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IN VITRO EFFECTS OF FIPRONIL ON NEURONAL EXCITABILITY IN MAMMALIAN AND MOLLUSCAN NERVOUS SYSTEMS

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Abstract: The effect of the insecticide fipronil on non-target organisms was studied on rat brain slices and identified giant neurons of the pond snail *Lymnaea stagnalis*. This compound acts as an antagonist on $GABA_A$ receptors. Although fipronil has moderate mammalian toxicity, our experiments confirmed that it modifies neuronal excitability in the rat somatosensory cortex. The amplitudes of evoked field potentials increased significantly after 30 min fipronil treatment. Short-term plasticity was examined with paired-pulse stimulation, this phenomenon was not affected by fipronil. On the other hand, the efficacy of LTP-induction was enhanced in the treated slices. Fipronil is highly toxic to freshwater invertebrates, especially molluscs. In *Lymnaea stagnalis*, the firing pattern of a GABA receptor-containing neuron (RPeD1) was studied. On this neuron, GABA has an excitatory, hypopolarizing effect. Fipronil treatment decreased the action potential frequency in a concentration-dependent manner. On the membrane potential of the cell, it had a slightly hyperpolarizing effect. These experiments confirmed that fipronil toxicity is mediated by GABA receptors in the nervous system of invertebrates as well as vertebrates. These types of experiments may help in establishing tolerance levels of pesticide residues and in finding proper treatment in case of eventual poisonings.

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INTRODUCTION

The phenylpyrazole insecticide fipronil, introduced in 1993, is widely used as a veterinary medicine against cat and dog fleas [4] and lice [21]. It is also effective against locusts, mosquitoes, and a number of crop pests, such as termites and Colorado potato beetles [7].

The first generation of insecticides, which act on γ-aminobutyric acid (GABA) receptors, include cyclodiene compounds such as lindane and dieldrin, which are very toxic. Fipronil belongs to the second generation of insecticides, which act on ionotropic GABA ($GABA_A$) receptors of the nervous system, and display higher selectivity for insects than cyclodienes. Fipronil may be also effective against dieldrin-resistant pests. Studies of its mechanism of action have revealed a non-competitive blockade of ionotropic GABA receptors [24].

Ionotropic GABA receptors are ligand-gated anion channels, composed of five subunits. Their basic structure is very similar among species belonging to various phyla of the animal kingdom. Opening of the channel generally results in an inhibitory effect, but in some cases it causes excitation, as in molluscs [28], or in young mammals [1], depending on the Cl^- equilibrium between the cell and its environment. The β subunit has been shown to play an important role in the binding of fipronil to the $GABA_A$ receptors [24]. An insect GABA receptor subunit (called LCCH3) displays 47% sequence identity to the vertebrate β subunit

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and 56% identity to a molluscan β-like subunit cloned from *Lymnaea stagnalis* [14]. In consequence of these differences between the channel proteins, the pharmacological properties of invertebrate and mammalian GABA receptors are slightly different [9]. The higher toxicity of fipronil in insects may be explained by a higher affinity for insect-type receptors [13, 30], though the mammalian toxicity is not negligible either. Moreover, the compound may additionally block inhibitory glutamate receptors, which are to be found in invertebrates, but not in vertebrates [16].

Despite posing a lower environmental risk than the previously used insecticides, fipronil is classified as a moderately hazardous chemical by the World Health Organization. It has been found to be highly toxic to bees [10], freshwater invertebrates (especially arthropods, but also molluscs) and some birds [27, 29]. There are concerns about its possible effects on the health of mammals. A multilevel neurobiological investigation, including electrophysiological experiments on rat neocortical slices, indicated that a single, large intragastric dose of fipronil caused a weak and transient increase in mammalian neuronal excitability, which returned to the normal level within 7 days [25]. Fipronil has negligible metabolic effect in the brain [31]. Two case reports have described the symptoms of fipronil intoxication in humans [8, 11].

The aim of the present study was to examine the direct neuronal effects of different doses of fipronil, the influence of systemic factors or metabolic processes being excluded. Fipronil was applied directly into the perfusion solution in two *in vitro* models. On rat neocortical brain slices, alterations in general neuronal excitability and synaptic plasticity were studied. On the identified snail neuron RPeD1, changes in firing pattern were studied.

