn-sensitive *Drosophila melanogaster* nicotinic acetylcholine receptor identified through chemically induced target site resistance, resistance gene identification, and heterologous expression. *Insect Biochem Mol Biol* **40**: 376-384 (2010).
11. Salgado VL, Studies on the mode of action of spinosad: insect symptoms and physiological correlates. *Pesticide Biochemistry and Physiology* **60**: 91-102 (1998).
12. Salgado VL, The modes of action of spinosad and other insect control products. *Down to Earth* **52**: 6-13 (1997).
13. Salgado VL and Saar R, Desensitizing and non-desensitizing subtypes of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons. *J Insect Physiol* **50**: 867-879 (2004).
14. Perry T, McKenzie JA and Batterham P, A *Dalpha6* knockout strain of *Drosophila melanogaster* confers a high level of resistance to spinosad. *Insect Biochem Mol Biol* **37**: 184-188 (2007).
15. Hsu JC, Feng HT, Wu WJ, Geib SM, Mao CH and Vontas J, Truncated transcripts of nicotinic acetylcholine subunit gene *Bdalpha6* are associated with spinosad resistance in *Bactrocera dorsalis*. *Insect Biochem Mol Biol* **42**: 806-815 (2012).
16. Wang X, Ma Y, Wang F, Yang Y, Wu S and Wu Y, Disruption of nicotinic acetylcholine receptor α6 mediated by CRISPR/Cas9 confers resistance to spinosyns in *Plutella xylostella*. *Pest Management Science* **76**: 1618-1625 (2020).
17. Silva WM, Berger M, Bass C, Williamson M, Moura DM, Ribeiro LM, et al., Mutation (G275E) of the nicotinic acetylcholine receptor alpha6 subunit is associated with high levels of resistance to spinosyns in *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *Pestic Biochem Physiol* **131**: 1-8 (2016).

18. Puinean AM, Lansdell SJ, Collins T, Bielza P and Millar NS, A nicotinic acetylcholine receptor transmembrane point mutation (G275E) associated with resistance to spinosad in *Frankliniella occidentalis*. *J Neurochem* **124**: 590-601 (2013).
19. Geng C, Watson GB and Sparks TC. Nicotinic Acetylcholine Receptors as Spinosyn Targets for Insect Pest Management. In *Advances in insect physiology*, ed. by Cohen E, pp. 2 volumes (2013).
20. Baxter SW, Chen M, Dawson A, Zhao JZ, Vogel H, Shelton AM, et al., Mis-spliced transcripts of nicotinic acetylcholine receptor alpha6 are associated with field evolved spinosad resistance in *Plutella xylostella* (L.). *PLoS Genet* **6**: e1000802 (2010).
21. Somers J, Nguyen J, Lumb C, Batterham P and Perry T, In vivo functional analysis of the *Drosophila melanogaster* nicotinic acetylcholine receptor Dalpha6 using the insecticide spinosad. *Insect Biochem Mol Biol* **64**: 116-127 (2015).
22. Ihara M, Furutani S, Shigetou S, Shimada S, Niki K, Komori Y, et al., Cofactor-enabled functional expression of fruit fly, honeybee, and bumblebee nicotinic receptors reveals picomolar neonicotinoid actions. *Proceedings of the National Academy of Sciences* **117**: 16283-16291 (2020).
23. Lansdell SJ, Collins T, Goodchild J and Millar NS, The Drosophila nicotinic acetylcholine receptor subunits Dalpha5 and Dalpha7 form functional homomeric and heteromeric ion channels. *BMC neuroscience* **13**: 73 (2012).
24. Brand AH and Perrimon N, Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**: 401-415 (1993).
25. Denecke S, Nowell CJ, Fournier-Level A, Perry T and Batterham P, The Wiggle Index: An Open Source Bioassay to Assess Sub-Lethal Insecticide Response in *Drosophila melanogaster*. *PLoS ONE* **10**: e0145051 (2015).
26. Livak KJ and Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-408 (2001).
27. Venken KJT, He YC, Hoskins RA and Bellen HJ, P[acman]: A BAC transgenic platform for targeted insertion of large DNA fragments in *D. melanogaster*. *Science* **314**: 1747-1751 (2006).
28. Bischof J, Maeda RK, Hediger M, Karch F and Basler K, An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 3312-3317 (2007).
29. Perry T, Somers J, Yang YT and Batterham P, Expression of insect alpha6-like nicotinic acetylcholine receptors in *Drosophila melanogaster* highlights a high level of conservation of the receptor:spinosyn interaction. *Insect Biochem Mol Biol* **64**: 106-115 (2015).
30. Rosenheim JA and Hoy MA, Confidence Intervals for the Abbott's Formula Correction of Bioassay Data for Control Response. *Journal of Economic Entomology* **82**: 331-335 (1989).
31. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al., Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**: 676-682 (2012).
32. Grauso M, Reenan RA, Culetto E and Sattelle DB, Novel putative nicotinic acetylcholine receptor subunit genes, *Dalpha5, Dalpha6 and Dalpha7,* in *Drosophila melanogaster* identify a new and highly conserved target of adenosine deaminase acting on RNA-mediated A-to-I pre-mRNA editing. *Genetics* **160**: 1519-1533 (2002).
33. Wolstenholme AJ and Rogers AT, Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. *Parasitology* **131 Suppl**: S85-95 (2005).
34. Cully DF, Vassilatis DK, Liu KK, Paress PS, Van der Ploeg LH, Schaeffer JM, et al., Cloning of an avermectin-sensitive glutamate-gated chloride channel from Caenorhabditis elegans. *Nature* **371**: 707-711 (1994).
35. Rezvani K, Teng Y and De Biasi M, The ubiquitin-proteasome system regulates the stability of neuronal nicotinic acetylcholine receptors. *J Mol Neurosci* **40**: 177-184 (2010).

