Concentration-dependent effects of acute and chronic neonicotinoid exposure on the behaviour and development of the nematode *Caenorhabditis elegans*

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Abbreviations:

Nicotinic acetylcholine receptor, nAChR; NGM, nematode growth medium

Abstract

BACKGROUND Neonicotinoids insecticides are under review due to emerging toxicity to non-target species. Interest has focused on biological pollinators whilst their effects on other organisms that are key contributors to the ecosystem remain largely unknown. To advance this we have tested the effects of representatives of three major classes of neonicotinoids, thiacloprid, clothianidin and nitenpyram on the free-living nematode *Caenorhabditis elegans* (*C. elegans*), as a representative of the Nematoda, an ecologically important phylum contributing to biomass.

RESULTS Concentrations that are several-fold higher than those with effects against target species had limited impact on locomotor function. However, increased potency was observed in a mutant with a hyper-permeable cuticle which shows that drug access limits the effects of the neonicotinoids in *C. elegans*. Thiacloprid was most potent (EC_{50} 714 μM). In addition, it selectively delayed larval development in wild-type worms at 1 mM.

CONCLUSIONS *C. elegans* is less susceptible to neonicotinoids than target species of pest insect. We discuss an approach in which this defined low sensitivity may be exploited by heterologous expression of insect nicotinic acetylcholine receptors from both pest and beneficial insects, in transgenic *C. elegans* with increased cuticle permeability to provide a whole organism assay for species-dependent neonicotinoid effects.

1 INTRODUCTION

Since launching into the agrochemical market in the 1990s, neonicotinoids became the most commonly used insecticides worldwide [1]. Currently, there are six compounds used as crop protectants and one used as external pest control agent. These are classified into N-nitroguanidine (imidacloprid, dinotefuran, thiamethoxam, and clothianidin), N-cyanoamidines (acetamiprid and thiacloprid) and nitromethylenes (nitenpyram). Several

factors contribute to their success, including high potency against a wide range of piercing and sucking pests [2], selective toxicity to insects over humans [3], distinct mode of action, and advantageous psychochemical properties which allow a diversity of application methods [4]. Nevertheless, there are emerging issues associated with their use which currently focus on their potential toxicity towards one of the most important biological pollinators, bees [5]. This focus has instigated many studies but the effects on other biomass important organisms e.g. other pollinators, molluscs [6] or nematodes have not been investigated in depth or systematically.

Pharmacological and electrophysiological data suggests that nicotinic acetylcholine receptors (nAChRs) are the principal site of action of neonicotinoids [7-15]. However, different neonicotinoids exhibit differential mode of action depending on the sub-type of receptor investigated. Some act as full, partial and some as super-agonists [16-19]. As an example, clothianidin is a super-agonist on hybrid chicken-*Drosophila* receptors [16] and on the native nAChRs expressed in *Drosophila* CNS [17] In contrast it acts as a partial agonist on cockroach CNS neurons [18].

The widespread use and issues associated with neonicotinoids requires better understanding of their specificity but even compounds with the same chemical pharmacophore target distinct nAChR subunits [14, 20]. An important limitation relates to the difficulty in achieving heterologous expression of insect nAChRs [21]. The desire to routinely express these receptors has led to search for co-factors that underpin their expression *in vivo* that can be transplanted to heterologous systems. An important step forward involved cross-referencing understanding of the molecular determinants of *C. elegans* receptor expression to the investigation of insect receptors. Thus, studies on the determinants of nAChR expression in *C. elegans in vivo*

identified a nematode molecular chaperone RIC-3 [22, 23]. Its conserved role is shown by the observation that it is obligatory to expression of insect receptors in *Xenopus* oocytes [24]. Therefore, transgenic approaches in the model organism *C. elegans* could serve as a platform to identify neonicotinoid-sensitive, insect nAChRs.

The *C. elegans* genome contains a predicted 29 nAChR subunits [25]. A subset of these are expressed at the neuromuscular junction of the pharyngeal and the body wall muscle and are involved in feeding, locomotion, and reproduction. In addition they have wide expression in sensory and integrating circuits implicated in higher function chemosensory driven behavioural responses and other environmentally cued behaviours underpinning plasticity [26]. This involvement of nAChRs in cholinergic pathways that regulate environmentally driven behavioural adaptation in *C. elegans* is similar to their role in pollinating insects where it has been shown that neonicotinoids have the ability to disrupt neural plasticity [27-29].

