Pesticides and pesticide combinations on brain neurochemistry

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Abstract

Pesticides have been suggested to play a role in the development of many neurodegerative diseases including Parkinson's disease and Alzheimer's disease. Additionally, it has been suggested that exposure to pesticides and other environmental chemicals during the early stages of life could result in an increased vulnerability to such substances that could lead to neurotoxicity and degeneration late in life. We hypothesized that exposure to mixtures of certain pesticides could change neurotransmitter levels and cellular oxidative stress and that this would be greater in mice exposed early and later in life than mice exposed only as adults. We studied the effects of permethrin (PR) (a pyrethroid type I) and endosulfan (EN) (an organochlorine) on the levels of catecholamines, indolamines, acetylcholinesterase, lipid peroxidation and α-synuclein in the brain of mice. These pesticides have different structures but both are known to modify the kinetics of voltage-sensitive ion channels and calcium ion flux/homeostasis that could affect the release of several neurotransmitters. The study consisted of two experiments: In the first experiment, adult C57Bl/6 mice (7-9 months old) were injected, intraperitoneally, with the following treatments: EN 4.3, 2.15 mg/kg; PR 150, 15 mg/kg and their mixtures EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg. Mice were sacrificed 24 hrs after the last injection. In the second experiment, doses consisted of EN 0.7, 1.4 mg/kg, PR 1.5, 15 mg/kg and their mixtures EN 0.7 + PR 1.5 mg/kg and EN 1.4 + PR 15 mg/kg were given to juvenile mice intraperitoneally daily during a period of two weeks from postnatal day 5 to 19. Mice were then, left undisturbed with their dams. Re-challenge was performed when mice were 7-9 months old and dosages of EN 4.3, 2.15 mg/kg, PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally every other day during a period of two weeks to match the treatments when pesticide exposure was only as adults. Mice were sacrificed 24 hrs after the last injection.

The corpora striatum was extracted and analyzed by HPLC for catecholamines (dopamine, DOPAC, homovalinic acid and norepinephrine) and indolamines (serotonin and

5-HIAA). In general low doses of permethrin and endosulfan alone and in combination (EN 2.15 + PR 15 mg/kg) altered the levels of catecholamines and indolamines in both studies with adult mice and mice dosed as juveniles and re-challenged as adults. Catecholamine and indolamines levels were affected to a greater extent in the adult mice than in mice dosed as juveniles and re-challenged as adults, when compared to controls.

Acetylcholinesterase was increased under both exposure situations but again adult mice seemed to be more affected than mice dosed as juveniles and re-challenged as adults.

Because reactive oxygen species have been implicated in the development of Parkinson's disease, and are known to cause degradation of certain neurotransmitters, we monitored the levels of lipid peroxides in brain cortex as an indicator of free radical tissue damage. The peroxide levels were measured by thiobarbituric acid reactive products (TBARS). Increased levels of lipid peroxides were significant in the low dose treatment groups of the adult study. However, there seemed to be a pattern between the levels of dopamine and DOPAC in the striatum and the levels of peroxidation in cortex. The presence of dopamine metabolites appeared to be related to high levels of peroxidation within the basal ganglia and up-regulation of proteins such as α-synuclein. Western blots of α-synuclein in both experiments of the study showed intense double and triple bands that corresponded to aggregated α-synuclein. In general, when compared with controls, mice dosed as juveniles and re-challenged as adults did not alter the above parameters as much as mice dosed only as adults. Instead, the mice first dosed as juveniles seemed to develop an adaptation response to the later exposure of these pesticides.

Taking all these results into account, early exposure and re-challenge with permethrin and endosulfan in this study appeared to induce a protective response against neurochemical changes in the brain of these mice. In addition, low doses of these pesticides and the low dose combination mixture seem to exert an effect on the parameters studied.

Therefore, exposure to pesticides such as endosulfan and permethrin and their combinations could make a contribution towards the initiation or aggravation of biochemical neurodegenerative diseases such as Parkinson and Alzheimer’s diseases.

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I, Carolina Aguilar, hereby declare that I dosed the mice, collected and stored the brain parts, conducted the experiments of acetylcholinesterase, TBARS and α-synuclein and prepared the samples for HPLC determination. Actual HPLC analyses were done by Geraldine Magnin and Barbara Wise. Daniel Ward did the statistical analysis.

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Abbreviations

1R – Isomer R (from latin rectus = right)

2S – Isomer S (from latin sinistrus = left)

5-HIAA – 5 -hydroxyindolacetic acid

5-HT – Serotonin

A30P – Alanine 30 to proline

A53T – Alanine 53 to threonine

Acetyl CoA – Acetyl coenzyme A

ACGIH – American Conference of Governamental Industrial Hygienists

ACh – Acetylcholine

AChE – Acetylcholinesterase

AD – Alzheimer's disease

ANOVA- Analysis of variance

APP – β amyloid precursor

ATP – Adenine triphosphate

Aβ – β amyloid

C-1 – Carbon 1

C-3 – Carbon 3

C57Bl/6 – Specific breed of mice

CA – California

Ca^{2+} - Calcium ion

ChAT – Choline acetyltransferase

CMMID – Center for Molecular Medicine and Infectious Disease

CNS – Central nervous system

CO – Corn oil

COMT – catechol-O-methyltransferase

CS syndrome – Choreoathetosis syndrome

CYP2D6 – Cytochrome P450 enzyme debrisoquine hydroxylase

D_1 – Dopamine 1 receptors

D_2 – Dopamine 2 receptors

DA – Dopamine

DAT – Dopamine transporter

DCC – Diethyldithiocarbamate

DE – Delaware

DNA – Desoxyribonucleic acid

DOPAC – Dihydroxyphenylacetic acid

DRN – dorsal raphe nucleus

DTNB – Dithio-bis-nitrobenzoic acid

EDTA – Ethylenediaminetetraacetic acid

EN – Endosulfan

FIFRA – Federal Insecticide, Fungicide and Rodenticide Act

g – Gravity acceleration

GABA – γ-Aminobutyric acid

GP – Globus pallidus

GPe – Globus pallidus external

GPi – Globus pallidus internal

HM – High mixture

HPLC – High performance liquid chromatography

HVA – Homovalinic acid

i.p – Intraperitoneal

Ig G – Inmunoglobulin G

IL – Illinois

kDa – Kilo Dalton

LBD – Lewy body disease

LD50 – Lethal dose 50

LM – Low mixture

MAO – Monoamino oxidase

MAO-B – Monoamino oxidase type B

MD – Maryland

MDA – Malondialdehyde

mg/kg – milligrams/kilograms

min – Minutes

MPP+ - 1-methyl-4-phenylpyridinium ion

MPTP-1-methyl-4-phenyl-1, 2,3,6-tethrahydropyridine

NAC – Non-amyloid component of Alzheimer's disease

nAChRs – nicotinic acetylcholine receptors

N – Nitrogen

NACP – Non-amyloid component precursor

NADH – Nicotinamide adenine dinucleotide hydrogen

NC – North Carolina

NE – Norepinephrine

NIOSH – National Institute for Occupational Safety and Health

nm – nanometers

NRC – National Research Council

NY – New York

O.T.A – Office of Technology Assessment

p – Probability

PA – Pennsylvania

PD – Parkinson's disease

PHF's – Paired helical filaments

pmol – picomoles

PR – Permethrin

Pro – Proline

PSD – Pesticides Safety Directorate

Put – Putamen

PVDF – Polyvinyldiene difluoride

REL – Recommended exposure limit

ROS – Reactive oxygen species

rpm – Revolutions per minute

SAS – Statistical Analysis System

SDS – Sodium lauryl sulfate

SEM – Standard error of the mean

SNc – Substantia nigra pars copacta

SNr – Substantia nigra pars reticulata

SNr – Substantia nigra

STN – Subthalamic nucleus

Syn/SYN – Synuclein

T syndrome – Tremor syndrome

TBA – Thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances

TBS – Tris buffered saline

TH - Tyrosine hydroxylase

TIQs - Tetrahydroisoquinolines

TLV – Threshold limit value

TWA – Time-weighted average

U.S.A – United States of America

V – Volts

VA – Virginia

VMAT2 – Vesicular monoamine transporter

WHO – World Health Organization

β-C – Beta -Carbolines

µl – microliter

PART 1: INTRODUCTION

1. STATEMENT OF HYPOTHESIS

A pesticide is any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any insects, rodents, nematodes, fungi, or weeds or any other form of life declared to be pests…and any substance or mixture of substances intended for use as a plant regulator, defoliant or desiccant. (Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) 1947 amended 1959.

Around 1 billion pounds of pesticides are used every year in the United States of America (USA) at a cost of approximately $7.2 billion. Human exposure to pesticides is likely to occur through many different sources such as drinking water, foods, household products, and occupational exposure. Many studies have shown that most of these pesticides reach non-target species when used for what they are intended. For this reason, several studies suggest that environmental exposure to pesticides may be related to the later impairment of nervous system functions. Exposure to pesticides and other environmental chemicals during the early stages of life could result in the production of a vulnerability to such substances that could mask a chronic silent toxicity that might lead to neurotoxicity and degeneration late in life (Thiruchelvam et al 2002).

The overall goal of this study was to determine the changes in the neurotransmitter levels as well as the cellular oxidative status following exposure of neonatal and adult mice to the insecticides permethrin and endosulfan as indication of their potential contribution to neurodegenerative diseases.

1.1. Hypothesis

Exposure to permethrin, endosulfan or its mixture could result in a clinical or sub-clinical disruption of the dopaminergic or cholinergic pathways, potentially contributing to neurodegenerative diseases later in life. These diseases include Parkinson's, Alzheimer's diseases or Lewy body disease among others.

Exposure to pesticides during early life may result in a higher susceptibility to subsequent environmental exposures, potentially unmasking a silent neurotoxicity later in life.

1.2. Specific aims

- Determine the effects of permethrin, endosulfan and its mixture on the dopaminergic system. This has implications for Parkinson's disease.
- Determine the effects of permethrin, endosulfan and its mixture on the cholinergic system. This has implications for Alzheimer's disease.
- Determine the effects of permethrin, endosulfan and its mixture on cellular oxidation and α-synuclein. This has implications on the above neurodegenerative diseases.
- Determine the effects of permethrin, endosulfan and its mixture after early exposure to juveniles and following re-challenge as adults. This has implications on the above neurodegenerative diseases

2. JUSTIFICATION OF THE HYPOTHESIS

Most of the neurodegenerative diseases are not considered as a single disease entity. In fact, most of them have been proposed to have multiple etiologies such as genetics, infections, trauma, and environmental factors (Litvam 1999). The discovery of MPTP (1-methyl-4-phenyl-1,2,3,6-tethrahydropyridine) as a neurotoxicant and a model of Parkinson's disease triggered the assumption that more chemical substances could be contributing to the

development of such diseases. Many studies suggest that the risk of Parkinson's disease and other related syndromes may be greater for agricultural workers in the rural environment, possibly because of the exposure to pesticides (Di Monte et al 2002). The pesticides classified as organochlorines and pyrethroids have been proposed as some of the possible compounds likely to target the nigrostriatal pathway (Karen et al 2001). Although organochlorines are not used as frequently as in the past, pyrethroids are highly used pesticides in agricultural and military environments. Mixtures of these pesticides could have a greater influence or a synergistic effect. There have been studies with pesticides like paraquat that demonstrated its ability to cause up-regulation and aggregation of α-synuclein, a protein that is suspected of playing a role in the pathogenesis of Alzheimer's and Parkinson's diseases (Manning-Bog et al 2002). No similar studies have been done with organochlorines and pyrethroids. Thus, we propose that the organochlorine endosulfan and the pyrethroid permethrin could affect the thalamocortical pathway and have an effect on the aggregation of α-synuclein that could eventually contribute to neurodegeneration. Also, some studies suggest that exposure to defined pesticides can lead to permanent alterations of several neurotransmitters in the brain (Ahlbom et al 1995). If this is the case, early exposure to some of these pesticides could increase the probability that neurodegenerative process could occur later in life especially if re-exposure occurs. Pesticides are believed to cause a higher risk to children than to adults. Children can be exposed really early in life to pesticides through many different routes, including prenatal maternal exposure, maternal milk or contact with pesticide residues on parent's clothing. Therefore, children are believed to be able to absorb higher amounts of pesticides per body weight than adults and their immature development could make them more susceptible to neurotoxicity (O.T.A 1992).

Gaps in the literature include the following:

- Very few studies have been performed that examine early exposure and later neurochemical effects.
- The effects of pesticides on the development of the nervous system are mostly unknown.
- Few studies had been performed that include an adequate follow-up to assess the length of injury, especially on the development of chronic neurodegenerative diseases.
- There are few or no studies assessing the interactions among these pesticides at long-periods after exposure.

Therefore, due to the importance of pesticides and their consequences in human health there is reason to perform this study. Thus, the purpose of this research is to determine the effect of the pesticides permethrin, endosulfan and mixtures of these substances on neurochemical processes that could contribute to neurodegeneration. The work also provides a better understanding of early exposure on the development of these diseases later in life. The literature review provides the necessary background for understanding the purpose of this work.

