PhD Thesis

FUNCTIONAL NEUROTOXICITY OF INSECTICIDE XENOBIOTICS IN RATS IN COMBINED EXPERIMENTAL EXPOSURE

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The Applicant's Relevant Publications

I. Papp, A., Szabó, A., *Lengyel, Zs*, Nagymajtényi, L. (2005) Biomarkers of certain environmental neurotoxicants: An overview. Centr. Eur. J. Occup. Envir. Med. 11, 135-143.

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III. *Lengyel, Zs.*, Fazakas, Z., Papp, A. (2005) Functional neurotoxic effects of combined oral administration of insecticides to rats during intra- and extrauterine development. Homeostasis 43, 179-183.

IV. *Lengyel, Zs.*, Fazakas, Z., Nagymajtényi, L. (2005) Changes in the central nervous activity of rats treated with dimethoate in combination with other neurotoxicants in different phases of ontogenesis. Arh. Hig. Rada. Toksikol. 56, 257-264.

V. *Lengyel, Zs.*, Lukács, A., Szabó, A., Institóris, L.(2006) Neurotoxic and general effects of combined subchronic exposure of rats to insecticides and heavy metals. Acta Biol. Hung. 57, 423-432. *Impact factor: 0.636* .

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Summary

Pest control is achieved today mainly by means of chemical pesticides. The majority of insecticides used today are toxic for a wide range of animals, and also for humans. The insecticides involved in this Thesis; belonging to the group of organophosphates, carmabates, pyrethroids and formamidines; are of this kind.

Organophosphates (OPs) are derived from one of the phosphorus acids. Their primary mode of action is inhibition of acetylcholinesterase (AChE). The OP used in the experiments was dimethoate (**D**), an agent used against a wide range of insect pests. Several preparations containing **D** are registered in Hungary. **D** is moderately toxic by ingestion, inhalation, and dermal absorption. **D** is of low persistence in the soil, and is rapidly broken down by soil microorganisms. Waterborne and foodborne exposure of the population by **D** cannot be excluded.

Carbamates inhibit cholinesterase as OPs do, and exposure to carbamates has been associated with human nervous system dysfunctions although their AChE inhibiting effect is transient. In the experiments presented, propoxur (**P**) was used. **P** is applied on a variety of insect pests in agricultural and non-agricultural settings. **P** is highly toxic orally; signs of **P** poisoning are those resulting from inhibition of AChE. **P** is of moderate to low persistence in the soil environment, is likely to enter groundwater, so that non-occupational human exposure may be waterborne.

Pyrethroids have low toxicity in mammals and man. They act on the Na^+ channels of axons by slowing down their inactivation. The pyrethroid used in the present work was cypermethrin (**C**), used in agricultural and hygienic insect control. **C**-based insect killers registered in Hungary are numerous. Due to the low human toxicity, no health risk arises from eventual environmental residues of **C**. Human **C** poisoning, a rare event, is manifested in hypersensitivity, choreoathetosis, tremors and paralysis.

Formamidines represent a small group of insecticides. Their proposed action is inhibition of monoamine oxidase, and an agonist-like effect on alpha2-adrenoceptors. In this study we used amitraz (**A**), an insecticide and acaricide. Two amitraz-based products were in use in Hungary up to 2005. In human poisonings with **A**, the leading symptom is depression of the CNS with drowsiness, coma and/or convulsions. **A** is broken down rapidly in well-oxygenated soil. The half-life in soils is less than one day.

The characteristics and environmental behaviour of the insecticides involved in this Thesis, together with their ways of application, make multiple or simultaneous human exposure by them a likely event, resulting in their combined presence and action in the human organism. There exists an ample body of data on the toxicity, including neurotoxicity, of single insecticides, but little is known about their combined effects. This lack of data has been recognised in the last years, resulting in research goals defined such as studies on the potential of combined actions, and assessment of the risks caused. The aims of the present study – to investigate the functional neurotoxicity of certain commonly used insecticides, and their combinations, in rats – were based on the above mentioned problems and on the research experiences of the Department.

The experiments were carried out on Wistar rats. When the treatment (detailed below) was over, the rats were prepared for electrophysiological recording in urethane anaesthesia. Electrocorticogram (ECoG) and sensory evoked potentials (EPs) were recorded form the primary somatosensory (SS), visual (VIS) and auditory (AUD) area, and compound action potentials were recorded from the tail nerve. From the ECoG records, frequency spectrum according to the standard EEG bands was calculated. On the EPs, latency and duration was measured, and from the tail nerve record, conduction velocity and refractory periods were calculated.

For acute administration (see below), young adult males were used, weighing 250-300 g. The insecticides or their combinations were given in a single dose (1/5, 1/25 or 1/100 LD_{50}), 24 hours before electrophysiological recording. The two main goals here were to test which of the combinations are of interest for pre- and postnatal exposure experiments, and to establish the dose range for later work.

When an acute 1/5 LD_{50} dose of the insecticides was given alone, the relative power of the delta band was significantly reduced by **D** and **A** in all three cortical areas. **A** also had a reducing effect on the theta and alpha bands. The effect of **P** was, surprisingly, opposite to that seen with **D**, and **C** caused practically no alteration in the ECoG spectrum. In the double combinations, significant increase was seen in the fast bands. The triple and quadruple combinations gave different results compared to the double combinations. **DPC** caused increase of the fast band activity, unlike **DP**, **DC** or **PC**. **DCA** had a similar effect, and the effect of **DPCA** was also similar and even more pronounced.

Of the lower doses, 1/100 LD_{50} had no noteworthy effect, and the effect of 1/25 LD_{50} was also below significance except **DPCA**.

The effect of the insecticides on the EPs was not very marked in single acute application: slight increase of the latency (mostly with **D** and **A**) and mild, mostly non-significant decrease of the duration was seen. With double combinations, the changes of the EP parameters were more pronounced. All combinations containing **D** increased significantly the EP latency. On the SS and AUD EP, also the **CA** treatment had a like effect. Compared to single administration, the effect of **DC** and **CA** on the latency was stronger than that of **C** alone in the SS area, of **DA** than **A** in the VIS area, as well as of **DC** than both components and of **DP** than **D** alone in the AUD area. With the triple and quadruple combinations, the general trend was increase of the latency, significant in all combinations in the SS and VIS area, and with **DPCA** in the AUD area. On the SS EP, all combinations except **DCA** had a stronger effect than the corresponding double combinations. On the VIS EP, the exception was **DPC**.

Of the parameters of the tail nerve, the relative refractory period (RRP) was the most sensitive to the insecticides. When given acutely in 1/5 LD_{50} dose, **D**, **C** and **A** induced significant increase of it the RRP, and the effect of the double combinations was also dominated by these agents. The triple and quadruple combinations were not always more efficient.

In the protocols involving intra- and extrauterine development, the substances were administered to pregnant female Wistar rats as follows: *P protocol*, dams daily treated from the 5^{th} to 15^{th} day of pregnancy; *P+L protocol*, dams treated as above and during the 4 weeks of lactation; *P+L+P protocol*: dams treated as above, and weaned male offspring treated for further 8 weeks in a 5 days per week schedule. Here, only the combinations **DP**, **DC**, **PC** and **DPC** were used.

In the rats exposed according to the *P protocol*, the alterations of the ECoG were slight. The only double combination with significant effect was **DC**, causing increase in the theta band in all areas and decrease in the gamma band in the VIS area. With **DPC**, similar decrease in the fast bands was seen. In the *P+L protocol*, the effects on the ECoG were partly dissimilar to that seen in the *P protocol*. **CP** caused some shift to slower frequencies. With **DC** and **DP**, however, decrease of the delta band was seen in all 3 areas (significant in the VIS and AUD area). The effect of the triple combination was similar to

that of the double combinations containing **D**, but was much more marked. In the *P+L+P protocol*, the general trend in the double combinations was increase in the fast and decrease in the slow bands. With **CP**, decreased alpha activity was obtained. In the **DC** group, decreased delta activity in the SS, and decreased alpha in the VIS, area was significant. The effect of **DPC** was opposite to that of the double combinations: slow bands increased. In the variation of the pre-and postnatal treatment where a single 1/5 LD_{50} dose of the insecticides and combinations was given on the 5^{th} day of pregnancy, no noteworthy effects were found.

There was hardly any latency change in the SS EP, when the insecticides were given in the *P protocol*. In the VIS area, all double combinations caused a significant latency increase, and in the AUD area, **DP**. In the rats treated according to the *P+L protocol*, latency increase in the SS area was significant in the **CP** and **DP** groups. In the AUD area, the latency lengthening was also stronger than in the *P protocol* and was significant in the **DC** group. When administered according to the *P+L+P protocol*, the trend of change of the EP latency was similar to hat seen with the P+L protocol but more changes were significant (indicating a summation of the effect of pre- and postnatal exposures).

In rats exposed according to the *P* protocol, the effects on the RRP were slight and non-significant. In case of the *P+L protocol*, the increase of the RRP was more pronounced, and significant with the triple combination, suggesting that the extent of change depended on the total time of exposure. In the *P+L+P protocol* the increase was significant in the treated groups except in **PC**. It was noteworthy that the combinations which caused significant RRP increase did the same also in acute application.

In hygienic toxicology, the final goal is to provide better health protection for those exposed to toxic chemicals. With any pesticide, unless it is absolutely specific for the target organisms, human toxicity is always a major concern. Production, transport and application of insecticides entails occupational exposure, while population-level exposure may result from residues in foods originating from the applied chemicals, or from the environmental presence of the agents. Especially in larval poisoning, health damage develops over a longer period of low-level exposure with no or minimal substance-specific symptoms. In such situations, early detection and follow-up is of great importance, and can be achieved by means of biomarkers. In case of xenobiotics acting on the CNS, biomarkers

of effect are far more practical than biomarkers of exposure, especially if the exposure is multiple. Although there is no consensus about the association of neurological signs and low-level insecticide exposure in humans, it was supposed that the results of this work may contribute to further development of exposure limits; and that certain functional alterations, detected in the course of the experiments, may be sufficiently specific and sensitive to allow a future application as effect biomarker.

The particular points of conclusion, derived from the above results, are the following:

- The neurotoxicity of amitraz, not well described from this aspect so far, could be demonstrated in our experimental system.
- The effects of dimethoate and amitraz were similar, but those of dimethoate and propoxur were not, in spite of the dissimilar and similar mode of action, respectively. No synergism between dimethoate and propoxur was observed.
- Dimethoate and amitraz dominated mostly in the effects obtained by the combinations.
- The changes in the spontaneous cortical activity were, with several agents applied, opposite when the substance was applied only prenatally, than when pre- and postnatal application was used.
- The comparison of changes of cortical evoked activity seen in the *P+L* vs. *P+L+P protocols* indicated summation of effects of indirect and direct exposure.
- Both spontaneous and evoked cortical activity was sensitive to the insecticide exposures but the changes in the latter could be more accurately assessed.

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Abbreviations

A	amitraz
ACh	acetylcholine
AChE	acetylcholinesterase
ADI	acceptable daily intake
AUD	auditory
BChE	butyrylcholinesterase
CNS	central nervous system
C	cypermethrin
D	dimethoate
dur.	duration
ECoG	electrocorticogram
EEG	electroencephalogram
EP	evoked potential
GABA	γ-aminobutyric acid
lat.	latency
MRL	maximal residue level
P	propoxur
RRP	relative refractory period
SS	somatosensory
VIS	visual

1. INTRODUCTION

1.1. The importance of pest control and its historical background

Agricultural production, the source of food and industrial raw materials, has always been playing an essential role in the support and development of human societies. An important aspect of agriculture is pest control, the process of promoting the sufficient quality and quantity of products, which has much evolved throughout history. A number of natural and – later on – artificial agents have been used in order to protect the plants against various damaging effects.

In 3000 BC, Sumerians burnt sulphur as a protection against insects and acarids. Chinese scripts of 2500 years describe the use of mercury- and arsenic-based chemicals against lice and other insects. Ancient Greeks and Romans used oily sprays, ash, mercury-based creams and lye to protect themselves, their animals and crops. Several of the conventional methods of pest control are based on organic materials or biological interaction. Romans burnt the rest of crops out in the fields after harvesting and rotated crops to minimize crop devastation. Chinese had the knowledge of insecticides of herbal origin, and used predator ants to liquidate worms in orchards 1200 years ago. Many farmers still use ducks and geese in pest control, because they eat up the insects and weeds. Nicotine, the alkaloid of tobacco, was also a wide-spread insecticide.

1.2. Modern pesticides and their types

Any chemical used by man to control pests – insects and acarids, fungi, weeds, nematodes, snails, slugs, etc. – can be regarded as pesticide. Insecticides are a thus major group within pesticides.

An insecticide may kill the insect on contact or after being swallowed. Insecticides called systemics may be absorbed, injected, or fed into the plant or animal to be protected, so that the insects feeding on this plant or animal will be killed.

The majority of insecticides used today have broad spectrum: these are toxic for a wide variety of animals, and also for humans; by attacking a system common to all, such as the nervous system. The insecticides involved in this Thesis; belonging to the group of

organophosphates, carmabates, pyrethroids and formamidines; are of this kind. Novel groups of insecticides are much more selective. The chitin synthesis inhibitors affect only arthropods, interfering with the development and molting. Growth regulators, acting on certain species that have a particular hormone, are even more specific. Pheromones are the most restrictive because they react with only one species or one sex of a single species, and they work by other mechanism than killing.

Insecticides also vary in their persistence after application. Some break down almost immediately into nontoxic compounds. These "short term" chemicals are advantageous in situations where the insects do not return or where long-term exposure could be harmful to humans or other non-target species, and are often used in homes and dwellings. Other insecticides remain active for a long period. Such "residual" pesticides are of use when the insects are a constant problem, and where and when they will not be an environmental and/or health hazard (Ware and Whitacre, 2004).

Further important groups of pesticides include fungicides (used to control the fungi which cause molds, rots, and plant diseases), herbicides (to control unwanted plants), rodenticides (to kill rats, mice and other rodents which occasionally damage cultivated plants or stored crops), as well as nematocides and molluscicides.

1.3. Insecticides investigated in the experiments

1.3.1. Organophosphates

Organophosphates (OPs) are derived from one of the phosphorus acids. These insecticides, generally, are the most toxic of all pesticides to vertebrates. The OPs' insecticidal qualities were first observed in Germany before World War II (which led to the development of the extremely toxic OP nerve gases sarin, soman, and tabun).