In the current study we have evaluated the impact of different chemical classes of neonicotinoids on *C.elegans* behaviours with a longer term goal of establishing this as a transgenic model for the expression of insect nAChRs and characterisation of their interaction with neonicotinoids. We investigated the effects of three classes of neonicotinoids: thiacloprid, clothianidin and nitenpyram, and compared them to the definitive nAChR agonist, nicotine. We exposed worms to high concentrations of drugs acutely (4 hours) and chronically (24 hours) and determined effects on locomotion and development. We indicate how the whole organism approaches are facilitated by mutants that enable drug dosing in the intact organism. This identifies an ability to report acute and chronic effects of these important compounds and a potential route to investigate their ecotoxicology. This work

provides a benchmark against which the genetic tractability of the worm can be used to investigate neonicotinoid mode of action.

2 METHODS

2.1 *C. elegans* maintenance

Wild-type (N2 Bristol strain) and strain CB7431 (Genotype: *bus-17*, (*br2*) X, outcrossed 4 times; obtained from CGC) *C. elegans* were cultured at 20°C on nematode growth medium (NGM) agar plates seeded with 50 µL lawn of OP50 *Escherichia coli* strain [30]. Experiments were performed on young hermaphrodite adults (L4+1 day). Drugs and reagents were purchased from Sigma Aldrich. Observations were made using a binocular microscope, unless otherwise stated. Results are expressed as mean ± SEM of '*N*' determinations. Graph generation and measurement of EC_{50} was performed in GraphPad (version 6.07).

2.2 Drug solutions

Thiacloprid and clothianidin were dissolved in 100% dimethyl sulfoxide (DMSO) and added to experiments so the drug vehicle concentration was 0.5% v/v. Nitenpyram and nicotine stocks were prepared by dissolving drugs in dH_20 and diluted to the indicated final concentrations. Thiacloprid, clothianidin and nicotine stocks were kept at -18°C for up to 1 month, whereas nitenpyram stock was made immediately prior to experiments. Nitenpyram containing solutions were protected from light between the measurements to prevent photo-degradation.

2.3 Acute exposure and effects on motility; thrashing assay

In liquid *C. elegans* make rhythmic stereotypical flexing movements centred on the midpoint of their body called 'thrashing'. To assess the impact of neonicotinoids on *C. elegans* motility thrashing was assayed. Experiments were performed in a 24-well plate in a final volume of 500µl of phosphate buffer (M9)[30] with 0.1% Bovine Serum Albumin buffer with either drug/solvent or solvent alone as control. Typically 2 (vehicle control) or 6 (treatment) worms were picked into a single well containing phosphate buffer and left for at least 5 minutes to equilibrate to the solution. Then thrashes, defined by a single inflection back and forth, were counted for a duration of 30 seconds, defined as time zero, to provide baseline measurement for each worm. Subsequently, either drug/solvent or solvent alone (50 µL) was added to the wells and carefully mixed by pipetting the bath volume up and down to give the final desired concentration. Thrashing rate was scored for 30 seconds at time points: 10, 30, 40, 60 and 120, and 240 minutes post-addition of the drug/drug vehicle.

2.4 Chronic exposure and effects on motility; body bends

To allow chronic exposure to drugs experiments were performed in 6-well plates on solid nematode growth medium (NGM agar) in which three of the wells were seeded with *E. coli* OP_{50} to allow 24 hour exposure of the young adult worm to drug in the presence of food and the remaining three wells were left *E. coli* free to provide an assay arena for motility. Clothianidin, thiacloprid and nicotine were incorporated in the medium by first making stock solutions in DMSO. Stock solutions or DMSO were added to molten NGM (55°C) to give the final indicated concentration in which DMSO (vehicle) did not exceed 0.5%. Each well of the 6-well plate was filled with 3 ml of molten NGM either with vehicle control or with drug/vehicle and left to solidify. 50 µL of *E. coli* OP_{50} (OD_{600nm}= 0.8-1) was added to three of the wells and the plates left in the fume hood for two hours to dry. The remaining 3 wells

were left without OP_{50}. One day old adult hermaphrodites were placed in each of the wells containing OP_{50} with either drug/vehicle or vehicle only. 24 hours later worms were transferred into the wells without OP_{50} containing either drug/vehicle or vehicle only. After 10 minutes acclimatisation period, the motility of worms on solid medium was measured by counting the number of body bends per minute.