PART 2: LITERATURE REVIEW

1. THE BASAL GANGLIA

The basal ganglia, the cerebral cortex and the cerebellum are the three major regions of the brain that control motor activity. The motor cortex is the area of the cerebral cortex from where the two major descending motor tracts originate. The connections among the motor cortex or sensorimotor cortex with the basal ganglia and the cerebellum influence the lower motor neurons through one of these two pathways, the pyramidal (corticospinal) system or the extra-pyramidal system. These systems control the innervation of skeletal muscles of the body and the motor nerve cells of the brain stem (Gilman et al 1996).

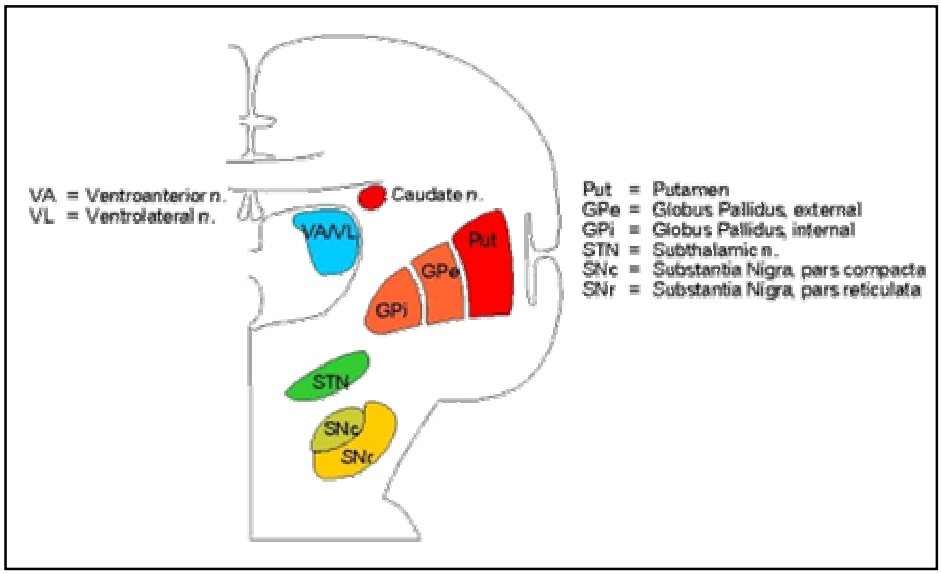
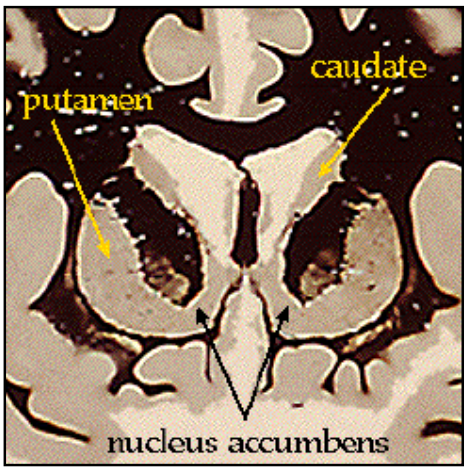


Figure 1: Structures of the basal ganglia.
http://w3.uokhsc.edu/human_physiology/Motor%20control/Basal%20Ganglia.html

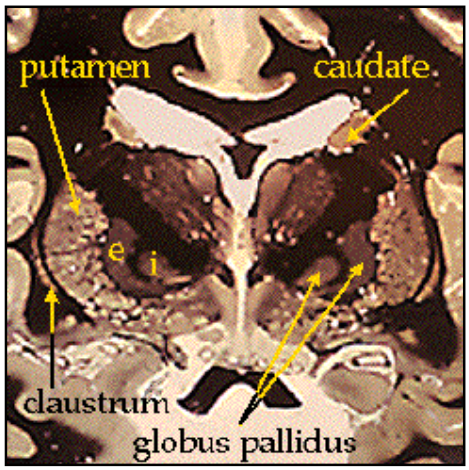
The basal ganglia are divided in two main parts: The striatum (caudate nucleus and putamen) and the paleostriatum (globus pallidum). The corpus striatum includes all the three parts together (caudate nucleus, putamen and globus pallidum) as well as two small structures

with reciprocal connections to the corpus striatum, the subthalamic nucleus (STN) and the substantia nigra (pars compacta and pars reticulata).

rostral section



middle section



caudal section:

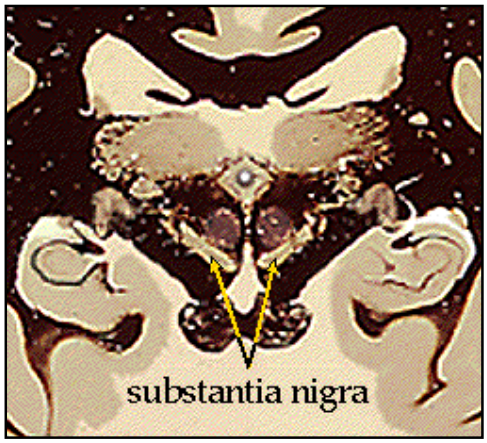


Figure 2: Different basal ganglia cerebral sections, showing the different structures forming the thalamocortical pathway. Pictures from Washington University School of Medicine, St. Louis, MO, USA. http://thalamus.wustl.edu/course/cerebell.html

In addition to these elements, which are associated with motor and associative functions, there is a ventral division of the basal ganglia (ventral striatum or nucleus accumbens, ventral pallidum and ventral tegmental area) that is related to limbic functions (Bolam et al 2000). All of these structures coordinate the nigrostriatal/basal ganglia system that

contributes to the initiation of voluntary movements and to postural adjustments (Slaughter 2001). Cortical information processed through this system is transferred to ponto-mesencephalic structures and, via thalamic nuclei, back to the frontal cortex (Kolomiets 2003).

The striatum in this system is the gateway and input structure for all the afferent connections received from the cerebral cortex, intralaminar thalamic nuclei, midbrain serotoninergic neurons and substantia nigra (Ciba Foundation Symposium 1983). The output structures consist of the globus pallidum interna and the substantia nigra pars reticulata. These innervate structures of the basal ganglia that regulate different cortical areas of the brain such as the motor, premotor and prefrontal cortical areas. Input and output structures are coupled among each other in an organized way to develop two "parallel" pathways: The direct and the indirect striato-nigral pathways. In the *direct pathway,* corticostriatal information is transmitted directly from the striatum to the output nuclei of the basal ganglia. In the *indirect pathway* corticostriatal information is transmitted indirectly to the output nuclei via a different network that interconnects the globus pallidus and the subthalamic nucleus. Activation of the direct pathway seems to be involved with the regulation of "desired motor behaviors" while the activation of the indirect pathway is thought to operate in the "suppression of undesirable motor processes" (Mink and Thach 1993).

1.1. Thalamocortical pathways of the basal ganglia. Direct and indirect pathways.

The striatum is the port of entry of all the input received from the cortical areas. The striatum thus, transmits to the output structures of the basal ganglia via two pathways:

- *Direct Inhibitory trans-striatal Pathway*. The direct pathway transmits cortical information directly from putamen neurons that contain γ-aminobutyric acid (GABA) and substance P. The information then goes to the output nuclei substance nigra pars

reticulata/globus pallidus interna (SNr/GPi). This pathway is regulated by dopamine class 1 (D_1) receptors.

- *Indirect Excitatory trans-striatal Pathway*. This pathway transmits cortical information indirectly from putamen neurons containing GABA and enkephalin to the globus pallidus pars externalis (GPe) and the subtalamic nucleus (STN) involving different connections.
- Inhibitory projection from striatum to globus pallidus externa (GPe).
- Inhibitory projection from globus pallidus externa (GPe) to subthalamic nucleus (STN).
- Excitatory projection from STN to globus pallidus interna (GPi) and substance nigra pars reticulata (SNr).
- Excitatory projection from GPi/SNr back to the cortex via thalamus.

The indirect pathway is regulated by dopamine class II receptors (D_2).

Because both of these pathways are under regulation of dopaminergic neurons from the Substantia Nigra pars compacta (SNc), progressive death of these SNc neurons unbalances the equilibrium between these pathways leading to the facilitation of the indirect pathway and understimulation of cortical areas (Bezard et al 1998). This decreased facilitation of cortical areas results in the development of motor disorders (Whichmann at al 1993).

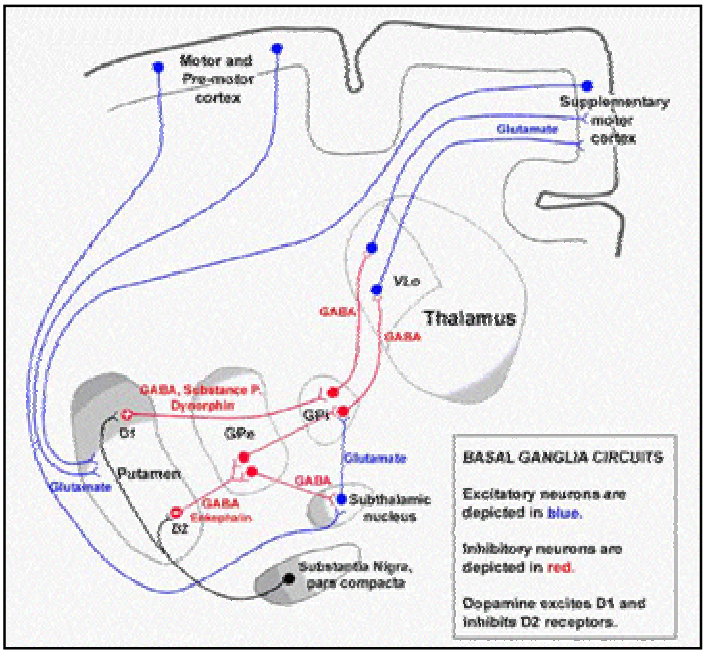


Figure 3: Circuits of the basal ganglia.
http://www.unifr.ch/biochem/DREYER/BG.html#BASAL%20GANGLIA%20CIRCUITS

In addition, lesions of the basal ganglia in humans cause:

- Disorders of the initiation of movement (akinesia).
- Difficulty continuing or stopping an ongoing movement.
- Abnormalities of muscle tone (rigidity).
- Development of involuntary movements (tremor).

Some of these symptoms are observed in many neurodegenerative maladies including Parkinson's disease, Alzheimer' s disease and Lewy body disease among others.

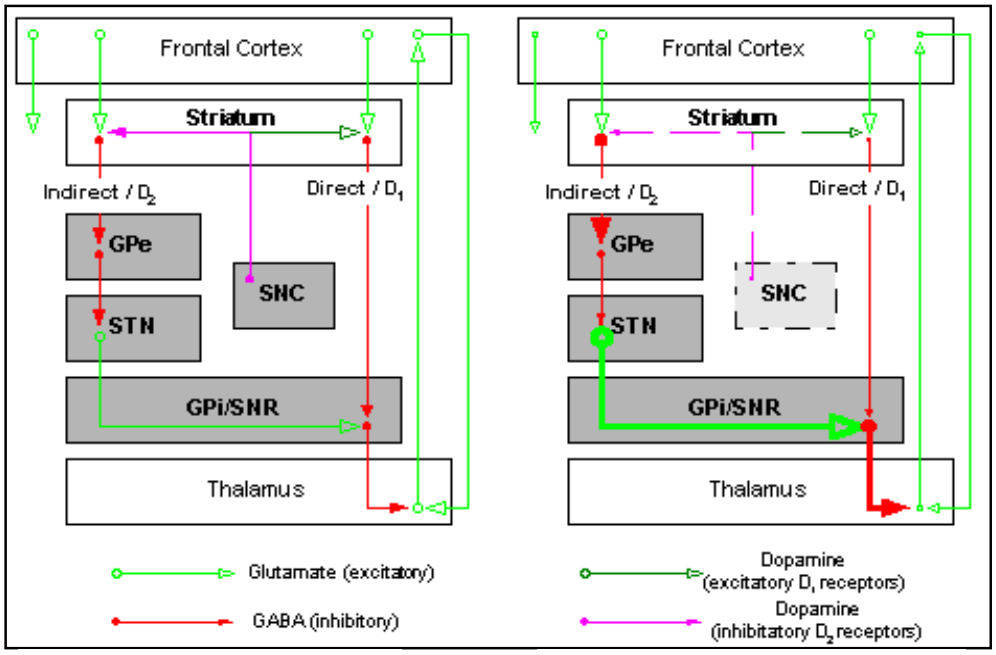
1.2. Neuroterminal components of the basal ganglia

1.2.1. Dopamine components

The main role performed by the basal ganglia is to process cortical signals coming from the cerebral cortex to the striatum. Two antagonistic pathways that involve different neuronal connections modulate all of these signals. The major types of neuronal cells involved in this regulation in the striatum are spiny projection neurons and interneurons, such as acetylcholinergic interneurons and γ-amino-butyric acid (GABA)ergic interneurons (Kemp and Powell 1971, Bolam and Bennett 2000). Spiny projection neurons have GABA as one of the main neurotransmitters and these neurons can be subdivided on the basis of where they project (Smith and Bolam 1990). There is one subpopulation that project toward the output nuclei of the basal ganglia that are regulated by dopamine D_1 receptors present on dynorphin/substance P-containing medium spiny neurons. The other subpopulation projects to the globus pallidus (GP) where regulation is influenced by dopamine D_2 receptors and enkephalin. Some studies suggest that corticostriatal terminals make synaptic contact with the heads of the spines of spiny projections giving rise to the direct and indirect pathways in the basal ganglia (Frostscher et al 1981, Dube et al 1988).