36. Chen D, Frezza M, Schmitt S, Kanwar J and Dou QP, Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Curr Cancer Drug Targets* **11**: 239-253 (2011).
37. Tsakiri EN, Sykiotis GP, Papassideri IS, Terpos E, Dimopoulos MA, Gorgoulis VG, et al., Proteasome dysfunction in Drosophila signals to an Nrf2-dependent regulatory circuit aiming to restore proteostasis and prevent premature aging. *Aging Cell* **12**: 802-813 (2013).
38. Sherman MY and Goldberg AL, Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* **29**: 15-32 (2001).
39. Nashmi R, Dickinson ME, McKinney S, Jareb M, Labarca C, Fraser SE, et al., Assembly of alpha4beta2 nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits: effects of localization, trafficking, and nicotine-induced upregulation in clonal mammalian cells and in cultured midbrain neurons. *J Neurosci* **23**: 11554-11567 (2003).
40. Gentry CL and Lukas RJ, Regulation of nicotinic acetylcholine receptor numbers and function by chronic nicotine exposure. *Curr Drug Targets CNS Neurol Disord* **1**: 359-385 (2002).
41. Benwell MEM and Balfour DJK, Evidence that Tobacco Smoking Increases the Density of (−)-[3H] Nicotine Binding Sites in Human Brain. *Journal of …* 1988).
42. Perry DC, Mao D, Gold AB, McIntosh JM, Pezzullo JC and Kellar KJ, Chronic nicotine differentially regulates alpha6- and beta3-containing nicotinic cholinergic receptors in rat brain. *J Pharmacol Exp Ther* **322**: 306-315 (2007).
43. Perez XA, McIntosh JM and Quik M, Long-term nicotine treatment down-regulates alpha6beta2* nicotinic receptor expression and function in nucleus accumbens. *J Neurochem* **127**: 762-771 (2013).
44. Liu MY and Casida JE, High Affinity Binding of [3H]Imidacloprid in the Insect Acetylcholine Receptor. *Pesticide Biochemistry and Physiology* **46**: 40-46 (1993).
45. Yang M, Wang B, Gao J, Zhang Y, Xu W and Tao L, Spinosad induces programmed cell death involves mitochondrial dysfunction and cytochrome C release in *Spodoptera frugiperda Sf9* cells. *Chemosphere* **169**: 155-161 (2017).
46. Xu W, Yang M, Gao J, Zhang Y and Tao L, Oxidative stress and DNA damage induced by spinosad exposure in S*podoptera frugiperda Sf9* cells. *Food and Agricultural Immunology* **29**: 171-181 (2017).
47. Aiken CT, Kaake RM, Wang X and Huang L, Oxidative stress-mediated regulation of proteasome complexes. *Mol Cell Proteomics* **10**: R110 006924 (2011).
48. Brinzer RA, Henderson L, Marchiondo AA, Woods DJ, Davies SA and Dow JA, Metabolomic profiling of permethrin-treated *Drosophila melanogaster* identifies a role for tryptophan catabolism in insecticide survival. *Insect Biochem Mol Biol* **67**: 74-86 (2015).
49. Vidau C, Diogon M, Aufauvre J, Fontbonne R, Vigues B, Brunet JL, et al., Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by *Nosema ceranae*. *PLoS ONE* **6**: e21550 (2011).
50. Ramirez NM, Effect of Sublethal Doses of Clothianidin and/or V. destructor on Honey Bee (*Apis mellifera L.*) Health, Behavior and Associated Gene Expression. 2018).
51. Doublet V, Labarussias M, de Miranda JR, Moritz RF and Paxton RJ, Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environ Microbiol* **17**: 969-983 (2015).
52. Perry T, Heckel DG, McKenzie JA and Batterham P, Mutations in Dalpha1 or Dbeta2 nicotinic acetylcholine receptor subunits can confer resistance to neonicotinoids in *Drosophila melanogaster*. *Insect Biochem Mol Biol* **38**: 520-528 (2008).