2.5 Development assay

One day old adult hermaphrodites (6-10) were placed into wells of a 6-well plate where the wells contained NGM, with or without drug or solvent, and seeded with OP_{50}, as described above. After 1 hour they were removed from the wells and the eggs they had laid were left behind. The larval development of hatched progeny was monitored. Developmental stages were identified following size/vulva/eggs present in the uterus according to criteria described by Karmacharya et al.[31] L1 were the smallest worms on the plate. L2 were slightly bigger, L3 bigger still, more mobile and displayed a pre-vulva space. L4 were identified by the appearance of the premature vulva, whereas gravid adults had a fully formed mature vulva and eggs present in their uterus. These observations were typically made at 60x magnification but when necessary, worms were viewed at higher magnification, under Nicon Eclipse E800 microscope at time point: 24, 30, 48, 54, 72, 80, 96, 120, 144 hours after eggs were laid. Results are represented as worms in each developmental stage as a mean % of total population size.

3 RESULTS

3.1 Nicotine and neonicotinoid effects on *C. elegans* motility

Wild-type N2 worms can sustain rhythmic thrashing in liquid for 4 hours and this is subject to a concentration-dependent inhibition in the presence of nicotine (Figure 1 a). At the

highest concentration tested, 100 mM, the inhibition is relatively rapid and complete inhibition of thrashing is observed at 10 minutes. Solubility restricted the concentrations that could be tested for thiacloprid and clothianidin to a maximum of 1 and 2.5mM, respectively, and neither of these compounds inhibited thrashing at these concentrations in contrast to 1mM nicotine (Figure 1a). Nitenpyram elicited an inhibition at 100 mM but unlike nicotine this was not a complete paralysis.

3.2 Effect of nicotine and neonicotinoids on mutant *C. elegans* with increased cuticle permeability

An important and poorly defined determinant of potency is the ability of a drug present in the external environment to access the interior worm, either by ingestion and absorption across the gut wall or diffusion, and perhaps transport, across the cuticle. In the case of nicotine and neonicotinoids this may be a limiting factor in terms of their biological activity as they require access to neurotransmitter receptors that are expressed on muscle and neurons. Worm mutants with aberrant cuticular integrity have enhanced sensitivity to drugs[32, 33] and provide a useful experimental model to investigate whether drug absorption across the nematode cuticle is a limiting factor in drug sensitivity. The baseline behaviour of the cuticle mutant, *bus-17*, is similar to N2 and it thrashes at a similar rate (Figure 1b). As before, nicotine and nitenpyram showed a rapid and dose dependent inhibition of thrashing, however the potency of both compounds was markedly shifted to lower concentrations (Figure 1b). Remarkably, the *bus-17* mutant revealed a susceptibility of *C. elegans* to thiacloprid and clothianidin that was not apparent in wild-type N2 worms with intact cuticle (Figure 1b). This increased potency is summarized in concentration-response curves for nicotine, thiacloprid, clothianidin and nitenpyram (Figure 2). In the *bus-17* mutant thiacloprid is the most potent of the compounds tested with an EC_{50} of 480 μM.

The thrashing assays revealed effects of the compounds on motility over a time-course of 4 hours but are not suited for longer drug exposure assays as they are conducted in the absence of the worm's food, *E. coli* OP_{50} and therefore over the course of a day control worms will no longer thrash. Therefore to test whether or not more protracted exposure to the compounds would further increase the sensitivity of *C. elegans* to the inhibitory effects on motility, i.e. 24 hours exposure rather than 4 hours exposure, we used an assay in which adult worms are exposed to the drugs on an agar lawn laced with *E. coli* OP_{50} (Figure 3) allowing chronic drug exposure via ingestion and through the cuticle. Again, the potency of nicotine was greater in the *bus-17* mutant compared to N2 and for clothianidin and thiacloprid revealed a sensitivity that was not evident in N2. An analysis of the concentration-response curves for nicotine, thiacloprid and clothianidin indicates that thiacloprid is the most potent inhibitor of body bends (Figure 4).