Dopamine D_1 and D_2 receptors have opposite effects on the modulation of the direct and indirect pathways in the basal ganglia (Gerfen 1992). Dopamine D_1 receptors have an excitatory effect on the direct pathway while D_2 receptors have an inhibitory effect on the indirect pathway (Slaughter et al 2001). Both effects increase the output of the thalamocortical system. In Parkinson's disease (PD), the progressive death of neurons from the SNc upsets D_1/D_2 equilibrium towards the stimulation of the indirect pathway leading to hyperexcitation of the globus pallidus and substantia nigra pars reticulata and therefore to the excitation of

GABAergic neurons (DeLong 1990). This, along with the reduction of cortical activation, leads eventually to Parkinsonism.



(A) NORMAL BRAIN (B) PARKINSONIAN BRAIN

Figure 4: Functional connectivity within the basal ganglia thalamo-cortical circuit. Direct and indirect pathways.

(A) Normal brain has normal connectivity.

(B) Parkinsonian brain with severe damage to the substantia nigra pars compacta (SNc). This results in an over inhibition of the thalamus and loss of excitatory activity controlling the fontal cortex. The death of the SNc neurons upsets the equilibrium between the direct and the indirect pathways (DeLong 1990).

http://sprojects.mmi.mcgill.ca/gait/parkinson/neurology.asp

1.2.2. Acetylcholine components

Acetylcholine (ACh) is present in less than 5% of the large spiny neurons in the striatum (Siegel et al 1999). The rest of the striatal neurons are spiny GABA (γ-aminobutyric acid) projection neurons (Marin et al 1997). However, cholinergic neurons are most likely the largest neurons within the striatum. These cholinergic interneurons have relatively short axons that are confined to the striatum and interact with medium spiny neurons that project to the globus pallidus interna and substantia nigra pars reticulata of the thalamocortical pathway (Izzo et al 1988).

These cholinergic neurons are modulated by both muscarinic and nicotinic receptors found in the striatum. Activation of the muscarinic receptors can decrease the release of the γ-aminobutyric acid (GABA) neurotransmitter, whereas activation of nicotinic receptors can result in GABA release (Siegel et al 1999).

Synthesis and termination of ACh activity are regulated by choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), respectively. Many pesticides are AChE inhibitors. Disruption of the cholinergic system with a decline in the activities of ChAT and AChE are common features of neurodegenerative processes such as Alzheimer's disease. Because ACh is one the main neurotransmitters in the cortex and because this structure is directly involved with the thalamocortical pathway, we believe that is important to study these cholinergic pathways along with the chosen pesticides.

1.2.3. Acetylcholine-dopamine interactions

Several studies suggest that the levels of dopamine and acetylcholine in the striatum are in a state of balance for normal striatal function (Stoof et al 1992). Therefore, the interaction between cholinergic and dopaminergic neurons appears to be central to the pharmacology of the basal ganglia (Lehmann et al 1983). Striatal cholinergic interneurons express D_2 DA

receptors (LeMoine et al. l990). Thus, it has been proposed that striatal acetylcholine release is under dopaminergic control through D_2 receptors (Stoof et al l992). The DA–ACh interaction hypothesis predicts that an elevation of extracellular DA levels is followed by a decrease in ACh levels due to the inhibition of ACh release as a result of the stimulation of D_2 but not D_1 receptors (Baud et al 1985, Scatton et al 1982). Recently, it has been suggested that dopamine D_1 receptor agonists are able to increase the output of striatal ACh release, whereas dopamine D_1 receptor antagonists attenuate the output of ACh (Consolo et al 1992). Therefore it has been proposed that dopamine controls the neurotransmission of acetylcholine in a reciprocally symmetric manner through stimulatory D_1 and inhibitory D_2 receptors (Di Chiara et al 1994). In addition, several histochemical studies showed that nicotinic acetylcholine receptors (nAChRs) are expressed on dopaminergic neurons in the striatum (Jones et al 2001, Colquhoun et al 1997). Stimulation of these receptors seems to modulate the release of dopamine from terminals (Fu-Ming et al 2001). Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. On the other hand, regulation of cortical ACh could influence cognitive processes and behavior. Cortical ACh can be found at the terminals of cholinergic neurons whose cell bodies are located on the basal forebrain (Materi and Semba 2001). The basal forebrain is a structure of the limbic system that shares nuclei such as the ventral striatum and the ventral pallidum with the basal ganglia. This means that this whole system regulates, in part, movement and the cognitive aspects of motor control. Although acetylcholinesterase does not appear to be related to the DA innervation of the limbic structures (Marshall et al 1983), there could be an association with the basal ganglia through the thalamocortical pathway.

1.2.4. Serotonin components

Serotonin neurons have been associated with the regulation of several types of behavior such as food intake, body temperature, neuroendocrine function, sleep, sexual activity and blood pressure (Franks et al 1990). The regulation of these many functions is due to the existence of different types of serotonin receptors. The major source of serotonin projections to many subcortical nuclei and to the hippocampus and cortex is provided by the dorsal raphe nucleus (DRN) or nucleus supra trochlearis (Olszewski and Baxter 1982). Several authors have reported damage and reduction of serotonin and its metabolites in many areas of the brain in patients with both Alzheimer's and Parkinson's diseases. This suggests considerable damage to the serotonin system in both disorders (Fahn et al 1971, Birkmayer and Riederer 1975, Bowen et al 1983).

1.3. Development of the basal ganglia and pesticide exposure of juveniles

The developing nervous system was proposed as a potential target for pesticide exposure in the 1993 National Research Council report entitled, Pesticides in the Diets of Infants and Children (NRC 1993). The nervous system has a defined schedule of development and there are specific processes of migration, proliferation and differentiation that occur throughout childhood and into adolescence. Disruption by chemical exposure at any point of this time line could adversely damage both current and subsequent processes (Moser et al 2001). In the basal ganglia, it has been suggested that the direct pathway matures earlier than the indirect pathway (Gibb et al 1997). In addition, some studies suggest that the activity of tyrosine hydroxylase (TH) is highest in early childhood and shows exponential diminution with age in the first three decades (McGeer and McGeer 1973). It has been already proven that a lesion in the basal ganglia during childhood leads to dystonia, while a lesion in the basal ganglia in adults produces parkinsonism (Swett 1975). It has been reported that perinatal

and/or adolescent exposure to the organochlorine heptachlor in rats produced an increase in dopamine transporter (DAT) binding as early as postnatal day 10 and that this variation persisted into adult life (Purkerson-Parker et al 2001). Ricceri et al (2003), demonstrated that postnatal exposure to the organophosphorous compound chlorpyrifos induced long-term behavioral alterations in the mouse and their study supported the involvement of cholinergic systems in the delayed behavioral toxicity of this pesticide. Thiruchelvam et al (2002, 2003) stated in several of their publications that exposure to pesticides during the postnatal period can produce permanent and progressive lesions of the nigrostriatal DA system, and enhance adult susceptibility to these pesticides if re-exposure occurs. In general, damage to nigrostriatal dopamine neurons and the basal ganglia in the ages of childhood to adulthood may result in clinical symptoms that depend on the state of development. A patient with a lesion in this structure might show noticeable clinical symptoms if it affects a mature region but he may be asymptomatic or only show abortive symptoms if it occurs in an immature region (Segawa 2000).

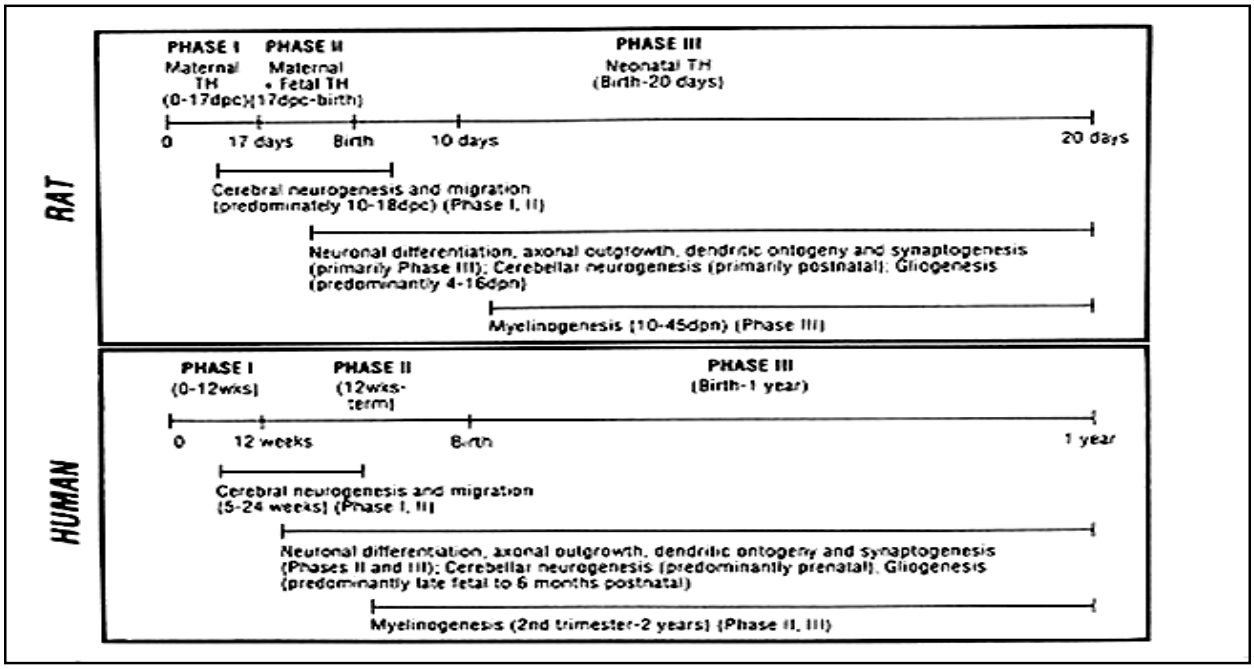


Figure 5: Comparison between the times for rat (rodent) and human brain development. http://www.thyroidmanager.org/Chapter15/15-2.htm

2. PARKINSON'S DISEASE

Parkinson's disease (PD) is one of the most common neurodegenerative diseases of aging. It is a chronic neurological disorder of likely multi-factorial origin named after Dr. James Parkinson in 1817. Its cardinal features are tremor, rigidity, bradykinesia and postural instability. These symptoms appear as the result of the death of dopaminergic neurons of the nigrostriatal system. Other symptoms observed are dementia and depression. Dementia is not unusual in Parkinson's disease and it has been reported to occur in 10-40% of cases. An important issue is the diagnosis of the disease. Many of these symptoms do not appear until there is a loss of 70-80% of dopaminergic neurons. It is generally accepted that there is a long presymptomatic phase in Parkinson's disease. The gradual manifestation of clinical symptoms could be due to compensatory mechanisms within the dopaminergic nigrostriatal neurones (Bezard 1998).

The neuropathology of PD has well defined histological identifiers. This condition is characterized by the loss of neurons of the substantia nigra that results in marked dopamine depletion in the striatum. Dopamine depletion is responsible for the production of the most motor symptomatology of the disease. Another important feature of the disease is the presence of Lewy bodies, small spherical inclusions with a granular core and a halo of radiating filaments, found in degenerating neurons.

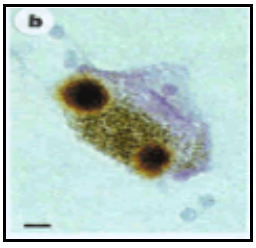


Figure 6: Pigmented nerve cell with two α-synuclein-positive Lewy bodies.
http://www.chemsoc.org/exemplarchem/entries/2003/nottingham_russell/1.html

Because Lewy bodies are not unique to PD, in order to make a diagnosis, both pathological and clinical features must be present (Langston 1995). α-Synuclein, a protein from the family of the synucleins, has been found as one of the main components of Lewy bodies.

The aggregation of this molecule has been proposed as a mechanism that could accelerate Lewy body formation leading to dopaminergic nerve terminal degeneration (Conway et al 1998). α-Synuclein is also found in Alzheimer's Disease (AD) and Lewy Body Disease (LBD). The main feature of AD is dementia, which can be found in PD as well. The neuropathological relationship among dementia, PD, Lewy bodies and AD remains unclear but of α-synuclein is common to all of them.

