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Title: Neonicotinoid insecticides inhibit cholinergic neurotransmission in a molluscan (Lymnaea stagnalis) nervous system

Article Type: Research Paper

Keywords: neuron; acetamiprid; imidacloprid; thiamethoxam; clothianidin; thiacloprid

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Abstract: Neonicotinoids are highly potent and selective systemic insecticides, but their widespread use also has a growing impact on non-target animals and contaminates the environment, including surface waters.
We tested the neonicotinoid insecticides commercially available in Hungary (acetamiprid, Mospilan; imidacloprid, Kohinor; thiamethoxam, Actara; clothianidin, Apacs; thiacloprid, Calypso) on cholinergic synapses that exist between the VD4 and RPeD1 neurons in the central nervous system of the pond snail Lymnaea stagnalis. In the concentration range used (0.01-1 mg/ml), neither chemical acted as an acetylcholine (ACh) agonist; instead, both displayed antagonist activity, inhibiting the cholinergic excitatory components of the VD4-RPeD1 connection. Thiacloprid (0.01 mg/ml) blocked almost 90 % of excitatory postsynaptic potentials (EPSPs), while the less effective thiamethoxam (0.1mg/ml) reduced the synaptic responses by about 15 %. The ACh-evoked membrane responses of the RPeD1 neuron were similarly inhibited by the neonicotinoids, confirming that the same ACh receptor (AChR) target was involved. We conclude that neonicotinoids act on nicotinergic acetylcholine receptors (nAChRs) in the snail CNS. This has been established previously in the insect CNS; however, our data indicate differences in the background mechanism or the nAChR binding site in the snail.
Here we provide the first results concerning neonicotinoid-related toxic effects on the neuronal connections in the molluscan nervous system. Aquatic animals, including molluscs, are at direct risk while facing contaminated surface waters, and snails may provide a suitable model for further studies of the behavioural/neuronal consequences of intoxication by neonicotinoids.

Tihany, Hungary, July 22, 2015

Dr. Mikko Nikinmaa
Editorial Office
Aquatic Toxicology

Dear Dr Nikinmaa,

Thank you very much for your response to our manuscript submission. We gratefully thank you and our reviewers for reading the manuscript, and we appreciate all the helpful comments made.

After carefully checking the Reviewers' comments we improved the manuscript on the basis of their suggestions. Please find our detailed answers to the Reviewers below.

Reviewer #1:

We greatly appreciate the Reviewer's comment **"***one of the best written manuscripts that I have reviewed*"

Minor points

1. Materials and Methods

How was Lymnaea saline obtained? Is this the modified HiDi saline? If not, what is HiDi?

For isolating the monosynaptic components of the synaptic responses between two neurons the perfusion chamber was filled with a modified saline which reduces the polysynaptically evoked postsynaptic responses and enhances the monosynaptic component (Berry and Pentreath, 1976). This modified saline contains **hig**her amount of **di**valent cations (Ca^{2+} and Mg^{2+}), therefore often nicknamed as HiDi saline (Brierly *et* al., 1997, Sivaramakrishnan *et* al., 2013, etc).

Berry MS, Pentreath VW (1976) Criteria for Distinguishing between Monosynaptic and Polysynaptic Transmission Brain Res 105:1-20 Doi 10.1016/0006-8993(76)90919-7)

Sivaramakrishnan S, Sanchez JT; Grimsley CA.. (2013). High concentrations of divalent cations isolate monosynaptic inputs from local circuits in the auditory midbrain. Frontiers in neuralcircuits: 7 Article Number: 175.

Brierley, MJ; Staras, K; Benjamin, PR (1997). Behavioral function of glutamatergic interneurons in the feeding system of Lymnaea: Plateauing properties and synaptic connections with motor neurons J. of Neurophysiol: 78 , 3386-3395.

We added details to the text accordingly

2.3 Electrophysiological recording

There is a Syed and Winlow 1991 and a Syed et al. 1990 in the reference section but not a Syed et al. 1991 as written in section 2.3.

We corrected the text (Syed and Winlow, 1991) accordingly

4. Discussion

This section has Hamlet et al. 2015 but the reference section has Hamlet et al. 2014. Which year is the correct year?

We corrected the text (Hamlet et al., 2014) accordingly.

5. Reference

This section has Hamlet et al. 2014 but the discussion section has Hamlet et al. 2015. Which year is the correct year? ?
This is correct, please see above.

Reviewer #2:

Major points
Data are presented as a collection of descriptive responses apparently established on the basis of single electrophysiological recordings in various conditions. No information was given concerning the number of replicates. A statistical analysis of response variability and significance is mandatory.
We added the necessary details including the number of experiments and statistics (see Materials and Methods 2.4. and text in the Results sections) and also inserted/created an extra figure (Fig. 6.) to present our results.

English and word accuracy should be refined in several places, in particular within introduction and discussion sections. Ex :
Introduction " Neonicotinoids are the newest generation of highly potent and selective systemic insecticides used as agrochemicals or protect plants" ; " but the positively charged nitrogen atom is replaced by other moieties resulting the nitro substituted imidacloprid" ;
A professional, native English corrector checked our manuscript and we corrected the text accordingly to her advices.

Minor points
Introduction
1/ p2, l.48, although comparisons of toxicological and ecotoxicological bioassays at different scales are justified in this section, the rationale given here is poor. Motivation for using various approaches in environmental risk assessment is rather inspired by the types of scientific information obtained (PNEC, EC50, mechanistic...)
We fully agree with the referee, that different disciplines and different methodologies are required to obtain data for environmental risk assessment. Toxicological and ecotoxicological bioassays may give informations regarding particular endpoints while physiological experiments may reveal target mechanisms and give tools for comparative studies.
We added this point to the revised text.

Results
1/ p4, Figure 1 : RPeD1 neurons exhibited spontaneous firing. Why ?
In normal saline members of the pattern generating neural networks (feeding, respiration) may display some spontaneous activity but without the synchronized pattern which charaterize their firing when the pattern- generating interneurons are activated. The identified RPeD1 cell is one of the respiratory interneurons which tonic activity in normal saline (as is seen on Fig 1.) shows that this cell does not receive synaptic inputs from the respiratory network.

What were the parameters of VD4 intracellular stimulation ?
We added the necessary details of the intracellular stimulation (see Materials and Methods and Results sections).
What was the value of synaptic delay ?
We added the values of the synaptic delay to the text accordingly (see Results section and Figure 1. legend).

2/ Figure 4 : authors should use the same time scale in graphs A & B to present more accurately kinetic differences.
We corrected the figure accordingly

Discussion
1/ p8, l.29 : "Our recent results...", this sentence should be followed by a reference na,
We corrected the text : “Our results presented above …”

3/ p8, l.37 : "the results suggested drug-induced changes of the nAchRs"; to me, the authors should draw more direct conclusions from both their experiments and literature discussion.
The most correct conclusion we could draw from our experiments are the following: 1./ the nicotinergic nature of the snail AChRs are confirmed (experiment with d-tubocurarine, see Fig 2.) and 2./ modulation of AChRs (both synaptic and extrasynaptic) in the presence of neonicotinoid insecticides (see Figs 3.-6.). We conclude, therefore, that the same target (nAChR) is involved in the neuronal effects of neonicotinoids both in the insect and snail.. For further details of the mechanisms additional (electrophysiological, pharmacological and molecular studies) will be required.

Finally, we would like to thank you again your time and kind assistance regarding our manuscript. We trust you will find the improved text to be suitable for publication.

Sincerely yours,

Dr Ágnes Vehovszky

Neonicotinoid insecticides inhibit cholinergic neurotransmission in a molluscan (*Lymnaea stagnalis)* nervous system

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Abstract

Neonicotinoids are highly potent and selective systemic insecticides, but their widespread use also has a growing impact on non-target animals and contaminates the environment, including surface waters.

