Neurotoxicology 72 (2019) 107–113

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

Neural conduction, visual motion detection, and insect flight behaviour are disrupted by low doses of imidacloprid and its metabolites

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ARTICLE INFO

Keywords:
Insect
Vision
Flight
Imidacloprid
Metabolites

ABSTRACT

While neonicotinoid insecticides impair visually guided behaviours, the effects of their metabolites are unknown and measurements of environmental concentrations of neonicotinoids, typically lower than those required to elicit toxic effects, tend to exclude metabolites. Here we examined the contributions of imidacloprid and two of its metabolites, imidacloprid-olefin and 5-hydroxy-imidacloprid, on neural conduction velocity, visual motion detection and flight in the locust (*Locusta migratoria*) using a combination of electrophysiological and behavioural assays. We show reduced visual motion detection and impaired flight behaviour following treatment of metabolite concentrations equal to sublethal doses of the parent compound. Additionally, we show for the first time that imidacloprid and its metabolites result in a decrease in conduction velocity along an unmyelinated axon. We suggest that secondary effects of the insecticide on the biophysical properties of the axon may result in decreased neural conduction. As these metabolites display neurotoxicity similar to the parent compound they should be considered when quantifying environmental concentrations.

1. Introduction

Flight behaviours, including orientation, navigation and collision avoidance are essential to the survival of many animals and are guided primarily by the processing of visual information (Ibbotson et al., 2017; Rind and Bramwell, 1996; Taylor and Krapp, 2007). Neurotoxic insecticides, including neonicotinoids (neonics) that target cholinergic neurotransmission in the insect central nervous system (Tomizawa and Casida, 2003), affect specific behaviours following sublethal exposure (Fischer et al., 2014; Parkinson et al., 2017). Significant concern over the effects of neonics on honey bees and other important pollinators has led to bans that are highly contested (Blacquière and van der Steen, 2017). Arguments against these bans point to discrepancies between laboratory and field-realistic doses (Walters, 2013). However, measurements in the field often exclude neonic metabolites that may accumulate in pollen and honey and pose the greatest risk to overwintering and nurse-aged worker bees that rely on these stores (Baines et al., 2017; Codling et al., 2016). Some neonic metabolites display similar or increased toxicity to the parent compounds (Nauen et al., 1998; Suchail et al., 2001a) suggesting a need for a greater understanding of these toxic effects.

The effects of the neonic imidacloprid (IMD) on visual processing and visually guided avoidance behaviours has previously been examined in the locust (*Locusta migratoria*) (Parkinson et al., 2017). This system is useful in examining neonic toxicity due to the presence of a tractable looming-sensitive visual interneuron, the Descending Contralateral Movement Detector (DCMD). The DCMD receives excitatory input from its presynaptic partner, the Lobula Giant Movement Detector (LGMD) at a 1:1 ratio and the LGMD receives both excitatory and inhibitory signals from the retinotopic units of the compound eye (Rind, 1984). The excitatory synapses in the optic lobes and specifically on the LGMD/DCMD are nicotinic cholinergic (Rind and Leitinger, 2000), which is a direct target for IMD. Although IMD is an nAChR agonist (Tomizawa, 2004), initial agonistic effects may be followed by neural silence, dependent on concentration (Zafeiridou and Theophilidis, 2004), an effect that may be due to receptor desensitization (Nauen et al., 2003; Oliveira et al., 2011). Previously we found that a single sublethal dose of IMD resulted in decreased burst firing of the DCMD and attenuated escape behaviours 2 and 24 h after treatment (Parkinson et al., 2017).

Here we examine the effects of IMD and two metabolites, imidacloprid-olefin (OLE) and 5-hydroxy-imidacloprid (5OH) on action potential propagation, DCMD responses to a looming stimulus, and flight behaviour within 1 h after treatment to determine the relative effects of the metabolites and parent compound. We hypothesized that the metabolites would display increased toxicity sooner after treatment than

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https://doi.org/10.1016/j.neuro.2019.02.012
Received 25 September 2018; Received in revised form 14 January 2019; Accepted 16 February 2019
Available online 18 February 2019
0161-813X/

the parent compound, due to effects on reduced DCMD firing resulting from the metabolism of IMD to OLE and/or 5OH. We show in this paper that IMD, OLE and 5OH affect flight behaviour and visual motion processing within 1 h of treatment, and additionally that these compounds affect conduction velocity along the axon, suggesting secondary effects of the insecticide on information transfer within a known pathway.

2. Methods

2.1. Animals

Adult male locusts (*Locusta migratoria*) two weeks past the last imaginal moult were selected for experiments. Locusts were fed a diet of wheat grass and bran flakes and maintained at 25-30 °C with a 12h:12 h light:dark cycle at the University of Saskatchewan, Canada. All experiments took place during the animals' light cycle. Experiments were performed at 25 °C.