The possible types of acid of phosphorus, and the presence of hetero atoms like sulphur and nitrogen, results in six different subclasses of OPs: phosphates, phosphonates, phosphorothioates, phosphorodithioates, phosphorothiolates and phosphoramidates. Depending on the substituents, the OPs are again generally divided into three groups- aliphatic, phenyl, and heterocyclic.

The primary mode of action of OPs is inhibition of cholinesterase (ChE; Koelle, 1992), an important enzyme in the central and peripheral nervous system. There exist, in

fact, two cholinesterases: acetylcholinesterase (AChE) and pseudo- or butyrylcholinesterase (BChE). AChE (acetylcholin hydrolase, E.C. 3.1.1.7) is typically found in cholinergic neuro-neuronal synapses and in the neuromuscular and neuroglandular junctions. BChE (E.C. 3.1.1.8) is present in the blood plasma, RES and fat tissue (Ballantyne and Marrs, 1992). Inhibition of the enzyme is achieved by phosphorylation of the hydroxyl group of a serine side chain in the active centre of AChE (and BChE). In the breakdown of ACh, the active serine is acetylated, but this bond spontaneously hydrolyses leaving an intact enzyme. The covalent bond with the phosphate group is, on the contrary, regarded as irreversible, which is the basis of enzyme inhibition. This irreversibility is not absolute – depending on the OP in question, spontaneous reactivation may occur within hours but may also be practically impossible (WHO, 1986b). Another possible fate of the phosphorylated enzyme is ageing, where one of the other (alkoxy) groups detaches from the central phosphorus atom, resulting in a non-reactivable enzyme. If this ageing affects not AChE but another serin hydrolase in the nervous system, neurotoxicity target esterase, the result is the so-called organophosphate-induced delayed neurotoxicity (Koelle, 1992). It is an open question whether there are other target enzymes, or, more generally, target proteins, of OPs in the nervous system and how phosphorylation of these contributes to the toxicity of OPs (Richards et al., 1999).

Inhibition of AChE results in the accumulation of acetylcholine (ACh) at interneuronal and neuromuscular synapses. The resulting effects of OPs (and other cholinesterase blockers like carbamates, see below) can be divided to three groups as shown in Table I. Since the introduction of OPs in farming, cases of human poisoning and research on the consequences has been on the agenda. Most of the early research on human OP toxicity was aimed at the metabolism of ACh (Kaloyanova, 1975). Effects on the central nervous system were described e.g. by Metcalf and Holmes (1969).

A variety of nervous system effects were found in human intoxications with different OPs. EEG abnormalities were described from humans following exposure to the nerve gas sarin (Duffy et al., 1979; Yokoyama et al., 1998) or to the agricultural OP oxydemethon methyl (Muttray et al., 1996). Such alterations were observed also in animal experiments (Duffy and Burchfiel, 1980; Dési et al., 1994; Gralewicz et al., 1991). In

animals, alterations in cortical evoked potentials, too, were described (Arakawa et al., 1993; Dési and Nagymajtényi, 1999).

Table I. The effects of cholinesterase blockers (OPs, carbamates, etc.)

Muscarinic effects (located at postganglional parasympathetic nerve endings)	• hypersecretion of salivary, lacrimal, sweat, bronchial etc. glands • miosis, pinpoint pupils • increased intestinal motility, defecation and vomiting • urination • bradycardia and hypotension
Nicotinic effects (located in the ganglia and at the neuromuscular junctions)	• fasciculation, cramps and finally paralysis of skeletal muscles • mydriasis • tachycardia and hypertension
Central nervous system effects	• convulsions • ataxia • respiratory failure • coma

(Ballantyne and Marrs, 1992)

1.3.1.1. Dimethoate

The OP used in the experiments was dimethoate (**D**), because it has a moderate human toxicity (EPA) and has been used in a lot of countries (WHO, 1989a). The chemical name of **D** is O,O-dimethyl S-(N-methylcarbamoylmethyl) phosphorodithioate. Empirical formula: $C_5H_{12}NO_3PS_2$ (Fig. 1).

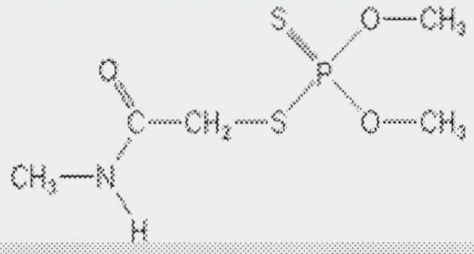

Figure 1. The chemical structure of dimethoate.

D as an insecticide is used to kill mites and insects systemically and on contact. It is used against a wide range of insect pests, including aphids, thripses, planthoppers, and whiteflies on ornamental plants, apples, corn, grapes, lemons, oranges, pears, tomatoes, watermelons etc. It is also used as a residual wall spray in farm buildings to control flies. **D** has been administered to livestock for control of botflies. Insecticidal preparations containing **D**, registered in Hungary, are the following: BI 58 EC, Danadim 40 EC, Rogor L-40 EC, and Sinoratox 40 EC (Szabadi, 2002).

D is moderately toxic by ingestion, inhalation, and dermal absorption. The reported acute oral LD_{50} values for the technical product range from 200 to 450 mg/kg in rats. The LD_{50} determined in our laboratory (Table II), 450 to 460 mg/kg, is within this range (Dési et al., 1991; Papp et al., 2004). Effects of acute exposure are those typical of organophosphates. Very high doses may result in unconsciousness, incontinence, and convulsions or fatality. Persons with respiratory ailments, recent exposure to cholinesterase inhibitors, impaired cholinesterase production, or liver malfunction may be at increased risk from exposure to **D**. High environmental temperatures or exposure of **D** to visible or UV light may enhance its toxicity.

In non-occupational settings, actual human exposure depends, beyond the toxicity of an insecticide per se, on the environmental fate of the insecticide agents. **D** is of low persistence in the soil, with half-life of about 20 (4 to 122) days. **D** is rapidly broken down by soil microorganisms. **D** is highly soluble in water, and it adsorbs only very weakly to soil particles so it may show considerable leaching. In water, **D** does not adsorb to sediments or suspended particles, nor does it bioaccumulate in aquatic organisms, and is subject to significant hydrolysis, especially in alkaline waters. The half-life for **D** in raw river water was 8 days, disappearance being due possibly to microbial or chemical degradation (WHO, 1986b). So, waterborne exposure of humans by **D** cannot be excluded. Due to the systemic character of **D**, foodborne exposure cannot be excluded either. Uptake of **D** was observed, e.g., in garden peas (Getenga et al., 2000) and in tomatoes and tomato products (Aysal et al., 2004). Based on the no observed adverse effect levels (NOAELs) in rats (0.05 mg/kg b.w. daily) and humans (0.2 mg/ kg b.w. daily), an acceptable daily intake (ADI) of 0.01 mg/kg b.w. was set by the WHO and FAO (FAO/WHO, 1996). In the EU regulation, maximum residue level (MRL) in foodstuffs for **D** is mostly 0.02 mg/kg, equal to the detection limit; interesting exceptions

are cherries and head cabbages (European Comission, 2003). The Hungarian regulation (Egészségügyi Minisztérium, 2005) is conform with the EU rules. Although the actual levels may be higher, even at 0.47 mg **D**/kg tomatoes (the maximum level found by Aysal et al.) an unlikely daily consumption of almost 1.5 kg of fresh tomatoes would be necessary to reach ADI by an average adult.

The safety provided by legal the MRL and ADI values is shown by human toxicity tests. Ingestion of 18 mg (about 0.26 mg/kg/day) of **D** for 21 days, or 2.5 mg/day (about 0.04 mg/kg/day) for 28 days induced no detectable AChE inhibition and no toxic signs. **D** is rapidly metabolized by mammals. Rats excreted about 50 to 60% of administered doses in urine, expired air and faeces within 24 hours. Human volunteers excreted 76 to 100% of the administered **D** within 24 hours. Following application of **D** to the backs of cows at 30 mg/kg, its concentration reached a maximum level of 0.02 ppm in blood and milk in about 3 hours, and decreased to 0.01 ppm within 9 hours (Ware and Whitacre, 2004).

1.3.2 Carbamates

The carbamate insecticides are derivatives of carbamic acid. Their mode of action is like that of the OPs, inhibition of ChE (WHO, 1986a). Carbamates inhibit cholinesterase (ChE) as OPs can, and they behave in almost identical way in biological systems (Alvares, 1992). Exposure to carbamates has been associated with human nervous system dysfunctions (Keifer and Mahurin, 1997; Goldman et al., 1985), although their AChE inhibiting effect is transient (see below). The symptoms following human exposure are identical to those seen after OP exposure (Table I). In rats, a single dose of ca. 1/10 LD_{50} caused a 60 % drop in cholinesterase activity and marked disturbances in higher nervous functions (Thiesen et al. 1999).

There are two main differences between the effect of OPs and carbamates: First, some carbamates strongly inhibit esterases other than ChE (the so-called aliesterases, miscellaneous aliphatic esterases of unknown exact functions), and they can be more selective against ChE in different species than OPs. The second and even more important difference is that the ChE inhibition by carbamates is reversible. The serin side chain in the active centre will be carbamylated on action of a carbamate. This covalent bond,

however, is less stable than in case of many OPs, and spontaneously hydrolyses, so that the enzyme will be reactivated (WHO, 1986a).

This difference used to have an important bearing in therapy of poisonings. For the treatment of OP poisoning, so-called enzyme reactivators were developed (Bismuth et al., 1992.). These pyridinium oxime derivatives had indeed an antidotal effect in laboratory tests and clinical use, provided that the poisoning was caused by an OP and not by a carbamate, and that the phosphorylated enzyme has not yet undergone aging. Notably, carbamate poisoning is aggravated by oximes. Hence, the up-to-date therapy of intoxication by AChE inhibitors – that is, OPs and carbamates – is based on atropine to counteract peripheral and central cholinergic hyperactivity, and on diazepam to stop central seizures and anxiety (Bencze and Gőbl, 1998).

1.3.2.1. Propoxur

In the experiments presented, propoxur was used as a carbamate. Propoxur (**P**) is a non-systemic insecticide, its chemical name is 2-isopropoxy-phenyl-N-methyl carbamate (Fig. 2). **P** is used on a variety of insect pests (chewing and sucking insects, ants, cockroaches, crickets, flies, and mosquitoes) and may be used for control of these both in agricultural and non-agricultural (e.g. private or public facilities and grounds) applications. Agricultural applications include cane, cocoa, fruits, grapes, maize, rice, sugar, vegetables, cotton, lucerne, forestry, and ornamentals.

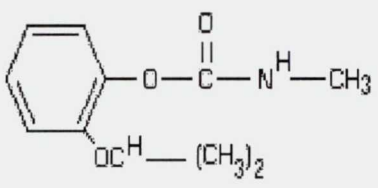

Figure 2. The chemical structure of propoxur

It has contact and stomach action that is long-acting when it is in direct contact with the target pest. In Hungary, no propoxur-based agricultural insecticides are registered (Szabadi, 2002). In spite of this, **P** has been chosen for the study because it has been in use worldwide in disease control and agricultural applications. In household insecticides, like Baygon, **P** was marketed also in Hungary.

P is highly toxic via the oral route, with reported LD_{50} values of approximately 85 mg/kg in rats and mice; the value determined previously in our laboratory is not different from that (Institóris et al., 2004). Its dermal and inhalational toxicity is slight, and it does not cause skin or eye irritation in rabbits. Signs of **P** intoxication are those resulting from inhibition of AChE (see Table I). Depending on the severity of exposure, this effect may be short-term and reversible. In rats, **P** poisoning resulted in brain pattern and learning ability changes at lower concentrations than those which caused cholinesterase-inhibition and/or organ weight changes (WHO/FAO, 1989). During wide-scale spraying of **P** in malarial control activities conducted by the WHO, only mild cases of poisoning were noted. Applicators who used **P** regularly showed a pronounced daily fall in whole blood cholinesterase activity and a distinct recovery after exposure stopped. No adverse cumulative effects on cholinesterase activity were demonstrated.

P is of moderate to low persistence in the soil environment, with reported field half-lives of 14 to 50 days. **P** may be mobile in many soils, and due to its high solubility in water and moderate persistence, **P** has a high potential for groundwater penetration (WHO/FAO, 1989). These properties of **P** indicate that non-occupational human exposure might be waterborne. The water solubility of **P** (ca. 2 g/l; WHO/FAO, 1976) and the half-life mentioned above allow considerable residual levels in groundwater. It is not a systemic insecticide, so the residues found and regulated in food (in the EU and Hungarian legislation, mostly 0.05 mg/kg: Egészségügyi Minisztérium, 2005) are primarily due to surface spraying.

P is very efficiently detoxified in humans (WHO 1986a). Human adults have ingested single doses of 50 mg of **P** without apparent symptoms. Prolonged or repeated exposure to **P** may cause symptoms similar to acute effects. In female rats given ca. 18 mg/kg/day of **P** as a part of a three-generation reproduction study, reduced parental food consumption, growth, lactation, and litter size were observed, while at 2.25 mg/kg/day only parental food intake and growth were depressed. Reproductive effects in humans are thus unlikely at expected exposure levels (Baron, 1991).

1.3.3. Pyrethroids

The development of this group of insecticides was largely influenced by the human health hazard concomitant to the use of OPs and carbamates. Their model substance, natural pyrethrin (extract of toxins contained in the flowers of some *Chrysanthemum* species) has seldom been used for agricultural purposes because of its costs and instability in sunlight. The synthetic pyrethrin-like materials are called pyrethroids.

Pyrethroids are "axonal poisons" acting on the Na^+ channels of axons by slowing down their inactivation (Kadous et al., 1994; Oortgiesen et al., 1989). On the basis of the consequences of prolonged channel opening, type I and type II pyrethroids are distinguished. The Na^+ channel opening prolongation with type I agents is moderate, leading to repetitive axonal firing. In case of type II pyrethroids, including cypermethrin, less channels remain open for a longer time, resulting in frequency-dependent depolarization and conduction block (WHO, 1989b).

Pyrethroids have low toxicity in mammals and man. In several field and laboratory studies, no health effects or neuro-functional alterations in pyrethroid-exposed workers were observed (WHO, 1989b).