MATERIALS AND METHODS

Electrophysiology on rat cortical slices

The experiments were performed on young adult, male Wistar rats (100–200 g, Toxicoop, Hungary). The experimental design was approved by the ELTE University Animal Care Committee and the Budapest Animal Health Care Authority. The rats were kept under a constant 12-h light/dark cycle and controlled temperature (22±2°C). Standard pellet food and tap water were available *ad libitum*.

Slice preparation and maintenance. The rats were decapitated in deep chloral-hydrate anaesthesia, the brains were quickly removed and coronal slices (400 μm thick) were cut from the somatosensory cortex with a vibratome. After incubation for 1 h in a HEPES-buffered (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and its sodium salt, pH 7.1–7.2) solution, a slice was placed into an interface-type recording chamber (FST, Canada) through which standard artificial cerebrospinal fluid (ACSF) was perfused (2.5 ml/min). The solution was saturated with carbogen (5% CO_2 + 95% O_2) at 33±1°C. The composition of this perfusion solution was (in mM): 126 NaCl; 26 $NaHCO_3$; 1.8 KCl; 1.25 KH_2PO_4; 1.3 $MgSO_4$; 2.4 $CaCl_2$; 10 glucose. Fipronil was dissolved in standard perfusion solution to give a final concentration of 4 or 8 mg/l.

Electrophysiological recording and data analysis. Evoked field potentials were recorded with extracellular glass microelectrodes (8–10 MΩ) filled with 1 M NaCl as in standard field potential recording experiments [12]. The recording electrodes were positioned into the lower part of layer III of the somatosensory cortex slices. Bipolar tungsten electrodes positioned immediately below the recording electrodes, at the border of the white and grey matter, were used for electrical stimulation. The duration of the square voltage pulses was 100 μs. Signals were amplified (Bioamp, Supertech, Hungary) and were displayed on a digital oscilloscope (Gould DSO 420, Gould Electronics, Cleveland, Ohio, USA). The recorded signals were digitalized with an A/D converter (VR-10, Instrutech Corp., Great Neck, New York) and stored on a VHS tape for further analysis.

Before the whole procedure was started, the viability of the slices was tested. Applying single shock stimulation, the characteristic field response was recorded. If the peak-to-peak amplitude of the maximal evoked response was <1 mV, the slice was excluded from the experiments. After determination of the threshold of the evoked field potential (T), a stimulus strength – amplitude (input – output (I-O)) curve was recorded by gradually increasing the stimulus intensity from T up to 3T in 8 steps. To test short-term plasticity, paired-pulse stimulation at a stimulus strength of 2T was then applied with interstimulus intervals of 500, 200, 100 and 50 ms. This was followed by the induction of long-term potentiation (LTP) by tetanic stimulation. The stimulation parameters were as follows: intensity 2T, frequency 100 Hz, duration 5 s, 4 times with 10-s breaks. Subsequently, test stimulation at 2T was used at 0.1 Hz during 30 min. Finally, an I-O curve was again determined.

To test the effect of fipronil after the first I-O curve recordings (reference) the perfusion solution was switched to insecticide-containing solution. During the first 30-min perfusion in 4 or 8 mg/l fipronil solution, the continuous stimulation was suspended to observe whether any spontaneous activity developed. Next, an I-O curve was recorded and the same procedure as above was followed.

Stored signals were digitalized and analysed with the S.P.E.L. Advanced Intrasys programme (Experimetria, Budapest, Hungary). The peak-to-peak amplitude of the first component of the evoked responses was measured. To compare I-O curves before and after treatment, the paired Student t-test (statistically significant at $p < 0.05$) was used for statistical analysis. To compare control and treated groups, ANOVA (statistically significant at $p < 0.05$) was applied. Data are presented as means ± S.E.

Electrophysiology on *Lymnaea stagnalis* neurons

These experiments were performed on identified neurons of the mollusc *Lymnaea stagnalis*. The animals were kept under 12-h light/dark cycle at 22–25°C, and fed *ad libitum*.

Preparation and maintenance of the ganglia. The CNS was dissected and incubated in 0.1% Protease solution (Sigma, Budapest, Hungary) dissolved in normal HEPES-buffered Ringer solution for 5 min. After incubation, the CNS was rinsed twice with Ringer solution and pinned down in a dish containing Ringer solution. The connective tissue sheaths covering the ganglia were removed, and the ganglia were allowed to regenerate at 4°C and then stored at room temperature for 30 min. All experiments were performed at room temperature (21±1°C). The perfusion solution (4 ml/min) was either normal Ringer solution or Ringer solution containing fipronil.

