Neonicotinoid and sulfoximine pesticides differentially impair insect escape behavior and motion detection

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Insect nervous systems offer unique advantages for studying interactions between sensory systems and behavior, given their complexity with high tractability. By examining the neural coding of salient environmental stimuli and resulting behavioral output in the context of environmental stressors, we gain an understanding of the effects of these stressors on brain and behavior and provide insight into normal function. The implication of neonicotinoid (neonic) pesticides in contributing to declines of nontarget species, such as bees, has motivated the development of new compounds that can potentially mitigate putative resistance in target species and declines of nontarget species. We used a neuroethologic approach, including behavioral assays and multineuronal recording techniques, to investigate effects of imidacloprid (IMD) and the novel insecticide sulfoxaflor (SFX) on visual motion-detection circuits and related escape behavior in the tractable locust system. Despite similar LD₅₀ values, IMD and SFX evoked different behavioral and physiological effects. IMD significantly attenuated collision avoidance behaviors and impaired responses of neural populations, including decreases in spontaneous firing and neural habituation. In contrast, SFX displayed no effect at a comparable sublethal dose. These results show that neonicos affect population responses and habituation of a visual motion detection system. We propose that differences in the sublethal effects of SFX reflect a different mode of action than that of IMD. More broadly, we suggest that neuroethologic assays for comparative neurotoxicology are valuable tools for fully addressing current issues regarding the proximal effects of environmental toxicity in nontarget species.

The use of agrochemicals has become increasingly important for sustaining large-scale monocultures that are vulnerable to pests (1). Many modern insecticides are applied as seed treatments to prophylactically address this threat, and these products are available as complex mixtures containing multiple insecticides and fungicides. Of the seed treatments, the most commonly used insecticidal group is the neonicotinoids (neonics), which are nicotinic acetylcholine receptor (nAChR) agonists with specificity for insect receptor subunits (2). Neonics have been implicated in contributing to declines of nontarget insects, with wild bee populations displaying the greatest sensitivity to these compounds (3–5). The sublethal effects of neonics are very complex, however, and it is difficult to link effects observed across levels of biological organization and to estimate the risk of exposure in the field.

The development of novel insecticides is necessary to contend with insecticidal resistance in target organisms that can arise from receptor polymorphisms and enhanced detoxification pathways (6). A novel group of insecticides, the sulfoximines, display a similar mechanism of action as the neonics but do not exhibit cross-resistance (7), related to the differential detoxification pathways of these insecticidal groups (8). While a sulfoximine insecticide, sulfoxaflor (SFX), is currently marketed in seed treatment mixtures, the range of sublethal effects on nontarget organisms is not fully understood. SFX has been shown to negatively affect reproductive success in bumblebees (9, 10) but does not affect olfactory conditioning (11). The introduction of new agrochemicals to the ecosystem before a complete understanding of the negative effects results in a repetitive pattern of ecological damage. To mitigate these effects, toxicologic assays should be developed that simultaneously address effects at multiple levels of biological organization.

The neonic imidacloprid (IMD) has previously been shown to affect visual motion processing and collision avoidance behavior, the descending contralateral movement detector (DCMD), which responds preferentially to objects approaching on a direct collision course (looming) (14, 15). This neuron displays bursting activity (16) and is important for generating escape behaviors (17, 18). In addition, this neuron habituates to repeated stimulus presentation (19), a phenomenon related to the inhibitory pathways in the optic lobe that are activated in tandem with excitatory pathways (14, 20, 21). Other descending interneurons can be recorded from the ventral nerve cord with differing response profiles to the DCMD in response to a looming stimulus; however, the population response of these neurons has been examined in only one study (22), and another neuron, the late DCMD, is known to habituate less than the DCMD (23). Here, using a combination of multichannel extracellular recordings, we defined the effects of IMD and the novel insecticide SFX on the population responses of descending neurons during visual motion detection and collision avoidance behavior, to investigate effects of imidacloprid (IMD) and the novel insecticide sulfoxaflor (SFX) on visual motion-detection circuits and related escape behavior in the tractable locust system. Despite similar LD₅₀ values, IMD and SFX evoked different behavioral and physiological effects. IMD significantly attenuated collision avoidance behaviors and impaired responses of neural populations, including decreases in spontaneous firing and neural habituation. In contrast, SFX displayed no effect at a comparable sublethal dose. These results show that neonicos affect population responses and habituation of a visual motion detection system. We propose that differences in the sublethal effects of SFX reflect a different mode of action than that of IMD. More broadly, we suggest that neuroethologic assays for comparative neurotoxicology are valuable tools for fully addressing current issues regarding the proximal effects of environmental toxicity in nontarget species.