53. Manhire-Heath R, Golenkina S, Saint R and Murray MJ, Netrin-dependent downregulation of Frazzled/DCC is required for the dissociation of the peripodial epithelium in Drosophila. *Nat Commun* **4**: 2790 (2013).

Tables

Table 1. *D. melanogaster* strains used in this study

Name	Genotype	Source
Armenia[14]	+;+;+	[52]
Dα6>GAL4	*w Dα6*>GAL4	[29]
Dα6>GAL4; *dα6^{nx}*	*w Dα6*>GAL4; *Dα6^{nx}*	[29]
φX-86Fb; d*α6^{nx}*	*y w M{eGFP.vas-int.Dm}ZH-2A; dα6^{nx}; M{RFP.attP}ZH-86Fb.*	[29]
dα6^{nx}; UAS-*Dα6^{YFP}*	*w; Dα6^{nx};* UAS-*Dα6^{YFP}*	This study
dα6^{nx};UAS-*Dα7^{YFP}*	*w; Dα6^{nx}*; UAS-*Dα7^{YFP}.*	This study
UAS-mCD8-GFP	*w ; UAS-mCD8-GFP*	[53]

Figure Legends

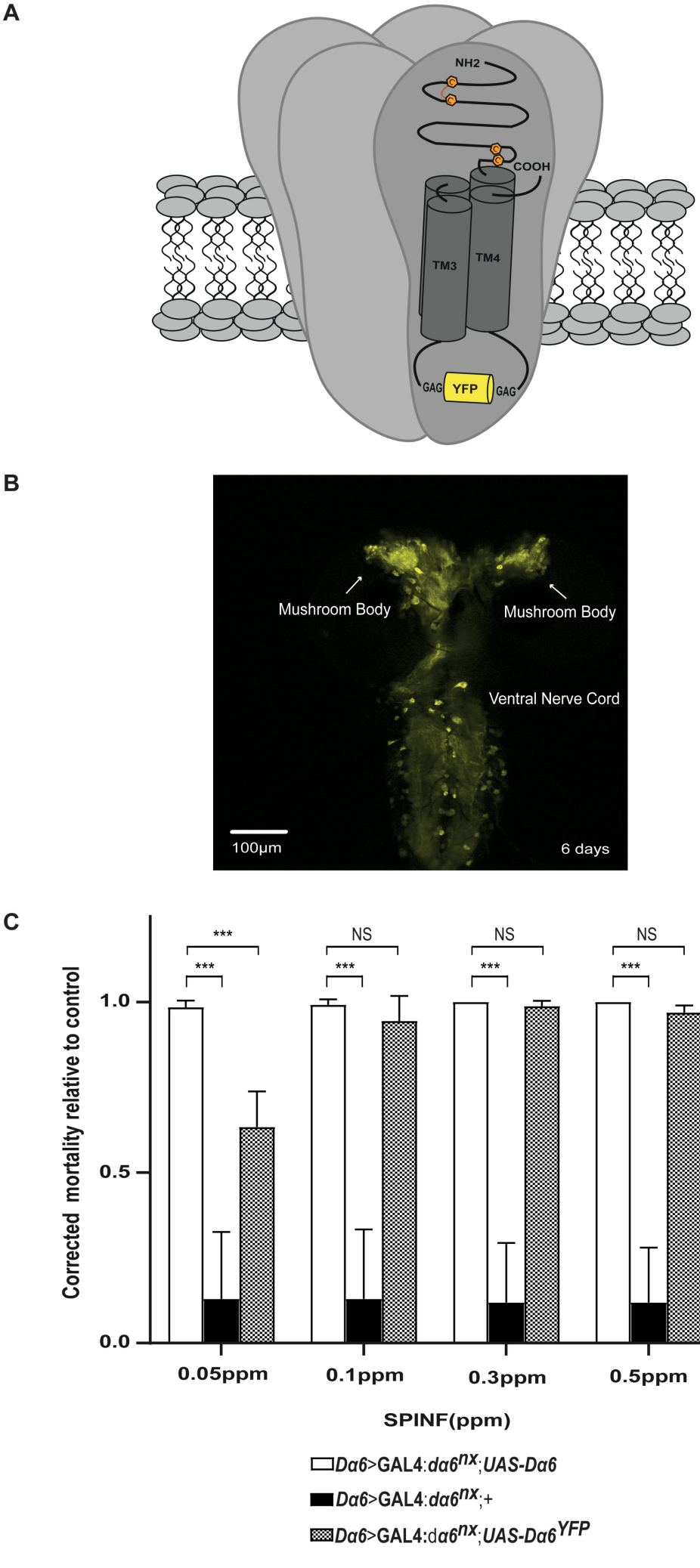
Figure 1. The effects of SPINA and SPINF exposure on *D. melanogaster* 3rd instar larvae. (A) Movement levels of wildtype larvae at 0, 5, 15, 30, 45, 60, and 90 minutes after exposure to 14ppm SPINF, relative to their initial movement levels. Prior to the assay, larvae were reared for six days on either control (CON - closed circles), 0.025ppm SPINF (closed triangles) or 0.08ppm IMI (closed squares) media. (* $p<0.05$, *** $p<0.001$ Student's t-test). (**B)** Expression of *dα6* transcript was quantified in *D. melanogaster* 3rd instar larval brains dissected from larvae that were raised for six days on undosed (CON) or insecticide dosed media (IMI - 0.08ppm and SPINF - 0.025ppm). There were no significant differences in *dα6* transcript levels between these conditions. Error bars for A represent 95% confidence intervals and error bars for B represent the standard error of the mean. For MIQE details see Table S1)

Figure 2. Visualizing and validating function of the Dα6^{YFP} subunit. (A) Schematic of the insertion site for the yellow fluorescent protein (YFP) tag in the Dα6^{YFP} and Dα7^{YFP} constructs used in this study. YFP was recombineered [27] into the intracellular loop between transmembrane domains 3 and 4 spaced by a GAG linker on either side of the YFP tag. (**B).** Expression of the Dα6^{YFP} subunit in dissected 3rd instar larval brains using the GAL4>UAS system [24], in a *dα6^{nx}* background by the *dα6* native enhancer "*dα6>GAL4*" [29]. Strong expression of the Dα6^{YFP} subunit was observed in the mushroom body and in the ventral nerve cord. (**C)** To assess function of Dα6^{YFP}, its ability to rescue SPINF susceptibility was assayed in comparison to expression of wildtype Dα6. The tagged *Dα6* and the wild type *Dα6* subunit were expressed by crossing to dα6>GAL4 in a *dα6^{nx}* mutant background as per [29]. Survival of flies after eclosion was recorded with data corrected for control mortality (Abbott's correction) plotted with 95% CIs. (*** $p<0.001$ Student's t-test).