We tested the neonicotinoid insecticides commercially available in Hungary (acetamiprid, *Mospilan;* imidacloprid, *Kohinor;* thiamethoxam, *Actara*; clothianidin, *Apacs;* thiacloprid, *Calypso*) on cholinergic synapses that exist between the VD4 and RPeD1 neurons in the central nervous system of the pond snail *Lymnaea stagnalis*. In the concentration range used (0.01-1 mg/ml), neither chemical acted as an acetylcholine (ACh) agonist; instead, both displayed antagonist activity, inhibiting the cholinergic excitatory components of the VD4-RPeD1 connection. Thiacloprid (0.01 mg/ml) blocked almost 90 % of excitatory postsynaptic potentials (EPSPs), while the less effective thiamethoxam (0.1mg/ml) reduced the synaptic responses by about 15 %. The ACh-evoked membrane responses of the RPeD1 neuron were similarly inhibited by the neonicotinoids, confirming that the same ACh receptor (AChR) target was involved. We conclude that neonicotinoids act on nicotinergic acetylcholine receptors (nAChRs) in the snail CNS. This has been established previously in the insect CNS; however, our data indicate differences in the background mechanism or the nAChR binding site in the snail.

Here we provide the first results concerning neonicotinoid-related toxic effects on the neuronal connections in the molluscan nervous system. Aquatic animals, including molluscs, are at direct risk while facing contaminated surface waters, and snails may provide a suitable model for further studies of the behavioural/neuronal consequences of intoxication by neonicotinoids.

Keywords: neuron, acetamiprid, imidacloprid, thiamethoxam, clothianidin, thiacloprid

Short title: Neonicotinoids inhibit cholinergic receptors of *Lymnaea* neurons

1. Introduction

Neonicotinoids are the newest generation of highly potent and selective systemic insecticides used as agrochemicals or to protect plants in the household from sucking insects (Tomizawa and Casida, 2005). Imidacloprid was the first neonicotinoid introduced to the market in the 1990s, followed by its homologues thiacloprid, thiamethoxam, nitenpyram, acetamiprid, clothianidin and dinotefuran. During the next 20 years neonicotinoids successfully replaced the carbamates and organophosphates as soil or seed treatments (Jeschke *et* al., 2011).

All neonicotinoid molecules are structurally related to nicotine, a natural alkaloid insecticide, but the positively charged nitrogen atom is replaced by other moieties, resulting in the nitro-substituted imidacloprid and thiamethoxam or the cyano-substituted acetamiprid and thiacloprid. The metabolites of some of these neonicotinoids also possess bioactivity; for example clothianidin, the active metabolite of thiamethoxam, has an even stronger effect in the insect CNS than thiamethoxam itself (Benzidane *et* al., 2010). The toxic effect of the neonicotinoids is based on their strong agonist binding to nicotinic acetylcholine receptors (nAChRs), which is confined to the CNS in the insect. While the binding is largely irreversible, it competes with natural acetylcholine (ACh) binding at the same receptors (Tomizawa and Casida, 2003). The selective effect of neonicotinoids on insects mainly results from differences between insect and mammalian nAChRs, but is also due to a structural feature of neonicotinoids, namely a pharmacophore which lacks a charged nitrogen and enables the molecule to more easily cross the brain-blood barrier in the insect nervous system (Tomizawa, 2013; Liu *et* al., 2010).

The widespread use of neonicotinoid type insecticides also triggers environmental concerns that spread beyond the exposed areas and the target organisms (insects). When used as a seed coating, the water solubility and systemic action of imidacloprid, thiamethoxam or clothianidin allow these chemicals to travel from the seedlings to other parts in the growing plant, causing them to appear in the guttation droplets, pollen or even honey made from the treated plants (Girolami *et* al., 2009; Chen *et* al., 2014). Neonicotinoids, therefore, also pose a potential risk for non-target pollinator insects and other organisms that come into contact with the treated plants. It is possible that the recent appearance of colony collapse disorder (CCD), resulting in a seriously decreased number of bees worldwide, can be linked to the intensive use of globally distributed neonicotinoid insecticides in agricultural areas (Gill *et* al., 2012; Cressey, 2013; Dicks, 2013; van der Sluijs *et* al., 2013). Recent data demonstrate that neonicotinoid chemicals and their metabolites persist and accumulate in soil (Goulson, 2013) and also appear in aquatic ecosystems, potentially affecting a number of invertebrate taxa initially considered as non-target organisms (Jeschke *et* al., 2011; Morrissey *et* al., 2015). Most recent studies suggest a declining abundance of macro-invertebrates (Van Dijk *et* al., 2013) and a shift of species composition, in particular in aquatic communities where neonicotinoid pesticides are present in the environment (Liess and Von Der Ohe, 2005; Beketov *et* al., 2013).

Acute and chronic toxicity assessment studies of neonicotinoids most often use aquatic arthropods (crustaceans and insects), which provide well established and budget sensitive models for toxicological testing (Jemec *et* al., 2007; Daam *et* al., 2013; Pisa et al. 2015). Toxicological bioassays usually give informations regarding particular endpoints while physiological experiments may reveal target mechanisms and also give tools for comparative studies. Direct physiological/pharmacological analysis of the cellular/neuronal changes behind the neuronal alterations, however, often requires a far more complex and sophisticated system (Matsuda *et* al., 2001; Deglise *et* al., 2002; Palmer *et* al., 2013).

In the isolated central nervous system (CNS) of selected gastropods (*i.e.* the pond snail, *Lymnaea stagnalis* or the edible snail, *Helix pomatia*) the identifiable giant neurons (with diameters up to 100 µm) allow potential toxic effects to be examined using intracellular electrophysiology techniques. Acetylcholine is a neurotransmitter and modulatory substance both in the CNS and the periphery of these organisms; moreover, cholinergic receptor subtypes including nAChRs have also been established (Walker *et* al., 1996; Vulfius *et* al., 2005; van Nierop *et* al., 2006; Krajcs *et* al., 2014). Therefore the identifiable snail neurons provide a suitable tool to characterize the interactions between the ACh receptors (AChRs) and potentially toxic substances including heavy metals, insecticides or mycotoxins (Arvanov *et* al., 1993; Gyori *et* al., 1994; Gyori *et* al., 2007). The roles of many identified neurons in controlling the relatively simple behavioural patterns of the animals have also been established (Chase, 2002), meaning that toxin-evoked functional changes of the nervous system will refer to known behavioural alterations of the intact animal (Dobranskyte *et* al., 2004; Vehovszky *et* al., 2007; Das and Khangarot , 2011).

The visceral VD4 neuron in the CNS of *Lymnaea stagnalis* provides monosynaptically transmitted inputs to a number of its followers including the symmetrically located pair of giant neurons (LPeD1 and RPeD1) of the pedal ganglia (Syed and Winlow, 1991). Both connections have also been shown to re-form between the isolated neurons when placed in culture conditions (Syed *et* al., 1990; Hamakawa *et* al., 1999). The first, excitatory component of these monosynaptic connections provides a suitable *in vitro* model while studying synaptogenesis (Feng *et* al., 1997; Woodin *et* al., 2002), or toxin-induced alterations of cholinergic neurotransmission (Woodall *et* al., 2003; Onizuka *et* al., 2012).

We tested the effects of commercially available insecticides that contain neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam) on the identified cholinergic synapses between VD4 and RPeD1 neurons in the isolated CNS. Our results confirm that neonicotinoid insecticides act on the AChRs in the molluscan CNS, and also demonstrate differences in sensitivity and kinetics between the AChRs of different locations (synaptic and extrasynaptic) on the same neuron.

This study provides the first data on the effects of neonicotinoids on molluscs, an example of non-target members of the aquatic ecosystem exposed to the harmful side effects of intensive pesticide use.