2.2. Treatments

Imidacloprid (IMD; Sigma-Aldrich, Oakville, Canada), imidacloprid-olefin (OLE; Toronto Research Chemicals, North York, Canada), and 5-hydroxy-imidacloprid (5OH; Toronto Research Chemicals, North York, Canada) were dissolved in DMSO and diluted in locust saline (147 mmol NaCl, 10 mmol CaCl2, 3 mmol NaOH, 10 mmol Hepes, pH 7.2) to produce a final concentration of 10 ng/µl with 0.2% (v/v) DMSO for all experiments. The vehicle control solution contained locust saline and 0.2% (v/v) DMSO.

2.3. Behaviour

Locusts were loosely tethered in the centre of a 0.9 × 0.9 × 3 m Plexiglas wind tunnel with a constant wind speed set to 3 m/s, which is the average flight speed of a flying locust (Baker et al., 1981), and presented with the image of a 14 cm disc looming perpendicularly to the axis of the animal (90° to head-on) to elicit collision avoidance behaviours (Parkinson et al., 2017). Flight behaviour was recorded before (PRE) and 1 h after treatment with 10 ng/g (of locust; mean locust mass = 1.5 g) of IMD (n = 10), OLE (n = 10), or 5OH (n = 10). Animals were treated by injecting 1 µl/g of locust of a solution containing 10 ng/µl of IMD, OLE or 5OH in locust saline plus 0.2% (v/v) DMSO into the hemolymph under the cuticle of the pronotum. We showed previously that the vehicle alone had no effect on behaviour and therefore did not use a vehicle control solution here (Parkinson et al., 2017). Flying animals were scored as responding (R) if animals responded to the stimulus with a turn, glide or stop, or not responding (NR) if they did not respond. Animals that were not able to fly were scored as not flying (NF).

2.4. Electrophysiology

Locusts were dissected dorsally to expose the ventral connectives and ganglia (Cross and Robertson, 2016), filled with 0.2 ml locust saline, and three suction electrodes were attached to the right ventral nerve cord: posterior to the prothoracic ganglion, as well as anterior and posterior to the mesothoracic ganglion (Fig. 1A). A ground wire was inserted into the abdomen and reference electrode inserted into the flight muscles. The image of a 7 cm diameter looming disc was presented to the left eye of the locust at 60 frames/s through a rear-projection screen 20 cm from the eye at an angle of 45 degrees from the front of the animal (Fig. 1B). The visual stimulus program was written in Python using Pyglet, a program used to write video graphics. An Arduino was coded to output a single 5 V pulse at the projected time of collision (TOC), or time the looming stimulus would have collided with the locust. Three repetitions of the looming stimulus with 3-minute inter stimulus intervals were presented for the pre-treatment control, after which saline was removed from the body cavity and replaced by 0.2 ml saline plus 10 ng/g of locust of IMD (n = 9), OLE (n = 6) or 5OH (n = 8), or the vehicle control (n = 7). Continuous neural data were amplified with a differential AC amplifier (A–M Systems, model no. 1700, 100 Hz high pass and 5 kHz low pass filters, gain = 100x). Amplified neural data and stimulus pulse were digitized using a Data Translation DT9818 data acquisition board (TechnaTron Instruments, Inc., Laval, QC) and recorded at 30 kHz with DataView version 11 (W.J. Heitler, University of St Andrews, Scotland).