Solutions. The composition of the standard Ringer solution was (in mM): 45 NaCl; 1.7 KCl; 4 $CaCl_2$; 2 $MgCl_2$; 5 HEPES; dissolved in distilled water; pH 7.8, adjusted with 2.5 mM NaOH. The fipronil-containing solution was freshly prepared; the chemical was dissolved in distilled water to give a 1 mg/ml stock solution, which was subsequently diluted with Ringer solution, to give a final fipronil concentration of 1, 2, 4, 20 or 50 mg/l.

Electrophysiological recording and data analysis. A respiratory interneuron known as right pedal interneuron (RPeD1 neuron) was used for electrophysiological recording. The neurons were identified according to their localization and size [3]. Conventional intracellular recordings were made using Axoclamp 2A amplifier (Axon Co., USA). Glass microelectrodes filled with 1.0 M KCl and 1.5 M K-acetate (10-15 MΩ) were used as recording electrodes. The action potentials were amplified, and then stored with the Dasylab 5.0 software. A 10-min control period recording was made from each cell, and the perfusion medium was then switched to fipronil-containing solutions of different concentrations, also recorded for 10 minutes.

The data were analyzed with the Orbital spike 3.9 software [26], interspike intervals were measured and their occurrence was calculated.

Chemicals. Fipronil was obtained from Rhône-Poulenc (Lyon, France) in a purity of 80%. All inorganic compounds were purchased from Sigma (Budapest, Hungary).

RESULTS

Electrophysiological recording on rat brain slices. 24 slices were included in the analysis, 8 were control slices, 8 treated with 4 mg/l fipronil and 8 treated with 8 mg/l fipronil, where the maximal evoked responses were larger than 1 mV.

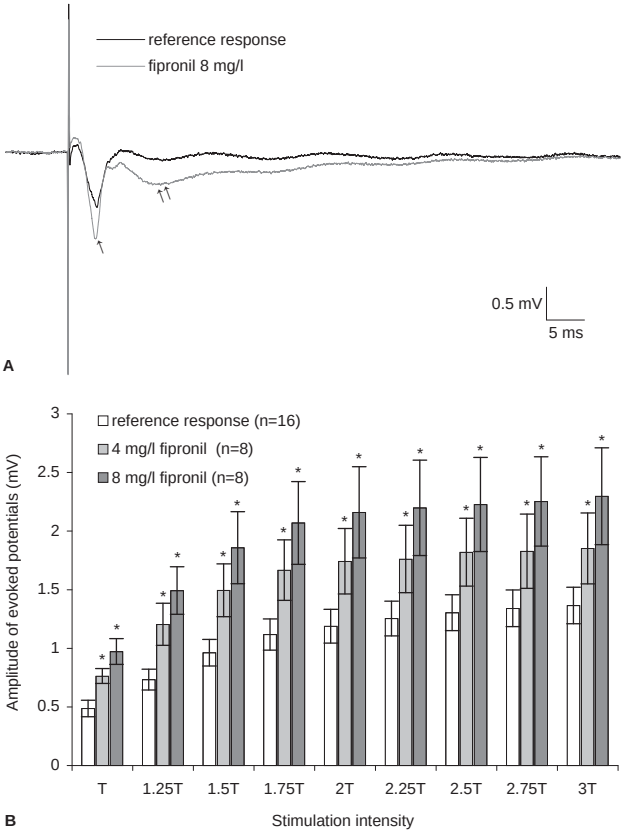

Figure 1. Effects of fipronil treatment on excitability of rat neocortex slices. (A) Characteristic field potentials evoked by electrical stimulation from the same slice before and after treatment with 8 mg/l fipronil. Fipronil increased the amplitude of the response (↑) and facilitated the development of a second component (↑↑). (B) Relation of stimulus intensity-evoked response amplitude in neocortex slices. T indicates the threshold stimulus intensity evoking a response; the intensity increases to 3T in equal steps. The "reference" data group depicts the evoked responses in the slices before treatment. After 4 or 8 mg/l fipronil application for 30 min, significant increases in the amplitude of the evoked potentials were observed at both concentrations; the changes were concentration-dependent (paired Student t-test, *: $p<0.05$). Data are presented as means ±SE.

No spontaneous activity was observed in either the control or the treated slices.