2.1. Etiology of Parkinson's disease

2.1.1. Aging

Parkinson's disease is commonly known as an age-related disorder because it is usually associated with individuals in their middle age or older. Changes that occur in the brain during aging, for example, include loss of weight. In fact, brain weight remains stable during maturity and starts declining after 70 years of age. A more extensive loss of neurons and therefore of brain weight occurs when normal brain is injured (Enna et al 1981). In regular aging conditions, brain weight loss, however, is small and occurs at a rate of 1.4-7% during the tenth to the eighth-decade (McGeer et al 1977, Mann et al 1982). Therefore, this is not enough itself to produce the 70-80% loss of neurons expected in the striatum of patients with PD. On the other hand, some studies report onset of the disease between 20-40 years of age (Yokochi et al 1979, 1984). For this reason, although age is an unequivocal risk for the onset of the disease, other causes such as genetics or environmental factors must trigger or accelerate this neuronal loss.

2.1.2. Genetics

Several studies in twins and siblings showed negative evidence of a genetic cause for Parkinson's disease (Ellenber et al 1995). These studies demonstrated that identical twins (monozygotic) are not likely to develop PD disease any more often than dizygotic twins or than two unrelated individuals. In addition, it has been demonstrated that familial parkinsonism is very rare (Marder et al 1996). More recently, it has been found that specific single-point gene mutations may lead to familial PD (Polymeropoulos et al 1997). Thus, cultured lymphoblastoid cells lines from patients with PD and AD were found to be especially sensitive to DNA damage from X-Rays (Robbins et al 1985). However, these mutations formed following X-ray exposure would not be inherited, unless there is damage in the DNA of the gonadal cells of the individual (Golbe 1993). Other single point gene mutations have been found in the genes encoding parkin and unbiquitin proteins that affect the gene for α–synuclein. Two mutations found in the gene encoding for α–synuclein have been associated with rare inherited forms of PD. One mutation was originally found in some Italian and Greek families (A53T); this is an alanine (Ala) 53 to threonine (Thr) substitution. The other mutation was found in a German family (A30P), which is an Ala-30 to proline (Pro) substitution (El-Agnaf et al 2002). Because α-synuclein is believed to be a major compoment of Lewy bodies, aggregation of this protein plays an important role in the development of those neurodegenerative diseases in which Lewy bodies are formed. Another important molecular genetic phenomenon is that mitochondrial genetic defects can cause DNA rearrangement during development. This genetic effect could lead to an imperfection in the complex I of the respiratory chain, increasing the toxicity of several environmental compounds such as MPTP which targets NADH-ubiquinone oxidoreductase, or complex I.

At present, genetics cannot be said to be responsible for the production of diseases such as PD and AD. Future cloning of the responsible genes in the large PD families however, could explain genetic contribution to these diseases.

2.1.3. Metabolic factors

Several metabolic irregularities have been associated with the development of PD. It has been suggested that asymptomatic enzyme dysfunction can enhance the susceptibility to the production of endogenous and exogenous toxins (Koller et al 1990). Abnormalities in the cytochrome P540 system have been proposed as one of the most common causes leading to detoxification deficiency. Impaired function of the hepatic cytochrome P450 enzyme debrisoquine hydroxylase (CYP2D6) seems to be increased in persons with PD, especially in people presenting a young onset of the disease (Barbeau A 1969). This enzyme is expressed in the substantia nigra of dopaminergic neurons and it could be implicated in the detoxification of MPTP (Gilham et al 1997) since it appears to function as a low-affinity dopamine reuptake system (Niznik et al 1990). Therefore, abnormalities in detoxification systems could trigger inability to cope with the neutralization of toxins of internal or external origin. Extra neuroprotective agents such as antioxidants could help in this task.

2.1.4. Environmental factors

- **MPTP**

Parkinson's disease is a chronic progressive neurodegenerative disease. Many authors suggest that the disease starts 20-30 years before the first symptom appears (Ellenberg et al 1995). Therefore, the late onset of the disease could be due to the chronic and progressive exposure to low doses of neurotoxicants that would slowly damage the neuronal terminals. It is also possible that limited contact could occur early in life and produce a latent toxicity that

could arise later in life (McGeer et al 1977). The discovery in 1983 that exposure to 1-methyl-4-phenyl-1,2,3,6-tethrahydropyridine (MPTP) damaged the substantia nigra and induced irreversible parkinsonism in humans, is an unequivocal demonstration of neurochemical toxicity. MPTP, after crossing the blood-brain barrier, is transformed by the monoamino oxidase type B (MAO-B) to its oxidized form 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ can then have access to dopaminergic neurons because it enters the cells though the dopamine transporter (DAT). Here it accumulates and concentrates in synaptic vesicles and mitochondria via the vesicular monoamine transporter (VMAT2) (Daniels and Reinhard 1988). Once in the mitochondria, MPP+ inhibits complex I of the mitochondrial chain and, therefore, decreases the levels of energy in the form of ATP. This lack of energy affects nigrostriatal neurons causing cell death. In addition, the interaction of MPP and complex I leads to free radical production through complex II. This, along with the lack of cell energy, increases the inability of the neurons to cope with oxidative damage and apoptosis occurs (Di Monte et al 1992). Free radical production could also be caused by the release and metabolic transformation of dopamine into 6-hydroxydopamine and hydrogen peroxide.

Additionally, MPTP could cause neurotoxicity through the production by nitric oxide synthase of nitric oxide. Nitric oxide has been suggested to contribute to nigrostriatal injury when it reacts with superoxide anion generating peroxynitrile, which is able to cause neuronal death (Lipton et al 1993). Several classes of heretocyclic molecules have been proposed as MPTP analogs. Substances such as tetrahydroisoquinolines (TIQs) and beta-carbolines (β-C) have been reported to cause nigrostriatal damage, showing a similar mechanism of action as MPTP (Ellenberg et al 1995). TIQs and β-Cs are naturally occurring alkaloid compounds present in a variety of foods (Makino et al 1988). They can also be produced in the brain through an array of different reactions involving biogenic amines such as condensation of the dopamine amine group with the carbonyl groups of α-ketoacids and aldehydes (Collins and

Origitano 1983). Both compounds have been found in the brain and cerebrospinal fluid of patients with PD (Matsubara et al 1995). In addition, the herbicide paraquat has a structural relationship with MPP. Paraquat's primary target is the lung where it causes a free-radical induced damage when it reacts with oxygen. However, this reaction can also occur in other tissues including the brain. Additionally, paraquat causes up-regulation of the genetic machinery responsible for the production of the protein α–synuclein, which is a major component of Lewy bodies. Thiruchelvam et al (2000) demonstrated synergistic dopaminergic toxicity when paraquat was administered in combination with maneb to C57Bl/6 mice. This indicated that, like paraquat, many other compounds with pesticide action could contribute to the onset of neurodegenerative processes.

- **Pesticides**

Pesticides have contributed to the improvement of our food supply and quality of our environment by controlling harmful pests and insects. However, many pesticides can be expected to reach non-target organisms. Toxic chemicals are likely to induce functional and morphological changes in the nervous system, including motor and sensory alterations, which affect integrative capabilities such as learning and memory. Many pesticides, such as organophosphorous compounds, carbamates, pyerthroids and organochlorines, have been proposed as some of the substances able to cause this kind of neurotoxicity. Although exposure to low doses of these pesticides is common, there are approximately 2.7 million agricultural workers and 1.3 million certified pesticide applicators in the United States that are at a higher risk (O.T.A. 1992). The severity of the toxicity caused by these chemicals depends on the amount absorbed and the inherent toxicity of the product.

The most frequent routes of exposure to pesticide are skin absorption and inhalation. Many studies suggest a link between exposure to pesticides and the development of

neurodegeneration. The strongest evidence of this association comes from the studies of Barbeau et al (1987). This investigation found a correlation of 0.97 between region-specific Parkinson's disease and the exposure of pesticide in these regions of Canada. Another study investigated the relationship between young onset Parkinson's disease and rural life. From a cohort of 557 patients that included 21 young-onset PD patients, 19 had been born and had lived their first 15 years in rural communities (Rajput et al 1986). Among commonly used pesticides implicated in PD is the herbicide paraquat. Paraquat has a chemical structure similar to that of MPTP. Several studies suggested that paraquat was able to reach and damage the nigrostriatal system in the mouse (Shimizu et al 2001). In addition, paraquat was reported to cause up-regulation and aggregate formation of α-synuclein, which is a major component of Lewy bodies observed in Parkinson's and Alzheimer's diseases (Manning-Bog 2002). Many other pesticides have been suggested to contribute to the development of such diseases.

Organochlorines such as dieldrin or heptachlor and pyrethroids such as permethrin have been associated with nigrostriatal damage and alteration of the dopamine transporter DAT, contributing towards these diseases (Miller at al 1999, Bloomquist 2002.).

Another important fact to take into account is interaction among pesticides. Many pesticides are not toxic alone but when associated with other compounds present an additive or synergistic effect. This is the case of diethyldithiocarbamate (DCC). When DCC is administered prior to MPTP, its neurotoxic properties increase enormously (Corsini et al 1985). Another important study of pesticides mixtures reported that there was 37% depletion of DA in mouse brain following exposure of paraquat and maneb in combination (Thiruchelvam et al 2002).

All these findings suggest that there are many substances that are potentially able to cause or contribute to neurodegeneration. Therefore, this research attempted to clarify some mechanisms associated with the production of this neurodegeneration.

2.2. Pesticides used in this investigation

Pesticides can be classified in many different ways, but usually they have been classified according to their biochemical action. Permethrin and endosulfan are synthetic organic insecticides and interrupt the nervous system by affecting ion permeability, leading to interference with axonal transmission (Abou-Donia 1992). Other forms of pesticide toxicity are: acetylcholinesterase (AChE) inhibition, interference with receptors of the nervous system or axon degeneration and myelin damage.

2.2.1. Endosulfan

Endosulfan is an organochlorine cyclodiene insecticide still used in the United States against a variety of crop pests that infect deciduous fruits, nuts, cotton, vegetables, tea and coffee.

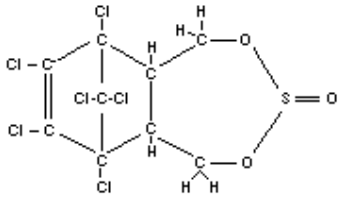


Figure 7: Chemical structure of endosulfan.
http://www.inchem.org/documents/jmpr/jmpmono/v065pr23.htm

Chlorinated hydrocarbon insecticides can be divided into the following sub-groups: (Abou-Donia 1992).

- DDT-Type compounds.
- Hexachlorocyclohexanes.
- Chlorocyclodienes.
- Mirex and Kepone
- Terpene Polychlorinated Insecticides.

Endosulfan is a brown crystalline chlorocyclodiene with a terpene odor. It consists of two isomers, alpha and beta, that are formulated in a ratio of approximately 70:30.

Technical endosulfan (6, 7, 8, 9, 10 10-hexachloro-1, 5, 5a, 6, 9, 9a, hexahydro 6, 9-methano- 2, 4, 3-benzodioxathiepin, 3-oxide) is used as a broad-spectrum contact and stomach insecticide mainly in agriculture and, depending on the country, in public health (WHO 1984). Both isomers are fairly resistant to photo-degradation and persistent in soil. Endosulfan has low or intermediate volatility in tropical regions with high temperatures. Endosulfan and similar cyclodiene pesticides evaporate and tend to be uniformly distributed through all parts of the environment. Due to their persistence, organochlorines accumulate in food chains. Once in the alimentary chain, these compounds and their metabolites bio-concentrate in the adipose tissue of different organisms. These tissues act as a reservoir from where they are slowly released and distributed to other systems in the body. One destination is milk lipids, so organochlorines can be transferred to babies during lactation (Waliszewski et al 2001). Because of its use in tobacco farming, smoking could be considered as an additional source of exposure.

Organochlorines act as central nervous system stimulants and acute intoxication could lead to hyperactivity, irritability, disorientation, tremors and convulsions followed by death (Medepalli et al 1994). Children may be especially sensitive to brain and nerve damage and may undergo long term behavioral and learning disabilities as a consequence of exposure (Echobichon and Joy 1982). Endosulfan and its main metabolite, endosulfan sulfate, rapidly penetrate into the brain. Of the two isomers, the alpha isomer seems to have the more toxic effect (Paul and Balasubramaniam 1997).

The mode of action of these insecticides in the brain is interference with axonal transmission of nerve impulses resulting in a disruption of functions of the central nervous system (CNS) (WHO 1984).

Disruption in the CNS is likely to be caused through three main mechanisms: (WHO 1984).

- Slowing the closure of sodium channels. This leads to prolonged action potentials by repetitive discharges.
- Blocking of GABA-chlorine channels ($GABA_A$), leading to hyper-excitation.
- Involvement of the serotonic system, probably inhibiting serotonin (5-HT) uptake.