3.3 Effects of nicotine and neonicotinoids on *C. elegans* development

During the time-frame of the chronic dosing experiments described above wild-type N2 worms will typically lay eggs which hatch into larva and then over the course of 3 further days will mature into adults. We noted during the chronic dosing of adult worms with neonicotinoids that this developmental programme appeared to be delayed by nicotine and thiacloprid and therefore investigated the impact of these compounds on larval development in more detail. To do this, we monitored the progression of larval development from a synchronized population of eggs through L1, L2, L3 and L4 by counting the relative proportion of each developmental stage after 1, 2, 3 and 6 days exposure to the compounds. Both nicotine and thiacloprid were found to slow larval development (Figure 5). The differential distribution of distinct larval stages following nicotine and thiacloprid dosing was

most marked at day 2, with the effect of thiacloprid the most marked. This suggests the drug impacts on L2 to L3 transition. Clothianidin and nitenpyram at 1 mM failed to shift the development (data not shown).

4 DISCUSSION AND CONCLUSIONS

To initiate investigations of poorly understood toxicity of neonicotinoids on important biomass organisms, nematodes, we have exposed the free-living nematode *C. elegans* to thiacloprid, clothianidin, nitenpyram and to the well-studied nAChR agonist, nicotine. Previous studies [34] report effects of low mM concentrations of thiacloprid and imidacloprid on reproduction. In our study we investigated effects on motility and in wild-type N2 worms we observed no significant impairment of locomotion upon acute or chronic exposure to the neonicotinoids thiacloprid or clothianidin although there was an inhibitory effect of nitenpyram at high millimolar concentrations. In contrast, nicotine paralysed *C. elegans* at lower millimolar concentrations and it was notable that the potency was ten times greater with chronic exposure compared to acute assays, consistent with the suggestion that nicotine does not permeate the worm's cuticle readily.

To investigate the role of cuticle in drugs permeability, we performed locomotion assays on *C. elegans* mutant, characterised by a more "leaky" cuticle (*bus-17*)[32]. We observed clear inhibitory effects of thiacloprid and clothianidin on motility at high micromolar concentrations, with the former being the most potent out of all compounds tested. Since the field levels of neonicotinoids in soil and water are orders of magnitude lower (low micromolar concentrations in water and 2 ng per g in soil)[35] at least short term exposure to neonicotinoids should have limited effect on related nematodes, assuming that N2 *C. elegans* is a predictor for wild isolates and nematode species. However, protracted incubation with

nicotine and thiacloprid delayed larval development of wild-type N2, suggesting compounds may accumulate in the nematode's tissues, highlighting the importance of bioaccumulation in determination of ecotoxicity. Moreover, neonicotinoids have been shown to disrupt aspects of movement in bees[36] and in *C. elegans* [37] (this study). In bumblebees there is also evidence for disruption of higher cognitive tasks by neonicotinoids [28] and therefore efforts into determining whether neonicotinoids impact on more intricate behavioural repertoires in *C. elegans* are also worthy of further investigation .

Overall our data highlight that *C. elegans* is not susceptible to field relevant concentrations of neonicotinoids despite the fact that susceptibility to high concentrations of all three of the neonicotinoids tested can be demonstrated in a mutant with increased cuticle permeability. Indeed, our data suggest that neonicotinoids are three orders of magnitude less potent on *C. elegans*, than on insects [27-29, 38-46]. This creates an opportunity to use a *C. elegans* transgenic model hopping approach which utilises the introduction of genes of interest, from either pest species or other non-target organisms, in to wild-type or mutant *C. elegans* to provide an experimentally tractable system to investigate mode of action and selective toxicity [47-49]. This approach will use manipulation of the insect genes encoding candidate neonicotinoid-sensitive nAChRs. As *C. elegans* possess essential biosynthetic machinery for invertebrate nAChR receptor [23, 24] this creates a favourable cellular environment for receptor expression. Potential candidate insect nAChR subunits are brown planthopper *Nilaparvata lugens α1* [50] green peach aphid *Myzus persicae* β1 subunits both associated with neonicotinoid-resistance, as well linked to neonicotinoid-susceptibility an α1 subunit of housefly *Musca domestica* [51]. The data shown in this study indicate that the *C. elegans bus-17* mutant will provide a useful genetic background for these studies by enhancing the access of neonicotinoid compounds to their target receptor expressed in the worm. Therefore, by expressing subunit combinations in

the *bus-17* background, one can apply cellular assays to investigate the functionality of the heterologous receptors [47] and compare neonicotinoid-sensitivity of transgenic versus control strains in behavioural assays. Such pharmacological and structural characterisation is crucial in understanding neonicotinoid-induced behavioural alterations and may contribute to the identification of new more selective neonicotinoids.