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Significance

Novel insecticides are developed and implemented in agriculture without a broad understanding of their sublethal effects. Although all act via nicotinic acetylcholine receptors, neonicotinoids and the novel sulfoxaflor insecticide exhibit differences in relative toxicity. In this study comparing the effects of these insecticides on visual motion detection and escape behavior, we show that sulfoxaflor displays decreased sublethal toxicity despite similar lethal endpoints of these insecticides. Imidacloprid reduces putative neural population code variability when responding to approaching objects, suggesting that neonics may constrain the tuning of visual sensory circuits. We suggest that neuroethologic methods are powerful tools to link toxic effects across levels of biological organization and further our understanding of how neural populations operate in complex sensory environments.

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presentation of a looming stimulus. Effects on the population of descending interneurons were correlated with effects on collision avoidance behavior. Although similar concentrations of SFX and IMD resulted in lethality, there were differences in sublethal effects. While sublethal doses of IMD resulted in decreased jumping escape behaviors, SFX had no effect on behavior at sublethal amounts. Correspondingly, we found that IMD had a significant effect on the population response, while SFX did not significantly affect the responses of these descending neurons to object motion. These results are significant, as they provide evidence of differences in the modes of action of two related insecticides and highlight the importance of comparing lethal and sublethal effects across compounds.

Results

SFX and IMD Display Comparable Lethal Toxicity 48 h after Acute Treatment. Across the range of doses tested (1 to 10,000 ng/g), IMD and SFX displayed comparable effects on locust mortality at 48 h after a single oral dose (Fig. 1A). LD_{50} values were ~320 ng/g of locust for SFX, and 780 ng/g for IMD, with doses ≥320 ng/g displaying significantly increased mortality compared with vehicle control (one-way ANOVA, SFX: F_{6,6} = 63.843, P < 0.001; IMD: F_{6,6} = 53.774, P < 0.001).

IMD, but Not SFX, Impairs Collision Avoidance Behavior 24 h after Sublethal Exposure. All animals, either before treatment (n = 324) or following treatment with vehicle control (n = 35), responded to the looming stimulus with a jumping escape behavior (Movie S1). Sublethal treatment with IMD resulted in animals that did not respond to the looming stimulus, while equal doses of SFX did not affect behavior (Movies S2 and S3). SFX did not affect collision avoidance behavior across the range of doses below the LD_{50} (one-way ANOVA, F_{6,6} = 0.034, P > 0.001), while this behavior was significantly affected with IMD doses ≥10 ng/g (one-way ANOVA on ranks, H_{6,6} = 30.954, P < 0.001) (Fig. 1A). This is illustrated by the dose–response curves, which show an ED_{50} of 5.2 ng/g (just 0.67% of the LD_{50}) for IMD, compared with 280 ng/g (87.5% of the LD_{50}) for SFX.

IMD Affects Multineuronal Responses to Object Motion. We selected a sublethal dose of 100 ng/g for both IMD and SFX to determine whether these insecticides affect neural population responses. Twelve animals were included in each treatment group (100 ng/g SFX, 100 ng/g IMD, and vehicle control), and we recorded neural responses to looming stimuli at 24 h after treatment. Using tetrode recordings of the ventral nerve cord, we sorted spikes from individual neurons (units) for each locust (Fig. 1B), which formed statistically distinct clusters in three-dimensional (3D) space (SI Appendix, Table S1). We discriminated 80 units in the vehicle control group (n = 12), 89 units in the IMD group (n = 12), and 77 units in the SFX group (n = 12), for a mean of 7 units per locust for the vehicle and IMD groups and 6 units per locust in the SFX group. Using peristimulus time histograms (PSTHs) and cumulative sum plots for each unit, we excluded units that did not show a significant change in firing rate during stimulus presentation (Fig. 1C). We found no significant difference in the mean number of units per animal per group, the number of units responding per animal per group, or the percentage of units responding per animal per group (Fig. 1D).