The evoked field potentials increased significantly following the 30-min fipronil treatment at both concentrations. Following 4 mg/l fipronil application, the mean amplitude of the first component of the evoked responses increased from 1.15±0.17 mV to 1.743±0.28 mV at a stimulation strength of 2T. At 8 mg/ml, the increase was more marked: from 1.23±0.12 mV to 2.16±0.39 mV. I-O curves of slices before and after treatment are presented in Fig. 1B; both doses of fipronil significantly enhanced the basic excitability of the neocortical slices. Fipronil treatment also facilitated the development of longer-lasting, complex evoked responses, with the appearance of a second component of the evoked response (Fig. 1A). Whereas none of the control slices displayed this second component of the evoked response, it appeared in 3 of the 8 slices after the 30-min perfusion with the 4 mg/l fipronil solution, and in 4

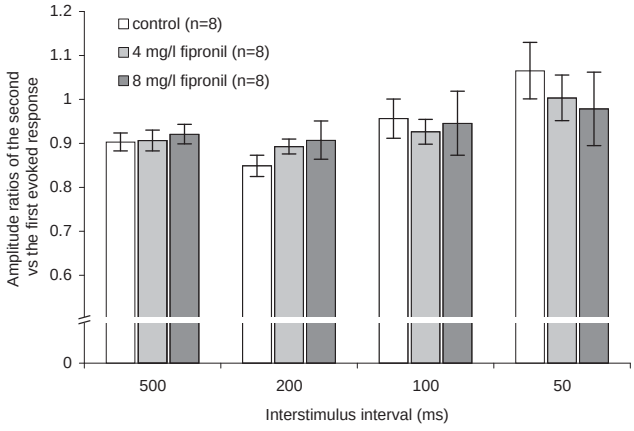

Figure 2. Short-term plasticity was tested by using paired-pulse stimulation at different interstimulus intervals with 2T intensity. The amplitude ratios of the second and the first evoked responses are shown. Inhibition of the second responses was observed, except for the 50 ms interstimulus interval, where the amplitude ratio, in case of the control, was slightly >1. Fipronil does not have an appreciable effect on short-term synaptic plasticity. Data are presented as means ±SE.

of the 8 slices following the 8 mg/l fipronil treatment. This reflects a higher excitability of the treated slices.

Short-term plasticity was tested by paired-pulse stimulation. The amplitude ratio of the second *vs* the first evoked response was calculated (Fig. 2). We nearly always observed a slight inhibition of the second evoked potential. The only exception was the 50 ms interstimulus interval, where the second response was slightly larger than the first one, in case of the control slices. The changes following fipronil treatment were not significant, and it may be stated that fipronil treatment did not exert any effect in the paired-pulse tests.

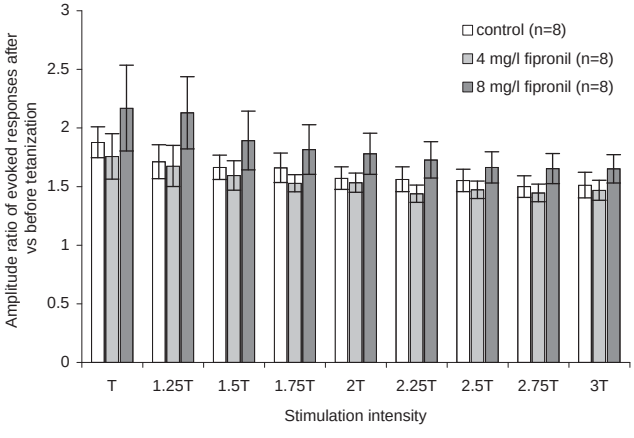

Figure 3. Alterations in long-term synaptic plasticity (LTP) were characterized by comparing the ratios of the amplitude of the responses evoked by a stimulation intensity of 2T before and after tetanization. When the effects of fipronil were analyzed, test stimuli before tetanization were recorded before switching the standard solution to fipronil-containing solution. Treatment with 4 mg/l fipronil did not exhibit a significant effect on LTP, whereas 8 mg/l fipronil increased the efficacy of LTP induction. Data are presented as means ±SE.