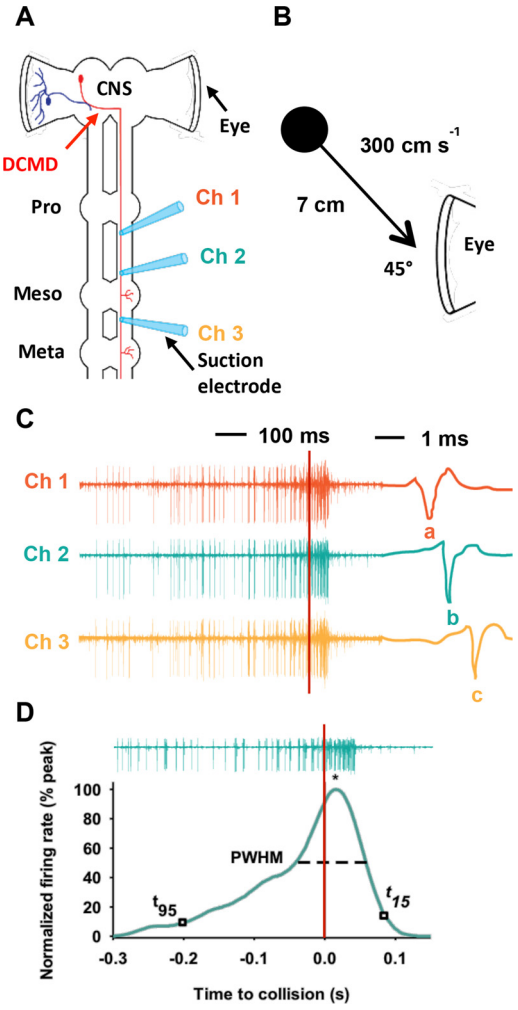

Fig. 1. Electrophysiology, experimental procedures and data analysis. A) Suction electrodes recorded the activity of the DCMD axon at three points along the ventral nerve cord: posterior to the prothoracic ganglion, and anterior and posterior to the mesothoracic ganglion. B) We presented locusts with the image of a 7 cm diameter disk looming at 300 cm/s at 45° from the front of the animal. C) Raw extracellular recordings from the ventral nerve cord during presentation of a looming stimulus. Waveforms to the right of the traces show individual DCMD spikes in a shorter time window, with the relative timing of letters highlighting the peak of each spike used to calculate conduction velocity. D) Raw recording and corresponding peristimulus time histogram (PSTH) constructed as the firing rate versus time using a 1 ms bin width and smoothed with a 50 ms Gaussian filter. Firing parameters including peak time and peak firing rate (*), peak width at half maximum (PWHM), rise phase (t_{95}, the time when the histogram last increased above the 95% confidence interval with a positive slope, to *) and decay phase (* to t_{15}, the time when the firing rate decayed to 15% of the peak) were measured from the PSTHs to compare responses before and after treatment.

2.5. Data analysis

Spike sorting of the three channels of continuous neural data was performed using DataView. Raw continuous data were upsampled to 90 kHz. The large amplitude DCMD spike times were extracted with a threshold analysis (Fig. 1C). Spike times were aligned to the stimulus pulse and exported into Neuroexplorer analysis software (NEX Technologies, Littleton, MA) to construct peristimulus time histograms (PSTHs) with a 1 ms bin width and smoothed with a 50 ms Gaussian filter (Fig. 1D). Matlab (The MathWorks, Natick, MA) was used to calculate DCMD firing parameters from the PSTHs. DCMD conduction velocity was calculated between the three channels as the reciprocal of the delay between spikes along the connective and across the mesothoracic ganglion and normalized as a proportion of the conduction velocity of the first spike of the first control recording for each animal (Cross and Robertson, 2016).

2.6. Statistical analysis

Statistical analyses were performed using SigmaStat 3.5, and figures were created with SigmaPlot 12.5 (Systat Software Inc., Richmond, CA) and Illustrator CS2 (Adobe Systems, San Jose, CA). Normally distributed variables were compared using two-way Repeated Measures ANOVA (two-way RM ANOVA) followed by a Holm-Sidak post-hoc analyses comparing across treatment and time to the control and pre-treatment, respectively. Variables that failed tests of normality or equal variance were compared using one-way ANOVA within time (pre-treatment, 30 min and 60 min), and treatments were compared post-hoc to the vehicle using Dunn's method. Parametric data were plotted as bar graphs including positive standard error of the mean bars (s.e.m.), and non-parametric data were plotted as boxplots showing the median value, 25th and 75th percentile as box boundaries and 10th and 90th percentiles as error bars. Significance was assessed at $P < 0.05$.

3. Results

3.1. Behaviour

Locust flight and escape behaviours were tested in a wind tunnel, using the image of a looming stimulus to elicit escape manoeuvres. While all pre-treatment locusts (n = 30) flew and responded to the looming stimulus with an escape manoeuvre (turn or glide), treatment with 10 ng/g IMD, OLE or 5OH reduced or abolished behavioural responses, resulting in locusts that either flew without responding to the looming stimulus or did not fly (Fig. 2). The parent compound, IMD had the smallest effect, with 40% of animals flying and responding to the stimulus, while 30% flew and did not respond and 30% could not fly 1 h after treatment. OLE resulted in 50% of animals unable to fly, 40% of animals flying but not responding to the stimulus, and 10% flying and responding with an escape manoeuvre. 5OH had the largest effect on flight, with 80% of animals unable to fly 1 h after injection, and the remaining 20% flew, but did not respond to the looming stimulus. These results show that, while all compounds affected flight behaviour at the concentration used, the effects varied across treatments.