Figure 3. The effect of sub-lethal exposure to SPINF and SPINA can be directly visualized from *dα6*>GAL4;*dα6^{nx}*; UAS-*Dα6^{YFP}* larvae. (**A**) Brains dissected from 3rd instar larvae raised for six days on control (DMSO only) media and (**B**) raised on 0.025ppm SPINA. (**C**) Results from quantification of fluorescence level changes observed in dissected brains from larvae raised for six days on either 0.025ppm SPINA; 0.025ppm SPINF; 0.08ppm IMI; or 0.0025ppm IVM (*** p<0.001, Student's t-test) relative to larvae raised on untreated media (95% confidence interval shown).

Figure 4. Quantification of the impact of SPINA exposure on *ex vivo* 3rd instar larval brains expressing Dα6^{YFP}. Images of dissected brains from *dα6*>GAL4;*dα6^{nx}*; UAS-*Dα6^{YFP}* after exposure to DMSO alone (**A,B,C**) or 30ppm of SPINA dissolved in DMSO (**D,E,F**). (**G**) Fluorescence measurements of Dα6^{YFP} levels from dissected brains exposed to DMSO (n=13) or DMSO+SPINA (n=11) over 20 minutes relative to the 0 minute timepoint. Error bars for G denote 95% CI.

Figure 5. Inhibition of the ubiquitination-proteasome system using PS-341 reduces the effect of SPINA on Dα6^{YFP} protein levels and SPIN on larval movement. Representative images from dissected larval brains following acute exposure to 6ppm SPINA pre-incubated for 30 minutes in DMSO at timepoints (**A**) 0, (**B**) 10 and (**C**) 20 minutes or PS-341 at (**D**) 0, (**E**) 10 and (**F**) 20 minutes (see methods). (**G**) Quantification of fluorescence levels relative to time 0 of Dα6^{YFP} in larval brains preincubated in PS-341 then exposed to SPINA. (**H**) Relative movement levels of wildtype larvae, pre-treated with ethanol (open triangles) or PS-341 (closed triangles) upon exposure to 2ppm SPIN (*** p<0.001, Student's t-test). Blank treatments were also tested (Fig. S4). Error bars for G and H denote 95% CI.



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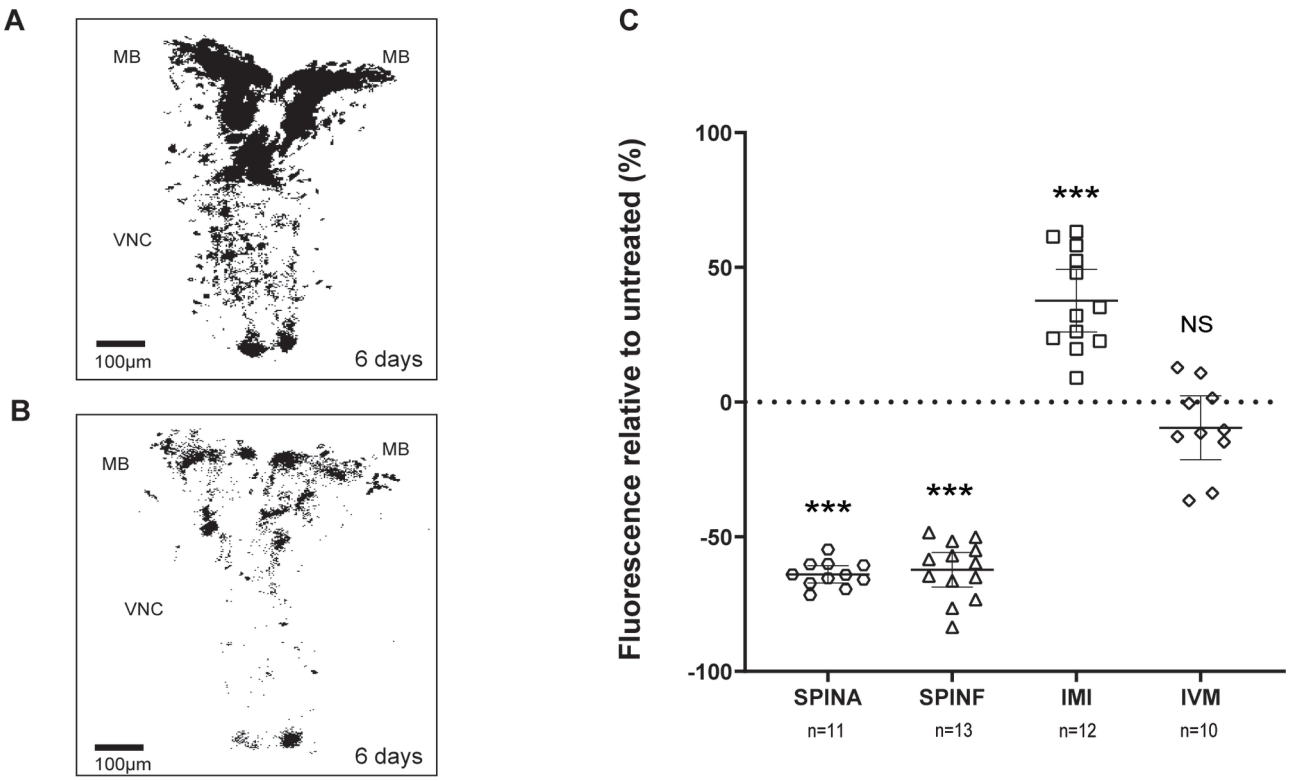