2. Materials and Methods

2.1. Animals

Adult specimens of the pond snail *Lymnaea stagnalis* were collected in the Balaton area (Hungary), kept in tanks filled with filtered Balaton water and fed on lettuce *ad libitum.*

2.2. Chemicals

The individual insecticides tested were used in the form of the commercially available products in Hungary, namely acetamiprid (*Mospilan*, Sumi Agro), imidacloprid (*Kohinor*, Makteshim Agan), thiamethoxam (*Actara*, Syngenta), clothianidin (*Apacs*, Arysta Life Science) and thiacloprid (*Calypso*, Bayer). Other chemicals were obtained from Sigma–Aldrich Chemie GmbH, Germany. All the chemicals were dissolved in normal *Lymnaea* saline immediately prior to the experiments. The accurate concentrations of the active ingredients in each neonicotinoid product were confirmed by GC/MS chromatography.

Electrophysiological experiments used physiological *Lymnaea* solution (normal saline) made from distilled water and containing NaCl (51.5 mM), KCl (1.7 mM), $CaCl_2$ (4.1 mM), $MgCl_2$ (1.5 mM), buffered with Hepes (5 mM) and set to pH= 7.9. In some experiments

a modified (HiDi) saline was used, with elevated amounts of the following divalent cations: $CaCl_2$ (24.6 mM) and $MgCl_2$ (5 mM).

2.3. Electrophysiological recording

The electrophysiological tests were carried out on isolated *Lymnaea* CNS preparations placed in a perfusion chamber filled with normal saline. The upper layer of the connective tissue covering the dorsal surface of the suboesophageal ganglion was removed mechanically first, and then the inner layer was digested with 0.1% protease treatment (Sigma type XIV) for 5 min before removing.

Both the pedal RPeD1 and the visceral VD4 giant neurons were visually identified by their size, position and colour (Syed and Winlow, 1991) before penetration by microelectrodes for electrophysiological recording. The RPeD1 neuron was impaled by two independent microelectrodes to inject current into the cell body for membrane polarization while simultaneously recording synaptically-evoked potentials or ACh-induced membrane responses from the same neuron. Controlled amounts of ACh were applied ionophoretically onto the cell surface of the RPeD1 neuron by placing a low resistance micropipette filled with 100 mM ACh in distilled water adjacent to the cell and passing positive current pulses (1 s duration, 10-50 nA amplitudes). Spontaneous leakage of ACh from the pipette was prevented by applying a constant retaining current of -0.5 nA between the application pulses. Both the recording electrodes and the injecting pipette were made from 1.2-1.4 mm diameter filamented borosilicate glass tubes (Harward Apparatus Ltd), pulled to a tip resistance of 6-10 MΩ.

For recording and storing electrophysiological data and also to control the current pulses for intracellular current injections, DasyLab software (version 5.63; http://www.dasylab.com/) was run on a PC through a National Instruments PC 6035E interface card (Newbury, UK).

2.4. Statistical analysis

Electrophysiological data are presented as mean values and standard deviation (S.D.) calculated from four to five independent experiments (the numbers of experiments are indicated in the text). Statistical evaluations were made between the mean EPSPs amplitudes (treatment versus control) using Student's paired t test. The level of significance was set to be at least $p< 0.05$.

3. Results

3.1. Synaptic connections between the VD4 and RPeD1 neurons

In the *Lymnaea stagnalis* CNS, the presynaptic VD4 neuron in the visceral ganglion and its postsynaptic target, the RPeD1 pedal neuron, are both members of the respiratory network forming rather complex, reciprocal synaptic connections with each other (Syed and Winlow, 1991). The individual postsynaptic components (single inhibitory or biphasic excitatory followed by inhibitory responses) may vary depending on the metabolic or seasonal status of the animal (Copping *et* al., 2000; Magoski and Bulloch, 2000). Pharmacological testing of the RPeD1 neuron therefore first requires isolation of the monosynaptic, cholinergic response (Skingsley *et* al., 1993).

In normal physiological saline, stimulation of the VD4 neuron by intracellular current pulses (1 s duration, 10-50 nA amplitudes) evoked a biphasic synaptic response, starting with a compound excitatory component (summated EPSP) followed by a longer inhibitory (hyperpolarizing) potential on the RPeD1 neuron (Fig. 1A.). By hyperpolarizing the postsynaptic membrane to -80 mV the second, inhibitory component was eliminated, and

VD4 stimulation by the same parameters evoked an excitatory response (Fig. 1B.). On replacing the bathing solution to a modified (HiDi) saline with an elevated concentration of Mg^{2+} and Ca^{2+}, the polysynaptic pathways activated by presynaptic stimulation were mostly inhibited (Berry and Pentreath, 1976). As a result, the synaptic responses on the RPeD1 follower were reduced, and each of the presynaptic action potentials evoked on the VD4 neuron were followed by single excitatory postsynaptic potentials (sEPSPs) on the postsynaptic neuron (Fig. 1C.). This 1:1 relationship between the presynaptic action potentials and the sEPSPs with enhanced (up to 16.6 mV) amplitudes and their short, rather constant synaptic delay (22.2 ± 2.41 ms; summarized from 8 randomly selected experiments) confirmed the monosynaptic nature of this component of the VD4-RPeD1 connection.

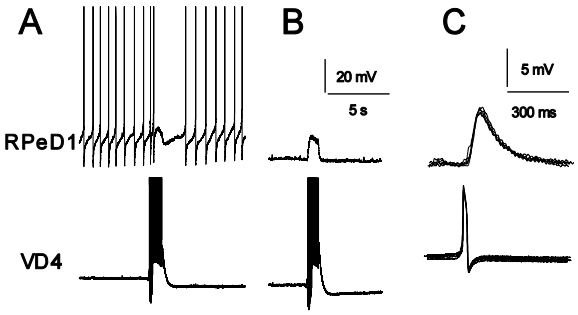

Fig. 1. Synaptic connections between the identified neurons in the *Lymnaea* suboesophageal ganglion. A. In normal saline, VD4 stimulation evokes a biphasic excitatory-inhibitory response (increased firing followed by temporal inhibition of action potentials). B. After the RPeD1 membrane was hyperpolarized to -80 mV, the excitatory component of the VD4 evoked response (summated postsynaptic potentials) is still visible without the hyperpolarizing phase. C. After 20 min perfusion in HiDi saline, each of the action potentials of the VD4 neuron is followed by a single excitatory postsynaptic potential (sEPSP) with constant synaptic delay (18.6 ± 2.069 ms; n= 10 in this individual experiment). The figure shows five superimposed traces of action potentials on VD4 and the corresponding EPSPs on the RPeD1 neuron hyperpolarized to -80 mV.

The cholinergic nature of the VD4 –RPeD1 synapse has previously been established (Woodin *et* al., 2002; Xu *et* al., 2009). Our experiments confirmed the involvement of nicotinergic ACh receptors in the monosynaptic excitatory component of the VD4 evoked synaptic responses on the RPeD1 neuron, by the almost complete (up to 90 %) inhibition of the sEPSP amplitudes in 50 µM d-tubocurarine applied in the bath (Fig. 2A.). This blocking effect of the postsynaptic responses was only partially reversible by longer (up to 30 min) washing out with the control (HiDi) saline (Fig. 2A,B). Simultaneous intracellular recording and intracellular stimulation of the presynaptic VD4 neuron during the experiments showed no alteration of its membrane potential or excitability (Fig. 2A.), confirming that tubocurarine inhibited the postsynaptic (nicotinergic) ACh receptors, resulting in decreased single EPSP amplitudes.