3.2. DCMD responses to looming stimuli

We examined the responses of the DCMD to a looming stimulus before treatment and over 1 h after treatment with 10 ng/g of IMD, OLE, or 5OH. The response profile of the DCMD was altered after treatment with IMD, OLE or 5OH compared to the vehicle control group (Fig. 3). The effect of IMD and metabolites is visible 30 min after treatment, with the peak of the PSTHs lowered to approximately 80% of pre-treatment levels. This effect was enhanced 60 min after treatment, with the peak firing rates reduced to 75% of the pre-treatment peak, and slight differences in the rising and falling slopes of the histograms between treatments.

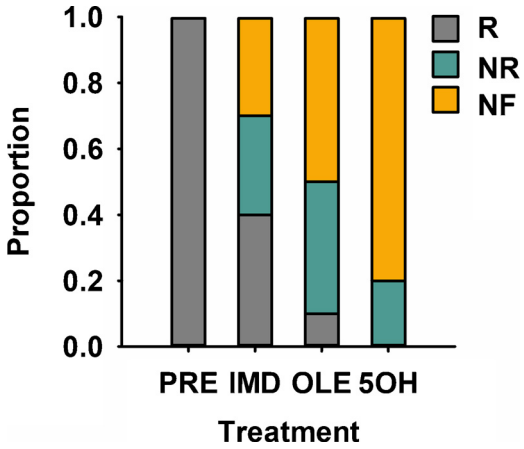

Fig. 2. Flight and escape behaviours before (PRE) and after treatment with IMD, OLE or 5OH. Loosely tethered flight behaviour of locusts was recorded in a wind tunnel during presentation of a looming disk before (PRE) and after treatment with IMD, OLE and 5OH. Animals that responded to the stimulus with a turn or a glide were scored as responding (R), and those that did not respond to the stimulus were scored as not responding (NR). Animals that were not able to fly in a coordinated manner after treatment were scored as not flying (NF).

Mean PSTH shape was compared between treatments to the vehicle and across time to pre-treatment values by examining individual response parameters (Fig. 4). The total number of DCMD spikes per stimulus presentation was significantly affected across treatments (two-way RM ANOVA, $F_{3,47} = 3.675$, $p = 0.025$). Post-hoc multiple comparisons (Holm-Sidak) showed that the number of spikes was significantly reduced at both 30 and 60 min after treatment with IMD and 5OH compared to the control (IMD: 30 min = 71.6%, 60 min = 71.3%; 5OH 30 min = 80.4%, 60 min = 68.6%). The peak firing rate was affected by dose (two-way RM ANOVA, $F_3 = 5.006$, $p = 0.007$) and time after treatment (two-way RM ANOVA, $F_3 = 4.263$, $p = 0.049$). Post-hoc analysis showed the peak firing rate was reduced for OLE and 5OH at 30 min after treatment (OLE = 81.5%; 5OH = 83.6%), and for all treatments at 60 min after treatment (IMD = 79.2%; OLE = 75.6%; 5OH = 74.3%). The reduction in peak firing rate was significantly lower for 5OH at 60 min compared to 30 min after treatment.

Despite a reduction in total number of spikes and peak firing rate, there was no effect on PWHM or peak time for any treatment ($p > 0.05$). Histogram shape was compared in higher resolution by examining the rise and decay phases. Rise phase was affected by dose at 60 min after treatment (one-way ANOVA, $F = 12.081$, $p < 0.001$), while there was no effect on rise phase at 30 min after treatment. Holm-Sidak multiple comparison versus the control group showed a significantly shortened rise phase with the IMD and 5OH treatments. Similarly, there was no effect of treatment on the decay phase of the histogram, while there were significant effects at 60 min (one-way ANOVA on Ranks, $H_3 = 9.504$, $p = 0.023$). Dunn's post hoc analysis showed the IMD treatment resulted in an increased decay phase 60 min after treatment.

In summary, these results show that the treatments reduced the total number of spikes and peak firing rate of the DCMD in response to a looming stimulus, and that the effects were on peak firing rate were present for the 5OH and OLE treatments already by 30 min after treatment. In addition, we see effects on the histogram shape, with a shortened rise phase and lengthened decay phase for the IMD treatment at 60 min, and a shortened rise phase for the 5OH treatment at 60 min after treatment.