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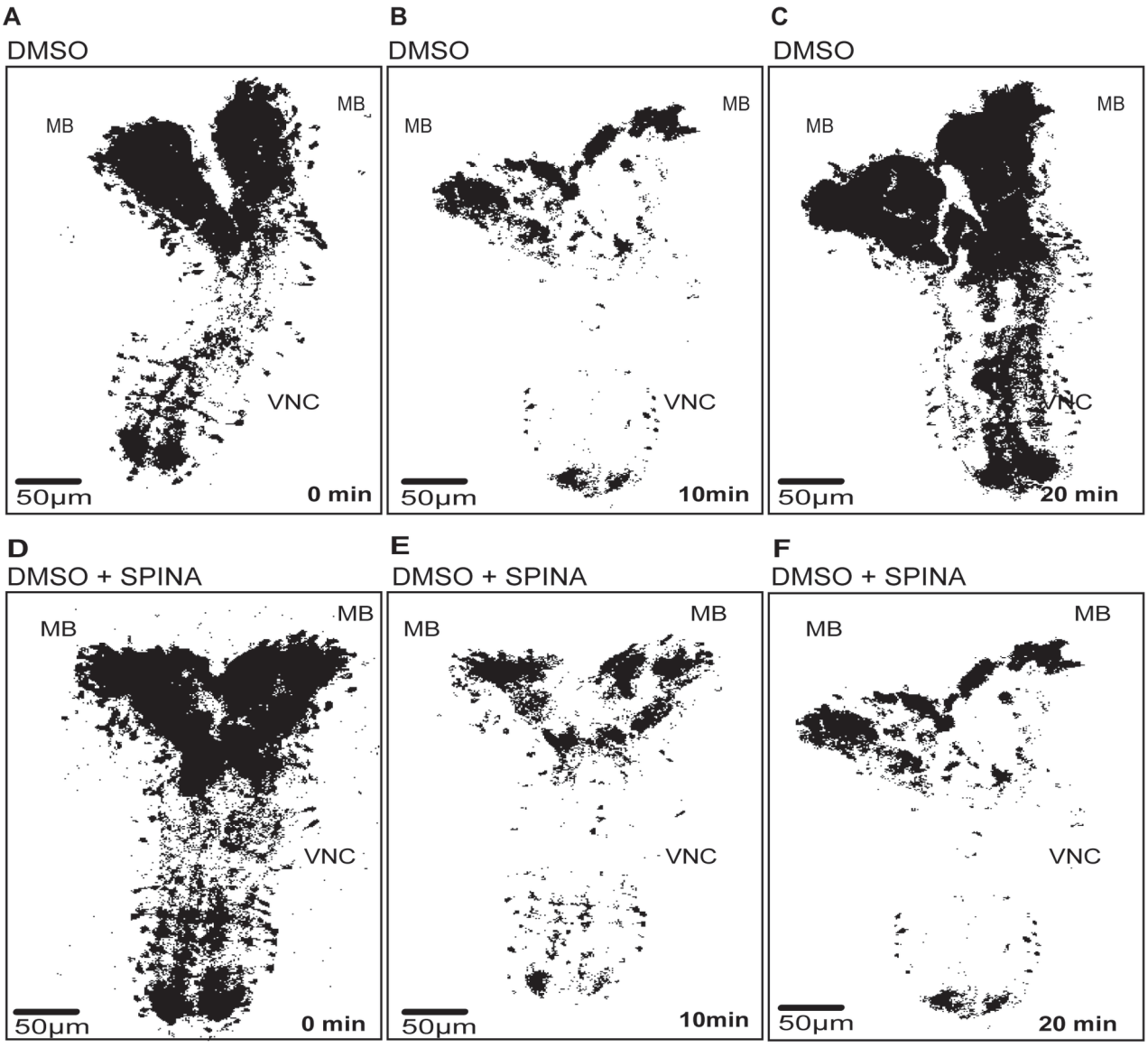