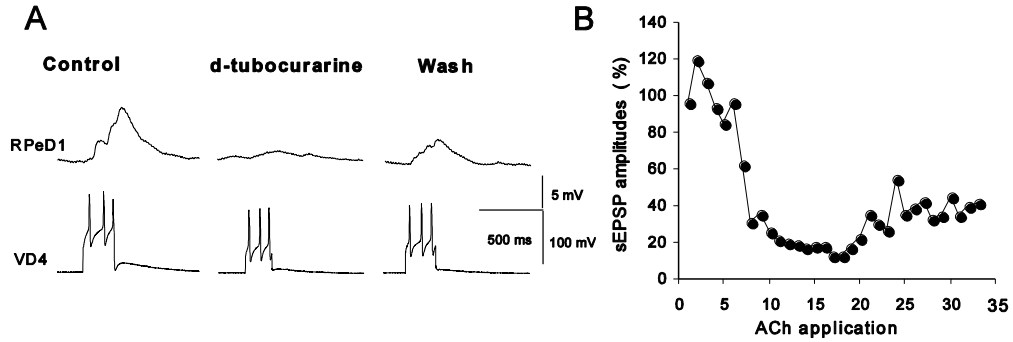

Fig. 2. D-tubocurarine in the bath blocks the monosynaptic connection between the VD4 and RPeD1 neurons. A. In control (HiDi) solution the amplitudes of the summated EPSPs on the postsynaptic neuron are decreased in 50 µM d-tubocurarine and only partially washed out by normal saline (upper trace). Intracellular injection of standard depolarizing pulses evokes the same intracellular responses (action potentials) from the VD4 neuron (lower trace). B. The amplitudes of the single EPSPs (expressed as percentage of control amplitudes) are rapidly reduced and only partially recover during wash out by control (HiDi) saline.

3.2. Effect of neonicotinoid insecticides on the excitatory responses

The monosynaptic connections between the VD4-RPeD1 were also tested in the presence of insecticides containing the commercially available formula of different neonicotinoids (see Materials and Methods). All chemical were dissolved in the test (HiDi) saline and the final concentrations (0.01- 0.1 mg/ml) were expressed in the amount of the active product in each of the insecticides (*e.g.* imidacloprid in *Kohinoor*), in the approximate concentration range usually recommended by the distributors (0.1-1%; see Discussion).

The experimental protocol for pharmacological testing of EPSPs was the same as that used in d-tubocurarine experiments above. Control responses were recorded and averaged after 4-6 stimulations in HiDi, then the perfusion system was switched to apply the test solutions (chemicals dissolved in HiDi) for a 15 minute period before washing out with standard saline. The EPSP amplitudes recorded on RPeD1 were measured after each of the presynaptic stimulations and finally expressed as a percentage of control amplitudes (see Fig. 2B, 4A,B). Each of the synaptic experiments was repeated in 4-5 different isolated CNS preparations. Additionally, we tested the neonicotinoid effects on the extrasynaptic ACh receptors of the RPeD1 neuron by locally injecting 100 mM ACh onto the cell body between each of the presynaptic stimulations. To prevent sensitization of the receptors involved, the current pulses used for intracellular stimulation or ACh application followed each other by at least one minute intervals while continuously perfusing the preparation with control or test saline.

In the concentration range used, aqueous solutions of neonicotinoids did not evoke any excitatory effects. However, both the monosynaptic excitatory component of the VD4-RPeD1 connection and the ACh-evoked membrane responses were inhibited in the presence of the neonicotinoids mentioned above. Moreover, we found that the synaptic and ACh evoked responses had the same relative potencies, as thiacloprid proved to be the most effective inhibitor of both responses at the lowest concentration (0.01 mg/ml). Thiacloprid (0.01 mg/ml) blocked the synaptically evoked EPSPs on the RPeD1 neuron by almost 90 % to 12 ± 4.2 % ; n=5 of the control response, and the membrane responses to locally applied ACh were also reduced (Fig. 3A.). An order of magnitude higher concentration (0.1 mg/ml) was required to generate about the same inhibitory effects using imidacloprid (19.5 ± 5.8 %; n=5) and chlotianidin (26.4 ± 4.6 %; n=4), demonstrated in Fig. 3B and Fig. 3C, respectively. Standard depolarizing pulses injected into the presynaptic VD4 neuron, however, evoked the same

number of action potentials in the presence of all neonicotinoids, confirming that their postsynaptic effects were most likely targeting the nACh receptors on the RPeD1 neurons.

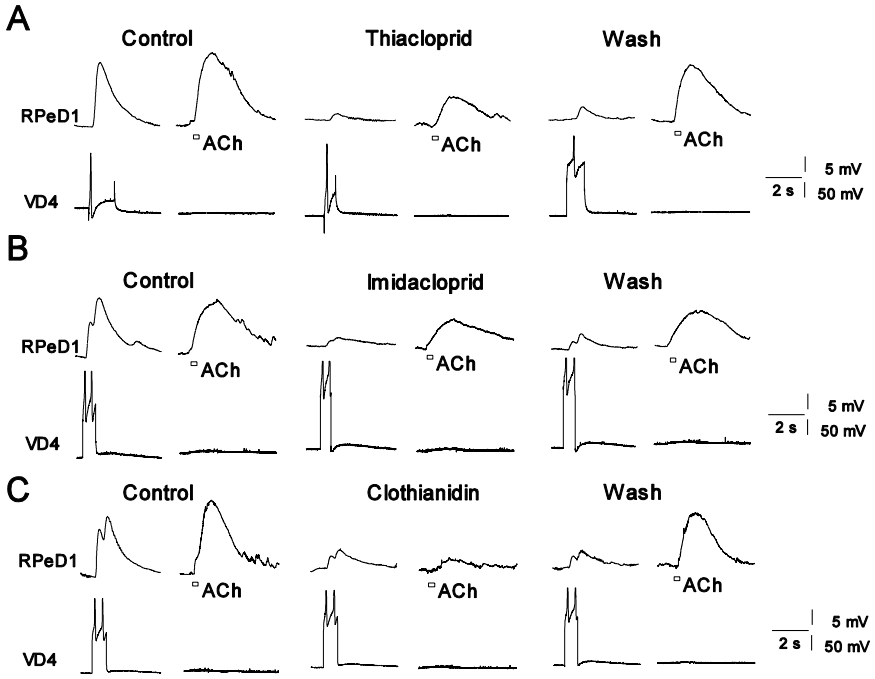

Fig 3. Neonicotinoids inhibit both the synaptic responses (VD4 evoked EPSPs) and the ACh evoked membrane depolarizations of the RPeD1 neuron. A. Both the single EPSPs and the ACh responses are reduced in the presence of 0.01 mg/ml thiacloprid in the bath. B. Imidacloprid (0.1 mg/ml) blocks the synaptic and ACh evoked responses of the RPeD1 neuron and both responses are partially washed out in normal saline. C. The ACh response is more sensitive to 0.1 mg/ml clothianidin in the bath but mostly recovered in normal saline, while the EPSPs are almost irreversibly blocked by the same treatment.

The differences between the synaptic *versus* extrasynaptic receptors in terms of sensitivity to and reversibility of the same chemicals were clearly demonstrated in these experiments, when presynaptic stimulations and ACh injections were applied alternatively during the course of the pharmacological experiments (Fig. 3,4.). 0.01 mg/ml thiacloprid reduced both EPSP amplitudes and ACh-evoked depolarizations (Fig. 3A.), and after washing for over one hour, only the ACh-evoked membrane response recovered up to about 80 % of the control response, while the synaptic connections (amplitudes of the single EPSPs) were still mostly inhibited (Fig. 3A, 4A.). Similarly, imidacloprid and clothianidin (0.1 mg/ml) each reduced both the synaptic and the ACh responses on the same RPeD1 neuron, but only the ACh response reversed partially after washing, while the synaptic response remained almost completely blocked (Fig. 3B,C, 4B.).

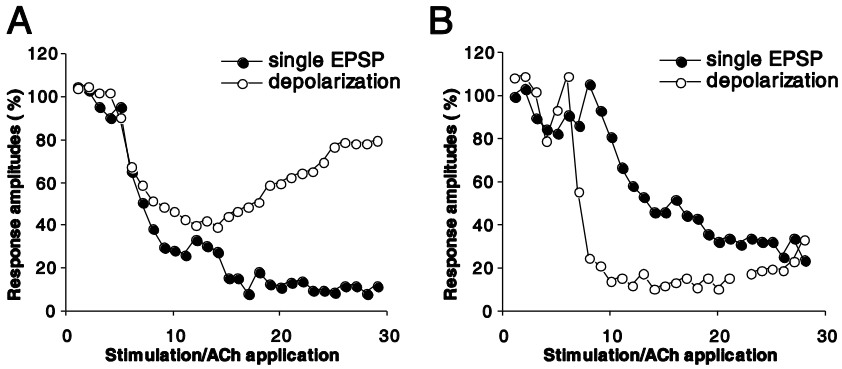

Fig. 4. Sensitivity and kinetic differences between the VD4 evoked synaptic effects and the ACh evoked membrane depolarizations. A. The EPSP amplitudes recorded on the RPeD1 neuron continuously decrease in 0.01 mg/ml thiacloprid, while the ACh evoked responses (depolarization amplitudes) start to recover during wash out. B. The synaptic responses (EPSP amplitudes) are irreversibly reduced reaching about 10 % of their initial value in 0.1 mg/ml clothianidin, while the ACh responses (depolarization amplitudes) are partially washed out in normal saline.