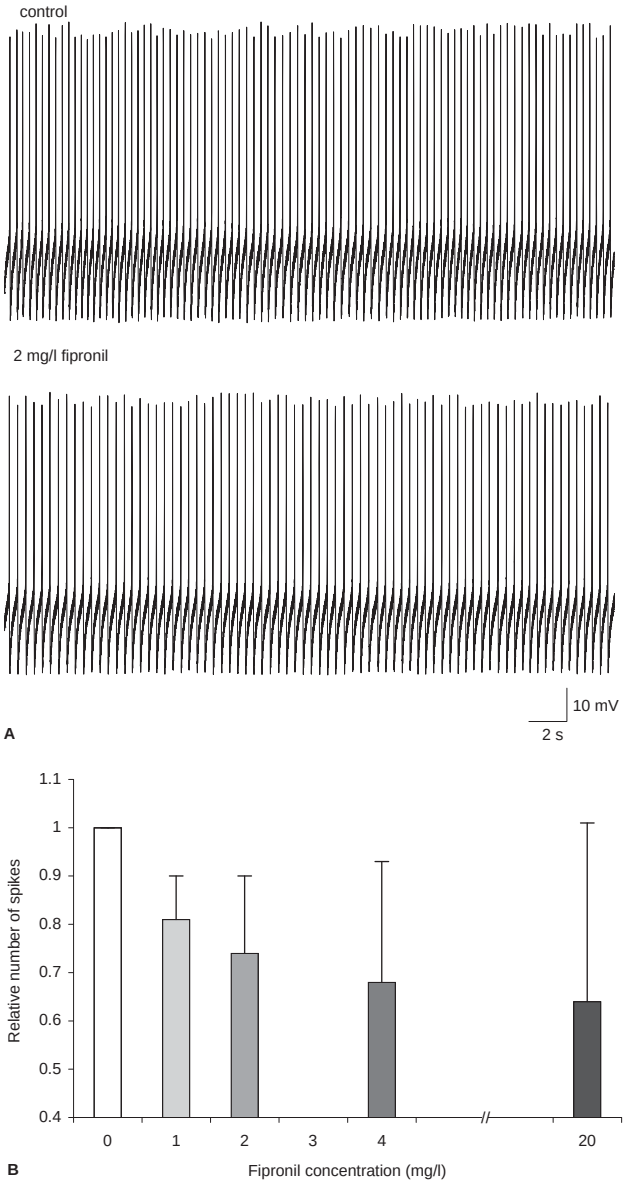

Figure 4. Electrophysiological recordings from RPeD1 giant neurons of the Lymnaea pedal ganglia. (A) Representative traces demonstrating the change in action potential (AP) firing frequency produced by perfusion of fipronil onto the central ring ganglia. Fipronil caused a decrease in AP firing frequency (upper trace: control, lower trace: effect of fipronil). (B) Bar graph summarizing the relative changes in AP firing frequency induced by different concentrations (0, 1, 2, 4 and 20 mg/l) of fipronil.

To analyze the effect of LTP induction, the amplitude ratios of the test responses evoked after *vs* before LTP induction were calculated. These ratios were slightly higher in the slices after the 8 mg/l fipronil application than in the control slices. At a stimulation intensity of 2T, the ratios for the control slices, the slices treated with 4 mg/l and with 8 mg/l fipronil were 1.57±0.10, 1.53±0.08 and 1.78±0.18, respectively (Fig. 3). After LTP induction, a second component of the evoked response appeared in 2 of the 8 control slices, in 5 of the 8 slices treated with 4 mg/l fipronil and in 6 of the 8 slices treated with 8 mg/l fipronil.

