DOCTORAL THESIS

Analysis of nervous system effects of insecticides using the *ex vivo* brain slice technique

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INTRODUCTION

Pesticides (herbicides, fungicides and insecticides) are used worldwide in great quantity. As these chemicals, designed to kill pest species, are toxic substances, it is very important to study their eventual harmful effects on human health and on the environment (non-target species). Pesticides may get in direct contact with workers during fabrication, packaging and application or may get into the food chain via treated crops. Among the many pesticide categories, it is insecticides that may cause the greatest risk for human health, as these chemicals act on insect receptors, ion channels and enzymes which are very similar in structure and function to those found in the human body.

The neuronal and neuromuscular junctions are the target of several important insecticide groups. Neurotransmission systems are fairly similar within the animal kingdom, so the chemicals designed against target insects are potentially neurotoxic for mammalian species and humans. Newly developed insecticides are more and more selective for insects, rendering mammals less sensitive to them, but they may have harmful effects in case of accidental or chronic exposition. During my work, I have analyzed the neuronal effects of three insecticides with different mode of action. These are Bancol® (active ingredient: bensultap), Regent® (active ingredient: fipronil) and Sumi-Alfa® (active ingredient: esfenvalerate). Experiments were carried out on surviving rat brain slices, using microelectrophysiological methods which are suitable to examine the effects of the chemicals on general excitability and basic synaptic functions of brain tissue.

Bensultap is a neonicotinoid type insecticide, it acts as a mainly inhibitory modulator on nicotinic acetyl-choline receptors (nACRs). In the central nervous system of insects, acetyl-choline is the main excitatory neurotransmitter, and the nicotinic receptor type is far more abundant than in mammals (Breer and Sattelle, 1987). In mammalian brain, nicotinic transmission has rather a modulatory role; it leads to increased release of other (mainly excitatory) transmitters, thus increasing general excitability (Dani, 2001).

Fipronil belongs to the phenylpyrazole group, it is an antagonist of ionotropic γ-amino-butyric acid (GABA)-receptors. GABA is an important inhibitory neurotransmitter both in invertebrate and vertebrate nervous system. It has a key role in setting the activity level of neuronal circuits and synchronizing activity (Mody and Pearce, 2004). Chemicals inhibiting GABA-receptors lead to the overexcitation of the nervous system (McCormick, 1989).

Esfenvalerate belongs to the pyrethroid insecticide group, these chemicals are agonists of the voltage-gated Na^+ channels, acting via prolongation of their opening time (Narahashi, 2000). Voltage-gated Na^+ channels enable the generation and propagation of action potentials in excitable tissues. The effect of esfenvalerate on brain slices is less easy to predict than the effect of receptor agonists/antagonists, as, on one hand, voltage-gated Na^+ channels are present on all neurons and, on the other hand, esfenvalerate may cause different levels of depolarization depending on concentration and treatment time, thus it may enhance or inhibit the activity of cells. For this reason, experiments concerning esfenvalerate were carried out, apart from cortical slices, on hippocampal slices as well, which can be used to simultaneously characterize the excitatory input arriving on pyramidal neurons (by measuring EPSPs- excitatory postsynaptic potentials) and also the firing properties of the same cells (by measuring POPS- population spikes). Thus, additional information can be gained concerning the effects of the chemical on neuronal circuit level. In this more complex study, also the examination of esfenvalerate's eventual effects on seizure susceptibility of cortical networks was included.

AIMS OF THE STUDY

The aim of my experiments was to examine the effects of three insecticides (bensultap, fipronil and esfenvalerate) on the central nervous system of a mammalian animal model and to determine how well these effects can be studied in an *in vitro* test system. The experiments were carried out on surviving rat brain slices, using the field potential recording technique in the somatosensory neocortex and the hippocampus. Previous experiments in our laboratory have shown that bensultap and fipronil can cross the blood-brain barrier in rats and that after *in vivo* treatment, characteristic electrophysiological changes can be detected in the neocortical brain slices prepared from treated animals (Dóczi et al., 1998; Szegedi et al., 2005). In the present experiments, their direct effects on brain slices were studied. Thus, in case of bensultap and fipronil, the chemicals were dissolved in the perfusion solution of the slices and directly applied on them (*in vitro* treatment); in case of esfenvalerate both *in vitro* and *in vivo* (intragastric) treatments were studied.

The exact questions were the following:

- Do the insecticides influence the general excitability of the examined brain areas? Does the treatment change the stimulation threshold voltage and the amplitude of the evoked potentials?
- Do the chemicals influence short-term synaptic plasticity? Does the paired-pulse ratio change after treatment?
- Do they modify long-term synaptic plasticity? Is the induction of long-term potentiation (LTP) as efficient in treated as in control slices?
- Do they elicit spontaneous epileptiform activity in the brain slices? If applying a convulsant, does insecticide treatment change the seizure susceptibility of the slices?