Among the neonicotinoids tested, acetamiprid (0.1 mg/ml) proved to be a less effective blocker of the synaptic responses, reducing the EPSPs by about 60 % (to 42.4 ± 10.9 %; n=4) as seen in Fig. 5A. Finally, thiamethoxam (0.1 mg/ml) resulted in a highly variable effect on the synaptic connections by reducing the VD4 evoked EPSP amplitudes to 89.7 ± 17.8 % in only three experiments out of five (Fig. 5B.). The summarized results on the inhibitory effects of the neonicotinoids on the VD4-RPeD1 synaptic connection (EPSP amplitudes) are summarized on Fig. 6.

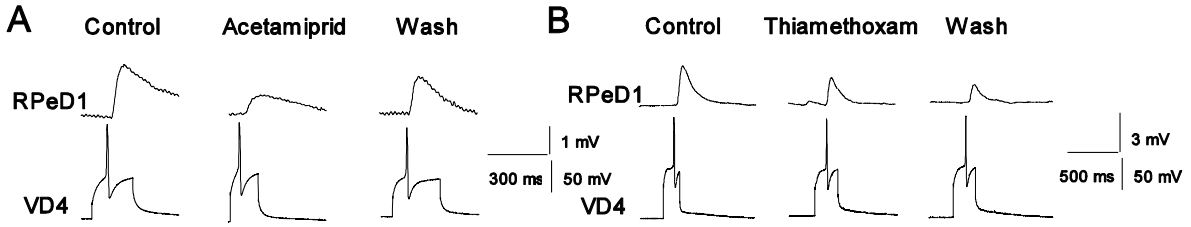

Fig. 5. Neonicotinoids inhibit the VD4 – RPeD1 synaptic connections. A. Perfusion by acetamiprid (0.1 mg/ml) reversibly decreases the synaptically evoked EPSP of the pedal RPeD1 neuron. B. The amplitude of the VD4-evoked EPSPs is reduced in the presence of thiamethoxam (0.1 mg/ml), and cannot be washed out in normal saline.

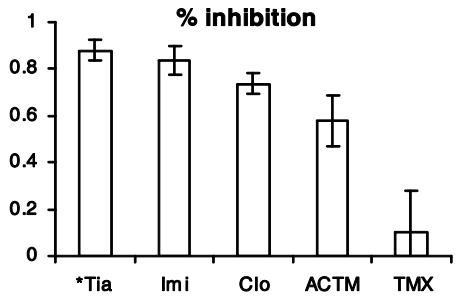

Fig. 6. Summary of the inhibitory effects of neonicotinoid insecticides on the EPSP amplitudes. Tia: thiacloprid; Imi: imidacloprid; Clo: clothianidin ACTM: acetamiprid; TMX:

thiamethoxam. Asterisc (*Tia) indicates that tiamethoxam was used at a magnitude lower (0.01 mg/ml) concentration than the other chemicals (0.1 mg/ml). Data are expressed as percentage of control responses.

4. Discussion

Target selectivity of a substance is the key element for its practical use either as a medicinal drug or an agrochemical. The acetylcholine receptors (AChRs) are particularly often involved in toxic effects of bioactive substances, like animal venoms, plant toxins, and also by neonicotinoid type insecticides (Daly 2005; Dutertre and Lewis 2006; Tomizawa and Casida 2003). The basic structural and pharmacological information on AChRs initially arose from vertebrates (Dani, 2001), but further studies suggest their much higher diversity in invertebrates (van Nierop *et* al., 2006; Wu and Lukas, 2011; Holden-Dye *et* al., 2013). In insects, moreover, molecular studies have also revealed taxonomical differences among the subclasses of nACh receptors (Dupuis *et* al., 2012; Liu *et* al., 2013).

In the molluscan CNS, ACh also acts as a neurotransmitter (Walker *et* al., 1996), but unlike in vertebrates, the molluscan cholinergic receptors may mediate both excitatory (cathionic) and inhibitory (anionic) postsynaptic effects (Kehoe and McIntosh, 1998; van Nierop *et* al., 2005; Vulfius *et* al., 2005). The identification of the molluscan AChR binding protein AChRBP, homologue of the ligand-binding extracellular loop of the nACh receptors (Smit *et* al., 2001), further facilitated the comparative studies including the evolutionary relationship of the nAChRs between taxa (van Nierop *et* al., 2006; van Nierop *et* al., 2005) and also the structure-binding analysis of neonicotinoids (Tomizawa, 2013).

Our results presented above showed inhibitory modulation of the VD4-RPeD1 synaptic connections by all the neonicotinoids tested. Drug-induced changes in synaptic efficacy may refer to a whole set of mechanisms of action, either presynaptic (changing excitability, vesicle mobilization, neurotransmitter release) or postsynaptic (changing biophysical properties of the postsynaptic membrane, enzymatic break down or reuptake of the neurotransmitter in the synaptic cleft). Here we simultaneously tested the cholinergic EPSPs (the excitatory component of the VD4-RPeD1 connection) and the ACh-evoked membrane responses on the follower RPeD1 neuron, and the results suggested drug-induced changes of the nAChRs, a similar mechanism to that which characterises the neonicotinoid effect in the insect CNS (Tomizawa and Casida, 2005).

The involvement of the nAChR type receptors in both synaptically and ACh-evoked membrane responses was confirmed by their reversible inhibition in 50 µM tubocurarine (see Fig 2.). We also demonstrated the inhibition of both the synaptically evoked EPSPs and ACh induced membrane responses on the same (RPeD1) neuron by neonicotinoids. In the concentration range used, 0.01 mg/ml thiacloprid proved to be the strongest blocker, while 0.1 mg/ml thiamethoxam resulted in the weakest inhibition. These results correspond with insect toxicological results, which consider thiamethoxam to be only a “moderately toxic” insecticide (Tan *et* al., 2007) and also by arthropod (crustacean and insect) assays (Anderson *et* al., 2015). We should also note, however, that thiamethoxam is the precursor of the more effective clothianidin (Benzidane *et* al., 2010) and likely metabolized in the treated plants or in the insects affected. We cannot rule out that thiamethoxam has a higher toxic potential under field conditions than we can assess by laboratory experiments.

We are aware that the commercial pesticide formulations we used contain a wide range of other ingredients (solvents, stabilisers etc), and non-specific side effects or synergisms between the different components cannot be avoided. However, using these formulations we get closer to the field situations when pesticides (a mixture of chemicals) appear in the environment. For the same reason, the insecticide concentration of the active

product (*e.g.* imidacloprid in *Kohinoor*) was used in the range (1 ‰ - 1%) usually recommended by the distributors for agricultural use, for example for foliar spray treatments (Bonmatin *et* al., 2015). A similar range of neonicotinoid concentrations (up to 100-300 mg/l) was measured in guttation drops of corn seedlings from coated seeds (Girolami *et* al., 2009; Tapparo *et* al., 2011). Compared with other laboratory studies, the concentration (300-400 µM) of neonicotinoids in our experiments represent the upper range of the values used in insect experiments (*in vitro* and *in vitr*o), which varied from 1-10 nM (Thany, 2009; Benzidane *et* al., 2011; Palmer *et* al., 2013), up to as high as 1 mM (Deglise *et* al., 2002).