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Figure legends

Figure 1. The concentration-dependence and time course for the inhibitory effect of nicotinic compounds on motility of *C. elegans*.

Wild type (a) and *bus-17* (b) worms were acutely exposed to varying concentrations of nicotine thiacloprid, clothianidin, nitenpyram or drug vehicle (control), in liquid medium. Their effect on thrashing over time was recorded. Data are mean ± SEM collected over ≥2 observations, with number of determinations specified in brackets. One way Anova (Kruskal-Wallis test) with Dunn's Corrections, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

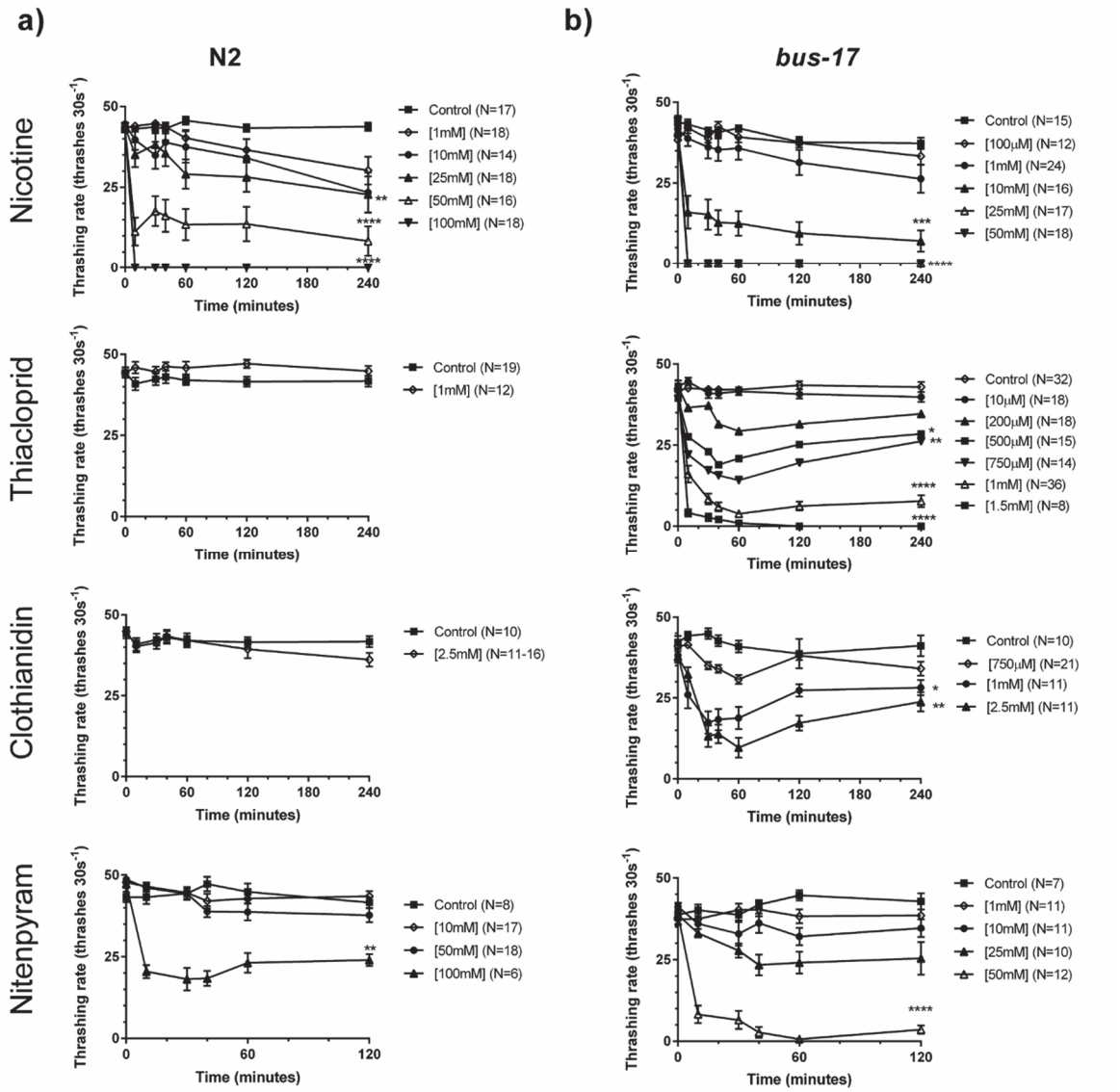
a)
b)
N2
bus-17
Nicotine
Thiacloprid
Clothianidin
Nitenpyram
Thrashing rate (thrashes 30s-1)
Time (minutes)
Control (N=17)
[1mM] (N=18)
[10mM] (N=14)
[25mM] (N=18)
[50mM] (N=16)
[100mM] (N=18)
Control (N=15)
[100µM] (N=12)
[1mM] (N=24)
[10mM] (N=16)
[25mM] (N=17)
[50mM] (N=18)
Control (N=19)
[1mM] (N=12)
Control (N=32)
[10µM] (N=18)
[200µM] (N=18)
[500µM] (N=15)
[750µM] (N=14)
[1mM] (N=36)
[1.5mM] (N=8)
Control (N=10)
[2.5mM] (N=11-16)
Control (N=10)
[750µM] (N=21)
[1mM] (N=11)
[2.5mM] (N=11)
Control (N=8)
[10mM] (N=17)
[50mM] (N=18)
[100mM] (N=6)
Control (N=7)
[1mM] (N=11)
[10mM] (N=11)
[25mM] (N=10)
[50mM] (N=12)