1.3.3.1. Cypermethrin

The pyrethroid used in the present work was cypermethrin (**C**). Its chemical name is [S,R]-alpha-cyano-3-phenoxybenzyl-2,2-dimethyl, [1R,1S, cis, trans]-3-(2,2-dichlorovinyl), cyclopropanecarboxylate (Fig. 3).

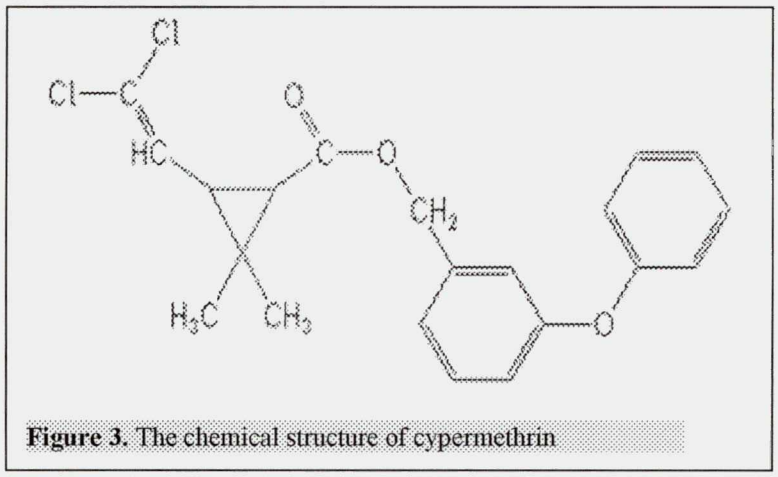

Figure 3. The chemical structure of cypermethrin

C is used to control a variety of pests, including moths of cotton, fruit and vegetable crops. It is also used for crack, crevice, and spot treatment to control insect pests in stores, industrial buildings, houses, laboratories etc.

Technical **C** is a mixture of eight different isomers, each of which may have its own chemical and biological properties. **C**-based insect killers registered in Hungary are: Alphaguard 100 EC, Chinmix 5 EC50, Cinmix turbo, Cyper 10 EM10 %, Cyperil 10 EC10 %, Cyperil-S ULV8 g/l, Cyperkill 25 EC, Ripcord 20 EC, Fury 10 EC, Lemagard 100 EC, Sherpa 250 g/l, Signal 300 ES, and Sumi-guard 10 %. The preparation called Nurelle-D 50/500 EC contains 50 g/l cypermethrin and 500 g/l chlorpyrifos, and is hence of especial interest (Szabadi, 2002).

C has a moderate persistence in soils, it degrades more rapidly on loose than on clay soils, and more rapidly in soils low in organic material. The half lives measured are equal to or longer than those for cholinesterase-inhibiting insecticides, and some bioaccumulation in water organisms has also been described. Due to the low human toxicity, however, no health risk arises from the environmental residues (WHO, 1989b).

Low toxicity for warm-blooded animals and humans is the most important advantage of pyrethroids over OPs and carbamates. In man, mammals and birds, the ester bond in the centre of the molecule is cleaved, giving two inactive products which are then excreted after conjugation, whereas in other taxonomic groups, metabolism is different. In line with that, **C** is moderately toxic by dermal absorption or ingestion (WHO 1989b). Human **C** poisoning, a rare event, is manifested in hypersensitivity, choreoathetosis, tremors and paralysis (Soderlund and Bloomquist, 1989; Vijverberg and van den Bercken, 1990). Symptoms of high dermal exposure include numbness, tingling, itching, burning sensation, loss of bladder control, incoordination and seizures; and those of high-dose ingestion, nausea, prolonged vomiting, stomach pains, and diarrhoea progressing to convulsions, unconsciousness, and coma (Bradberry et al., 2005). In animal experiments, **C** has been used even for generating chemically induced epileptic activity (Condés-Lara et al., 1999). Rats fed high doses (37.5 mg/kg) of the cis-isomer of **C** for five weeks exhibited severe motor incoordination, while 20 to 30% of rats fed 85 mg/kg died 4 to 17 days after treatment began. Pathological changes in the cortex of the thymus, liver, adrenal glands, lungs, and skin were observed in rabbits repeatedly fed high doses of **C** (Soderlund and Bloomquist, 1989; Vijverberg and van den Bercken, 1990).

C is also a slight skin or eye irritant, and may cause allergic skin reaction. In several field studies, occupationally exposed individuals had no or minimal and transient symptoms in spite of considerable urinary levels of **C** metabolites (WHO, 1989b).

EPA reported an oral LD_{50} of 187 to 326 mg/kg in male rats and 150 to 500 mg/kg in female rats; the broad range caused by differences in sex, vehicle (oily or aqueous) and the ratio of cis/trans isomers present (Ware and Whitacre, 2004). In previous works of the Department, 554 mg/kg was found (Institóris et al., 1999).

1.3.4. Formamidine-type insecticides

The formamidines represent a small group of insecticides. Their current value lies in the control of OP- and carbamate-resistant pests. Formamidine poisoning symptoms are distinctly different from other insecticides. Their proposed action is inhibition of monoamine oxidase, the enzyme responsible for degrading the neurotransmitters norepinephrine and serotonin (Moser and McPhail, 1989). The resulting accumulation of these compounds and other biogenic amines inactivates and kills the sprayed insects. In mammals, an agonist-like effect on alpha2-adrenoceptors was also found (Altobelli et al., 2001).

1.3.4.1. Amitraz

In this study we used amitraz (**A**; Fig. 4). It is an insecticide and acaricide used to control red spider mites, leaf miners, scale insects, and aphids.

On cotton it is used to control bollworms, white fly, and leaf worms. On animals, it is used to control ticks, mites, lice and other animal pests. **A** is slightly toxic to mammals if ingested orally (Atabek et al., 2002). It is not permitted on apples to prevent the appearance of its residues in processed apples or apple waste fed to animals, potentially leading to foodborne human exposure (Meister, 1992).

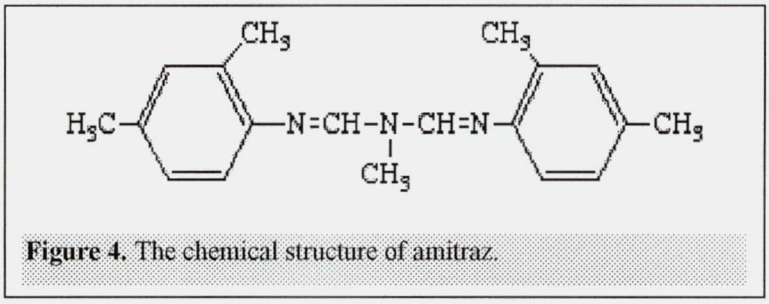

Figure 4. The chemical structure of amitraz.

Two amitraz-based products, Bybye 200 EC and Mitac 20, were in use in Hungary (Szabadi, 2002), up to 2005.

In human poisonings with **A**, the leading symptom is depression of the CNS with drowsiness, coma and/or convulsions; first of all in children (Yilmaz and Yildizdas, 2003). Respiratory depression bradycardia and abnormal blood pressure were also frequently reported. In rats, motor hypoactivity (Florio et al., 1993) and reduced arousal (Moser, 1991) were seen, together with other behavioural alterations and changes in evoked cortical activity (Boyes and Moser, 1987; Moser et al., 1987, Moser and McPhail, 1989). Reproductive toxic effects of **A** in animals were also described (Al-Thani et al., 2003).

A is broken down rapidly in well-oxygenated soil. The half-life in soils is less than one day. Degradation occurs more rapidly in acidic soils than in alkaline or neutral soils.

1.4. Aims of the study

The characteristics and environmental behaviour of the insecticides involved in this thesis, detailed above, together with their ways of application, make multiple or simultaneous human exposure by them a likely event, resulting in their combined presence and action in the human organism. First of all, formulations with more than one pesticide agent are to be mentioned, such as the above-mentioned Nurelle-D 50/500 EC. Furthermore, the activity spectrum of individual insecticides may require the combination of several agents (in one spray solution or sequentially) for an efficient plant protection. For example, **A** is often used together with C, and is sometimes mixed with **D** on fruit trees heavily infested with mites.

In the literature, there is an ample body of data on the toxicity, including neurotoxicity, of single insecticides, but little is known about their combined effects. This lack of data has been recognised in the last years. In Great Britain, a "Working Group on Risk Assessment of Mixtures of Pesticides" was set up. This body identified a number of areas of concern, among them the interaction of various anticholinesterases, subgroups of elevated risk such as young children, and the implication of experimental data to human health (Beaumont and Buffin, 2002). Among the research requirements stipulated, studies on the potential of combined actions, and assessment of the risks caused, are mentioned.

The aims of the present study were shaped by consideration of the open questions of combined pesticide effects, referred to above, and the research experiences of the Department. The general aim was to investigate the functional neurotoxicity of certain commonly used insecticides, and their combinations, in rats.

In the first experiments, the four agents **D**, **P**, **C** and **A** were given – one by one, and in double, triple and quadruple combinations – acutely, and the effects were investigated in 24 hours. Beyond obtaining information on the acute effects, this also showed what combinations were worth studying in the subsequent experiments, in other exposure schemes, longer in time and involving the phases of ontogenesis. These were supposed to be more realistic models of human exposure.

It was supposed that the results obtained may contribute to further development of exposure limit values in occupational applications and in terms of food, water etc. Existing limits refer to single substances only and do not reflect possible interactions. Further it was supposed that certain functional alterations, detected in the course of the experiments, may be sufficiently specific and sensitive to the insecticides and their combinations to allow a future application as effect biomarker.

2. METHODS

2.1. Animals, housing, chemicals

The experiments were carried out on Wistar rats. For the acute administration (see below), young adult males were used, weighing 250-300 g. In the protocols involving intra- and extrauterine development, the substances were administered to pregnant female Wistar rats. All animals were obtained from the Breeding Centre of the University, and were kept in the animal house of the Department under conventional conditions (22 to 24 °C, 12 hours light/dark cycle with light starting at 6:00 a.m.). Four adult males or one pregnant or nursing female was put in a cage. Standard rodent chow and drinking water was given ad libitum. The facilities for keeping the animals and the electrophysiological recording set-up used in the experiments (see 2.3.) are GLP-certified (certificate No. No. 3011/48/2003, issued by the Hungarian National Institute of Pharmacy).

Of the insecticides, dimethoate (**D**) was purchased from Cheminova (Lemvig, Denmark), and cypermethrin (**C**) from Agrochemie (Budapest, Hungary). Propoxur (**P**) was kindly donated by Bayer AG (Leverkusen, Germany) and amitraz (**A**), by Hockley International Ltd (Stockport, UK). All were of more than 95 % purity. Urethane was from Reanal (Budapest, Hungary) and all other materials, from the pharmacy of the Medical Faculty. The agents were administered orally by gavage. **D**, **P** and **C** were dissolved in sunflower oil of pharmaceutical quality to 0.5 ml/kg b.w. **A** was suspended in 2.5 % methylcellulose mucus (5 ml/kg b.w.) and was, in the combinations, given separately 30 min before the other substances. The control animals received 1 mg/kg b.w. sunflower oil. Each group, treatment and control, consisted of 10 rats.

2.2. Treatment protocols

2.2.1. Acute treatment

In the acute treatment, the insecticides or their combinations were given in a single dose, 24 hours before electrophysiological recording. The doses applied were fractions of LD_{50} (Table II) determined, based on literature data (WHO 1989a,b; WHO/FAO 1989), in previous studies of the laboratory (**D**: Dési et al., 1991; **P**: Institóris et al., 2000; **C**: Institóris et al., 1999; **A**: Institóris et al., 2006). A variety of doses and combinations was tested as shown in Table III.

With the acute administration, the two main goals were to test which of the combinations are of interest for more complicated protocols (see below), and to establish the dose range to be used in later experiments. In order to reduce the number of animals used, only the double, triple and quadruple combinations were tested in doses lower than 1/5 LD_{50}.

Table II. LD_{50} values of the insecticides.

Agent	LD_{50} (mg/kg b.w.)	1/5 LD_{50} (mg/kg b.w.)	1/25 LD_{50} (mg/kg b.w.)
dimethoate (D)	460.0	92.0	18.4
propoxur (P)	85.0	17.0	3.4
cypermethrin (C)	554.0	111.0	22.2
amitraz (A)	529.0	105.8	21.2

Table III. The doses and the combinations of acute treatment.

	Fractions of LD_{50}			
Treatment groups	**1/1**	**1/5**	**1/25**	**1/100**
D	+	+		
P	+	+		
C	+	+		
A	+	+	+	+
DP (dimethoate+propoxur)		+	+	
DC (dimethoate+cypermethrin)		+	+	
DA (dimethoate+amitraz)		+	+	
PC (propoxur+cypermethrin)		+	+	
PA (propoxur+amitraz)		+	+	
CA (cypermethrin+amitraz)		+	+	
DPC (dimethoate+propoxur+cypermethrin)		+	+	+
DPA (dimethoate+propoxur+amitraz)		+	+	+
DCA (dimethoate+cypermethrin+amitraz)		+	+	+
PCA (propoxur+cypermethrin+amitraz)		+	+	+
DPCA (dimethoate+propoxur+ cypermethrin+amitraz)		+	+	+

2.2.2. Pre- and postnatal treatment

In this protocol, the insecticides were given according to Table IV. In all three protocols, the number of pups per mother rat was adjusted to 8, with 5 males maximum. The treatment scheme, based on OECD Guideline No. 414 (OECD, 2001), includes the period of organogenesis (*P protocol*), and simulates the milk-borne exposure of babies from the mother's exposure during rapid postnatal development (*P+L protocol*) and the later exposure experienced in individual life (*P+L+P protocol*).

Table IV. Pre- and postnatal treatment.

	Administration		
Protocols	**To pregnant females, daily on the 5th to 15th day of pregnancy**	**To suckling females, during the 4 weeks of lactation**	**To the male offspring, for 8 weeks after weaning**
P	+	-	-
P+L	+	+	-
P+L+P	+	+	+

In previous works, this method proved to be useful in detecting the developmental effects on the nervous system by several environmental chemicals including dichlorvos (Dési and Nagymajtényi, 1999), the combination of **D** and lead (Lengyel et al., 2005; Nagymajtényi et al., 1998), and mercury (Papp et al., 2005). These experiences, and the results of acute application in the present work, were relied upon in determining the dose, which had to be set so that it induced functional alterations in the CNS without gross general toxicity. As described above, 1/25 LD_{50} was finally chosen.