Neonicotinoid insecticides are primarily regarded as ACh agonists in insects, which act by over stimulating the cholinergic receptors (Tomizawa and Casida, 2005; Palmer *et* al., 2013). In the concentration range we used (0.01-0.1 mg/ml), however, no ACh agonist excitatory effect (*i.e.* increased firing or membrane depolarization) was recorded either on the presynaptic VD4 or the postsynaptic RPeD1 neurons. Our results, therefore more likely suggest different background mechanisms, and probably a different molecular target site on the nACh receptor in the snail CNS.

The gastropod nervous system with its giant neurons also has the advantage of allowing studies of nACh receptors on the same identified neuron with distinct synaptic or extrasynaptic locations. Our results demonstrated that the synaptic responses (EPSPs) had higher sensitivity to neonicotinoids and the inhibition was less reversible compared to the membrane responses (depolarization) evoked by ACh on the same follower, RPeD1 (as seen on Fig 3 A,B and Fig 4. A,B in the presence of thiacloprid, imidacloprid, and clothianidin, respectively). Similar results have been obtained on the VD4-LPeD1 connection *in vitro;* Onizuka *et* al. demonstrated that the synaptically evoked responses are more sensitive and their inhibition is less reversible when compared with the extracellularly applied ACh effects (Onizuka *et* al., 2012). These results confirm a potential functional heterogeneity, in terms of different sensitivities or coupling mechanisms, of the nACh receptors which are located synaptically versus extrasynaptically on the same neuron. We should note that most of the pharmacological and kinetic analyses of cholinergic neurotoxins are carried out by extracellular application of acetylcholine on neuronal preparations or cloned nACh receptors, and that cholinergic synapses are rarely tested directly. We cannot exclude, therefore, that most toxicity assessment studies based on extracellular ACh applications likely underestimate the impairment of the neuronal functions (synaptic uncoupling, network or behavioural alterations) caused by neuroactive chemicals.

Both the identified VD4 and RPeD1 interneurons we studied are key members of the central pattern generator network controlling *Lymnaea* respiratory behaviour (Syed *et* al., 1990; Syed and Winlow, 1991). The cyclic rhythm of *Lymnaea* feeding is also organized by higher order cholinergic interneurons (Elliott and Kemenes, 1992; Yeoman *et* al., 1993; Vehovszky and Elliott, 1995) suggesting a major function of AChR mediated neurotransmission in pattern-generating central networks. Synaptic inhibition by cholinergic neurotoxins (including neonicotinoids), therefore, may also result in functional alteration of the pattern generating respiratory and feeding networks and finally modulate the behavior of the animal concerned.

Neonicotinoids contaminate surface waters and aquatic animals are at direct risk of intoxication (Anderson *et* al., 2015; Morrissey *et* al., 2015). Although molluscs are generally used in environmental toxicology studies (Salanki et al. 2003; Rittschof and McClellan-Green, 2005; Gust *et* al., 2011), only very few data are available on the potential toxic effects of neonicotinoids on these animals (Dondero *et* al., 2010; Hamlet *et* al., 2014). Here we propose the pond snail *Lymnaea stagnalis* as a molluscan model to study behavioural and neuronal changes evoked by neonicotinoids. Their relatively simple behavioural patterns may provide sensitive functional indicators of sublethal effects while the anatomical features of