Figure 2. Dose-response curves for the effects of nicotinic compounds on motility in liquid medium of *C. elegans*.

Concentration-response curves for the effects of a) nicotine, b) thiacloprid, c) clothianidin and d) nitenpyram on thrashing of *bus-17* (grey) and wild-type N2 (black) *C. elegans*. Thrashing rates were generated by taking 60 (nicotine and nitenpyram) or 120 minutes (thiacloprid and clothianidin) time points; that is when the steady-state was reached (Figure 1), and expressed as a percentage of the control thrashing activity. EC_{50} values (concentration of the drug that produced 50% paralysis) are shown in black for N2 and grey for *bus-17*. Data are mean ± SEM. The EC_{50} for clothianidin is an approximation as at the highest concentration tested (2.5mM) the maximum inhibition observed was 50%.

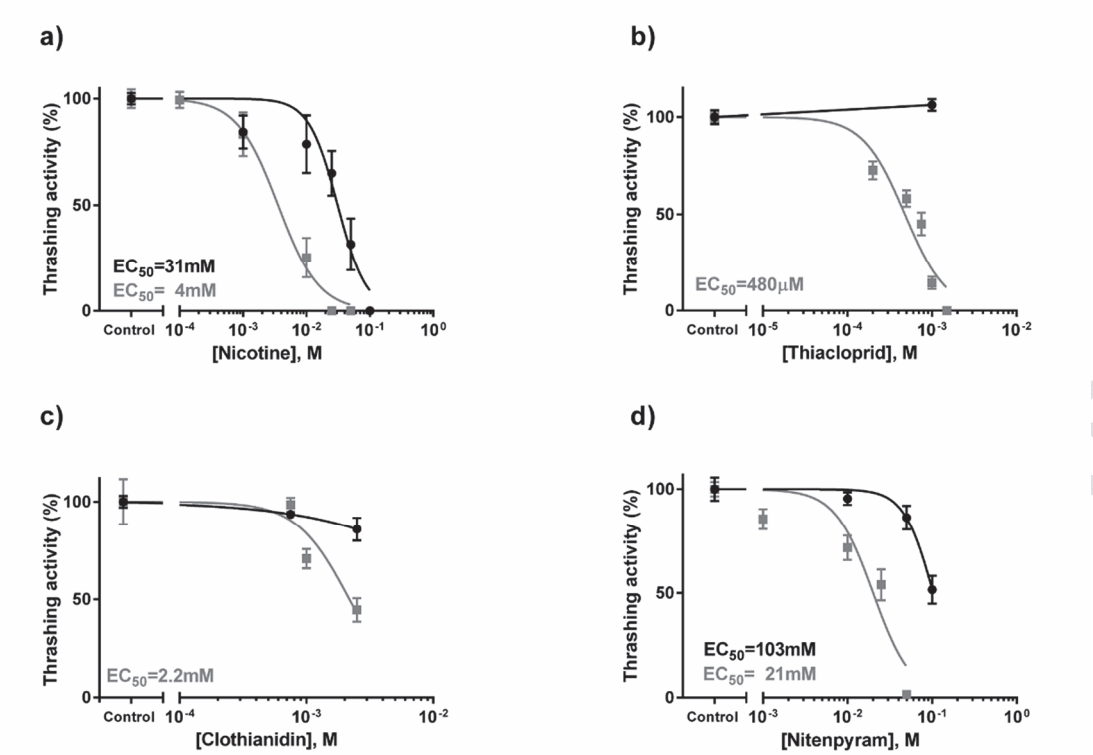

Figure 3. The concentration-dependence for the effects of chronic exposure to nicotinic compounds on locomotion of *C. elegans*.

Wild type (a) and *bus-17* (b) worms were exposed for 24 hours to varying concentrations of nicotine, thiacloprid, clothianidin or drug vehicle (control), incorporated into solid medium. Body bends were counted by visual observation. Data are mean ± SEM, collected over ≥3 observations number of replicates are given in graph bars. One way Anova (Kruskal-Wallis test) with Dunn's Corrections, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

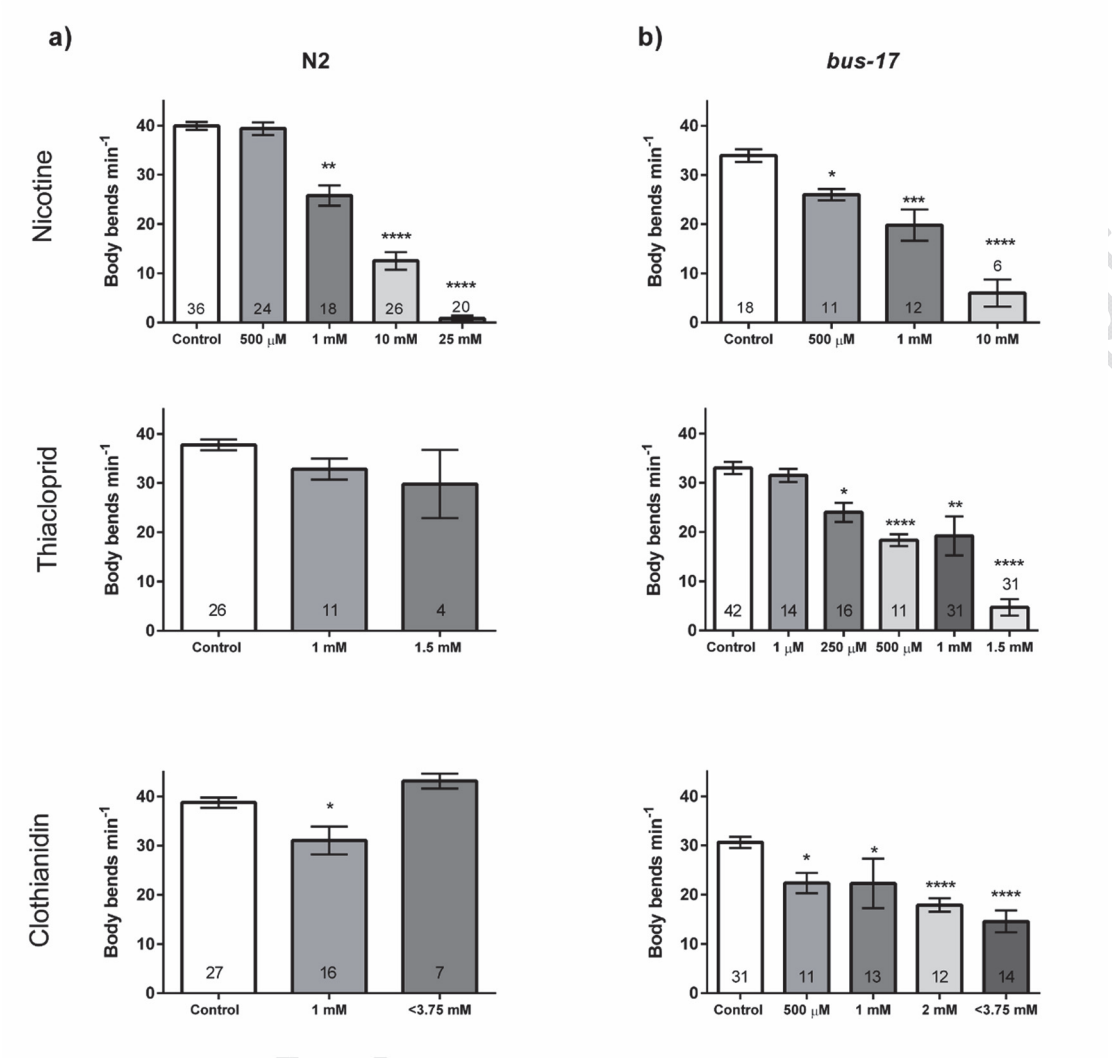
a)
N2
b)
bus-17
Nicotine
Thiacloprid
Clothianidin
Body bends min-1
Control
500 µM
1 mM
10 mM
25 mM
1.5 mM
2 mM
<3.75 mM
250 µM