In a variation of this protocol, the pregnant female rats were treated only once, on the 5th day of pregnancy, with 1/5 LD_{50} of the same combinations which were also given in the *P+L+P* scheme.

In these treatment protocols, the starting day of pregnancy was of crucial importance. In the present work, this was determined so that the day when the so-called vaginal plug appeared in the mated females was considered as day 0.

2.3. Cortical and peripheral sensory systems of the rat. Techniques of stimulation, recording and evaluation

In the experiments described the thesis, the somatosensory, visual and auditory systems of the rat were involved.

Within the somatosensory (SS) system, the projection of the whiskers was used. For rats, the whiskers are very important sensory organs. The follicles of the whiskers (vibrissae) are innervated by the intraorbital branch of the trigeminal nerve. The central axons project to the principal nucleus in the trigeminal complex of the brainstem, which

sends projections to the medial ventral posterior nucleus of the thalamus. Thalamocortical projection terminates a special region of the somatosensory cortex, characterised by peculiar cytoarchitecture and called "barrel field" (described by Woolsey and Van der Loos, 1970; Tracey and Waite, 1995). In the experiments the barrel field was localized as area Par1 according to the stereotaxic atlas of Zilles (1984). To obtain evoked activity, a pair of needles was inserted in the whisker pad contralateral to the recording site (see below) and electric shocks (3 - 4 V, 0.05 ms) were applied at 1, 2 and 10 Hz repetition frequency.

Visual (VIS) information is transported from the retina by the axons of the retinal ganglion cells, via the optic nerve and optic tract, to the lateral geniculate nucleus of the thalamus. From this relay station the optic radiation conducts the action potentials to the primary visual cortex (Oc1B; Zilles, 1984). For visual stimulation, flashes (ca. 60 lux, 1 Hz repetition rate) of a flashbulb were directed to the contralateral eye via an optical conductor.

In the auditory (AUD) pathway, the excitation of the cochlear hair cells is transferred by the auditory nerve to the cochlear nucleus. This in turn projects – both contra- and ipsilaterally – to the other auditory nuclei of the brainstem: the superior olivary complex and inferior colliculus. Auditory information, then, goes through the pathways of the medial geniculate body of the thalamus and projects to the primary and secondary auditory fields of the cortex (Te1; Zilles, 1984). To obtain acoustic stimuli, a miniature earphone was driven by short (ca. 5 ms, 1 Hz) square pulses, and the clicks generated (40 dB) were led through the hollow ear bar into the contralateral ear of the rat.

The tail nerve of the rat is a suitable substrate for examination of reactions of the peripheral nerves. There is a pair of dorsolateral and ventrolateral mixed nerve bundles in the tail. In its compound action potential, the spike of the motor and fast sensory fibres predominates. The tail nerve was stimulated at the base of tail by electric shocks (4 - 6 V, 0.05 ms, 1 Hz) delivered via a pair of needles, and the compound action potential was recorded 50 mm distally by another pair of needle electrodes. The sensory stimuli were generated by a digital time base and stimulator unit (Experimetria Kft., Budapest). All stimuli were of just supramaximal strength (meaning that, e.g., the stimulus voltage was increased until the SS evoked response reached maximal amplitude, and ca. 5% was added) and well above background.

In acute treatment (2.2.1), recording was done 24 hours after administration of the agents, and in the other treatment schemes (2.2.2.), on the day following the last administration. For recording, the rats were anaesthetized by intraperitoneal injection of 1000 mg/kg urethane (Bowman and Rand, 1980). The animal's head was then fixed in a stereotaxic frame, the scull was exposed by a mid-sagittal cut through the head skin and removal of the muscles and connective tissues adhering to the skull, and the left hemisphere was made accessible by removal of the parietal bone by means of a mini drill. Lidocaine (10 %) was applied on the wounds and liquid paraffin on the exposed dura. The rat, wrapped in a warm cloth, was put aside for at least 30 min for recovery. After that, the rat was laid into the stereotaxic instrument of the electrophysiological apparatus. A thermostated (+36.5 °C) base plate secured the animal's normal body temperature during the recording procedure. To record spontaneous and evoked cortical activity, ball-tipped silver recording electrodes were placed on the dura over the primary SS, VIS and AUD areas as described above. A stainless steel clamp was attached to the cut skin as an indifferent electrode.

Each recording cycle began with the recording of spontaneous cortical activity (electrocorticogram, ECoG) for 6 minutes, simultaneously from the three cortical sites. From the ECoG records, the relative spectral power by frequency bands: delta, 0.5-4 Hz; theta, 4-7 Hz; alpha, 8-13 Hz; beta1, 13-20 Hz; beta2, 20-30 Hz; gamma, 30-50 Hz (Kandel and Schwartz, 1985) was determined by the NEUROSYS 1.11 software (Experimetria Kft., Budapest). From the band activity data, the so-called ECoG index (the ratio of [delta+theta]/[beta1+beta2], showing ECoG band spectrum changes as a single variable) was calculated (Dési and Nagymajtényi, 1999).

Following the recording of ECoG, sensory stimulation was applied and cortical evoked potentials (EPs) recorded. Of each modality, a series of 50 stimuli were applied, first SS, then VIS and finally AUD. The recorded evoked responses were averaged automatically, and their parameters were measured with the help of screen cursors of the software.

On the somatosensory EP, onset latency was measured between the stimulus artefact (marked by 0 in Fig. 5) and onset of the first peak (A in Fig. 5). Duration of the EP was calculated as the difference of the 0-D and 0-A times. In case of the visual and auditory EPs, onset latency and duration was measured in an analogous way.

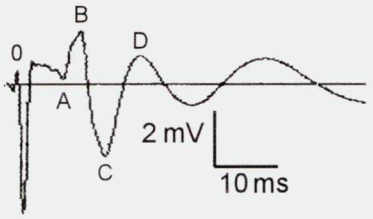

Figure 5.
Typical example of the somatosensory evoked potential with the specific measuring points. See text for details.

Finally, the tail nerve was stimulated and the compound nerve action potentials recorded. The stimuli were applied in trains of 10, first as single pulses and then in form of double pulses with varied inter-stimulus time. The tail nerve action potential had also a biphasic shape, and onset latency was defined analogously with the 0-A distance in Fig. 5. Tail nerve conduction velocity was calculated from the onset latency and the distance of the electrodes, according to Miyoshi and Goto (1973). From the double-pulse records, relative and absolute refractory periods (as described by Dési and Nagymajtényi, 1999; Anda et al., 1984) were calculated. The usefulness of relative refractory period was stressed e.g. by Anderson (1983). All recording and analysis was PC-based, using the NEUROSYS 1.11 software (Experimetria Kft). After finishing all recordings, the rats were sacrificed by an overdose of urethane.

2.4. Statistical evaluation

The primary data were compared by one-way ANOVA, separately for each cortical area, after the Kolmogorov-Smirnov normality test. Then, ECoG index, EP amplitude and EP duration were normalized, on the basis of the control mean in each area, and compared by one-way ANOVA. For post hoc analysis, LSD was used with $p<0.05$ as criterion of significance throughout.

During the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University of Szeged (based on the EU-harmonized animal welfare act of Hungary) were strictly followed

3. RESULTS

3.1 Effects on the spontaneous activity

3.1.1 Acute effects of the insecticides

When the rats received an acute 1/5 LD_{50} dose of the insecticides given alone, some clear-cut effects developed on the ECoG in 24 hours. The relative power of the delta band was significantly reduced by **D** and **A** in all three cortical areas (Fig. 6). **A** also had a reducing effect on the theta (in each area) and alpha (only in the SS area) bands. The effect of **P** was, surprisingly, opposite to that seen with **D** (although the changes were below significance), and **C** caused practically no alteration in the ECoG spectrum.

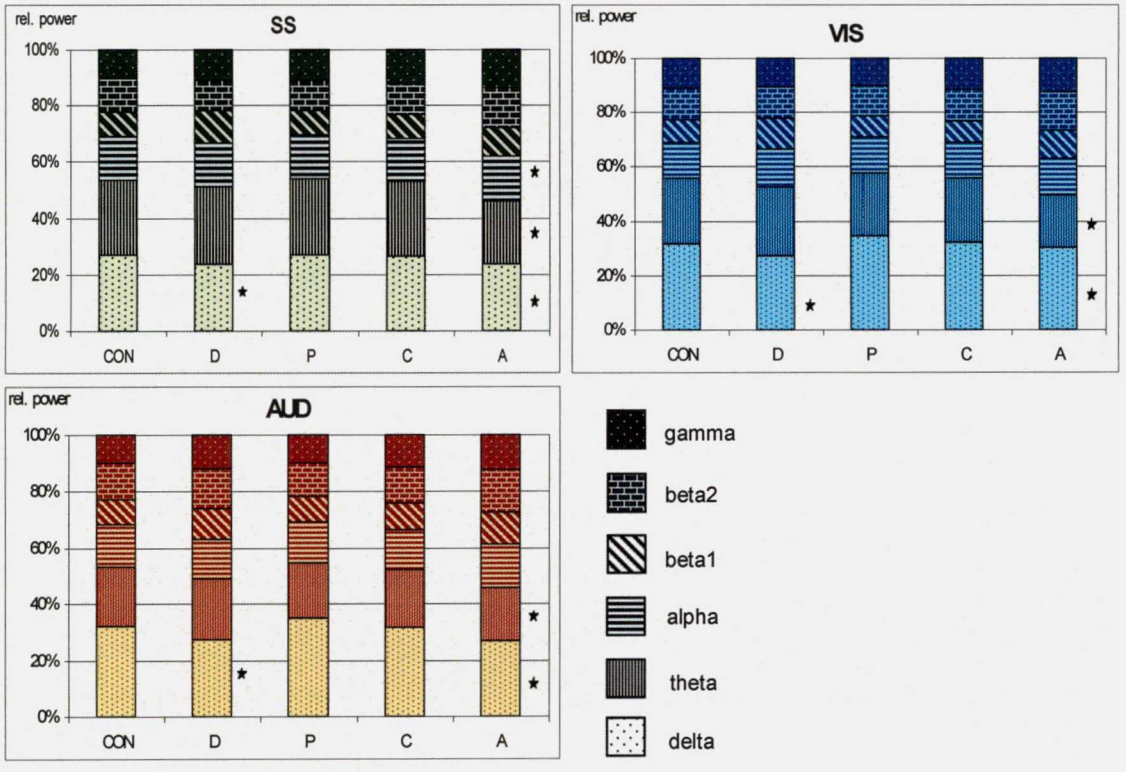

Figure 6.
Spectral distribution of the ECoG in the somatosensory (SS), visual (VIS) and auditory (AUD) cortical center after acute application of 1/5 LD_{50} of the insecticides.
Abscissa: treatment groups - CON, control; D, dimethoate; P, propoxur; C, cypermethrin; A, amitraz.
Ordinate: percents within the total ECoG power (mean, n=10). Insert: bar fill pattern for the frequency bands.
*: $p<0.05$ vs. control in the same band.

When the double combinations (see Table IV) were administered acutely, some decrease in the delta band was present in the groups containing **D** but this was not significant (Fig. 7). Significant alterations were seen, however, in the fast bands. In the SS area, the **DA** combination caused significant increase of the beta1, and **CA** in the beta1 and beta2, bands. The **DC** and **PC** combination had a similar but non-significant effect.

In the VIS area, gamma activity was increased in the groups containing **D** (significantly in **DC** and **DA**). In the **CA** group, beta1 and beta2 were increased. In each of the mentioned group, a corresponding, but non-significant decrease of the delta band power was also observed.

In the AUD area, **DA** caused significant increase in the gamma, and **CA** in the alpha and beta2 bands. The increase of delta in the **PA** group, also indicated in the SS and VIS area, was in this area significant. There was no clear difference between the trends seen in the three areas but the changes were of dissimilar strength.

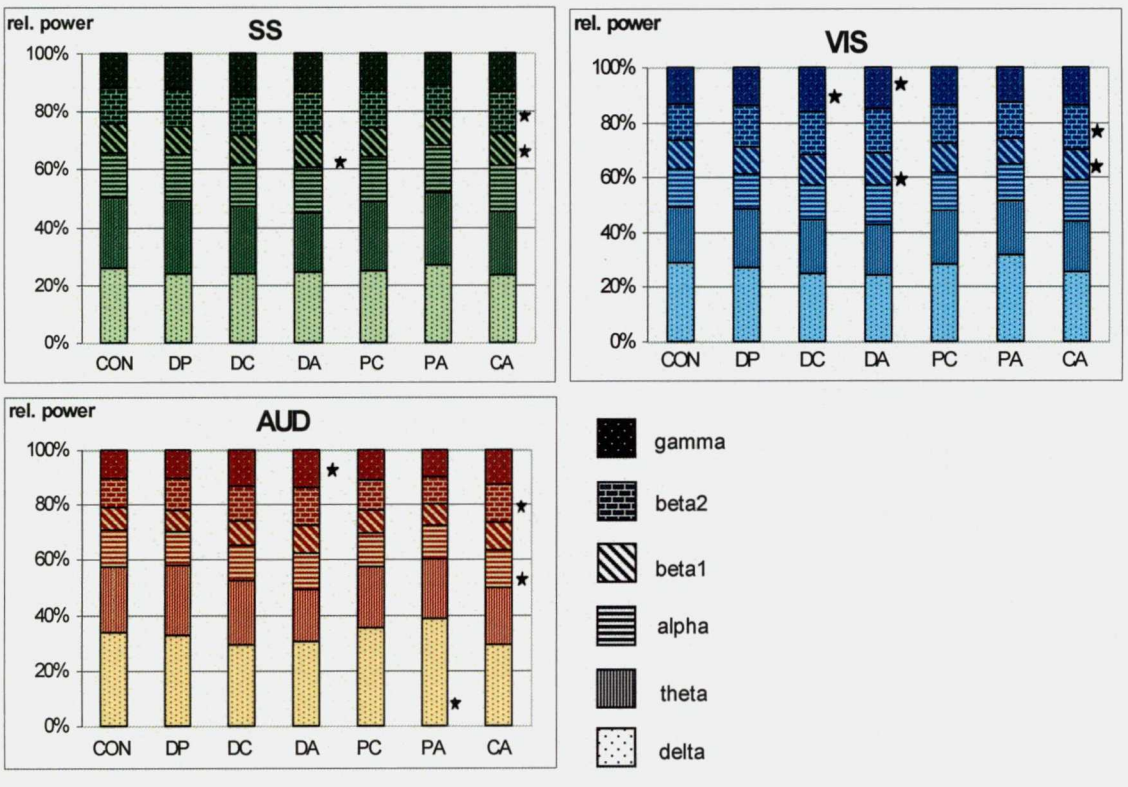

Figure 7.
Spectral distribution of the ECoG in the somatosensory (SS), visual (VIS) and auditory (AUD) cortical center after acute application of double combinations of the insecticides in 1/5 LD_{50}.
Abscissa: treatment groups - CON, control; DP, dimethoate-propoxur; DC, dimethoate-cypermethrin; DA, dimethoate-amitraz; PC, propoxur-cypermethrin; PA, propoxur-amitraz; CA, cypermethrin-amitraz.
The same display as in Fig. 6.