Endosulfan is considered to be a moderately to highly toxic insecticide according to the Hodge and Sterner scale (1956). The oral LD_{50} is 18 to 160 mg/kg in rats and 7.4 mg/kg in mice (NIOSH 1993, National Institute for Occupational Safety and Health as noted on the extoxnet webpage). Acute intoxication can happen after ingestion, inhalation or absorption through the skin.

Some exposure limits for endosulfan are addressed by the National Institute for Occupational Safety and Health (NIOSH) and the American Conference of Governmental Industrial Hygienists (ACGIH):

- NIOSH, recommended exposure limit (REL): 0.1 mg/m^3 as a Time-Weighted Average (TWA) for up to a 10-hr workday and a 40-hr workweek.
- ACGIH, threshold limit value (TLV): 0.1 mg/m^3 as a TWA for a normal 8-hr workday and a 40-hr workweek.

Both bear a "skin" notation indicating that skin absorption exposure occurs.

2.2.2. Permethrin

Permethrin is a pyrethroid insecticide. Pyrethroids are synthetic compounds derived structurally from the six natural pyrethrins that constitute the pyrethrum extract of the *Chrysanthemum* flowers (Soderlund et al 2002). Natural pyrethrums were used since 1800. In

the past 50 years, the chemical structure of the pyrethrins has been constantly modified in order to produce new insecticides both more effective and with long-lasting action. Because pyrethroids are more selectively toxic to insects than mammals, they are extensively used and they are considered potent and safe pesticides.

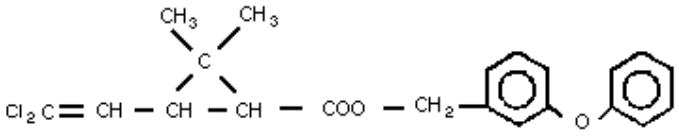


Figure 8: Chemical structure of permethrin.
http://www.inchem.org/documents/hsg/hsg/hsg033.htm

The acute oral LD_{50} of permethin is 490 mg/kg in the mouse (Casida 1983), but pyrethroids have different insecticidal activity depending on their structure. They usually contain an acid and an alcohol moiety. In many pyrethroid molecules, the presence of two pairs of diasteroisomers at the C-1 and C-3 carbons has shown that at the C-1 in the cyclopropane, only the 1R-*trans* chrisanthemic acid moiety presented insecticidal activity.

Also, it has been shown that the mammal neurotoxicity of pyrethroids depends on the stereochemical configuration at the C-1 of the cyclopropane or the same position in compounds lacking the cyclopropanecarboxylate moiety. Thus, only esters of the 1R cyclopropanecarboxylates and the isosteric 2S isomers of the non-cyclopropane acids are neurotoxic (Soderlund et al 2002). In addition, pyrethroids with an absolute 1R-*cis* configuration at C-3 of the cyclopropanecarboxylate esters of primary alcohols are both insecticidal and toxic to mammals. On the other hand, the pyrethroids with a 1R-*trans* configuration lack acute toxicity to mammals (Soderlund et al 2002). Finally, the presence of a α-cyano substituent in S configuration in the 3-phenoxybenzyl alcohol moiety increases acute neurotoxicity.

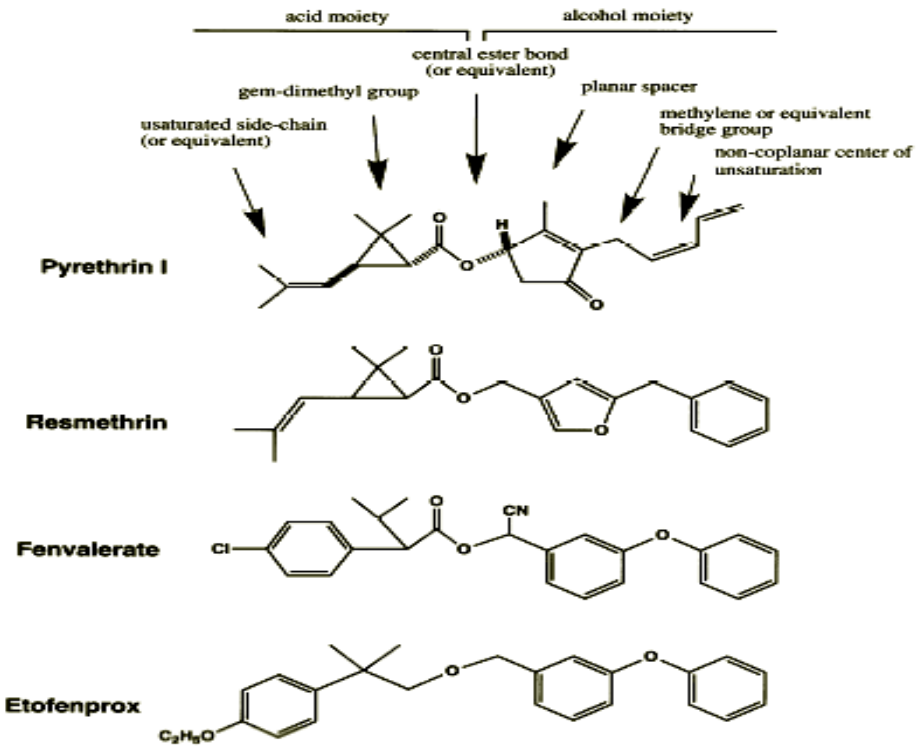


Figure 9: Chemical structures of different pyrethroids.
Soderlund et al 2002

Pyrethroids are classified in two groups based on chemical structure and signs of toxicity (Abou-Donia 1992):

- *Type I pyrethroids*:

  No cyano group.

  Produce the T (Tremor) syndrome of intoxication (Lawrence and Casida 1983).

  5-10 times more potent than Type II pyrethroids.

  Permethrin is an example of a Type I pyrethroid.

- *Type II pyerthroids*:

  Alpha-cyano group in the alcohol moiety.

Produce CS syndrome (choreoathetosis and salivation).

May affect GABA receptors in addition to the sodium channels affected by all insecticidal pyrethroids.

The mechanism of action for pyrethroids resides in the modification of the gating kinetics of fast sodium channels. This mediates the sodium permeability of the membrane and the production of the action potential. Nevertheless, there are other targets affected by the action of pyrethroids. These include potassium and chloride channels as well as acetylcholine receptors (Ford et al 1986).

- Type I pyrethroids prolong the opening of sodium channels, producing repetitive discharges and a large membrane depolarization leading to hyperexcitation, ataxia, tremors and convulsions. This is related to the release of Ca^{2+} and therefore the release of several neurotransmitters.
- Type II pyrethroids cause an extreme prolonged sodium channel opening that results in depolarization but not repetitive firing. This long depolarization is followed by a great release of neurotransmitters, including acetylcholine, leading to the production of cholinergic symptoms such as salivation and choreoathetosis.

Many studies suggest that pyrethroids can affect the turnover of dopamine in the striatum (Husain et al 1991). Gillette and Bloomquist (2003) provide evidence as well of permethrin modulating the dopaminergic system at low doses, in a persistent manner, leading to neurotoxicity.

2.3. Pesticide interactions

Around 1 billion pounds of pesticides are used every year in the United States of America (USA) at a cost of approximately $7.2 billion. Because of this extensive use of pesticides, exposure to multiple chemicals acutely or chronically, over the course of an organism's lifetime is likely to happen. Human exposure to pesticides and their mixtures is likely to occur through different sources, especially food and water. Up until recently, about 95% of all chemical toxicity studies were performed on individual chemicals (Simmons 1995, Groten et al 1998). Nowadays, many pests develop resistances to certain pesticides and tank mixtures have to be used to protect crops efficiently and avoid such resistances (PSD 2001). Therefore, because the use of pesticide mixtures in different formulations is becoming more and more common, studies involving chemical mixtures are of interest due to concerns of occupational and public health (Simmons 1995).

Chemicals in mixtures may interact with one another and alter the magnitude and sometimes the nature of the toxic effect. These changes are defined in Casarett and Doull's Toxicology: The Basic Science of Poisons (Klaassen and Eaton 1996).

- *Additivity* is when the combined effect of two chemicals is equal to the sum of the effect of each given agent alone (i.e. 2+2=4).
- *Synergism* is when the combined effect of two chemicals is greater than the sum of the effects of each agent given alone (i.e. 2+2=20).
- *Potentiation* occurs when one substance does not have a toxic effect on a certain organ or system but when added to another chemical makes that chemical much more toxic (i.e. 0+2=10).
- *Antagonism* occurs when two chemicals are applied together and interfere with each other's actions or one interferes with the action of the other (i.e. 4+6=8; 4+0=1).

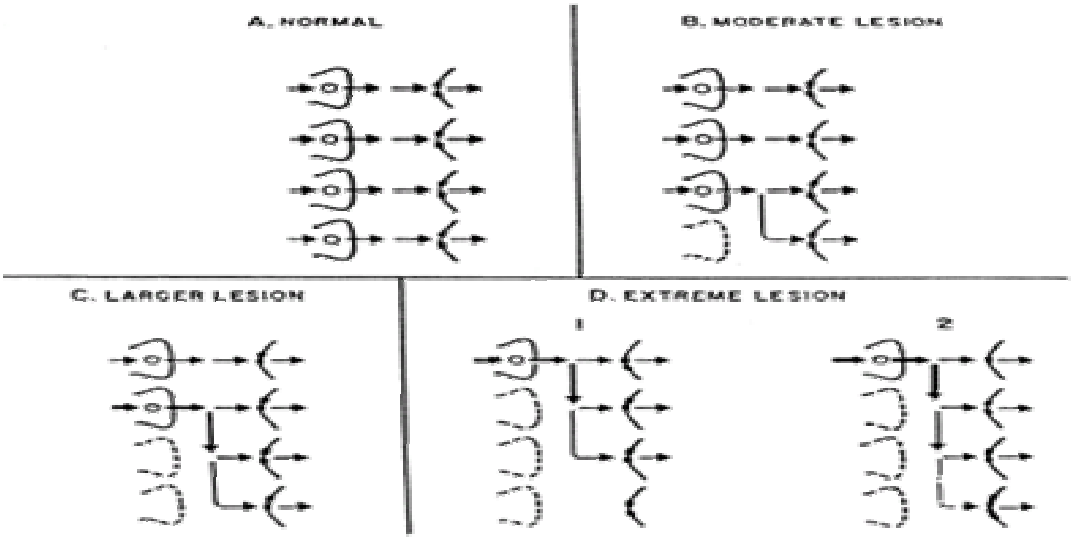
In our studies permethrin and endosulfan were chosen as the pesticides mixed at different concentrations. Endosulfan is compatible with many pesticides and may be found in formulations in many combinations. Endosulfan is not compatible with alkaline chemicals (Extoxnet). The pH of permethrin is 4. Therefore these two compounds are chemically compatible with each other for the mixture. These two compounds have different structures but both are known to modify the kinetics of voltage-sensitive sodium channels and calcium ion flux/homeostasis, which could lead to the release of several neurotransmitters (Ford et al 1986, WHO 1984). For this reason, it was hypothesized that the effects of these two pesticides could be increased or antagonized in combination.

2.4. Compensatory mechanisms in Parkinson's disease

Parkinsons's disease is a progressive neurodegenerative disorder for which symptoms appear when dopaminergic neuronal death is in excess of a critical threshold of 70-80% (Scherman et al 1989, Agid 1991). This gradual appearance of clinical signs seems to be due to the existence of compensatory mechanisms within the basal ganglia that mask the appearance of the first clinical symptoms (Bezard et al 1998). In fact, normal concentrations of extracellular dopamine are maintained in the partially denervated striatum without any kind of compensatory changes in dopamine uptake or release (Garris et al 1997).

In the maintenance of dopamine homeostasis, several factors are thought to be involved:

- The intrinsic properties of dopaminergic neurons develop adaptative responses within the nigrostriatal pathways to cope with a new situation created by the acute or progressive lesion of the sustantia nigra pars compacta (SNc).
- Neuronal plasticity.
- The different inputs to the sustantia nigra pars compacta (SNc) (Bezard 2003).



Bezard E et al 1998

Figure 10: Compensatory mechanisms model: (A) Under regular conditions, there are little synaptic interactions with neighbor terminals. (B) Moderate lesions may show no funct onal alteration because neurotrasmitters released may act at a denervated site. (C) Larger lesions may force the increase in the synthesis and release of DA to keep proper postsynaptic function. (D) After bigger lesions, there could be an initial period of failure and then compensation due to the increment of tyrosine hydroxylase among other mechanisms (Bezard et al 1998).

Two different types of compensatory mechanisms, slow and rapid, have been reported to be associated with the maintenance of DA homeostasis (Zigmond and Stricker 1989, Zigmond et al 1990, Zigmond 1997).

- *Rapid compensatory mechanisms:*

  -Increase of remaining dopamine neurons

  -Increase release of dopamine per pulse

  -Increase in the amount of dopamine

- *Slow compensatory mechanisms:*

  -Supplementary induction of tyrosine hydroxylase and synthesis of dopamine

  -Increase in the responsiveness of striatal neurones to dopamine.