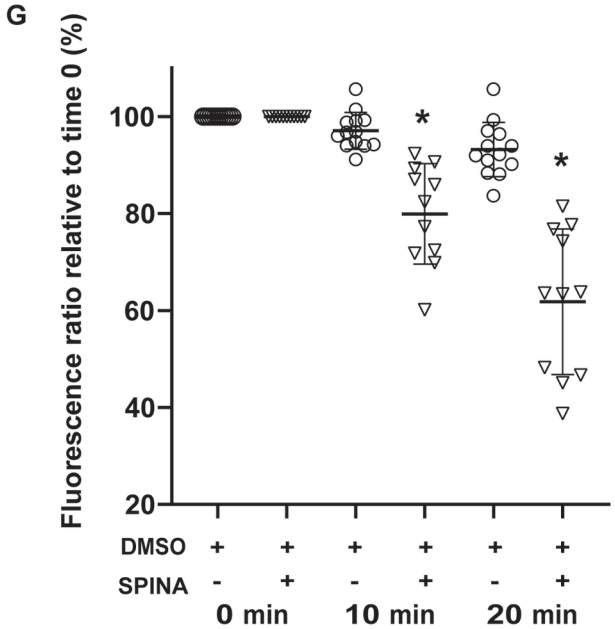

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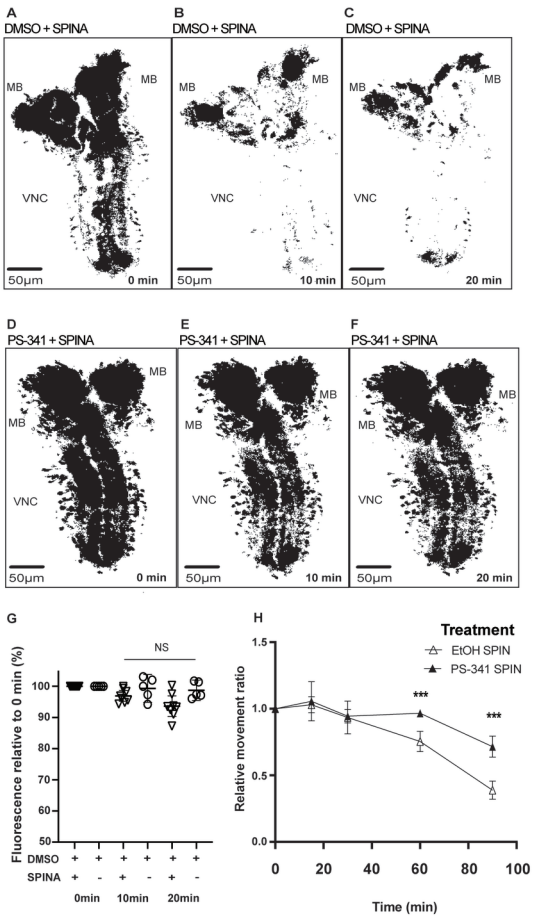