Acute application of the triple and quadruple combinations gave different results compared to the double combinations. For example, **DPC** caused increase of the fast band activity, unlike **DP**, **DC** or **PC**. In the SS area, beta1, beta2 and gamma were increased; in the VIS area, the same bands plus alpha; and in the AUD area, beta2 (Fig. 8). In the SS area, **DCA** had a similar but non-significant effect, and the effect of **DPCA** was also similar but even more pronounced. In the VIS and AUD area, the effect of **DCA** was significant and similar to that seen in the SS focus. In these areas, the effect of the quadruple combination was like in the SS area.

Of the lower doses, 1/100 LD_{50} had no noteworthy effect, and the effect of 1/25 LD_{50} was also below significance except **DPCA**.

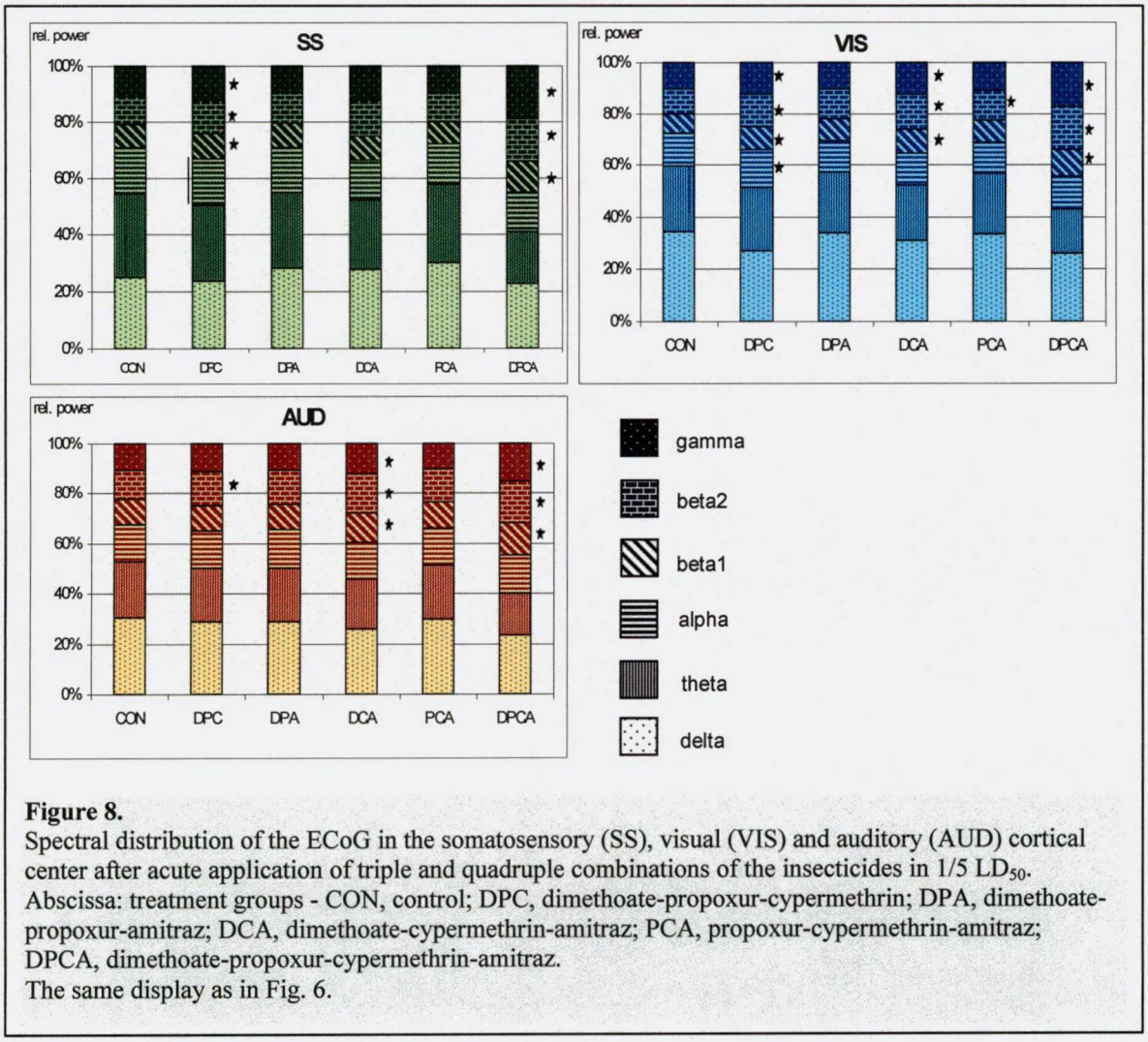

Figure 8.
Spectral distribution of the ECoG in the somatosensory (SS), visual (VIS) and auditory (AUD) cortical center after acute application of triple and quadruple combinations of the insecticides in 1/5 LD_{50}.
Abscissa: treatment groups - CON, control; DPC, dimethoate-propoxur-cypermethrin; DPA, dimethoate-propoxur-amitraz; DCA, dimethoate-cypermethrin-amitraz; PCA, propoxur-cypermethrin-amitraz; DPCA, dimethoate-propoxur-cypermethrin-amitraz.
The same display as in Fig. 6.

3.1.2. Effects of the insecticides in the ontogenesis model

In this study, first only the pregnant dams were treated with the double and triple insecticide combinations (*P protocol*, 2.2.2.), and the recordings were done in the F1 generation male rats (Fig. 9).

The alterations of the ECoG were not marked in this protocol. The only double combination with significant effect was **DC**, causing increase in the theta band in all areas and decrease in the gamma band in the VIS area. With the triple combination **DPC**, similar decrease in the fast bands (SS: beta2, VIS: gamma, AUD: beta1 and gamma) was seen. The theta band was somewhat increased in all three areas.

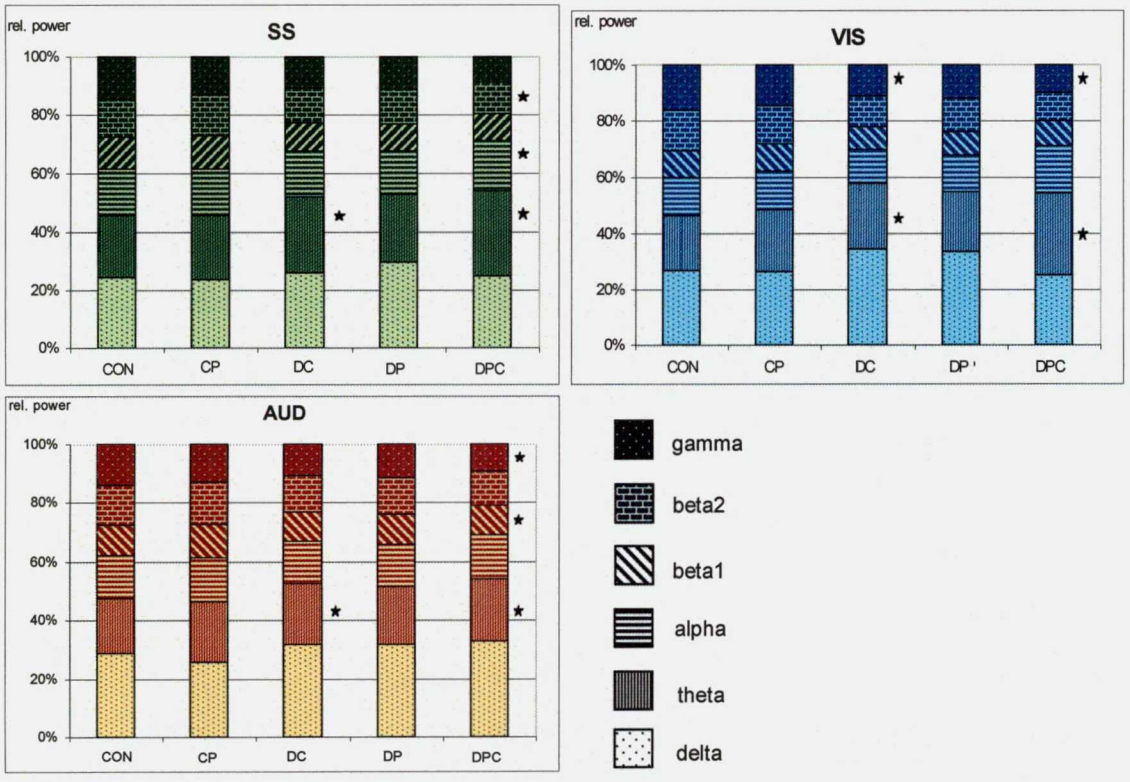

Figure 9.
Spectral distribution of the ECoG in the somatosensory (SS), visual (VIS) and auditory (AUD) cortical center after application of the double and triple combinations of the insecticides according to the *P protocol*.
Abscissa: treatment groups.
Ordinate: percents within the total ECoG power (mean, n=10). Insert: frequency bands.
*: $p<0.05$ vs. control in the same band.

In the *P+L protocol*, the dams were treated during pregnancy and lactation (see 2.2.2.) so the offspring could have had pre- and postnatal (indirect) exposure. The effects on the ECoG were partly dissimilar to that seen in the *P protocol* (Fig. 10). The effect of the double combinations was in most cases weak. **CP** caused some shift to slower frequencies but only the increase of delta band in the SS area was significant. **DC** and **DP** had an opposite effect, and decrease of the delta band was seen in all 3 areas (significant in the VIS and AUD area). Theta band decreased in the treated groups, significantly in case of **DC** and VIS. The effect of the triple combination was similar to that of the double combinations containing **D**, but was much more marked. Decrease of the delta band was significant in all areas, and of the theta band, in the VIS and AUD area; while the increase of the fast bands beta1, beta2 and bands, in the SS and AUD area was also significant.

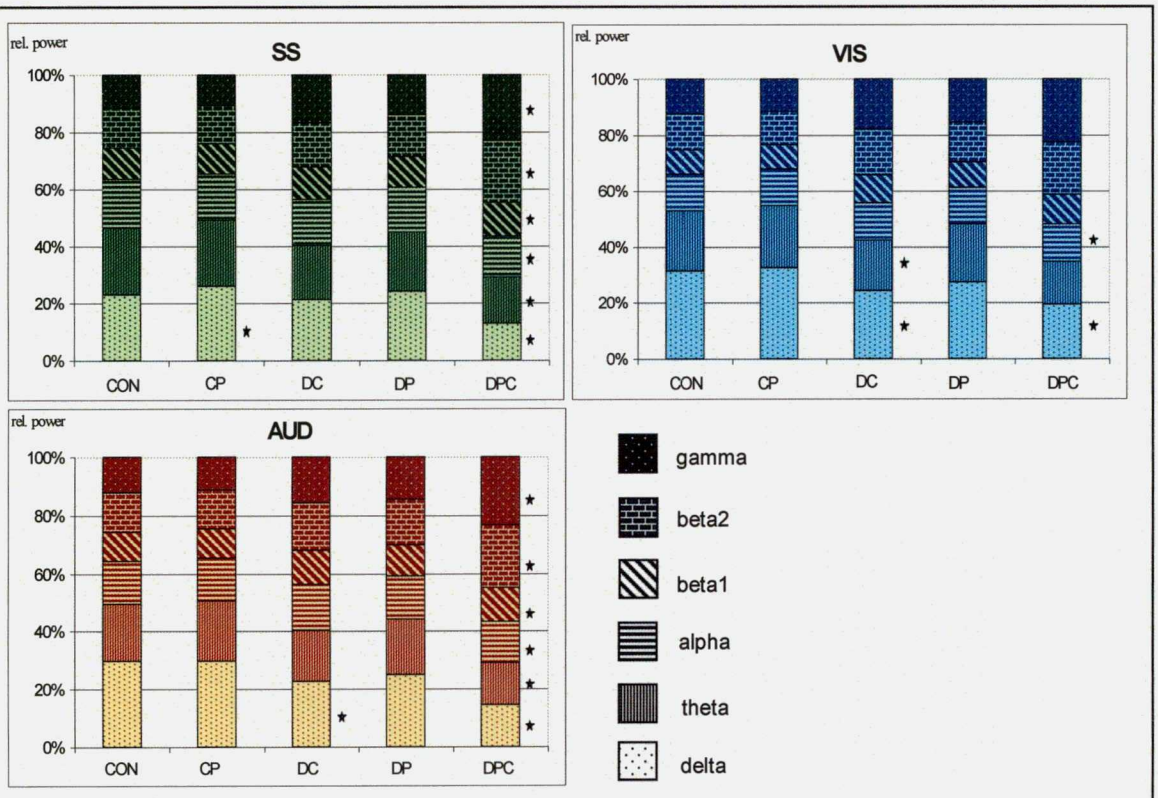

Figure 10.
Spectral distribution of the ECoG in the somatosensory (SS), visual (VIS) and auditory (AUD) cortical center after application of the double and triple combinations of the insecticides according to the *P+L protocol.*
The same display as in Fig. 9.

In the *P+L+P protocol*, the F1 males had eight weeks of direct exposure to the insecticides. In the double combinations, the general trend was increase in the fast and decrease in the slow bands (Fig. 11). With the combination **CP**, decreased alpha activity was obtained in the VIS, and increased gamma in the AUD, area. In the **DC** group, decreased delta activity in the SS, and decreased alpha in the VIS, area was significant. The effect of **DP** was similar but non-significant. The effect of the triple, **DPC**, combination was opposite to that of the double combinations: slow bands increased (significantly in the delta band in the SS and AUD, and theta band in the AUD, area). The decrease of the beta1 band was significant in the SS and AUD, and of the alpha band, in the VIS and AUD, area.