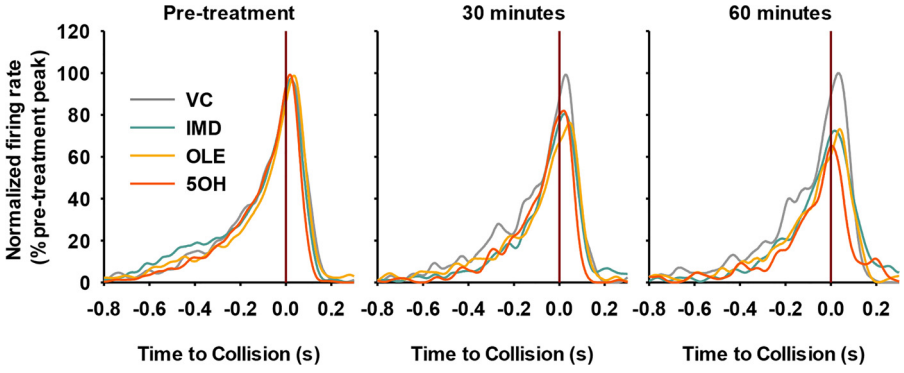

Fig. 3. Mean peristimulus time histograms (PSTHs). Data are from all animals in each treatment group normalized as a percent of the pre-treatment peak firing rate before treatment (pre-treatment), 30 min, and 60 min after treatment with 10 ng/g of locust of IMD (n = 9), OLE (n = 6) or 5OH (n = 8), or the vehicle control (n = 7). Vertical red lines at 0 s represent the projected time of collision of the looming stimulus.

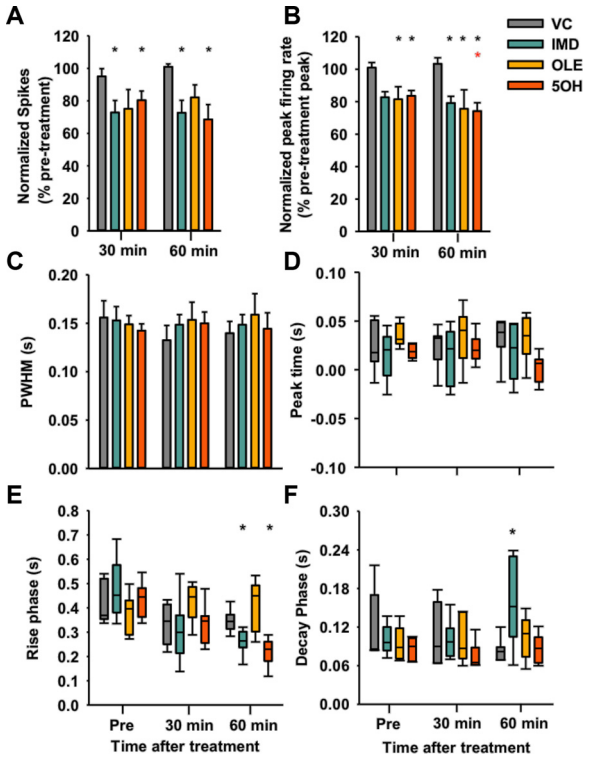

Fig. 4. Comparison of DCMD response parameters. Measurements included: A) total number of spikes per stimulus presentation, B) normalized peak firing rate, C) peak width at half the maximum firing rate, D) peak time, E) rise and F) decay phases of the histograms. Bars display positive s.e.m. and boxplots display the median line and 25th and 75th percentiles, with whiskers at the 5th and 95th percentiles. Asterisks above bars represent significance from a Two-Way Repeated Measures ANOVA. Black asterisks compare treatments to the vehicle (VC) within time (pre-treatment, 30 min and 60 min after treatment), and coloured asterisks compare within treatment over time. Asterisks above box plots denote significance of treatment compared to VC within time after treatment from One Way ANOVA tests.

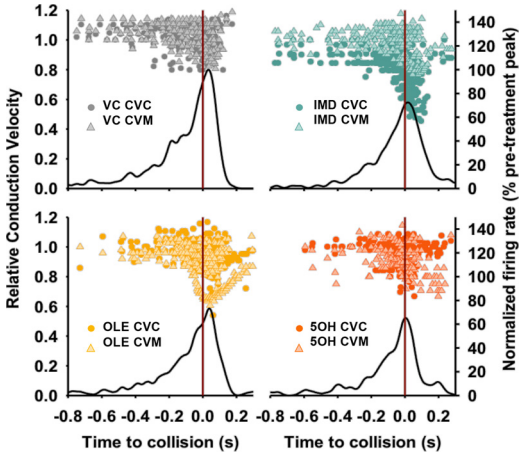

Fig. 5. Effects of treatment on conduction velocity. Relative DCMD conduction velocity versus time to collision for all DCMD spikes recorded along the ventral connective (CVC) and across the mesothoracic ganglion (CVM) for all animals in each treatment group during the approach of a looming stimulus at 60 min after treatment, and the corresponding mean PSTHs (black lines). Projected time of collision is marked with a red vertical line. Darker colours and circles represent measurements along the ventral connective, while triangles and lighter colours represent measurements across the mesothoracic ganglion.