The slow type of compensatory mechanism could be the responsible for the spontaneous recuperation frequently observed after partial lesion in experimental Parkinsonism (Bezard et al 1997).

Recent studies suggest that dopamine class II receptors (D_2) might be upregulated to compensate for dopamine loss in PD, because they are present in both the presynaptic and postsynaptic striatal neurons (Bezard et al 2003). Another novel compensatory mechanism suggested is the expression of enkephalin. Increased enkephalin release in the globus pallidus externa (GPe) would reduce γ-amino butyric acid (GABA) release through activation of δ-opioid receptors by met-enkephalin and this would keep the normal activity of GPe (Maneuf et al 1994). It has been proposed as well, that structures outside the basal ganglia could also compensate for the loss of dopamine in PD. In any case, the existence of compensatory mechanisms within the basal ganglia must be taken into account when interpreting results in progressive neurodegenerative syndromes such as Parkinson's disease.

3. ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is one of the most common causes of the loss of mental function, characterized by a progressive decline in cognitive function and neurodegeneration.

This pathology was first described by the German neuropathologist Alois Alzheimer in 1906. It has been also called "*senile dementia*" because is one of the most important degenerative diseases affecting older people. Dementia has become a major public health problem. Its incidence increases enormously with age and its prevalence doubles every 5 years (Jorm et al 1998, Hofman et al 1991). Alzheimer's disease (AD) patients experience a progressive cognitive decline and memory loss and finally completely loss of functional abilities (Terry et al 1999). The progression of the disease can be summarized within three broad clinical phases: (Reigsberg 1983, Terry et al 1999).

- *Forgetfulness or mild phase*. In this phase the individual is perfectly capable of performing as usual in social and employment situations. The patient usually starts forgetting names of places and feels ashamed for it.
- *Confusional or moderate dementia phase*. Individuals in this phase cannot function normally in social and employment environments. Patients also become less emotionally responsive. New information is rapidly forgotten.
- *Dementia or severe stage of AD.* Patients at this point cannot longer survive without external care. They are unable of washing themselves, become incontinent and lose phychomotor and speaking abilities.

Numerous studies have shown a strong correlation between the severity of dementia and the degree of neuropathological alterations in patients with AD (DeKosky et al 1990, Terry et al 1991). Neuropathologically, AD is characterized by neocortical atrophy, extensive neuron, synapse loss, abnormal depositions of senile plaques and neurofibrillary tangles (Terry et al 1981, 1983, 1991). These neurodegenerative changes are found primarily in the hippocampus and etorhinal cortex but also in the pariental, temporal and frontal lobes of the cortex (Braak and Braak 1999, Reisberg 1983). Other cortical and subcortical areas, such as the primary sensory cortex, motor cortex and basal ganglia, tend to be relatively preserved or only become affected in the latter stages of the disease (Terry et al 1999). The most important changes observed in these areas that are believed to correlate with dementia are neuritic plaques or senile plaques and neurofibrillary tangles. The neurofibrillary tangles are also called paired helical filaments (PHF's) of Alzheimer's disease type. In addition, we also find granulovacuolar bodies (Reisberg 1983).

- *Senile (neuritic) plaques* are found in the cortex (especially in the third layer of the frontotemporal cortex) (Reisberg 1983) and are composed of a central core of amyloid

protein surrounded by degenerating neurite fragments. These plaques are rich in aggregated amyloid β (Aβ) peptide and include 40-42 residues from the larger β amyloid precursor (APP) (Wirths et al 2003). Thus, these plaques can be of two types: plaques with and without abnormal neurites. The ones without neurites are called diffuse plaques (Terry et al 1999). However, senile plaques are not specific for AD, and diffuse plaques have been found as well in cognitively normal elderly people (Katzman et al 1988).

- *Neurofibrillary tangles or paired helical filaments (PHF's)* are found in the cerebral cortex, hippocampus, locus coeruleus and dorsal raphe of AD patients. However, they are very unsual in the neocortex of normal elderly people (Tomlinson 1977, Terry et al 1987). These filaments are dense tangles of neurofibrils contained in the neuronal perykaria of surviving neurons. Like senile plaques, these tangles are not specific for AD but they are usually found in greater amount in the above mentioned locations in AD patients.

- *Granulovacuolar bodies* are also found in the hippocampus of AD patients and they seem to be co-localized with PHF's (Tomlinson 1977). The cells with these inclusions are relatively common in normal aging people but there is an increase in number in AD patients (Ball et al 1977).

In addition to all these pathological changes, there is subcortical neuron loss in the nucleus basalis of Meynert and the nucleus locus coeruleus. This loss is associated with the reduction of neocortical levels of cholinergic and noradrenergic neurotransmitters, respectively (Whitehouse et al 1982). Thus, the brain structure that sustains the greatest damage in AD is

the cerebral cortex, although there are also other areas affected, such as the thalamus, caudate and putamen (Foster et al 1984). However, these subcortical structures of the basal ganglia show damage only in the latter stages of the disease (Terry et al 1999).

AD is believed to be caused by deterioration of more than a single neurotransmitter system. Even so, studies on this disease focus on the acetylcholine system. Acetylcholine is synthesized from choline and acetyl CoA in a presynaptic neuron by the enzyme choline acetyltransferase (ChAT) (Siegel et al 1999). The activity of ChAT in the cortex and the hippocampus is 70 to 90% lower in AD patients than in aged-normal controls (Enna et al 1981). Once acetylcholine has been produced, stored and performed its action in the brain, it is degraded at the synapse by acetylcholinesterase (AChE) enzyme. Dale predicted the existence of this esterase in 1914 (Dale et al 1914). Afferent acetycholinesterase containing fibers are broadly distributed throughout different cortical areas. In pathological conditions, a reduction of cortical AChE activity occurs, along with several other abnormalities. These abnormalities include deterioration of cholinergic processes, inappropriate anterograde or retrograde axonal transport and excessive release of the enzyme from different terminals. It is believed that in AD there is a correlation between the extent of AChE reduction and the severity of the disease (Reisberg 1983). AChE reduction is not noticeable at early stages of the disease and, in some cases, even in more severe stages. The diminution of AChE is not as apparent as the reduction of choline acetyltransferase. Like acetylcholine, other neurotransmitters are also affected in AD. Catecholamines such as dopamine, 3-methoxytyramine and homovalinic acid (HVA) levels are reduced in the caudate nucleus of the basal ganglia. Norepinephrine and serotonin (5-HT) are also decreased (Carlsson et al 1980).

4. α-SYNUCLEIN: THE LINK BETWEEN PARKINSON'S AND ALZHEIMER'S DISEASES

Many neurotransmitters decreased in AD are also decreased in PD. The comorbidity of Parkinson's and Alzheimer's diseases is not uncommon since both maladies are age dependent and they share many important clinical factors.

One of the emerging factors linking neurodegenerative diseases such as Parkinson's, Alzheimer's and Lewy Body diseases is the presence of brain aggregates of α-synuclein. Because these diseases seem to share common mechanisms, they have been called synucleinopathies (Trojanowski and Lee 2002).

α-Synuclein is a small protein of the family of the synucleins, αSYN, βSYN and γSYN, that is highly expressed in various brain regions, especially in presynaptic terminals (Jakes et al 1994, Lavedan 1998). A 35 amino acid peptide, named the non-amyloid β/A4 protein (Aβ) component of Alzheimer's disease (NAC), was found in the SDS-insoluble fraction of brain homogenates of patients with Alzheimer's disease. Later studies showed that these 35 amino acids were the residues (61-95) of the larger non-amyloid component precursor (NACP), also called α-synuclein (140 amino acids) (Wirths and Bayer 2003).

Structurally, α-synuclein possesses three modular domains, which include an amino-terminal lipid binding α-helix, a β-amyloid binding domain that encodes the non-amyloid component (NAC) of the Alzheimer's disease plaques, and an acidic tail with a carboxy-terminal (Lee et al 2001). This structure suggests that α-synuclein may be involved in the vesicle trafficking of synaptic terminals (Narayanan and Scarlata 2001). It may also be involved in many lipid interactions that regulate fatty acid transport between aqueous and phospholipid compartments in neuronal cells (Jo et al 2000, Sharon et al 2001). The N-terminal region of α-synuclein hosts the lipid binding domains that interact with phospholipid membranes altering the α-helicity of the protein (Davidson et al 1998). Additionally, α-

synuclein seems to be involved in the protein ubiquitination process and regulation of chaperone molecules. It may also be a possible substrate or inhibitor of protein kinase-dependent pathways (Lee et al 2001). *α*–Synuclein has a high intrinsic propensity to form fibrils in vitro (Kahle et al 2002).

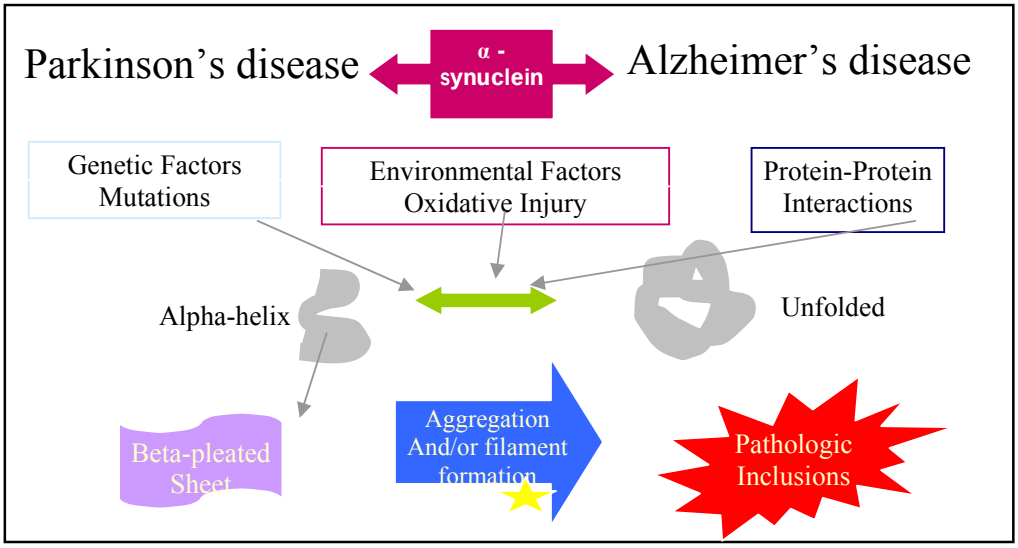


Figure 11: Model for α-synuclein aggregation. Duda et al 2000.

There are several mechanisms by which α-synuclein could contribute to neurodegenerative diseases:

Missense mutations A53T and A30P in the gene partially responsible for the onset of PD seem to accelerate the oligomerization of α-synuclein into protofibrillar intermediates and increase the transformation of these intermediates into amyloid-like fibrils (Sharon et al 2001). These PD-linked mutations self-aggregate α-synuclein creating a β sheeted structure that seems to relate to the inclusions found in Lewy Body Disease (LBD) (El-Agnaf 1998, Miake et al 2002).

Because α-synuclein is a protein involved in the nigrostriatal dopaminergic neurotrasmission, it may play a role in the binding with the dopamine transporter DAT. DAT mediates the uptake of synaptically released dopamine in the substantia nigra (Horn 1990).

Because dopamine can be itself metabolized to form free radical species (Ben-Shachar et al 1995), increased DAT activity could increase the amount of DA and therefore lead to oxidative stress, and enhanced aggregation of α-synuclein. Thus, this would induce neurodegeneration of dopamine cells and therefore would have implication in PD (Lee et al 2001).

In AD, one of the main pathological features is the presence of amyloid plaques. Several studies have confirmed that α-synuclein is not related to mature amyloid plaque cores in this disease (Bayer 1999, Culvenor 1999). In fact, it seems that α-synuclein accumulation is limited to dystrophic neurites at intracellular compartments and therefore, the extracellular amyloid plaque cores lack of α-synuclein accumulation (Wirths and Bayer 2003). However, α-synuclein and Aβ interact in vitro (Jensen et al 1997).

In any case, α-synuclein plays a role in the formation of such brain plaques in AD.

We mentioned before that α-synuclein seemed to control vesicle trafficking of synaptic terminals. Altered trafficking and abnormal processing of the larger β-amyloid precursor protein (APP) lead to the production and deposition of Aβ peptides (Wirths and Bayer 2003). This again, is one of the hallmarks of AD.

PART 3: EXPERIMENTAL DESIGN

1. CHOICE OF DOSAGES

Because these studies with permethrin and endosulfan have not been performed in the past, dosages were chosen based on similar or related studies so we would be able to compare results (Karen et al 2001, Bloomquist et al 2002, Anand et al 1985, Madepalli et al 1994, Kiran and Varma 1988, Paul et al 1994).