The mean PSTHs from pooled spikes in all units within each treatment revealed a similarity between the control and SFX treatments, whereas IMD attenuated the response (Fig. 2A). We measured the mean frequency of all units within 0.5-s epochs from −1.5 s before the time of collision (TOC) of the looming stimulus to 0.5 s after the TOC (Fig. 1A) and confirmed that for all epochs preceding the TOC, the mean frequency was lower for units in the IMD group (one-way ANOVA on ranks: −1.5 to −1 s, H_{2} = 16.455, P < 0.001; −1 to −0.5 s, H_{2} = 20.855, P < 0.001; −0.5 to 0 s, H_{2} = 16.358, P < 0.001), whereas after the TOC, there was no significant difference between treatments.

To further assess the attenuation of firing rate modulation across units following IMD treatment, we categorized the responses of each unit into distinct response groups based on several histogram parameters (Fig. 2A). For units that displayed a clear peak firing rate around the TOC, we categorized whether the peak firing rate was >100 spikes/s (group A), between 50 and 100 spikes/s (group B), between 25 and 50 spikes/s (group C), or <25 spikes/s (group D). The remainder of units displayed baseline tonic firing that either decreased around the TOC (group E) or increased during object approach with no distinct peak (group F). Despite no significant difference in the number
of responding units per treatment group, we found a significant difference in the distribution of the units among response groups ($\chi^2_{10} = 20.252, P < 0.05$) (Fig. 2B). A similar distribution of units in response groups for the control and SFX treatments were contrasted with a shift toward the low and moderate frequency peak groups (groups B to D) and away from groups with tonic spontaneous firing that included increases or decreases around the TOC (groups E and F) for the IMD treatment. We also measured the rise and decay phases of the histograms of units displaying a distinctive peak (groups A to C; Fig. 2C) (24). The rise phase was significantly shorter with lower peak firing rates, and IMD enhanced this effect, displaying the shortest rising phases (two-way ANOVA: by treatment, $F_2 = 9.733, P < 0.001$; by unit group, $F_2 = 71.690, P < 0.001$). Measurements of the decay phases of the PSTHs of units from groups A to C (Fig. 2D) showed a longer decay for units within group A treated with IMD (one-way ANOVA on ranks, $H_2 = 8.497, P < 0.05$).

**IMD Reduces Variation among Correlated Groups of Motion-Sensitive Neurons.** We performed a dynamic factor analysis (DFA) to identify common trends among time series data (25) using 50-ms bins with units pooled within each treatment group (vehicle control, 69 units; SFX, 72 units; IMD, 75 units) using Brodgar 2.7.9 (Highland Statistics). DFA is a multivariate analysis that can be used in place of a principal component analysis to reduce the dimensionality of data that occur in a time series. When selecting the number of common trends to include in the model, criteria included Akaike information criterion (AIC), an estimator of model prediction error, distribution of the residuals, and biological interpretation (25). We tested DFA models that included four, six, and eight trends, and although the AIC value was lowest for the model with eight trends (SI Appendix, Table S2), we identified the six-trend model as the most biologically relevant, given our initial analysis of the PSTHs that motivated grouping neural responses into six unit response groups (Fig. 3).

Qualitative visual examination of the six common trends within each treatment group showed properties supporting our classification of individual units. With the IMD treatment group, we found no common trend showing a sharp decrease in the standardized firing rate near the TOC, which we observed in the vehicle control and SFX treatment groups and which was similar to that in unit responses from group E. The common trend in the IMD treatment all displayed a peak firing rate either before or after the TOC (Fig. 3B). In addition, we observed little variation in the standardized firing rate before ~0.5 s (before collision) in the IMD group, while the SFX and vehicle control groups showed broad variation between trends.