3.3. Conduction velocity

The relative conduction velocity along the DCMD axon was altered by treatment of IMD, OLE or 5OH, and these effects depended on firing rate and location (along the ventral connective versus across the mesothoracic ganglion). Fig. 5 shows the relationship between conduction velocity, both along the ventral connective (CVC) and across the mesothoracic ganglion (CVM), and the firing rate of the DCMD during the approach of the looming stimulus. The firing rate peaked close to TOC and was associated with reduced conduction velocity for all treatments, including the vehicle, which was reduced by approximately 20% at peak firing rates. IMD, OLE, and 5OH also displayed a reduced conduction velocity at peak firing rates, but these were also associated with firing rates that were reduced to approximately 70% of pre-treatment levels.

The relationship between conduction velocity and firing rate appears to have been affected by treatments differentially along the connective or across the mesothoracic ganglion (Fig. 6A). Conduction velocity decreased with increasing firing rate for all treatments (including vehicle). We compared conduction velocity among treatments with the firing rate binned into three categories: < 100 spikes/s, 100–200 spikes/s, and > 200 spikes/s (Fig. 6B). Conduction velocity was binned to isolate the effect of firing rate (Cross and Robertson, 2016). All spike rates above 350 spikes/s were removed for this analysis since IMD, OLE, and 5OH reduced peak firing rates to below this level. There was a significant effect of treatment and frequency group on CVC (two-way RM ANOVA, treatment $F_3 = 7.024$, $p = 0.003$; frequency $F_3 = 28.620$, $p < 0.001$). Comparison of CVC within firing rate bin shows a significant effect of IMD and OLE at all firing rate bins. At firing rates > 200 spikes/s, CVC was also significantly reduced with 5OH. CVC was significantly reduced with 5OH in the 100–200 spikes/s bin compared to the < 100 spikes/s bin, and in the > 200 spikes/s bin the conduction velocity of all treatments was significantly lower than values in the < 100 bin within each treatment.

Conduction velocity was also affected by treatment (two-way RM ANOVA, $F_3 = 4.773$, $p = 0.014$) and was firing rate bin (two-way RM ANOVA, $F_3 = 48.947$, $p < 0.001$) as the axon crossed the mesothoracic

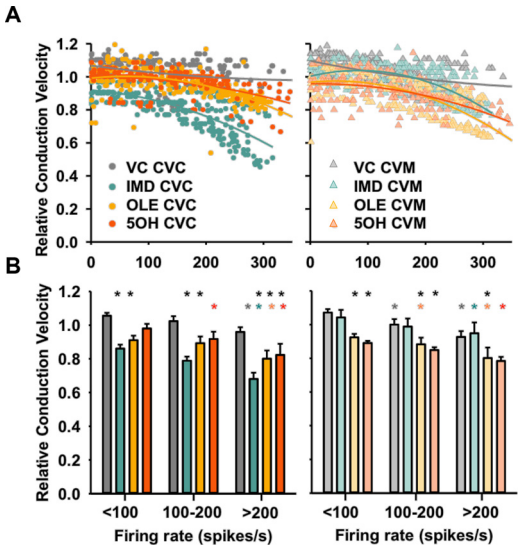

Fig. 6. Relative conduction velocity as a function of instantaneous firing rate along the ventral connective (CVC) and across the mesothoracic ganglion (CVM). A) Conduction velocity of each DCMD spike recorded from all animals 60 min after treatment within each treatment group during the approach of a looming stimulus versus instantaneous firing rate along the connective (left) or across the mesothoracic ganglion (right). Nonlinear regressions were fit for data within each treatment using a polynomial quadratic equation. B) Bar graphs (with positive s.e.m.) illustrating the effects of treatment and firing rate on CVC (left) and CVM (right). Asterisks denote results of two-way Repeated Measures ANOVA with black asterisks comparing treatments to the vehicle (VC) within each firing rate group and coloured asterisks comparing within treatment to the < 100 spikes/s firing rate group.

ganglion (CVM, Fig. 6B, right panel). Holm-Sidak post hoc analysis showed that OLE had a significant effect on CVM, resulting in a lower velocity compared to the vehicle within each firing rate bin. Additionally, the decrease in CVM with OLE was significantly greater at the 100–200 and > 200 spikes/s bins compared to the < 100 bin 5OH significantly decreased CVM compared to the vehicle in the lowest bin only, but CVM was significantly reduced in the > 200 bin compared to the < 100 bin. In the > 200 bin the vehicle and IMD CVM was significantly reduced compared to the < 100 bin within each treatment.

Overall, while conduction velocity was reduced for all treatments (including vehicle) at the highest firing rates (> 200 spikes/s), both along the ventral connective and across the mesothoracic ganglion, there was a significant decrease in conduction velocity compared to the vehicle within each frequency group that resulted from treatment with IMD, OLE and 5OH. OLE had a significant effect across all firing rates and locations whereas IMD produced the most dramatic effect along the ventral connective only.