Bloomquist and colleagues used C57Bl/6 male mice 7-9 month old treated with permethrin in doses ranging from 0-200 mg/kg with administration in 50 µl of corn oil (Karen et al 2001). In this study dopamine uptake was increased up to 134% of control at a dose of 1.5 mg/kg. Because an effect was evident at this dose level, this was one of the doses chosen for the study in which mice were exposed both as juveniles and adults. The other doses used in this study, 15 and 150 mg/kg, were also in this dose range and were chosen in order to compare experiments. Bloomquist and colleagues observed that 200 mg/kg of permethrin reduced DA uptake by 50% when compared with control (Karen et al 2001). Therefore, doses used in the present study for juveniles and adults ranged between 1.5-150 mg/kg.

For endosulfan, Anand et al (1985) noted elevated foot-shock fighting behavior in adult rats treated intraperitoneally with 3 mg/kg of endosulfan on 10 consecutive days. Additionally, these investigators showed an increase in the levels of dopamine and a decrease in the levels of serotonin (Anand et al 1985). In another study with endosulfan Wistar rat pups were dosed by gastric intubation with 6 mg/kg of endosulfan from postnatal days 2-25. DA levels were decreased, while serotonin levels were increased (Madepalli et al 1994). Kiran and Varma (1988) orally dosed rats of different age groups with 12.5 mg/kg of endosulfan. They observed age dependent decreases in brain acetylcholinesterase inhibition. In general, we have to take into account that oral doses are less toxic than intraperitoneal doses (Aune et al 2002). Because

the doses of endosulfan that we chose fit in this range and because endosulfan was previously administered to juvenile mice in our laboratory as 1/8 of the LD50 (Merck Index), we considered doses in the range of 0.7-4.3 mg/kg appropriate for our studies.

The pyrethroid permethrin was purchased as a mixture of four R, S-cis and R,S-trans isomers at the following concentrations, 20% cis and 75% trans. Endosulfan was provided as a white powder of 99.9% purity. Both pesticides were acquired from ChemService, Inc. (West Chester, PA, USA).

2. TREATMENTS

Mice dosed as adults

For the adult exposure to pesticides, we used male C57BL/6 retired breeder mice, 7-9 months old and weighing 30-45 g, purchased from Harlan Sprague-Dawley, Dublin, VA, USA. Mice were housed one per cage, fed *ad libitum,* and kept under environmentally controlled conditions of temperature (22 °C ± 1°C), humidity (40% to 60%) and light cycles (12/12-hour light/dark cycle), in accordance with Virginia Polytechnic Institute and State University guidelines for animal care.

Treatments consisted of the following: endosulfan (EN) 2.15, 4.3 mg/kg, permethrin (PR) 15, 150 mg/kg, and their mixtures, EN 2.15 + PR 15 mg/kg and EN 4.3 + PR150 mg/kg. Both pesticides were dissolved in corn oil as the vehicle, which was used as the negative control.

Pesticides were administered intraperitoneally (i.p) every other day at the same time over a 2-week period. Each group consisted of 6 mice per treatment. Mice were sacrificed 24 hours after the last injection by cervical dislocation and tissues were collected and frozen at -70 ^{o}C immediately.

Mice dosed as juveniles and re-challenged as adults

Female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and bred in our animal facilities at the Center for Molecular Medicine and Infectious Diseases (CMMID). Pups were left undisturbed with their dams for 4 days after birth. At the 5th day, mice were weaned and males were identified and injected, as only they were used for the experiment. Treatments were assigned randomly to the pups as follows: Endosulfan (EN) 0.7, 1.4 mg/kg, permethrin (PR) 1.5, 15 mg/kg and their mixtures EN 0.7 + PR 1.5 mg/kg and EN 1.4 + PR 15 mg/kg. The pesticides and the vehicle control were provided in a volume of 15 µl using a Hamilton syringe. Pups were injected daily intraperitoneally (i.p) during a 2-week period and weighed every week to ensure they were following a normal growing rate. Pups were then kept *ad libitum* in our facilities for 8 months under environmentally controlled conditions of temperature (22 °C ± 1°C), humidity (40% to 60%) and light cycles (12/12-hour light/dark cycle). At this point, they were re-challenged, using the regimen described above for the adult animals. Therefore, these treatments consisted of the following: endosulfan (EN) 2.15, 4.3 mg/kg, permethrin (PR) 15, 150 mg/kg, and their mixture, EN 2.15 + PR 15 mg/kg and EN 4.3 + PR150 mg/kg. Both pesticides were dissolved in 50 µl corn oil as the vehicle, which was used as the negative control. Pesticides were again administered intraperitoneally (i.p), this time every other day over a 2-week period to match the treatments given previously to adults as their first exposure to pesticides. By providing all mice with the same dosages of pesticides at the time of the second administration or re-challenge, we had potential to compare these groups of animals for the possibility of greater neurotoxicity in the mice that received post-natal and re-challenge treatments versus the ones that just were exposed to the pesticides as adults.

The possibility for comparison also provided opportunity to detect a possible silent toxicity that could have developed but that remained hidden until a second contact with pesticides occurred.

3. EXPERIMENTS

Brain dissection

Mice were euthanized by cervical dislocation and afterwards decapitated. Dissection of the brain was performed on ice at all times. A cut was made on the top of the mouse head and skin was separated leaving the entire skull free. The skull was removed and the brain was placed on a plastic surface with ice underneath. The olfactory bulb and cerebellum were removed and discarded. With a blade, we cut the brain by the middle sulc dividing it in its two hemispheres. This way we had a parasaggital view of the mouse brain.

Opening the pocket formed by the lateral ventricle we could find the corpora striata. Corpora striata were then extracted from both brain hemispheres, weighed and immediately placed in –70 °C. Cortex was separated from the rest of the remaining structures, weighed and immediately placed in –70 °C as well.

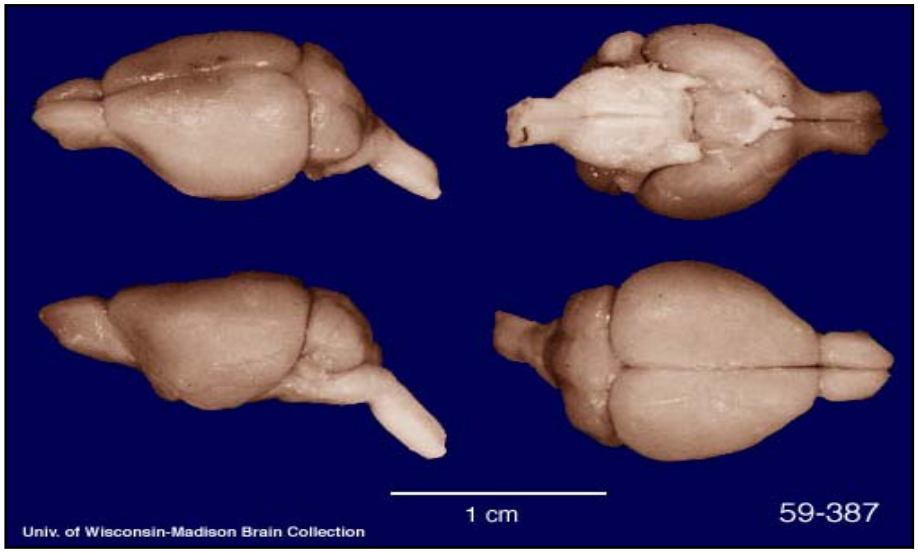


Figure 12: Mouse brain.
http://brainmuseum.org/Specimens/rodentia/mouse/

3.1 Catecholamines and indolamines determination

Dopamine, DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovalinic acid), norepinephrine (NE), serotonin (ST) and 5-HIAA (5-hydroxyindolacetic acid), were determined by reversed-phased high performance liquid chromatography (HPLC) and electrochemical detection (Jussofie et al 1993).

Tissues containing the corpora striata were homogenized in 250 μl of citric acid buffer pH 4.7 (mobile phase) containing isoproterenol as an internal standard. For the homogenization a sonicator was set at a speed of 6 cycles for 30 seconds. After centrifugation in a Beckman Microfuge Lite® at 12000 rpm for 10 min at 4°C, supernatants were kept at –20 °C until assayed.

Just prior the analysis samples were filtered through an Acrodisc LC 13mm syringe filter with 0.2 μm PVDF membrane into the HPLC vials.

The chromatographic instruments consisted of the following:

- Hewlet Packard Quat Pump with a degasser and autosampler (Agilent Technologies, Wilmington, DE, USA).
- Guard column, Nucleosil 100 C18 3 μ 8x4 mm (Macherey-Nagel, Easton, PA, USA)
- Reversed-phased C18 analytical column Nucleosil 100 3 μ, 250x4 mm (Macherey-Nagel, Easton, PA, USA).

Electrochemical detection is performed at the oxidation mode with + 0.35 Volt. Isocratic elution is executed at a flow rate 0.6 ml/min and a maximum pressure of 300 bars.

The mobile phase consisted of a citric acid buffer pH 4.7 composed of: sodium acetate 0.1 M, citric acid 25 mM, ethylenediaminetetraacetic acid (EDTA) 134 μM, octanesulfonic acid 230 μM, and methanol 6%.

3.2 Acetylcholinesterase (AChE) determination

Acetylcholinesterase activity in brain cortices was analyzed by spectrophotometry at 412 nm using acetylthiocholine as a substrate (Ellman et al 1961, Correll and Ehrich 1991).

Brain cortices were removed from storage at -70 °C, placed on ice and their weight was multiplied by 6.5 to determine the volume of phosphate buffer pH 8.0 in which the tissues were homogenized. Once tissues were homogenized by a sonicator set at speed 6, samples were diluted 1:10 with phosphate pH 8.00 buffer. Homogenized samples were again diluted 1:10 in pH 8.00 buffer to have the final working solution. The working solution was added to microplate wells along with 150 µl of 0.1 M phosphate buffer pH 8.00 and 50 µl of prepared solutions of 6 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and 4.5mM acetylthiocholine in triplicate. The absorbance of wells in the microplate was measured by spectrophotometry at 412 nm at time 0 and then again following incubation for 60 min. The difference of measurements at time 60 minus time 0 indicated activity of acetylcholinesterase as acetylthiocoline was hydrolyzed and a yellow color was formed as the hydrolysis product reacted with DTNB. Activity was expressed as micromole/min/mg protein. Protein was determined using a Biorad assay kit (Bio-Rad Laboratories, CA, USA).

3.3. TBARS determination

Cortex tissues were removed from storage at -70 °C, thawed and homogenized in 500 µl of phosphate buffered saline (PBS).

Reactive Oxygen Species (ROS) were assessed by thiobarbituric acid reactive products (TBARS) (Oxi-Tek, Zeptometrix, Buffalo, NY). The determination was performed by spectophotometry. Results were expressed in nmol MDA/ml.

This method consisted of the following:

Malondialdehyde (MDA) forms a 1:2 complex with thiobarbituric acid, developing a colored compound that can be measured by fluorometry or spectophotometry.

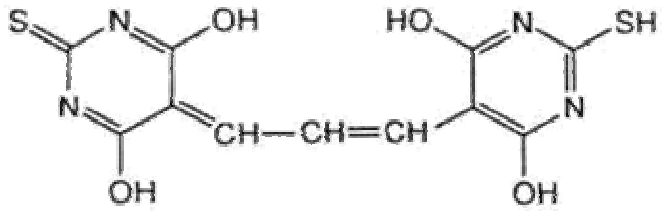


Figure 13: Malondialdehyde (MDA)-thiobarbituric acid colored complex. Zeptometrix

A standard curve was prepared at the following concentrations:

STANDARD NUMBER	MDA CONCENTRATION	VOLUME OF MDA STANDARD	VOLUME OF DILUENT
4	100 nmol/ml	1000 µl	0 µl
3	50 nmol/ml	500 µl	500 µl
2	25 nmol/ml	250 µl	750 µl
1	12.5 nmol/ml	125 µl	875 µl
0	0 nmol/ml	0 µl	1000 µl

Table 1: Preparation of standard curve for malondialdehyde (MDA)

Tubes were properly labeled and 100 µl of sample or standard were added to each tube. 100 µl of SDS (sodium lauryl sulfate) was added to each tube and tubes were swirled to mix. The thiobarbituric acid (TBA) vial was dissolved in a mix of sodium hydroxide and acetic acid (TBA diluent 1 and 2). 2.5 ml of this mix (TBA/Buffer reagent) was added by pouring down the side of each tube.

Tubes were covered with a glass marble and were incubated at 95 °C for 60 minutes.

After the incubation period, tubes were removed and cooled to room temperature in an ice bath for 10 minutes.

Tubes were then centrifuged at 3000 rpm for 15 minutes.

Pink colored supernatants were read at 532 nm in a Shimadzu spectrophotometer (Shimadzu Scientific Instruments. Inc. MD, USA).

Samples were assessed rapidly and tubes were always kept on ice before they were incubated at 95 °C for 60 minutes.

Results were obtained by extrapolation from the standard curve.

3.4. α-Synuclein determination

α-Synuclein was analyzed by western blot.