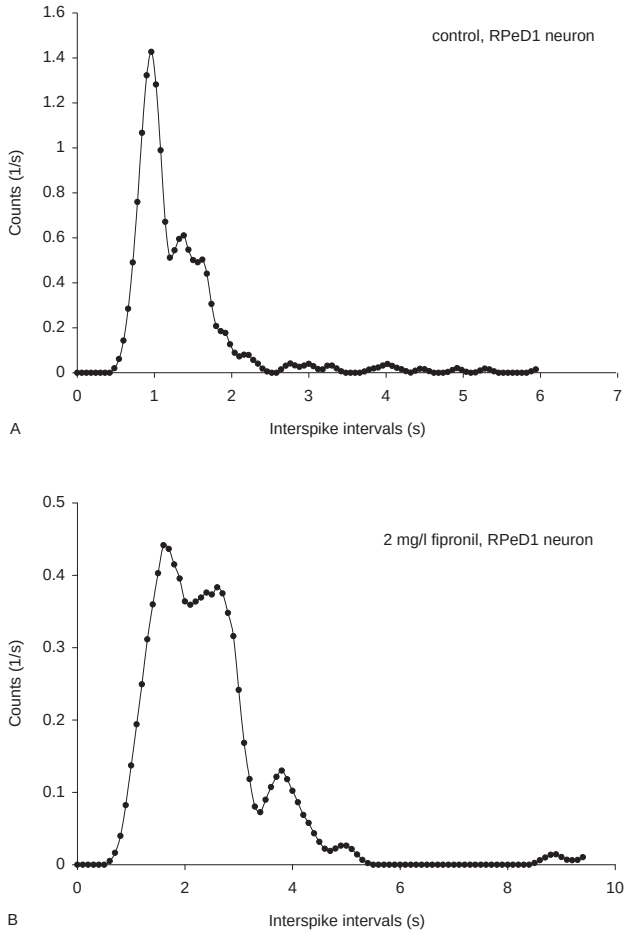

Figure 5. Interspike-interval (ISI) histograms in the absence and presence of fipronil. Detailed analysis of APs recorded from an RPeD1 giant neuron of Lymnaea in control saline (A) and in 2 mg/l fipronil-containing solution (B). The appearance of two, close-lying peaks is characteristic for the control ISI histogram of the tested giant neurons. Fipronil caused a marked shift in the ISI in the direction of higher values; the amplitude of each peak decreased.

Electrophysiological recording on identified *Lymnaea* neurons. The effects of fipronil on the neuronal activity were determined by monitoring the action potential (AP) firing frequency and the change in membrane potential (Vm). Intracellular recordings were obtained from 26 visualized RPeD1 giant neurons. Most of the neurons were initially spontaneously active at their resting Vm, demonstrating APs (Fig. 4A, control). Under control conditions, there were no significant changes in AP frequency during over 100 min of recording. Switching from normal saline to 1 mg/l fipronil-containing solution induced a sustained 18 % decrease in AP frequency: normal *vs* fipronil-treated 43.5±22.7 *vs* 36.1±22.6 spikes/min (18%, n=8); 2 mg/l fipronil caused a 26% decrease in AP frequency (n=7), and 4 mg/l fipronil a 32% decrease (n=7) (Fig. 4B). Application of a relatively high dose of fipronil (25–50 mg/l) did not result in any further decrease. After a 10-min exposure to fipronil, normal conditions were resumed, but the original AP frequency was not restored within 1 h after switching back from fipronil solution to normal. Vm shifted slightly towards hyperpolarization, but the changes relative to the control were not significant at any dose of fipronil (control: –45.3±8.2 mV and fipronil: –46.5±9.5 mV; n=14).

Thus, the neurons in the isolated central ring ganglia of *Lymnaea* responded to fipronil application dose-dependently, with a significant reduction in AP frequency but only a barely hyperpolarized Vm. Detailed analysis of the APs revealed marked changes in shape of the interspike-interval (ISI) histograms (Fig. 5). For the control cells, two individual peaks usually appeared, at 1 s and 1.3 s. Fipronil increased the ISIs, and the discrete peaks disappeared.

DISCUSSION

Our *in vitro* study revealed that the phenylpyrazole insecticide fipronil exerts marked neuronal effects on different non-target species. A clear increase in general excitability and an enhanced neuronal plasticity were observed in neocortical slices of rats. The mean amplitude of the evoked potentials was higher in the presence of fipronil (51% and 76% increases at 4 and 8 mg/l, respectively), and a second component of the evoked potentials often appeared. Fipronil slightly enhanced the effect of high-frequency stimulation to induce LTP, while it had only marginal effects on short-term synaptic events. These findings are compatible with the hypothesis that fipronil exerts its direct neuronal effects predominantly via an inhibitory action on $GABA_A$ receptors since $GABA_A$ antagonists, such as picrotoxin or bicuculline, cause similar alterations in neuronal excitability at low concentrations [20]. When a small dose of bicuculline was applied into the incubation medium of rat cortex slices, it was observed that low-frequency electrical stimuli usually evoked doubled spikes or complex longer-lasting responses, while in the control solution generally a single field response appeared [6]. Similar long-lasting complex responses to single stimuli were observed in our rat neocortical slices following fipronil application. The appearance of these epileptiform evoked potentials is thought to be a consequence of synaptic disinhibition.