4. Discussion

While a large body of research exists highlighting the toxicological effects of neonics, relatively few studies examine the contributions of the neurotoxic metabolites even though some metabolites persist longer *in vivo* and in the environment (Codling et al., 2016; Suchail et al., 2004). Identification of the specific contributions of neonic metabolites to toxicity enables a greater understanding of primary and secondary effects of these insecticides and highlight the significance of their presence in the environment.

Potential limitations of the current study included the method of administering the treatments differing between behavioural and electrophysiological assays, and the metabolism of IMD that would naturally occur within the time frames tested. For electrophysiological assays the locust was dissected dorsally and filled with saline, into which the chemicals were added, and during behavioural assays the chemicals were injected with a small volume of saline containing the dose directly into the hemolymph. Despite these differences the total amount of each chemical used was the same between experiments (10 ng/g), thus comparison of electrophysiological and behavioural assays is valid. Future studies should consider the time required for IMD to be metabolized in vivo in the locust and determine relative concentrations of OLE and 5OH within 1 h of treatment.

For behaviour and electrophysiology, we used a consistent dose of 10 ng/g of locust, which we showed previously to be sublethal at 0.04% of the LD50 (2500 ng/g) when injected into the hemolymph (Parkinson et al., 2017). In *Apis mellifera* imidacloprid has an acute toxicity (LD50) value of 570 ng/g for imidacloprid, 280 ng/g for imidacloprid-olefin, and 2580 ng/g for the 5-hydroxy metabolite (Suchail et al., 2001b), roughly ranging across an order of magnitude. Our previous findings used a concentration of imidacloprid that was two orders below the LD50 for locusts. Therefore, assuming a similar range of metabolite toxicity in bees and locusts, we suggest that the metabolite doses we used here were also sublethal.

4.1. Behaviour

We found that IMD, OLE and 5OH affected collision avoidance behaviour and flight 1 h after treatment. The effects were greatest for the 5OH metabolite, and IMD had the smallest effect overall. Sublethal doses of IMD are known to decrease collision avoidance behaviours in locusts 2 and 24 h after treatment (Parkinson et al., 2017) and here we confirm that this effect is already present 1 h after treatment, although there is more variability between animals.

Effects on forward flight versus flight steering suggest toxicity is occurring at different locations in the nervous system. Animals that were able to fly but did not respond (NR) suggest toxicity is targeted to visual interneurons, like the DCMD, that convey information about the looming stimulus, while motor control of flight, such as the thoracic central pattern generators, were unaffected. Toxic effects extended to the control of central pattern generators or directly to the innervation of flight muscles for animals unable to fly. Contrasting vertebrates, the synapses at neuromuscular junctions in insects are glutamatergic (Usherwood, 1977), while acetylcholine is the primary neurotransmitter within the insect central nervous system, mediating excitatory and inhibitory synapses via nicotinic and muscarinic receptors, respectively (Trimmer, 1995). However, insect motorneurons contain nAChRs as a primary excitatory synapse (Parker and Newland, 1995), and inactivation of motorneurons by IMD or its metabolites would result in animals that are unable to fly.

It is plausible that the effects on flight behaviour are caused by the metabolites of imidacloprid rather than the parent compound, as there is a greater effect of the metabolites when administered at the same dose. Imidacloprid is rapidly metabolized to OLE, 5OH and other metabolites, with OLE and 5OH measurable in the head, thorax, abdomen and hemolymph within 20 min of treatment in honeybees (Suchail et al., 2004). Given the quick metabolism of IMD in vivo, it is likely that the behavioural effects observed here are due partially to the metabolites, which could explain the enhanced effects of the metabolite treatments.

4.2. Visual motion detection

Visual motion detection was affected by IMD, OLE and 5OH 30 and 60 min after treatment, measured primarily as a decreased peak firing rate and total number of spikes, with no effect on the time of the peak and PWHM. Reduction in the total number of spikes and peak firing rate are measures of decreased DCMD excitation in response to the looming stimulus. As there was little or no effect on PWHM or peak time, it is likely that inhibitory neurons upstream of the LGMD/DCMD are also affected by IMD and its metabolites, resulting in decreased activity of

these neurons as well. Inhibitory synapses in the locust optic lobe are primarily muscarinic cholinergic (Rind and Leitinger, 2000; Zhu et al., 2018), and IMD has not been shown to affect these synapses (Buckingham et al., 1997). However, dendritic receptors activating inhibitory neurons may be nicotinic cholinergic, so it is possible that IMD and its metabolites are affecting these neurons in the same way as the DCMD, resulting in an overall reduced firing rate with less effect on the slope of the response.