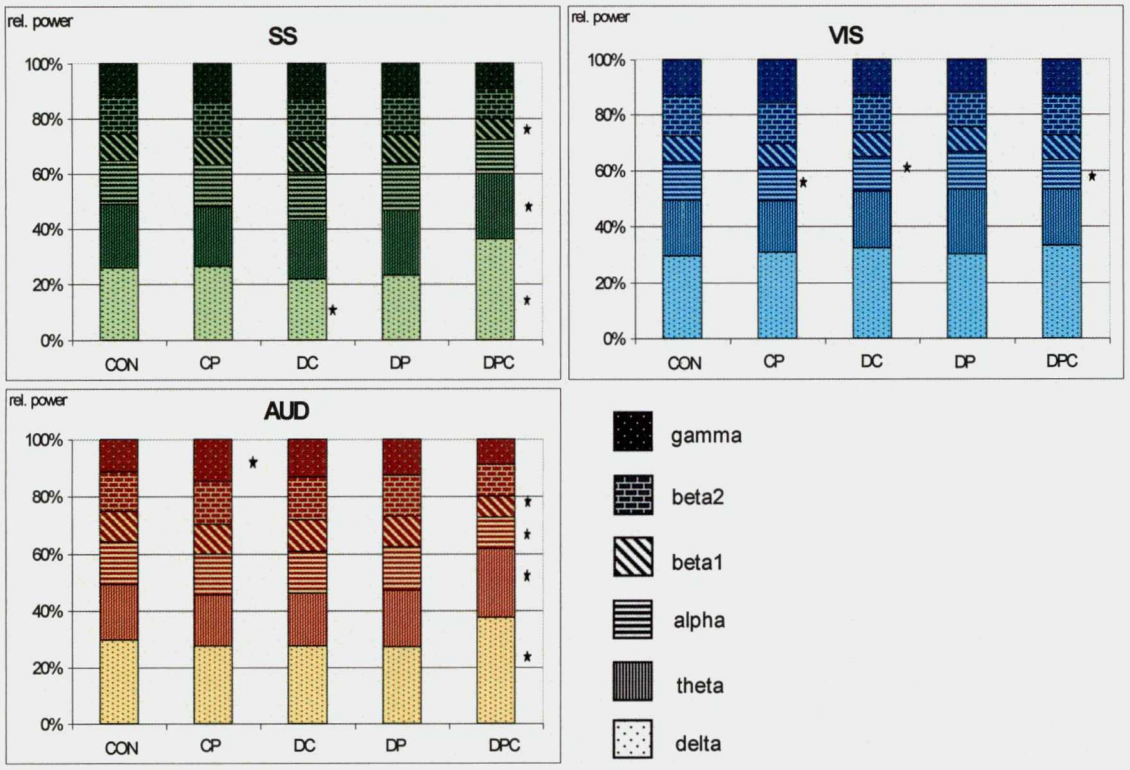

Figure 11.
Spectral distribution of the ECoG in the somatosensory (SS), visual (VIS) and auditory (AUD) cortical center after application of the double and triple combinations of the insecticides according to the *P+L+P protocol*.
The same display as in Fig. 9.

In the variation of the pre-and postnatal treatment where a single 1/5 LD_{50} dose of the insecticides and combinations was given on the 5th day of pregnancy, no noteworthy effects were found in the F1 males.

It seemed that although the spontaneous cortical electrical activity reacted sensitively on the administration of insecticides, the alterations did not always have a clear trend. It was supposed that evoked cortical activity, being a phenomenon of more circumscribed origin and better defined measurable parameters, would show more clear alterations on administration of the insecticides.

3.2 Effects on the cortical evoked potentials

3.2.1. Acute effects of the insecticides

In single acute application, the effect of the insecticides on the EPs was not very marked (Fig. 12). In the SS area, slight increase of the latency (but significant with **D** and **A**) and mild, mostly non-significant decrease of the duration was seen in the treated groups. In the VIS area, the increase of latency was significant in each treated group, and in the AUD area, in the groups treated with **P**, **C** and **A**. The duration decreased in the treated groups in the VIS area but mostly increased in the AUD area.

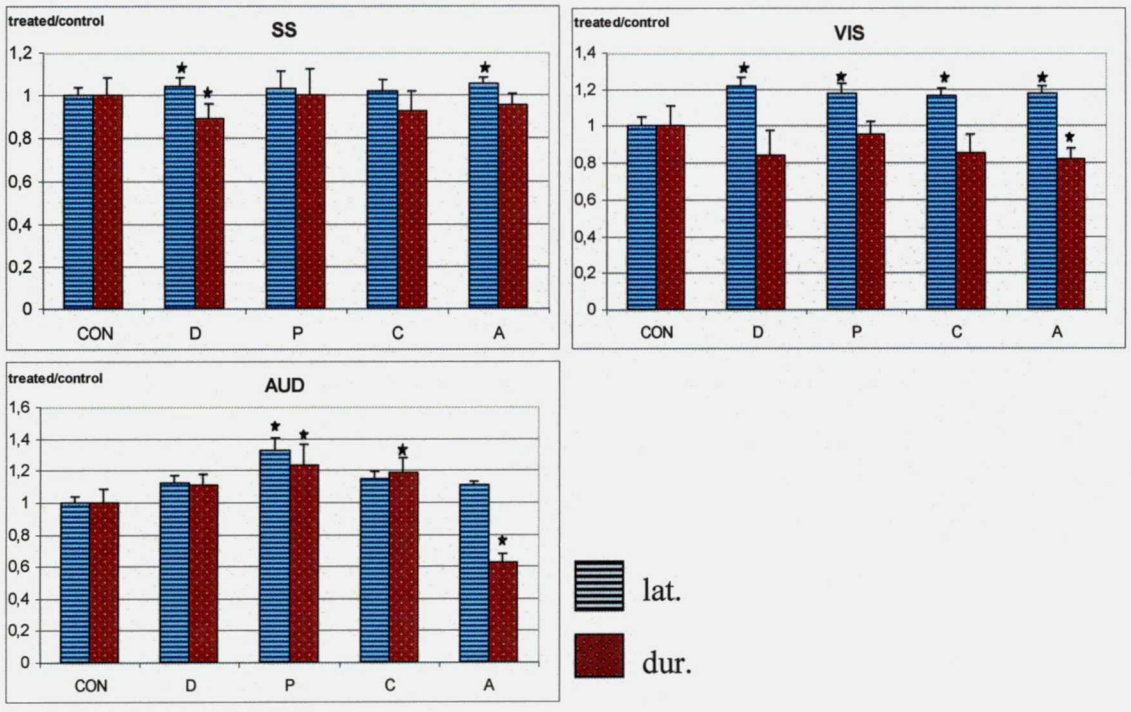

Figure 12.
Latency (lat.) and duration (dur.) of the somatosensory (SS), visual (VIS) and auditory (AUD) evoked potential after acute application of 1/5 LD_{50} of the insecticides.
All mean and SD values were normalized to the mean of control and represent thus relative changes.
Abscissa: treatment groups.
Ordinate: relative change (mean+SD, n=10)
*: $p<0.05$ vs. control.

When the agents were given in double combinations (Fig. 13), the changes of the EP parameters were similar to those obtained with single administration but more marked. All combinations containing **D** increased significantly the EP latency (except **DA** in the AUD area). On the SS and AUD EP, also the **CA** treatment had a like effect. Compared to single administration, the effect of **DC** and **CA** on the latency was stronger than that of **C** alone in the SS area, of **DA** than **A** in the VIS area, as well as of **DC** than both components and of **DP** than **D** alone in the AUD area. The alterations in the EP duration were, with a few exceptions, below significance and had no clear trend.

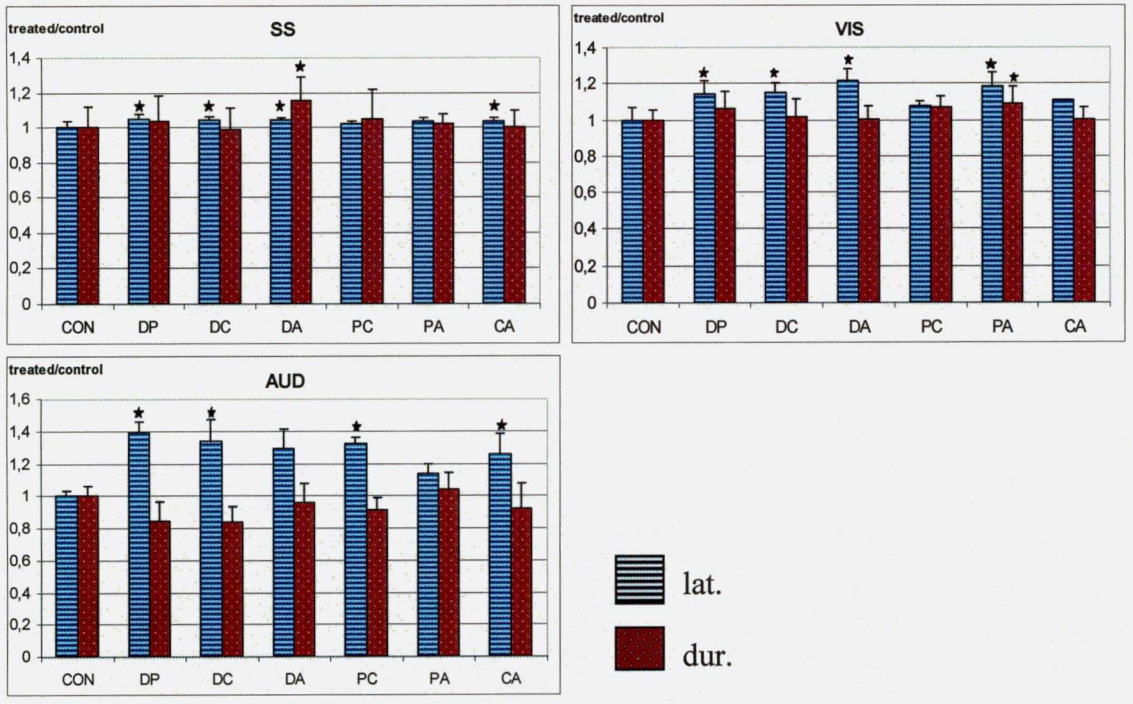

Figure 13.
Latency (lat.) and duration (dur.) of the somatosensory (SS), visual (VIS) and auditory (AUD) evoked potential after acute application of double combinations of the insecticides in 1/5 LD_{50}.
All mean and SD values were normalized to the mean of control and represent thus relative changes.
The same display as in Fig. 12.

The more complex, triple and quadruple, combinations of the insecticides had mostly marked effects on the EPs in acute application (Fig. 14). The general trend was increase of the latency, which was significant with all combinations in the SS and VIS area, and with **DPCA** in the AUD area. Other combinations also increased the AUD EP latency but significance was spoiled by too high standard deviation. On the SS EP, all combinations except **DCA** had a stronger effect than the corresponding double combinations. On the VIS EP, the exception was **DPC**. The changes of the duration had no clear trend, in spite of significant effect with some combinations.

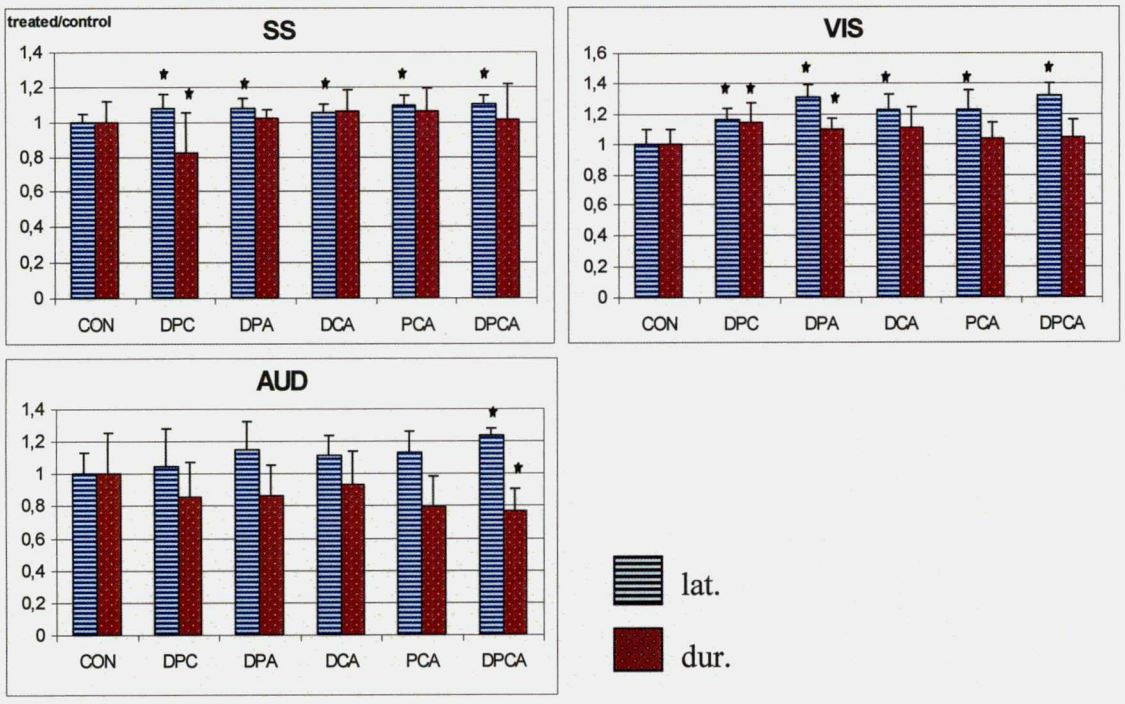

Figure 14.
Latency (lat.) and duration (dur.) of the somatosensory (SS), visual (VIS) and auditory (AUD) evoked potentials after acute application of triple and quadruple combinations of the insecticides in 1/5 LD_{50}. All mean and SD values were normalized to the mean of control and represent thus relative changes. The same display as in Fig. 12.

3.2.2. Effects of the insecticides in the ontogenesis model

When the insecticide combinations were given only to the pregnant females according to the *P protocol* (Fig. 15) there was practically no change in the latency of the SS EP. In the VIS area, all double combinations caused a significant latency increase, and in the AUD area, **DP**. In the AUD area, significant increase of the EP duration was also observed – in contrast to the acute treatment where in this area the EP duration mostly decreased.