their nervous system also allow for the study of toxin-evoked alterations at the cellular and network level.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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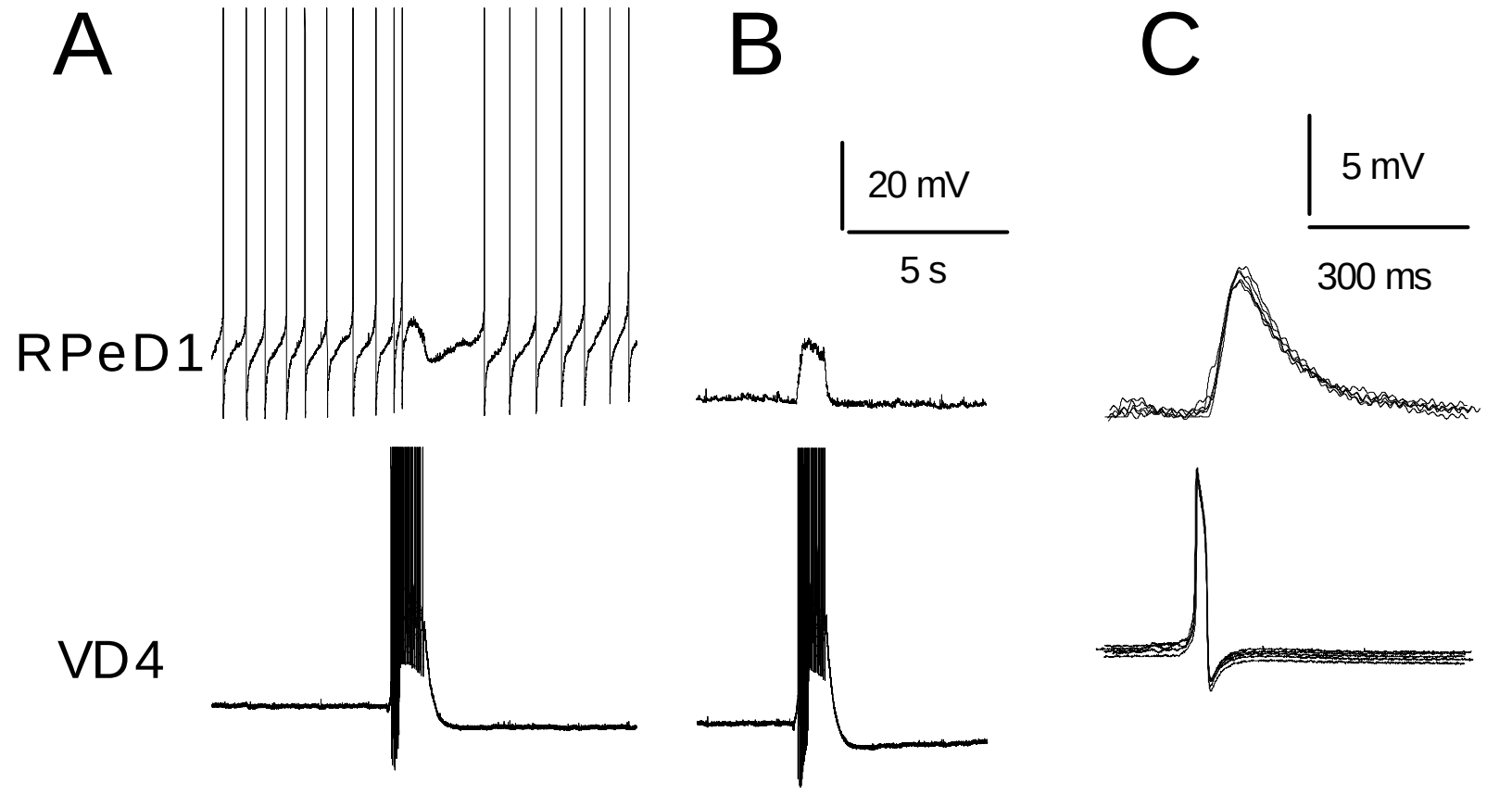
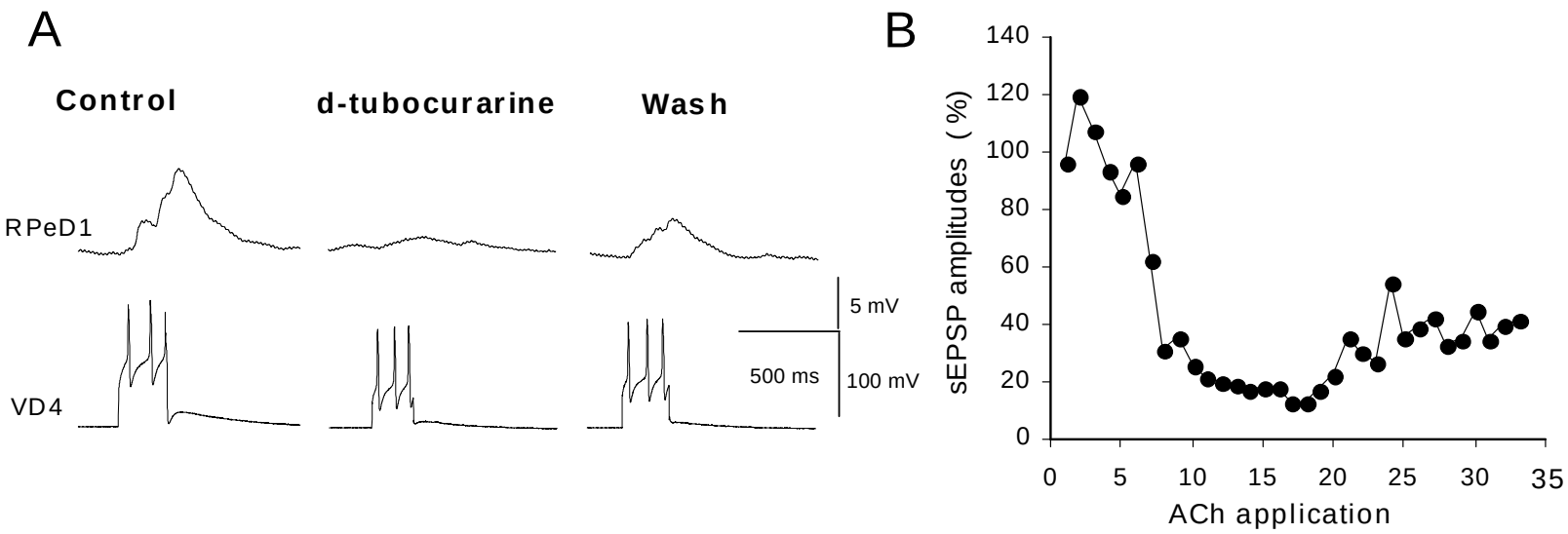
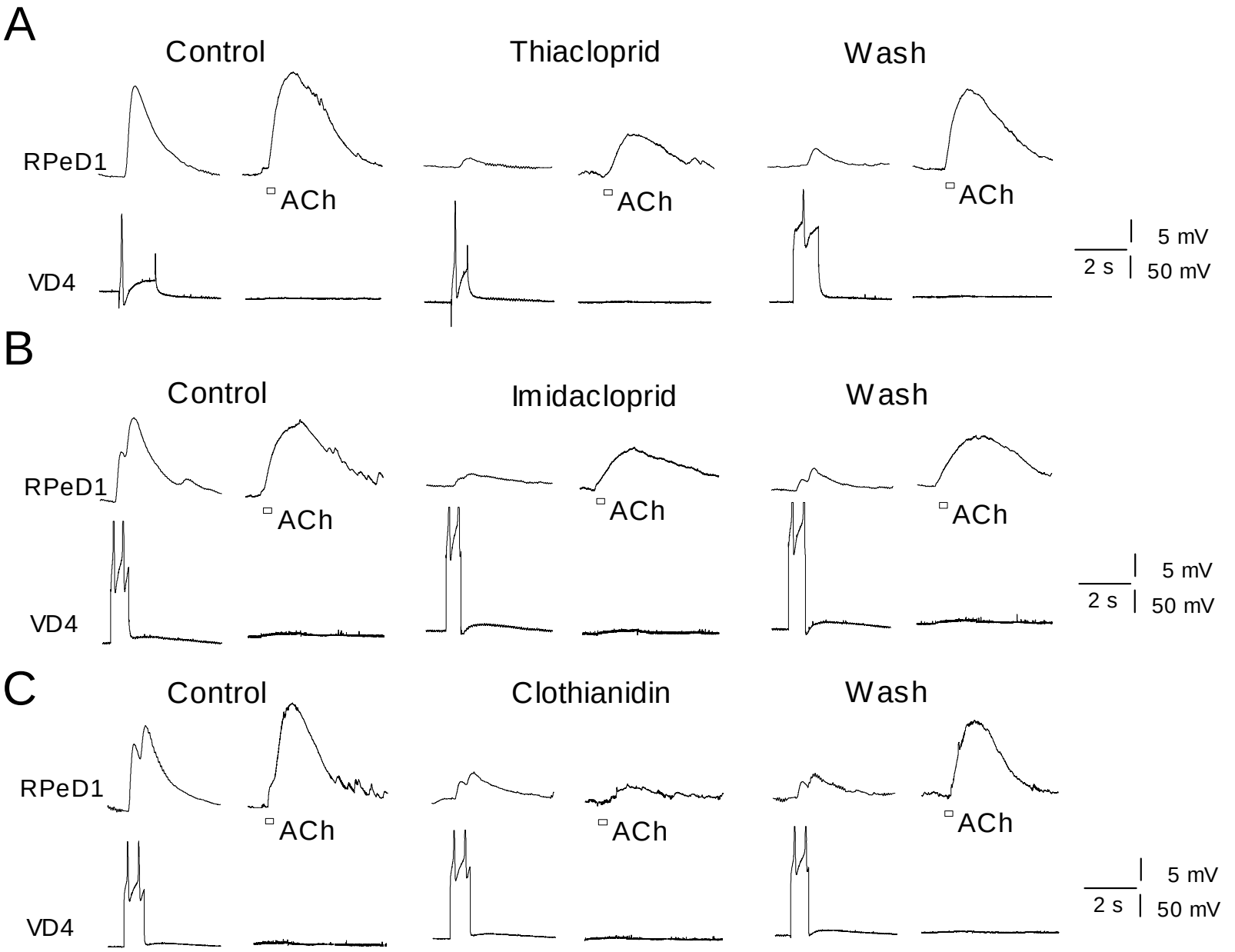
Figure(s)



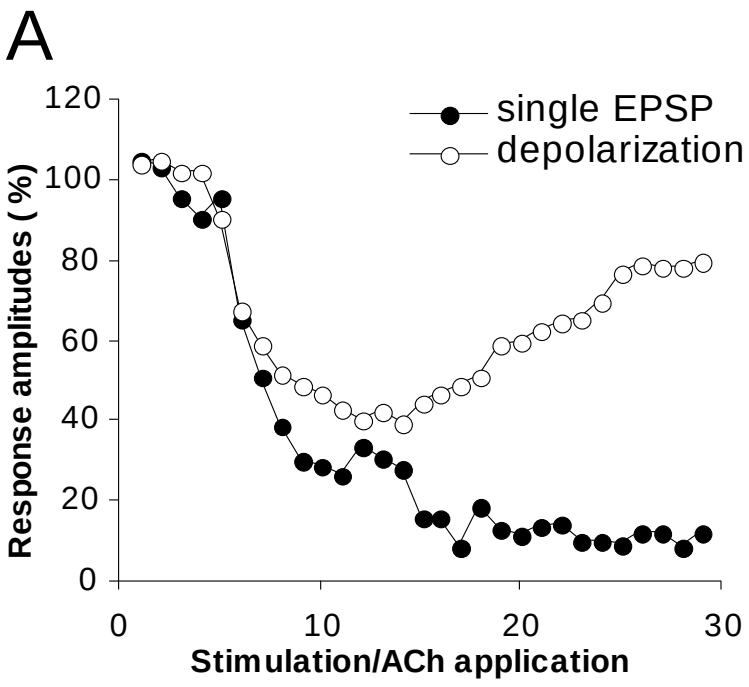
Figure(s)