We propose two potential mechanisms for the inactivation of nAChRs by IMD and its metabolites. The first is via the direct desensitization of the receptors resulting from prolonged exposure to the agonists (Nauen et al., 2003; Oliveira et al., 2011). An alternative mechanism is that IMD-mediated increased intracellular calcium ($[Ca^{2+}]_i$) results in the inhibition of nAChRs via phosphorylation-dependent mechanisms, such as the cAMP-mediated pathway. Insect nAChRs are regulated via second messengers including cAMP, which is activated by adenylyl cyclase and may be regulated directly by intracellular calcium (Thany et al., 2007). Neonics have been shown to directly alter $[Ca^{2+}]_i$ via interactions of nAChRs with voltage-gated calcium channels that amplify IMD-induced increases in $[Ca^{2+}]_i$ (Jepson et al., 2006). Furthermore, desnitro-IMD, a form of IMD highly toxic to mammals, activates the extracellular signal-regulated kinase cascade, which may result in deficiencies in cell survival/cell death via alterations in $[Ca^{2+}]_i$ resulting from activation of the nAChR (Tomizawa and Casida, 2003). Taken together, it is likely that the reduction in DCMD firing shown here results from decreased activity of nAChR caused both directly and indirectly by the binding of IMD and its metabolites to the receptors.

4.3. Waveform propagation

We report that the conduction velocity along the DCMD axon is reduced by IMD, OLE and 5OH, and that these effects depended on firing rate and location along the axon, the latter likely due to physical properties of the axon. The DCMD axon runs along the dorsomedial region of the connective and decreases in diameter as it crosses the mesothoracic ganglion, with collateral projections to the medial, lateral and ventral regions of the neuropil (Gray et al., 2010). At high firing rates, the intracellular ion concentrations would likely be altered due to the many synapses between the DCMD and other neurons in the ganglion. High frequency firing can result in decreased conduction velocity in dorsal root ganglion cells and is associated with an increase in $[Ca^{2+}]_i$ (Luscher et al., 1994). The effect may be responsible for the observed decrease in conduction velocity at high firing rates across the ganglion, but not along the ventral connective. Given that IMD had no effect on conduction velocity across the ganglion, but a very large effect (mean reduction of 32%) at high frequencies along the connective, we suggest that the isolation provided by the neuropil may reduce the effects of IMD. The mechanism by which IMD, OLE and 5OH decrease conduction velocity in the unmyelinated DCMD axon has not been examined directly, but it is possible this effect is caused by alterations to $[Ca^{2+}]_i$.

4.4. Conclusions

In summary, we found that exposure to 10 ng/g (100 µg/kg) imidacloprid and two of its metabolites, imidacloprid-olefin and 5-hydroxy-imidacloprid, affected flight and collision avoidance behaviours, with greater effects for 5OH than OLE, and for OLE than IMD. Additionally, we found that IMD and its metabolites decreased visual motion detection, as measured from the activity of the DCMD neuron, and that effects on the peak DCMD firing rate occurred sooner following direct treatment with the metabolites. These results support our hypothesis and suggest that the main effects on behaviour and neural coding are due, primarily to exposure to metabolites. One hour after treatment, IMD would be metabolized partly to 5OH and OLE, but at lower concentrations given the breakdown of IMD to 5OH, OLE and other, non-neurotoxic metabolites (Nauen et al., 2001). Given that the metabolites have the same or greater effect than the parent compound on both behaviour and neural processing highlights the importance of considering the environmental longevity and concentrations of these metabolites as well as the parent compound.

We additionally found that conduction velocity was significantly reduced with IMD, OLE and 5OH, which corresponds with effects measured in humans exposed to agricultural neonics (Huang et al., 2016; Zhang et al., 2018). Additional research is needed to uncover the mechanism affecting conduction velocity of neonics. Overall, we have described and compared the effects of two neonic metabolites on flight behaviour and visual processing and found that these metabolites display similar toxicity to the parent compound. This study should inform future studies examining ecological presence of neonics, as the presence of certain metabolites may be equally or more toxic to non-target organisms including important pollinators.

Competing interests

We have no competing interests.

Authors' contributions

R.H.P. designed and carried out experiments, analyzed the data, interpreted the results, prepared the figures, and wrote and revised the manuscript. J.R.G. conceived and designed experiments, interpreted the results, revised the manuscript, and approved the final version of the manuscript.

Funding

Funding was provided by the Natural Sciences and Engineering Research Council of Canada (Award number: RGPIN-2014-05269), the Canada Foundation for Innovation (CFI), and the University of Saskatchewan.

Acknowledgements

We thank C. Manchester and S. Zhang for assistance collecting pilot behavioural data, and E. Olson for designing the stimulus generation system used for the electrophysiological assays.

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