The degree of the observed enhancement in excitability is comparable to those described by Szegedi *et al.* [25] in a multilevel study, indicating that the acute oral administration of 100 mg/kg fipronil to rats may result in a brain concentration of approximately 9.1–18.2 nM (which corresponds to 4–8 mg/l in the perfusion solution). [^{3}H]Ethynylbicycloorthobenzoate ([^{3}H]EBOB) is a recently developed noncompetitive blocker of the GABA-gated chloride channel; fipronil concentrations causing half-maximal inhibition of ([^{3}H]EBOB) binding were 1.6 μM in rat dorsal root ganglion cells and human membrane preparations [15], and 2.4 μM or 5.3 μM for recombinant human receptors [17, 22]. On this basis, much less-than 50% occupancy of the receptors is expected in our experiments, which already resulted in a significant physiological alteration. It must also be considered that the real concentration

of the drug may well be substantially lower inside the slice (i.e. at the site of action) than in the incubation medium. This dose range was sufficient to cause enhanced responsiveness to electrical stimulation, but not to induce seizure activity.

Short-term neuronal plasticity depends considerably on the probability of transmitter release from the presynaptic terminals. Using the paired-pulse stimulation paradigm, in most cases we observed an inhibition of the second evoked response, which is characteristic in the neocortex. It has been reported that under control conditions, neocortical excitatory synapses release relatively high amounts of vesicular transmitters. The depletion of synaptic vesicles following electrical stimulation results in a depression of the response to a second stimulus shortly following the first one [5]. Decreasing GABAergic inhibition by fipronil should further enhance paired-pulse inhibition by increasing the release of excitatory transmitters. Indeed, when afferents to pyramidal neurons were stimulated by double pulses, a tendency to enhanced paired-pulse inhibition was seen when the interpulse interval was 50 ms. However, rather the opposite tendency was observed when the pulses were separated by 200 ms. This may be due to a weaker feed-back (postsynaptic) inhibition in the presence of fipronil. However, none of these differences was significant statistically.

Long-term change in synaptic strength is widely assumed to be the mechanism by which memory traces are encoded and stored in the brain [19]. There are a number of phenomena which have been presumed to underlie the development of LTP in the neocortex [18]. Among others, the activation of NMDA-receptors and voltage-gated Ca^{2+} channels play a critical role. All these processes lead to an increase in the postsynaptic intracellular Ca^{2+} level. Further, GABAergic transmission can influence the degree of postsynaptic depolarization and hence the changes in cytoplasmic Ca^{2+} level. In adult animals, LTP often cannot be elicited in the neocortex without the use of a low concentration of GABA-receptor antagonists. As we used relatively young rats, LTP-induction was also successful in the control slices, but the enhancement was less than for the slices treated with 8 mg/l fipronil.

The effects of fipronil on the firing pattern of identified neurons from the pulmonate pond snail *Lymnaea stagnalis* were studied by making intracellular recordings. The RPeD1 neuron is a member of a central pattern generating network controlling the aerial respiratory behavior [2]. We observed that fipronil does not have a hyperpolarizing effect on the Vm, but at 4 mg/l or higher concentration it decreases the AP firing frequency by 32%. Thus, in contrast with the observations in the rat neocortex slices, a decrease, rather than an increase in membrane excitability was caused by fipronil. As Vm does not change in response to fipronil treatment, the above alterations may be a consequence of an increased inhibitory or a decreased excitatory synaptic transmission.

In molluscan neurons, the responses to GABA can be either excitatory or inhibitory, producing an efflux or influx of Cl^- ions, depending on the Cl^- gradient between the cell interior and the environment. In the RPeD1 neuron, the investigated GABA responses were eliminated completely by picrotoxin, which proves the involvement of $GABA_A$ receptors [23]. GABA evokes a membrane depolarization in the majority of *Lymnaea* neurons, including the RPeD1 neuron [23]. Our results are consistent with the findings of these previously reported studies, and the inhibition of GABA action by fipronil is suggested in molluscs.

It may be concluded that fipronil exerts a direct effect not only on target animals, but also on non-target organisms. In a dose-dependent manner, it influences the basic excitability of the mammalian and the molluscan brain. As fipronil significantly increases the basic excitability of cortical neurons in mammals, a larger dose of fipronil (as contamination) might cause serious behavioural consequences in humans. A negative environmental effect must also be taken into consideration concerning its influence on the neuronal activity of non-target invertebrates. More precise toxicological determinations are required to increase our knowledge regarding the environmental and health effects of chemical compounds used in agriculture.

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