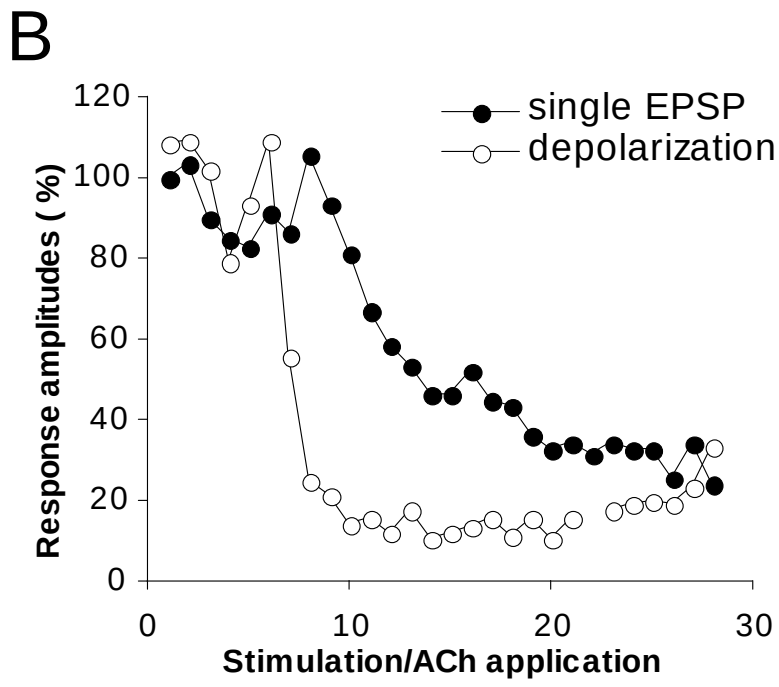

A
Control
d-tubocurarine
Wash
RPeD1
VD4
5 mV
500 ms
100 mV
B
sEPSP amplitudes (%)
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
ACh application

Figure(s)

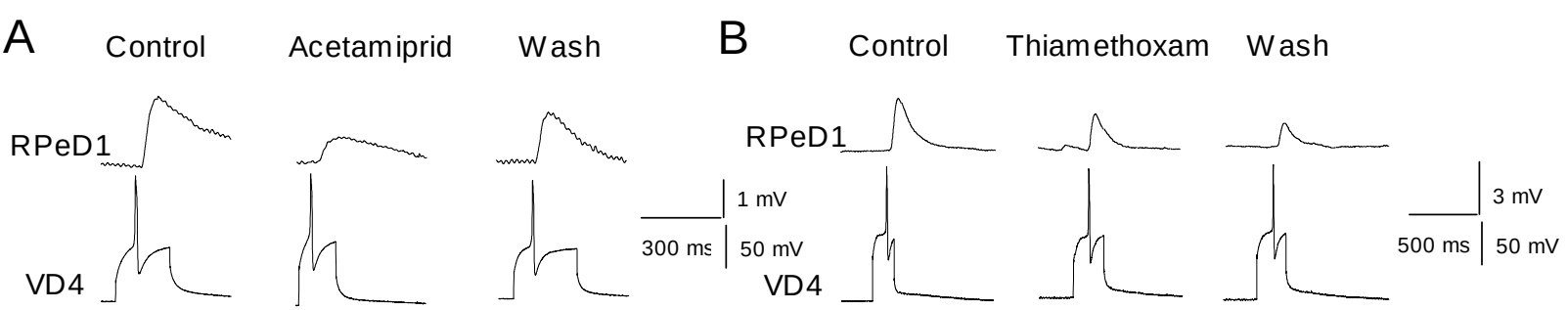

A
Control
Thiacloprid
Wash
RPeD1
VD4
ACh
ACh
ACh
5 mV
2 s
50 mV
B
Control
Imidacloprid
Wash
RPeD1
VD4
ACh
ACh
ACh
5 mV
2 s
50 mV
C
Control
Clothianidin
Wash
RPeD1
VD4
ACh
ACh
ACh
5 mV
2 s
50 mV

Figure(s)

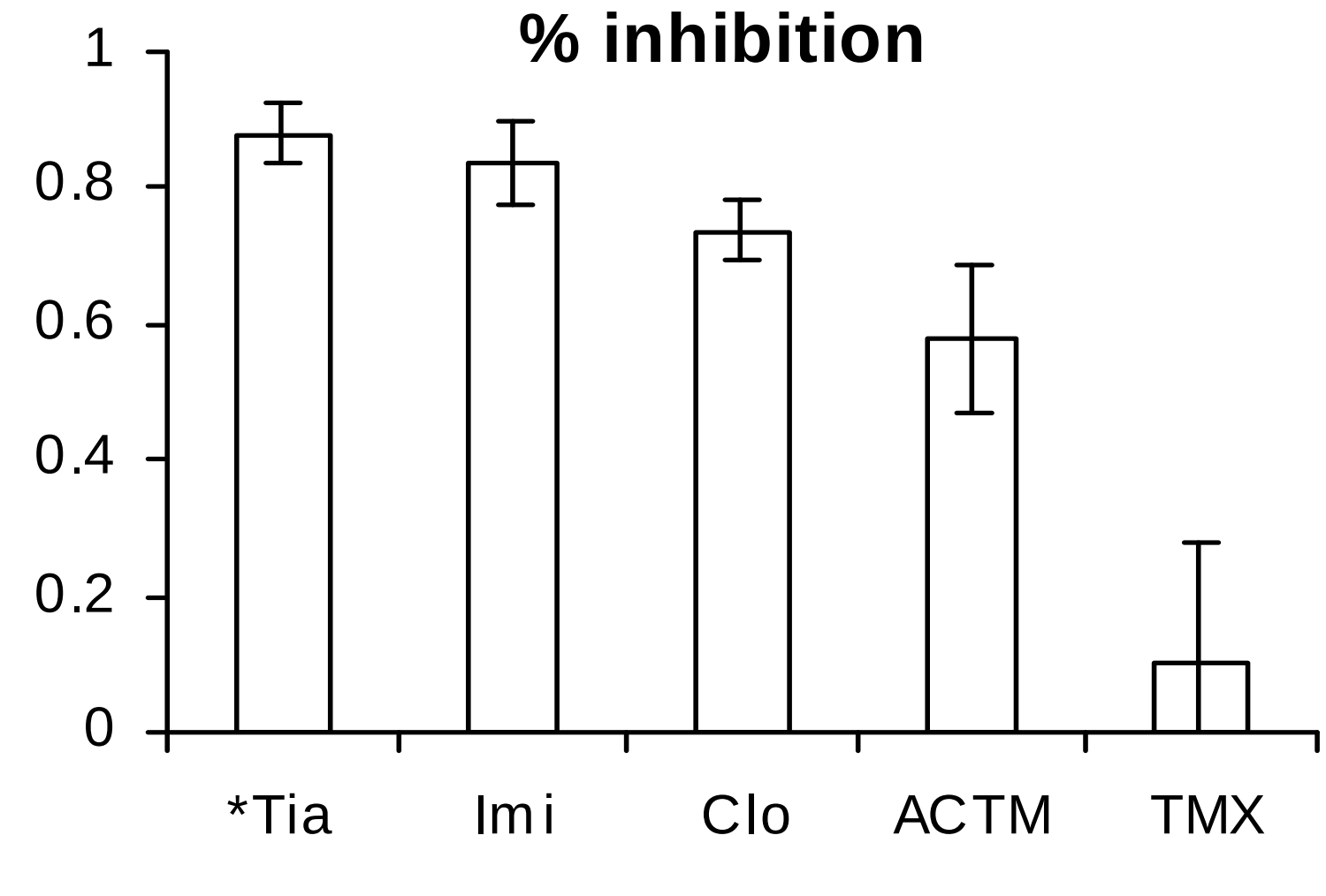

A
single EPSP
depolarization
Response amplitudes (%)
120
100
80
60
40
20
0
0
10
20
30
Stimulation/ACh application


B
single EPSP
depolarization
Response amplitudes (%)
120
100
80
60
40
20
0
0
10
20
30
Stimulation/ACh application

Figure(s)


A
Control
Acetamiprid
Wash
RPeD1
VD4
1 mV
300 ms
50 mV
B
Control
Thiamethoxam
Wash
RPeD1
VD4
3 mV
500 ms
50 mV

Figure(s)



Highlights

1. Neonicotinoid insecticides affect non-target animals and contaminate surface waters.
2. Electrophysiological tests confirmed the neuronal effect of commercial formulations.
3. Neonicotinoids in the snail CNS modulate the nicotinergic acetylcholine receptors