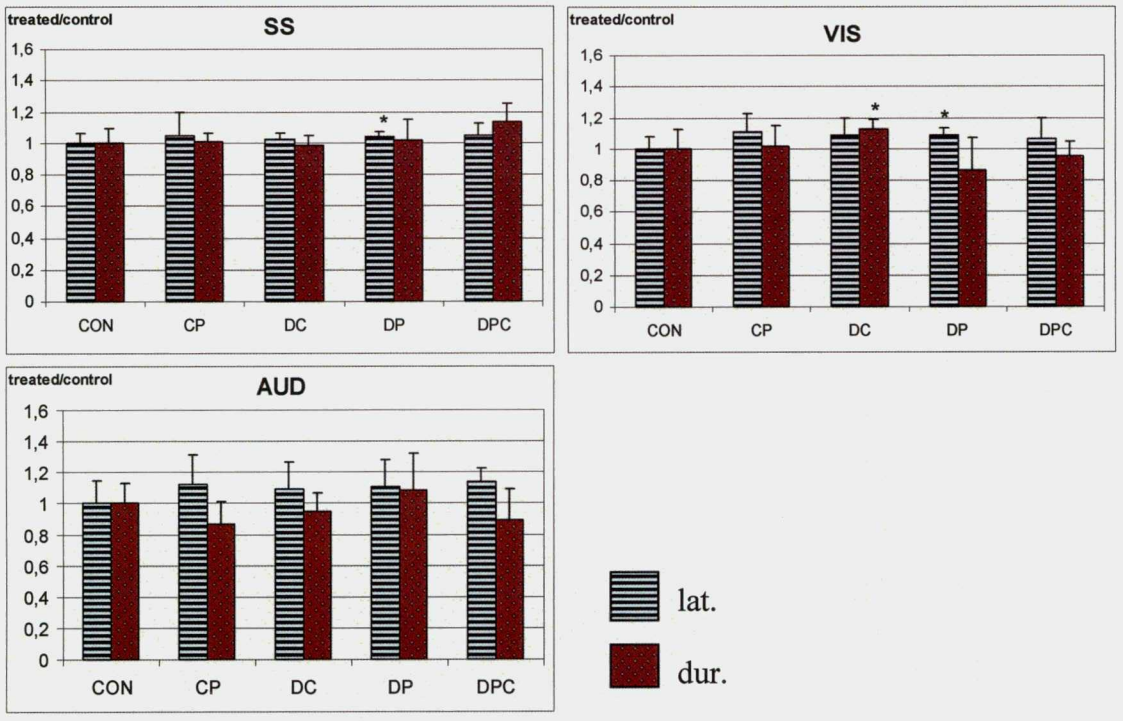

Figure 15.
Latency (lat.) and duration (dur.) of the somatosensory (SS), visual (VIS) and auditory (AUD) evoked potential after application of the double and triple combinations of the insecticides according to the *P protocol*.
All mean and SD values were normalized to the mean of control and represent thus relative changes.
Abscissa: treatment groups.
The same display as in Fig. 12.

In the rats treated according to the *P+L protocol* (Fig. 16) the changes in the EP parameters were not much more pronounced than in those treated by the *P protocol*. All the same, the latency increase in the SS area was significant in the **CP** and **DP** groups. In the VIS area, there were less significant alterations than in the *P protocol*, but in the AUD area the latency lengthening was stronger than in the *P protocol* and was significant in the **DC** group.

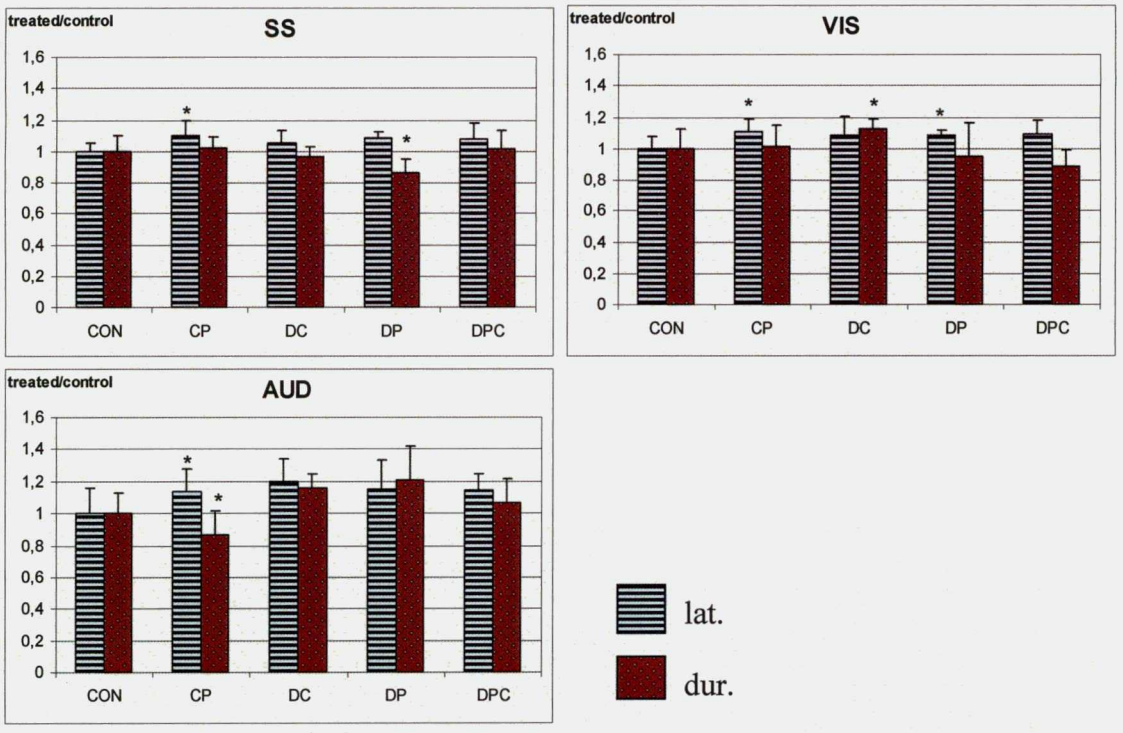

Figure 16.
Latency (lat.) and duration (dur.) of the somatosensory (SS), visual (VIS) and auditory (AUD) evoked potential after application of the double and triple combinations of the insecticides according to the *P+L protocol*.
All mean and SD values were normalized to the mean of control and represent thus relative changes.
The same display as in Fig. 12.

When the insecticides were administered according to the *P+L+P protocol* (Fig. 17), the trend of change of the EP latency was similar to hat seen with the P+L protocol but more changes were significant (which indicated a summation of the effect of pre- and postnatal exposures). On the SS EP, the latency lengthening caused by **DP** and **DPC** was stronger than in the corresponding groups in the *P+L protocol* and were significant. The same combinations induced significant latency lengthening also in the VIS area. In the AUD area, the latency increase was significant with all double combination. In this protocol, duration of the EPs was also increased in most of the treated groups, but this reached significance only in a few cases. Similarly to the ECoG, the EPs showed no noteworthy effects when a single 1/5 LD_{50} dose of the insecticides and combinations was given to the dams on the 5^{th} day of pregnancy.

3.3. Effects on the tail nerve compound action potential

Among the calculated parameters of the tail nerve, the relative refractory period (RRP) proved to be the most sensitive to the insecticides. When given acutely in 1/5 LD_{50} dose, **D**, **C** and **A** induced significant increase of the RRP (Fig. 18 I).

In case of the double combinations (Fig. 18 II), the change of the RRP was dominated by those agents, which had significant effect also in single application; that is, all treatments containing **D**, and the **CA** combination, had significant effect on the restitution of the action potential mechanism of the nerve.

The more complex combinations (Fig. 18 III) were not always more efficient. The non-significance of the effect of **DPA** might have been due partly to the high standard deviation, whereas the low effect of **PCA** was in line with the effect of the **PC** and **PA** (but not **CA**) combinations.

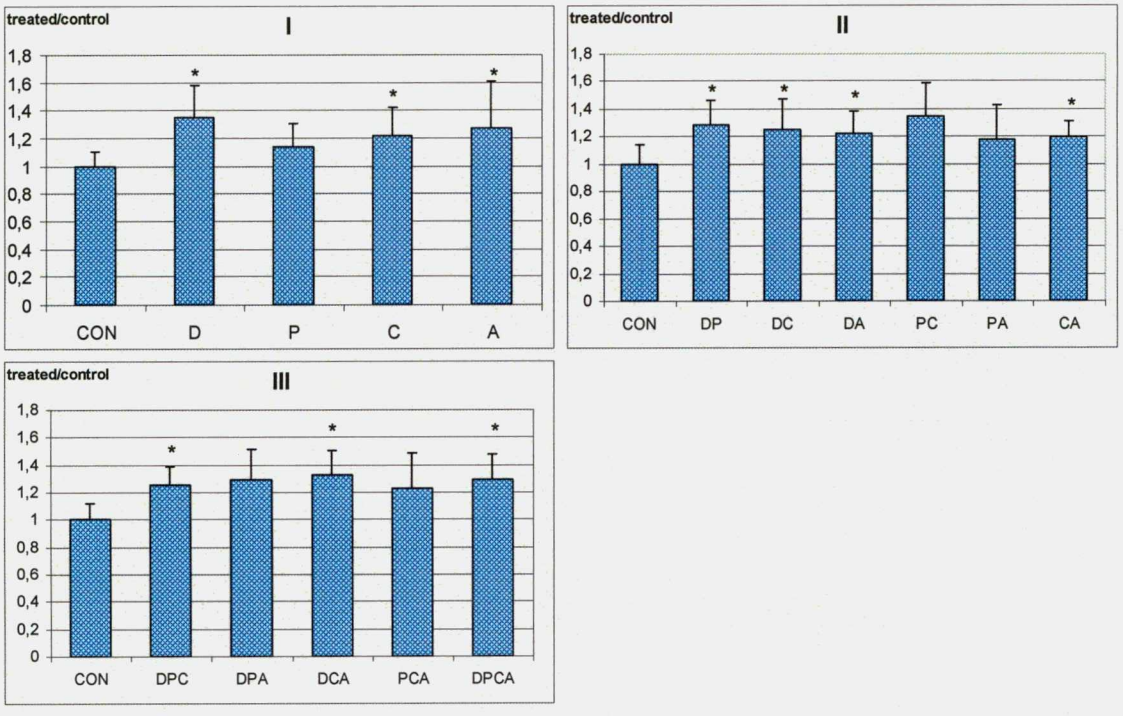

Figure 18.
Relative refractory period of the rats' tail nerve after acute application of single insecticides (I), double combinations (II), and triple and quadruple combinations (III).
All mean and DS values were normalized to the mean of control and represent thus relative changes.
Abscissa: treatment groups.
Ordinate. relative change (mean+SD).
*: $p<0.05$ vs. control.

The changes of the RRP in the developmental model are shown in Fig. 19. When the same double and triple combinations as mentioned earlier were applied according to the *P* protocol (Fig. 19 I), the effects were slight and non-significant. In case of the *P+L protocol* (Fig. 19 II), the increase of the RRP was more pronounced, and significant with the triple combination, suggesting that the extent of change depended on the total time of exposure. This trend developed further in the *P+L+P protocol* where the increase of the RRP was significant in the treated groups except in **PC** (Fig. 19 III). It was noteworthy that the combinations which caused significant RRP increase did the same also in acute application (Fig. 18).

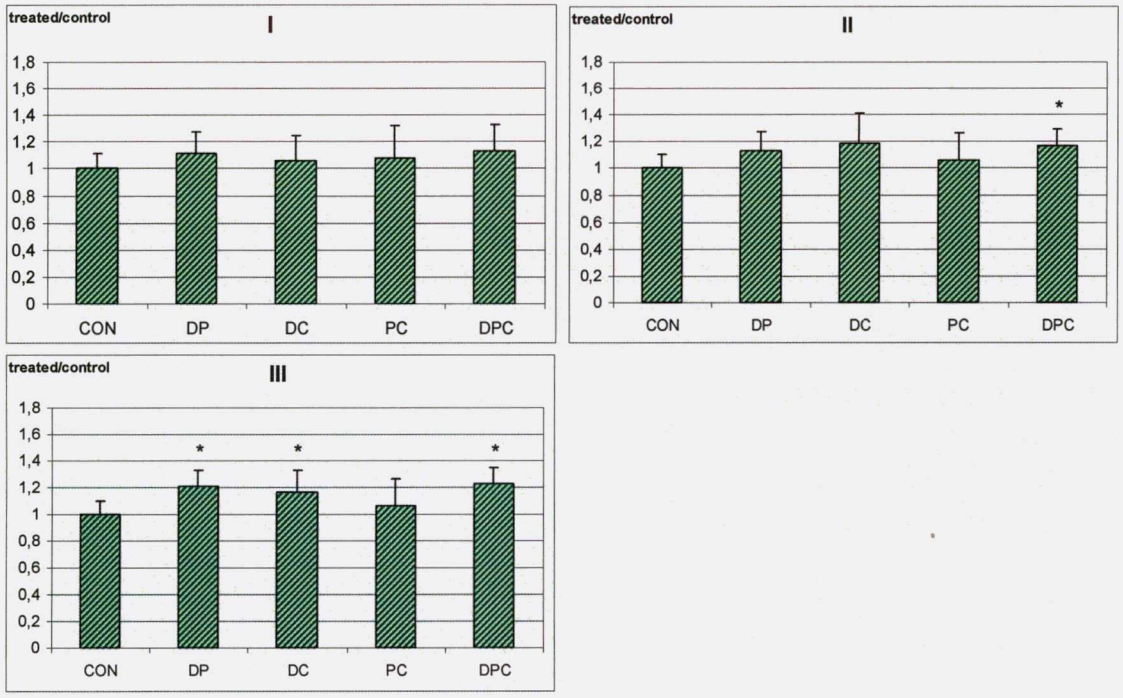

Figure 19.
Relative refractory period of the rats' tail nerve after application of double and triple insecticide combinations in the *P* (I), *P+L* (II), and *P+L+P* (III) protocol.
All mean and DS values were normalized to the mean of control and represent thus relative changes.
Abscissa: treatment groups.
Ordinate. relative change (mean+SD).
*: $p<0.05$ vs. control.