METHODS

- Preparation of coronal surviving brain slices from young male rats.
- *In vitro* treatment of brain slices with commercial insecticides dissolved in the perfusion solution: Bancol 50 WP® (active ingredient: 50% bensultap), applied concentrations: 30 and 60 μM active ingredient; Regent 80 WG® (80 % fipronil), applied concentrations: 10 and 20 μM; Sumi-Alfa 5 EC® (5% esfenvalerate), applied concentrations: 10, 20 and 40 μM.
- *In vivo* treatment of rats with intragastrically applied Sumi-Alfa® -solution, at a dose of 20 mg/kgbw (LD_{50}/4). Preparation of brain slices at 1, 2 and 7 days after treatment.
- Recording of field potentials evoked by electrical stimulation in layer II/III of somatosensory neocortex and hippocampus CA1 pyramidal layer.
- Examination of basic excitability of the slices by measuring amplitude and slope of evoked potentials (in neocortex, early and late component of EPSPs, in hippocampus, EPSP slope and POPS amplitude).
- Analysis of short-term synaptic plasticity with paired-pulse stimulation of the slices.
- Induction of long-term potentiation (LTP) in the slices by high-frequency stimulation.
- Seizure susceptibility of the slices characterized by analyzing spontaneous epileptiform activity evoked with 4-aminopiridine (after *in vivo* esfenvalerate treatment).

EXPERIMENTAL RESULTS

Effect of bensultap

In neocortical slices treated for 30 min with solution containing 30 μM bensultap, both the early and late component of the EPSPs increased significantly, while 60 μM bensultap caused only a slight increase. Short-term plasticity was not influenced by bensultap treatment. After induction of LTP, the amplitude of the EPSP increased less in the treated slices than in control ones.

Effect of fipronil

After perfusion with fipronil-containing solution, both the early and late components of the EPSPs increased significantly in neocortical slices, in case of both applied concentrations. Short-term plasticity was not influenced by fipronil. The increase in amplitude caused by LTP-induction was higher in the slices treated with the higher fipronil-concentration than in control slices.

Effect of *in vitro* treatment with esfenvalerate

In **neocortical slices**, perfusion with esfenvalerate-containing solution had different effects on EPSP amplitude depending on concentration. Values of slices treated with the 10 μM-solution did not differ from those of the control. In case of the 20 μM-solution, the amplitude of the early component of the EPSP slightly increased compared to the control, while in case of the slices treated with the 40 μM-solution, both early and late components of the EPSPs decreased significantly. Short-term plasticity was not influenced by esfenvalerate treatment. After induction of LTP, the increase of EPSP amplitude was smaller in slices treated with the two lower concentrations than in control slices. In case of the highest dose, however, EPSP amplitude decreased, long-term synaptic depression (LTD) occurred.

In **hippocampal slices**, a biphasic effect was observed also, EPSP slope increased after treatment with 20 μM esfenvalerate, while it decreased after treatment with 40 μM-solution. In contrast, the amplitude of the population spike decreased, compared to the control, at all three concentrations. Also the amplitude of a second POPS occurring in some of the slices was smaller in the slices treated with the two higher doses. During paired-pulse stimulation, in the slices treated with 40 μM esfenvalerate, the facilitation of the second response was stronger than in control slices. After induction of LTP, the increase in amplitude of the POPS was smaller in all three treated groups than in the control.

Effect of *in vivo* treatment with esfenvalerate

Due to *in vivo* treatment with esfenvalerate, severe acute convulsions developed in ca. 30% of the animals, displaying characteristic symptoms of pyrethroid intoxication. These animals were euthanized, not included in the analysis. In **neocortical slices**, one and two days after treatment, the voltage threshold necessary to evoke EPSPs was higher than in slices prepared from control animals. The early component

of the EPSP was slightly increased in treated slices, the amplitude of the late component was significantly higher two days after treatment. Concerning short-term plasticity, no differences between the slices originating from control and treated animals was found. Due to LTP-induction, a smaller increase in amplitude occurred at all three latencies after treatment than in the control animals. Also the seizure susceptibility of cortical slices was studied by analyzing the pattern and frequency of spontaneous epileptiform activity evoked with 4-aminopiridine. There was no significant difference between the slices originating from control and treated rats, but the slices prepared two days after treatment seemed more susceptible, spontaneous activity develops faster and more episodes occur than in the other groups.

In **hippocampal slices**, there was no difference between the voltage threshold values of the groups, nor the EPSP slopes. The amplitude of the POPS, however, was significantly lower two days and seven days after treatment than in the control group. In the same treated groups, the occurrence of a second POPs was more frequent, its amplitude was higher than in the control group. Concerning paired-pulse stimulation, in vivo esfenvalerate treatment did not have a clear effect. The increase in amplitude due to induction of LTP was smaller in the slices prepared seven days after treatment than in controls.