**IMD, but Not SFX, Affects Habituation of Individual Motion-Sensitive Neurons.** We used a series of 10 stimuli presented consecutively at 8-s intervals to observe how the discriminated visual neurons habituate. The DCMD habituates primarily with reductions in the peak firing rate and total number of spikes (19). We found that units with a medium- or high-frequency peak (from groups A and B) display a sharp decrease in firing from the first to tenth stimulus presentation, while those with more tonic firing patterns do not habituate (Fig. 4A). High variability of responses between stimulus presentations excluded group D units (<25 spikes/s peak) from habituation analysis.

To quantify habituation irrespective of response type, we calculated a habituation index using the total number of spikes and mean frequency, both normalized to the responses to the first (i.e., unhabituated) stimulus. A habituation index of 0 represents a response that did not habituate, while increasingly negative values represent stronger habituation (Fig. 4B). Values >0 may represent neural facilitation. Across treatment groups, the habituation index decreased sharply from the first to the second stimulus presentation and plateaued by the fourth or...
fifth stimulus presentation (Fig. 4C), and for all pooled units within each treatment, the habituation index significantly decreased by the 10th presentation (Wilcoxon signed-rank test, $Z = -5.794, P < 0.001$). Treatment did not affect the habituation index of approach 10 across units (Fig. 4D); however, we found that units from groups A to C (with a defined peak) habituated to a greater degree than those from groups E and F (one-way ANOVA on ranks, $H_4 = 66.210, P < 0.001$) and that within unit groups, IMD treatment produced less habituation, with significantly greater habituation index values (compared with controls) for units from group C (one-way ANOVA, $F_2 = 4.372, P < 0.05$) and group F (one-way ANOVA, $F_2 = 4.622, P < 0.05$).

Discussion
We have shown stark differences in the sublethal effects of IMD and SFX, despite their similar mortality curves. Our results suggest differences in the action of these compounds in the locust nervous system. While sublethal oral treatment with IMD resulted in robust effects on collision avoidance behavior, as seen previously with an injected dose (13), SFX did not affect this behavior at sublethal concentrations. This unanticipated result suggests that compared with SFX, IMD may bind more readily to the nAChR subtypes found in the locust optic lobes and central nervous system, or that the metabolites of IMD heighten its sublethal effects (12). The differences in sublethal toxicity between IMD and SFX were also reflected in their effects on the population responses of descending visual interneurons.

While there were no differences in the number of responding units per animal across treatments, IMD affected the distribution of units into putative functional groups. The overall attenuation of firing rate and spontaneous firing suggests that excitatory synapses are being affected similarly across various visual pathways, resulting in a bulk decrease in neural firing, as observed across the pooled responses of visual units recorded here. Results from the DFA further highlight the effects of IMD on spontaneous firing and attenuation of putative neural population responses to looming. More broadly, this finding suggests that neonicots may constrain the tuning of sensory systems that must operate within a variable natural environment. Decreased excitation in the optic lobes would result in decreased sensitivity for object motion early in the approach and accurate encoding only as the objects get larger (i.e., closer). Decreased early sensitivity is reflected in a shortened rising phase of units with peak firing near the TOC. Inhibitory neurons may also be attenuated after treatment with IMD but to a lesser degree, as suggested by a longer decay phase for units in group A. Inhibitory neurons may contain nAChRs on the dendrites, so their activation could be affected by IMD, whereas the inhibitory synapses themselves would be unaffected, because they are muscarinic (26, 27). Our findings are consistent with a previous study showing that excitation is mediated by acetylcholine in locust optic lobes (28).

Habituation of a population of visual interneurons had not previously been examined in the locust. Habituation of the DCMD is likely caused by the activity of inhibitory neurons in the optic lobes (19). We found reduced habituation of units in the IMD treatment, further supporting our hypothesis that the activity of both inhibitory neurons that contain dendritic nAChRs and excitatory neurons are attenuated. However, this effect was less pronounced for the inhibitory neurons, which are not under nicotinic cholinergic control.