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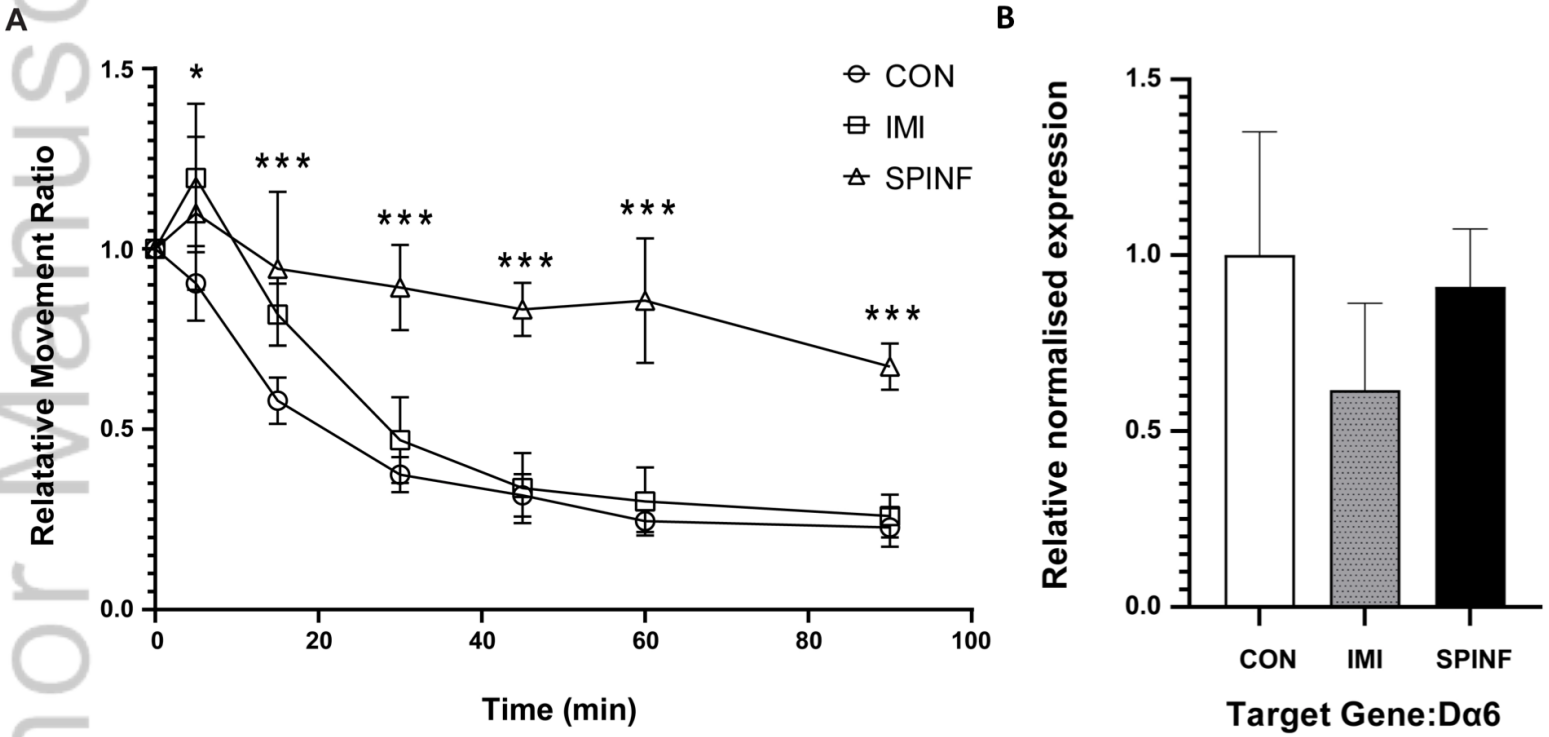

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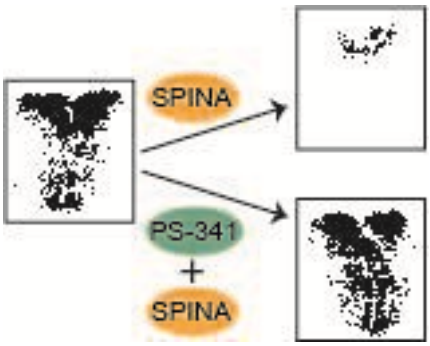

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Inhibiting the proteasome reduces molecular and biological impacts of the natural product insecticide, spinosad

Joseph Nguyen, Razi Ghazali, Philip Batterham and Trent Perry*

Fluorescently tagged nicotinic acetylcholine receptor subunits reveal spinosad exposure leads to degradation of Dα6. Chemically inhibiting this degradation reduces spinosad's effects, indicating a potential involvement in spinosad's mode of action