DISCUSSION

Bensultap is neonicotinoid type insecticide, it inhibits nicotinic acetyl-choline receptors. In rat neocortex, nAChRs occur frequently on pyramidal cells, mostly in presynaptic position. The activation of these receptors promotes the release of excitatory neurotransmitters, thus enhancing the excitability of the neocortex (McGehee and Role, 1996). A nAChR antagonist is expected to decrease general excitability and synaptic plasticity of the brain tissue. In contrast with these expectations, 30 μM bensultap in perfusion significantly increased the amplitude of field potentials, though 60 μM increased them only to a smaller extent. The efficacy of LTP-induction was decreased as expected. This contradiction can probably be explained by the fact that bensultap may not be a purely nicotinic antagonist. A basal molecule of neonicotinoids, nereistoxin was shown to exert a dual effect on nAChRs; at low concentrations it acts as a partial agonist, while at high concentrations it is an antagonist (Eldefrawi et al., 1980). The effect of bensultap on brain slices may be explained by this phenomenon. In previous experiments performed in our laboratory, *in vivo* treatment with bensultap was applied and mostly inhibitory effects were described. After chronic oral intake, the amplitude of evoked responses and seizure susceptibility decreased in cortical slices (Dóczi et al., 1998). After high-dose acute oral exposure, paired-pulse depression was enhanced and LTP-efficacy was decreased (Szegedi et al., 2005), the latter was also seen after *in vitro* treatment.

Fipronil of the phenylpyrazole group is an antagonist of ionotropic GABA receptors (Cole et al., 1993). In mammalian brain, GABA is the main inhibitory neurotransmitter; released mainly by

interneurons, it decreases the activity of principal cells. Chemicals having the same mechanism of action as fipronil (e.g. bicuculline, picrotoxin) increase the excitability of neuronal networks, in brain slices they increase the amplitude of evoked potentials, promote the appearance of their late components (McCormick, 1989). In accordance with this, in *vitro* treatment of neocortical slices with 10 and 20 μM fipronil increased both early and late components of the EPSPs. In a previous experiment performed in our laboratory, it was also shown that acute oral treatment with fipronil temporarily increased the amplitude of EPSPs in rat neocortical slices (Szegedi et al., 2005). The efficacy of LTP-induction, the extent of the increase in amplitude of evoked potentials depends on the basic activity level of the neuronal network (Teyler, 1999). In several studies, GABAA receptor-antagonists are applied to facilitate the development of LTP (Malenka, 1995). Thus, it is easy to interpret the finding that after treatment with 20 μM fipronil, a higher increase in amplitude was observed than in control slices.

Esfenvalerate is a pyrethroid type insecticide; it acts as an agonist of voltage-gated Na^+ channels, prolonging their opening time. This leads to the depolarization of neurons, first resulting in overexcitation and repetitive discharges, but to depolarization block beyond a certain membrane potential value. For this reason, pyrethroid effects may differ depending on concentration, treatment time and preparation (Narahashi, 2000). In case of ***in vitro* treatment**, this biphasic effect could be seen both in neocortical and hippocampal slices; after treatment with 10 and 20 μM esfenvalerate, the amplitude/slope of the EPSPs increased, but the POPS decreased, while at 40 μM esfenvalerate, both the EPSP and POPS amplitude was strongly decreased. It has been shown that other pyrethroids decrease the firing activity of neurons in brain slices (Rekling and Theophilidis, 1995). Treatment of hippocampal slices with 40 μM esfenvalerate increased paired-pulse facilitation; this may be explained with the decrease of the basic transmission level (Zucker and Regehr, 2002). The smaller effect of LTP-induction also follows from this fact (Teyler, 1999).

In brain slices originating from animals **treated *in vivo***, signs indicating increased and decreased synaptic activity could both be observed. The latter is suggested by increased stimulation voltage threshold and lower POPS amplitude; while the occurrence of longer latency components in evoked potentials suggests increased excitability. Owing to the lack of similar publications, these results cannot be directly compared with other studies, but it has been shown that *in vivo* pyrethroid treatment cause epileptiform activity in EEG, while decreasing the amplitude of sensory evoked potentials (Vijverberg and van den Bercken, 1990). Similarly to the *in vitro* treatment, one characteristic effect of *in vivo* treatment was the decrease of long-term synaptic plasticity. It has been described it several studies that pyrethroid treatment alters the performance of experimental animals in certain tests evaluating learning and memory (e.g. Zhang et al., 2008). Concerning the seizure susceptibility of neocortical slices, our experiments did not reveal clear effects, presumably due to the variability of data. Although no

spontaneous activity should occur 1-7 days after treatment, it can be assumed that the susceptibility of neuronal networks to convulsants changes at least temporarily.

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