Contrary to our predictions, SFX did not affect collision avoidance behavior or responses of motion-sensitive visual neurons at a sublethal concentration. This is a significant finding, as SFX and IMD act on the same target, the nAChR, although they are metabolized through different pathways (8). We previously demonstrated that metabolites of IMD, including IMD-olefin and 5-hydroxy IMD, display toxic effects on this collision avoidance pathway equal to or greater than those of the parent compound (12). The detoxification pathway of SFX may result in metabolites that do not bind to the nAChR or can be more readily excreted, suggesting that SFX would cause toxicity by binding to the nAChR before metabolism. However, SFX is not metabolized by the same
cytochrome p450 enzymes, such as the CYP6G1 monooxygenase, that mediate neonic metabolism and resistance in insects (8), and the effects of its metabolites remain unclear. Another explanation for the reduced toxicity of SFX compared with IMD observed here is that SFX may bind with low affinity to the nAChR subunits expressed in the locust optic lobes. In other species, SFX and IMD display differential binding at the agonist binding site, with SFX displaying lower affinity than various neonics (29). It is possible, however, that the benefit of metabolic stability outweighs the cost of reduced receptor affinity for use against insects resistant to neonics (30). Additional research is needed to determine whether the reduced toxicity of SFX compared with IMD seen here is due to reduced receptor affinity of SFX or whether these differences result from reduced metabolism of SFX or metabolites that do not display toxicity within this collision avoidance pathway.

Overall, our results offer evidence that a neonicotinoid insecticide causes reduced firing of neurons innervated through nicotinic cholinergic synapses located in the central nervous system. We show a widespread alteration of the neural responses transmitted by a putative population of visual interneurons which is associated with impaired escape behavior. Interestingly, we found no significant effect on either behavior or neural firing resulting from an equal dose of SFX, despite similarities in LD50 values with IMD. Although our results suggest that SFX may represent a preferable alternative to IMD given the reduced sublethal effects, repeat experiments with vulnerable nontarget organisms are necessary. This study highlights an interesting case in which lethality and sublethal effects do not follow the same patterns for two insecticides that share the same receptor target. We propose that a combination of toxicologic and neuroethologic methods should be used to fully understand the scope of toxicity of a given compound, with LD50 curves alone insufficient to capture this scope.

Materials and Methods

Percent mortality at 48 h after acute oral exposure was measured across a range of IMD and SFX doses (Fig. 1A). To quantify the effects of sublethal treatment of IMD and SFX on jumping escape behaviors in *L. migratoria*, locusts were shown the image of a 7-cm looming disk on a computer monitor at 24 h after the oral treatment. Responses were compared with those of the vehicle control group. A sublethal dose of 100 ng/g IMD or SFX or the vehicle control was used to examine effects on neural population responses in 12 animals per treatment. At 24 h after treatment, the locusts were dissected dorsally, and a twisted wire tetrode was inserted into the righthand ventral nerve cord. The looming stimulus was presented to the left eye five times at 3-min intervals to record unhabituated population responses of descending neurons, followed by 10 consecutive stimuli presented at 8-s intervals. Spike times of individual neurons (units) were obtained with Offline Sorter v 4.4 (Plexon) using a semiautomatic sorting method based on the k-means algorithm. In total, we discriminated 246 units across 36 animals (SI Appendix, Table S1). Raw spike times for individual units were used to construct PSTHs, smoothed with a 50-ms Gaussian filter, and aligned to the projected TOC of the stimulus.

Using these PSTHs, we determined which units were responding to the stimulus by plotting the cumulative sum of spike counts over an ellipse representing the 99% confidence level. A cumulative sum that did not pass outside or touch the edge of the 99% confidence level ellipse represented a firing rate that showed no significant change as a result of the stimulus, while
those that touched or expanded past the edge of the ellipse were considered to have a significant stimulus-evoked firing rate change (22). Units that were not responding were removed from subsequent analysis. Fig. 1C shows an example of the PSTHs and cumulative sum from a unit responding to the visual stimulus and a unit not responding to the visual stimulus. For stimuli spaced at 8-s intervals, we calculated a habitation index (H) of all responding units, using the proportions of the total number of spikes and mean frequency of each stimulus presentation normalized to the first stimulus. More details are provided in SI Appendix, Materials and Methods.

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Data Availability. All neural recordings and custom-written MATLAB code are available at https://datadryad.org/stash/dataset/doi:10.5061/dryad.mcvdncjwp.

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