5. DISCUSSION

In the Tables V and VI, demonstrating the changes of all measured parameters of the cortical activity in all treatment schemes and insecticide combinations, certain trends become visible.

Applied acutely, **D**, **A**, and most of the combinations containing either of them caused a shift in the ECoG to higher frequencies. The most conspicuous was the decrease of relative spectral power in the delta, and increase in the beta1, bands. This effect of **D** was in line with our previous observations in which **D**, and other OPs, were given in similar doses in various timing from acute to subchronic (Dési and Nagymajtényi, 1999; Nagymajtényi et al., 1988, 1994). Most probably, this phenomenon is explained by the cholinergic mechanism of the ascending cortical activation (Metherate et al., 1992). Ascending cholinergic activation is one of the primary determinants of the cortical spontaneous activity. The basal forebrain cholinergic system, induced by stimulation of the midbrain reticular system which receives collateral input from the sensory systems, can act as a "final common pathway" to modulate cortical EEG activity (Dringenberg and Vanderwolf, 1996, 1997). It is reasonable to suppose that cholinesterase inhibitors enhance the effect of activation, and shift the ECoG spectrum to higher frequencies, similarly to the mechanism of physiological cortical activation by sensory stimuli. Such an effect has been observed in rats in earlier studies of the laboratory (e.g., Dési et al., 1991, 1994; Papp et al., 2004), and was described as a finding on exposed humans (Duffy et al., 1979; Duffy and Burchfiel, 1980; Yokoyama et al., 1998). Given the known common mechanism (Alvares, 1992; Koelle, 1992) a similar effect of **D** and the carbamate **P** could have been expected. This, however, was not seen, and also the acute effect of **DP** on the ECoG was weaker than that of **D** alone. Similar findings were reported also by Institóris et al (2004). It is hard to give an exact explanation to this difference but it is possible that the doses, equitoxic in general outcome (i.e., identical fractions of LD_{50}) were not equitoxic on a specific endpoint such as cortical activity.

Table V. Summary table of the direction and significance of the changes in the numerical data of the ECoG and the EPs recorded from rats after acute treatment.

Treatment	ECoG																		Evoked potential					
	SS						VIS						AUD						SS		VIS		AUD	
	d	t	a	b1	b2	g	d	t	a	b1	b2	g	d	t	a	b1	b2	g	lat.	dur.	lat.	dur.	lat.	dur.
D	↓	↑	↑	↑	↓	↑	↓	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑	↓	↑	↓	↑	↑
P	≈	≈	≈	≈	≈	≈	↑	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↓	↑	≈	↑	↓	↑	↑
C	↓	↓	↓	↓	↓	↑	≈	≈	↓	↑	↓	↑	↓	↓	≈	↑	↑	↑	≈	↓	↑	↓	↑	↑
A	↓	↓	↓	≈	↑	↑	↓	↓	↓	↑	↑	↑	↓	↓	↓	↑	↑	↑	↑	↓	↑	↓	↑	↓
DP	↓	↑	≈	↑	↑	↑	↓	↑	≈	↑	↑	↑	≈	↑	↓	↑	↑	≈	↑	↑	↑	↑	↑	↓
DC	↓	↑	≈	↑	↑	↑	↑	↑	↓	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑	≈	↑	↓
DA	≈	↓	↑	↑	↑	↑	↓	≈	↑	↑	↑	↑	↓	↓	≈	↑	↑	↑	↑	↑	↑	≈	↑	↓
PC	≈	≈	≈	≈	≈	≈	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	≈	↑	↑	↑	↑	↓
PA	≈	≈	≈	≈	≈	≈	↑	↓	≈	≈	≈	≈	↑	≈	↑	↓	≈	≈	↑	↑	↑	↑	↑	≈
CA	↓	↓	↑	↑	↑	↑	↓	≈	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	≈	↑	↓	↑	↓
DPC	↓	↓	≈	↑	↑	↑	↓	↓	↑	↑	↑	↑	↓	↓	≈	≈	↑	↑	↑	↓	↑	↑	↑	↓
DPA	↑	↓	↓	↑	↑	↓	↑	↓	≈	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	≈	↑	↑	↑	↓
DCA	≈	↓	↓	↑	↑	↑	↓	↓	↑	↑	↑	↑	↓	↓	≈	↑	↑	↑	↑	≈	↑	↑	↑	↓
PCA	↑	≈	↓	≈	↑	↓	≈	↓	≈	↑	↑	↑	≈	↓	≈	≈	≈	≈	↑	↑	↑	↑	↑	↓
DPCA	↓	↓	↓	↑	↑	↑	↓	↓	≈	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	≈	↑	↑	↑	↓

The ECoG bands are marked by the first letter of their corresponding names (a: alpha, etc). The arrows show the direction of change, red arrows indicate significant change, ≈: no change.

Table VI. Summary table of the direction and significance of the changes in the numerical data of the ECoG and the EPs recorded from rats after treatment during the phases of ontogenesis.

	Treatment	ECoG																	Evoked potentials					
		SS						VIS						AUD						SS		VIS		AUD	
		d	t	a	b1	b2	g	d	t	a	b1	b2	g	d	t	a	b1	b2	g	lat.	dur.	lat.	dur.	lat.	dur.
P	PC	↓	↑	↑	↑	↑	↓	≈	↑	↑	↓	↑	↓	↓	↑	↓	↑	↑	↓	↑	≈	↑	≈	↑	↓
	DC	↑	↑	↑	↓	≈	↓	↑	↑	↓	↓	↓	↓	↑	↑	↑	≈	↓	↓	≈	≈	↑	↑	↑	↓
	DP	↑	↑	↑	↓	↓	↓	↑	≈	↓	↓	↓	↓	↑	≈	↓	↓	↓	↓	↑	≈	↑	↓	↑	↑
	DPC	≈	↑	↑	↓	↓	↓	↓	↑	↑	↓	↓	↓	↑	↑	↑	↓	≈	↓	↑	↑	↑	↓	↑	↓
PL	PC	↑	≈	↓	↓	↓	↓	↑	↑	≈	↓	↓	↓	≈	↑	↑	↓	↓	↓	↑	↑	↑	≈	↑	↓
	DC	↓	↓	≈	↓	↑	↑	↓	↓	↓	↑	↑	↑	↓	↓	≈	↑	↑	↑	↑	↓	↑	↑	↑	↓
	DP	≈	↓	↓	↑	↑	↑	↓	↓	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↓	↑	↓	↑	↑
	DPC	↓	↓	↓	↓	↑	↑	↓	↓	↑	↑	↑	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↑	↑
PLP	PC	≈	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↑	↓	↓	≈	↓	↑	↑	↑	↑	↑	↑	↑	↑
	DC	↓	↓	≈	↑	↑	↑	↑	↓	↓	↓	↓	≈	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑
	DP	↓	↓	↑	≈	↓	↓	≈	↑	↓	↓	↓	↓	↓	≈	↓	↓	≈	↑	↑	↓	↑	↑	↑	↑
	DPC	↑	↑	↓	↓	↓	↓	↑	↑	↓	↓	↑	≈	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑

The same display as in Table V.

In the pre- and postnatal treatment scheme (Table VI), administration of the combinations **DP**, **DC** and **DPC** by the *P protocol* induced ECoG changes which were generally opposite to those seen in acute application. Inhibition of foetal brain AChE may negatively influence CNS development due to the developmental regulatory role of ACh (Lassiter et al., 1998). Others, however, had doubts about such a role of the cholinergic system (Mehl et al., 1994). In our case, an explanation may be the increased ACh level during foetal development, resulting in decreased sensitivity of the receptors later during life. In the rats treated according to the *P+L* and *P+L+P protocols*, the changes of the ECoG bands showed a trend similar to that seen after acute application, although there were also some characteristic differences, like the delta band in all three cortical areas in the *P+L+P protocol*.

Applied acutely, the effect of **A** on the ECoG bands was similar to that of an OP. In human poisonings, the leading CNS symptom was "depression" (Atabek et al., 2002). In adults, altered consciousness, drowsiness, hypotension and myosis were observed (Avsarogullari et al., 2006). In children, the same symptoms were seen, together with bradycardia and generalized seizures (Yilmaz and Yildizdas, 2003). None of the reports so far described EEG, or other electrophysiological, signs. In animal experiments, increased spectral power synchronization was described (Sebban et al., 1999). This seemed to contradict with our findings, decreased delta and theta power, but the relative power changes calculated by our evaluation software are not directly comparable to the absolute changes found in the mentioned paper. Moreover, Gilbert and Dyer (1988) achieved increased excitation with a dose of **A** comparable to our 1/5 LD_{50} dose (100 mg/kg). **A** does not influence cholinesterase (Moser and McPhail, 1989), its main influence on CNS regulation is by acting on the monoaminergic control. **A** inhibits monoamine oxidase (Florio et al., 1993) and acts as an alpha-2-adrenergic agonist (Costa et al., 1989). Both effects can result in increased noradrenergic activation of the cholinergic neurons (in the nu. basalis) contributing to cortical activation, what was demonstrated indeed (Cape and Jones, 1998). So it seems that ascending cholinergic cortical activation is a final common pathway in the effect of **D** and **A**, raising the possibility of synergistic effects.

C had no noteworthy effect on the spontaneous cortical activity, either alone or in combinations. The primary site of action of **C** are the Na^+ channels (Narahashi, 1996).

These are, of course, present in the CNS, as are further targets of **C** (nicotinic ACh receptors: Abassy et al., 1983; GABA receptors: Lawrence and Cassida, 1983). An effect on AChE, increased activity, was also reported (Rao and Rao, 1995). All the same, no functional effects in the CNS have been described in animal experiments applying doses relevant to human toxicology (Dési et al., 1986). Electrographic and motor convulsions, such as those described in rats by Condés-Lara et al. (1999) were evoked by doses well above the 1/5 LD_{50} used in our acute experiments. In rats treated subacutely with the components of Nurelle 550 EC, chlorpyrifos and cypermethrin, no interaction of **C** with the OP was found (Latuszynska et al., 2001). This may be in contrast to our results in the pre-and postnatal treatment protocols. In rats treated according to the *P+L protocol*, there was practically no change of ECoG band power in the **PC** group, a shift to higher frequencies in the **DC** group, and a much more expressed similar change in the **DPC** group (Table VI). Apparently, this was a synergistic interaction. In the *P+L+P protocol*, the effect of **DPC** was again quite strong, and significant on several ECoG bands in the three cortical areas, but the direction was opposite to that obtained with the relevant double combinations (**DC** and **PC**), and to that seen in the *P+L protocol*. The literature available does not permit a conclusive interpretation of this phenomenon. However, there exists a common effect of OPs and pyrethroids: induction of convulsions (Bradberry et al., 2005; Koelle, 1992). Whether this is manifested in increased high-frequency oscillations or in high-voltage, low-frequency bursts, possibly represented on the ECoG band spectrum with opposite shift of relative power, may depend on a number of factors.

The most typical change in the measured parameters of cortical EPs was increased latency. Theoretically, a sensory cortical EP represents all and any effects between the sensory organ and the cortical focus recorded, including the afferent nerves and paths, and phenomena within the cortex. An influencing mechanism, taking place within the brain, may also have an effect on the spontaneous cortical activity, and, conversely, alterations in the afferent nerves may be reflected in the functional parameters of the tail nerve.

There exists a well-known relationship between spontaneous and stimulus-evoked cortical activity, according to which evoked responses tend to be depressed under conditions of increased spontaneous activity. This relationship has been demonstrated in animals (Herz et al., 1967) and humans (Corletto et al., 1967; Hegerl et al., 1996; Rémond and Lesévre, 1967). In our results, depression of the EPs was indicated by the increased

latency, and increased spontaneous cortical activity, by the higher relative power of the fast bands. This relationship was visible in the results of acute treatment, and also in the majority of the results obtained by treatment during ontogenesis.

The effect of the insecticides on nerves was indicated by the changes of the RRP in the treated rats. This parameter has no clear relationship to the EP parameters dealt with in this thesis, but the fact that the dependence of the EP latency on the frequency of stimulation was dissimilar in control and treated rats (Lengyel et al., 2006) indicated that altered nerve conduction also may have contributed to the EP latency increase. In case of **C**, being an "axonal poison" (Ware and Whitacre, 2004), the mechanism is quite clear. Of axonal effects of OPs, a few, inconclusive data have been published so far (Anderson and Dunham, 1985; Deshpande et al., 1996).

In hygienic toxicology, the final goal is to provide better health protection of those exposed to toxic chemicals. With any pesticide, unless it is absolutely specific for the target organisms, human toxicity is always a major concern because these substances are deliberately brought to the environment in high amounts. Production, transport and application of pesticides bears the risk of occupational exposure, while population-level exposure may result from residues in foods originating from the applied chemicals, or from the environmental presence of the agents (before decay) which then may appear in, e.g., the drinking water. This represents a health risk, dealt with in most of the countries by adequate regulation. The legal limits, however, are given for single substances, without taking possible interactions into account.

Especially the use of insecticides has entailed numerous cases of poisoning, both manifest and larval. In the latter, health damage develops over a longer period of low-level exposure with no or minimal substance-specific symptoms. In such situations, early detection and follow-up of the amount of exposure and the appearance of potentially harmful effects is of great importance, and can be achieved by means of so-called biomarkers, defined as "measurements that indicate exposure to a chemical, the effect of such exposure, or susceptibility to effect (usually toxic) of such an exposure" (Hayes, 2001). In case of xenobiotics acting on the CNS, biomarkers of effect are far more practical than biomarkers of exposure (Grandjean et al., 1994), e.g. because substance concentrations and biochemical changes in available human samples (such as blood or urine) do not necessarily reflect what is going on in the CNS (Manzo et al., 1996), especially if the

exposure is multiple. This holds true also for OPs/carbamates and cholinesterase activity (Dési et al., 1991; Gralewicz et al., 1991; Rosenstock et al., 1991; Savage et al, 1988). Although research up to now has brought no conclusive evidence – association of neurological signs and insecticide exposure was demonstrated in some studies (Muttray et al., 1996) but not in many others (Engel et al., 1998; London et al., 1998) – human neuro-functional biomarkers, based on recording and analysis methods similar to those described in this Thesis, may be a better approach of the problem of early detection. This, in turn, indicates the need of further studies.

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7. APPENDIX

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