Figure 4. Concentration-response curves for the effects of chronic nicotine and neonicotinoid compound treatment on locomotion of *C. elegans*.

Concentration-response curves for the effects of a) nicotine, b) thiacloprid, and c) clothianidin on body bends rates of *bus-17* mutant (grey) and wild-type N2 (black) *C. elegans*. Body bends rates are expressed as a percentage of the control activity. EC_{50} values (concentration of the drug that produced 50% paralysis) are shown in black for N2 and grey for *bus-17*. Data are mean ± SEM.

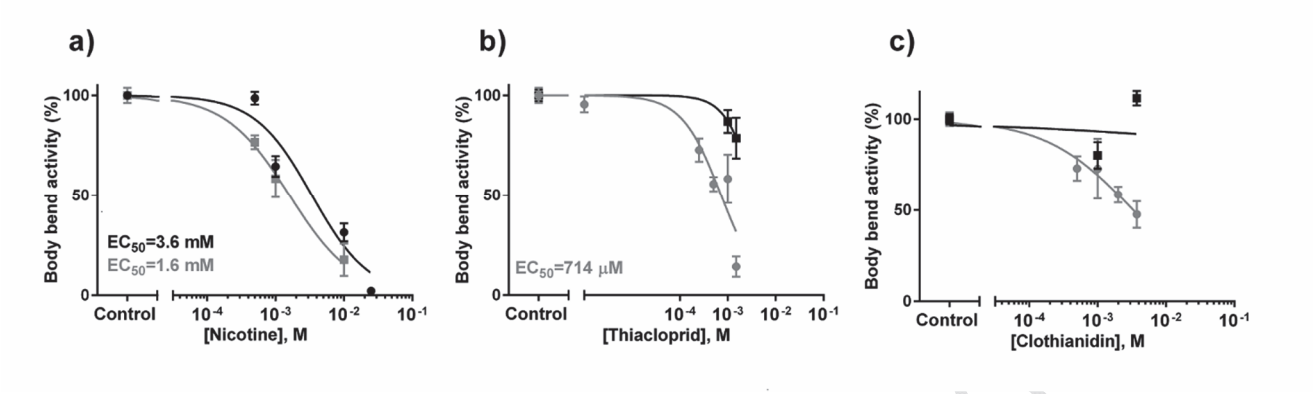

Figure 5. Effects of nicotine and thiacloprid on larval development of wild type *C. elegans.* Wild-type young hermaphrodites laid eggs on plates dosed with 1mM thiacloprid, 1mM nicotine or drug vehicle (control). Larval development in the presence of drugs was monitored over time. Worms were assigned to each one of 5 life-stages, namely L1, L2, L3, L4 and gravid adults. The fraction of worms in each stage as a % of total population at time point: 30, (day 1), 48 hours (day 2), 72 hours (day 3), 144 hours (day 6) was measured. Data are shown as the mean of $N \geq 3$.