Cortex tissues were removed from storage at -70 °C, placed on ice, thawed and sonicated in PBS buffer with 0.5 % Triton x 100 (lysis buffer) and a protease inhibitor cocktail (P8340 Sigma, Saint Louis, Missouri). Homogenates were incubated on ice for 30 min at this point to allow action of the proteases. Samples then were centrifuged at 12000 x g or 8500 rpm for 5 min at 15 °C and supernatants were kept for western blot determination.

Supernatants were diluted 1:1 with Laemelli loading buffer (0.5M Tris-Cl pH 6.5, 10% SDS (sodium lauryl sulfate), 1% bromophenol blue, glycerol, 2-mercaptoethanol) and heated for 5 min at 95 °C. After heating, 7 µl of standard (Bio Rad dual color standard from Bio-Rad Laboratories, CA, USA), 10 µl of positive control (rat cerebrum lysate, BD Transduction Laboratories, San Diego, CA, USA) and 10 µl of each sample were loaded on the gel and processed for SDS-polyacrylamide electrophoresis. Gels consisted of 15% acrylamide (Bio-Rad Laboratories, CA, USA) of 0.75 mm thickness. Gels were run using a Biorad apparatus (Bio-Rad Laboratories, CA, USA) at 190 V (constant voltage) for 60 min. After

electrophoresis was completed, gel, pads and membranes were soaked for 15 min in transblot buffer (tris 0.025 M, glycine 0.192 M, SDS 0.001%, 20% methanol in water). Proteins were then transferred from the gel to PVDF (polyvinyldiene difluoride) membranes and subjected to a current of 20 V during 15 min using a semi-dry transfer unit. Blots were then blocked with 5% nonfat milk in 0.1% TBS Tween for 2 hours at room temperature and incubated first with anti α-synuclein (Syn-1 from BD Transduction Laboratories) and mouse IgG as a secondary antibody, each for 2 hours. Between each of these processes, the membranes were washed two times with TBS Tween 0.1% (TBS + Tween 20 0.1%) and one time with TBS buffer pH 7.5 (Tris-HCL 20 mM, NaCl 500 mM, distilled water).

Incubating the blots for a minute with 5.5 ml of horseradish peroxidase substrate showed the results (Pierce Biotechnology, Rockford, IL).

Gels not transferred to a PVDF membrane were stained for 1 hour with 10 ml of Commassie Blue Stain (Gelcode blue from Pierce Biotechnology, Rockford, IL) to visualize all the protein bands. Incubating the gel in water during one hour enhanced visualization of the bands.

4. DATA ANALYSIS

4.1. Catecholamines and indolamines

Peak heights of dopamine, DOPAC, HVA, serotonin, 5-HIAA and norepinephrine (NE) were analyzed by linear regression analysis, based on a standard curve constructed from known concentrations of each of the catecholamines (catecholamine standards) and the use of isoproterenol as the internal standard. Thus, striatum catecholamine concentrations were calculated from their respective standard curves and expressed as pmol/mg protein.

Single determinations were performed. The results were expressed as the mean ±SEM from the results per treatment ($n = 6$ per treatment). Statistical significance ($p < 0.05$) of the

means of each of the catecholamines was determined using one way analysis of variance (ANOVA) with single degree of freedom contrasts with the SAS system version 8.2 (SAS Institute Inc, Cary, NC, USA). Data were graphed as the mean ± SEM.

4.2. Acetylcholinesterase

Acetylcholinesterase concentrations were quantified by spectrophotometry and results were expressed as means ± SEM (n = 6 mice per treatment). Experiment included three observations of readings per mouse. Units representing results were micromole/min/mg protein. Statistical significance ($p < 0.05$) of mean values was determined using one way analysis of variance (ANOVA) with single degree of freedom contrasts with the SAS system version 8.2 (SAS Institute Inc, Cary, NC, USA). Data were graphed as the mean ± SEM.

A positive control was included for validation of the assay. This positive control was not included in statistical analyses.

4.3. TBARS

Thiobarbituric acid reactive species were quantified as the mean ± SEM of concentrations obtained from two spectrophotometric readings per mouse (n = 6 mice per treatment) after extrapolation with a standard curve of known malonyldialdehyde (MDA) concentrations. Statistical significance ($p < 0.05$) of mean values was determined using one-way analysis of variance (ANOVA) with single degree of freedom contrasts with the SAS system version 8.2 (SAS Institute Inc, Cary, NC, USA).

4.4. α-Synuclein

Western blot bands were photographed and quantitatively calculated using the Kodak image station 440 CF. Band net intensity was analyzed per lane. Each lane corresponded to a

different treatment. Band number and intensity per lane depended on the concentration of normal protein (non aggregated) and the proportion of aggregated protein (double, triple bands) expressed by each treatment. Results were shown as the mean ± SEM of net intensity from two different repeated experiments. Two different factors were analyzed: For the bands expressing normal α-synuclein (protein non aggregated = ~17 kDa lower band of the western blot), the amount of protein was determined. In addition, we calculated the proportion of α-synuclein aggregated compared to control (Protein aggregated / protein non-aggregated +protein aggregated). This calculation corresponds to the double and triple bands observed above the normal synuclein (non aggregated) on the western blot. The net intensity of each of the bands was compared to control. Statistical significance ($p < 0.05$) of mean values was determined using one-way analysis of variance (ANOVA) with single degree of freedom contrasts with the SAS system version 8.2 (SAS Institute Inc, Cary, NC, USA). The standard was not included in the statistical calculations.

4.5. Pesticide interactions

Pesticide interactions were determined using the means ± SEM of both of the mixture treatment groups (n = 6 mice per treatment). Therefore, interactions were assessed for all the parameters studied for the high mixture treatment (EN 4.3 + PR 150 mg /kg) and the low mixture treatment (EN 2.15 + PR 15 mg/kg). Results of these interactions were obtained using one-way analysis of variance (ANOVA) with single degree of freedom contrasts with the SAS system version 8.2 (SAS Institute Inc, Cary, NC, USA). Statistically significant values ($p < 0.05$) were graphed as the mean of each of the components of the mixture for each of the parameters evaluated. In other words, no endosulfan (0) and endosulfan concentrations (low or high) were used as the reference on the X axis to assess the changes that pesticide induced on the paramenters studied when permethrin was incorporated to the mixture.

PART 4: RESULTS

1. OVERVIEW OF RESULTS

A summary of the results of HPLC analysis for catecholamines and indolamine levels, and their metabolites as well as the results for acetylcholinesterase, thiobarbituric acid reactive species and α-synuclein, are provided in Table 2 and Table 3. Table 2 provides the data attained when the pesticides were given to adult mice and Table 3 provides the data attained when mice where dosed as juveniles and re-challenged again as adults. The data for catecholamines and indolamines are also presented graphically in Figures 14-17.

ADULT MICE (Table 2)

SAS	DOPAMINE pmols/mg tis	DOPAC pmols/mg tis	HVA pmols/mg tis	NE pmols/mg tis	SEROTONIN pmols/mg tis	5-HIAA pmols/mg tis	ACHESTERASE nmols/min/mg prot	TBARS nmolsMDA/ml	α-Syn
CONTROLS (CO)	53±4.7	18±3.3	6.9±0.5	4.7±0.23	0.7±0.09	2..0±0.2	47.3±4.0	7.0±1.6	
HIGH VALUES mg/kg									
EN 4.3	45±4.7	19±3.3	5.6±0.5	4.1±0.25 P = 0.08	0.5±0.09 P = 0.06	1.6±0.2	55.8±4.4	8.4±1	⇧
PR 150	58±4.7	19±3.3	6.3±0.5	0.03±0.5 ⇩	1.3±0.09 ⇧	0.5±0.2 ⇩	59.4±4.0 ⇧	8.9±0.9	⇧
Mix EN4.3+PR150	65±4.7	27±3.3	8.5±0.5 ⇧	ND	1.3±0.09 ⇧	1.2±0.2 ⇩	57.1±4.0	11.2±0.9 ⇧	⇧⇧⇧
LOW VALUES mg/kg									
EN 2..15	47±4.7	15±3.3	5.4±0.5 ⇩	0.3±0.23 ⇩	1.2±0.09 ⇧	0.9±0.2 ⇩	62.1±4.0 ⇧	10.5±0.9 ⇧	⇧
PR 15	57±4.7	16±3.3	6.2±0.5	0.4±0.23 ⇩	1.2±0.09 ⇧	1.2±0.2 ⇩	56.5±4.0	12.6±0.9 ⇧	⇧
Mix EN2.15+PR15	38±5.2 ⇩	11±3.7	4.5±0.5 ⇩	0.9±0.25	1.1±0.09 ⇧	1.3±0.2 ⇩	61.3±4.0 ⇧	10.2±1.1	⇧
ANOVA F value	☆	0.09	☆	☆	☆	☆		☆	

Table 2: Brain neurochemistry of adult mice dosed with the pesticides endosulfan and permethrin. Results expressed as means ± SEM from C57BL/6 mice 7-9 months old dosed with the following pesticides: endosulfan EN 4.3, 2.15 mg/kg; permethrin PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR150 and EN 2.15 + PR 15 mg/kg (n = 6 mice per treatment).

⇧, ⇩ Arrows indicate higher or lower significant differences ($p < 0.05$) when compared to control CO = Corn oil

⇧ ⇧ ⇧ Triple arrows in the α-synuclein column indicate a very significant triple band corresponding to the proportion of α-synuclein aggregated.

☆ Stars indicate significant F ANOVA values within the treatments.

Abbreviations: DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovalinic acid; NE = norepinephrine; 5-HIAA = 5 hydroxyindolacetic acid; ACHESTERASE = acetylcholinesterase; TBARS = thiobarbituric acid reactive species; MDA = malondialdehyde; α-Syn = α-synuclein protein, CO = Corn Oil (Control); ND = Not detectable; mg tis = mg of tissue; mg prot = mg of protein.

MICE DOSED AS JUVENILES AND RE-CHALLENGED AS ADULTS (Table 3)

SAS	DOPAMINE pmols/mg tis	DOPAC pmols/mg tis	HVA pmols/mg tis	NE pmols/mg tis	SEROTONIN pmols/mg tis	5-HIAA pmols/mg tis	ACHESTERASE nmols/min/mg prot	TBARS nmolMDA/ml	α-Syn
CONTROLS (CO)	41±3.4	9.6±2	4.0±0.4	0.9±0.1	1.7±0.1	1.2±0.1	88.7±7.9	7.3±0.7	
HIGH VALUES									
EN 4.3	34±3.7	9.0±2	4.4±0.4	0.9±0.1	1.6±0.1	1.2±0.1	122±8.6 ⇧	8.6±0.7	⇧
PR 150	36±3.4	11±2	4.2±0.4	1.0±0.1	1.7±0.1	1.3±0.1	102±7.9	8.9±0.7	⇧
Mx EN 4.3+PR 150	43±3.7	14±2	5.1±0.4	0.9±0.1	1.8±0.1	1.6±0.1 ⇧	91.5±8.6	9.0±0.7	⇧
LOW VALUES									
EN 2.15	20±3.4 ⇩	11±2	3.0±0.4	0.7±0.1 ⇩	1.1±0.1 ⇩	1.1±0.1	95.5±7.9	7.1±0.7	⇧
PR 15	38±3.7	13±2	4.4±0.4	0.7±0.1 ⇩	1.6±0.1	1.4±0.1	84.4±8.6	7.7±0.7	⇧
Mx EN 2.15+PR 15	27±3.7 ⇩	17±2 ⇧	4.1±0.4	0.8±0.1	1.3±0.1 ⇩	1.5±0.1 ⇧	75.3±8.6	9.3±0.7	⇧⇧
ANOVA F value	☆			☆	☆	☆	☆		

Table 3: Brain neurochemistry of mice dosed with the pesticides endosulfan and permethrin as juveniles and re-challenged as adults. Results expressed as means ± SEM from C57BL/6 mice dosed as juveniles with endosulfan (EN) 0.7, 1.4 mg/kg, permethrin (PR) 1.5, 15 mg/kg and their mixtures EN 0.7 + PR 1.5 mg/kg and EN 1.4 + PR 15 mg/kg. Re-challenge of these mice was performed at 7-9 months of age with the following treatments listed as the final dose on the table above: endosulfan (EN) 2.15, 4.3 mg/kg, permethrin (PR) 15, 150 mg/kg, and their mixture, EN 2.15 + PR 15 mg/kg and EN 4.3 + PR150 mg/kg.

⇧, ⇩ Arrows indicate higher or lower significant differences ($p < 0.05$) when compared to control.

⇧⇧ Double arrows in the α-synuclein column indicate a very significant double band corresponding to the proportion of α-synuclein aggregated.

☆ Stars indicate significant F ANOVA values within the treatments.

Abbreviations: DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovalinic acid; NE = norepinephrine; 5-HIAA = 5 hydroxyindolacetic acid; ACHESTERASE = acetylcholinesterase; TBARS = thiobarbituric acid reactive species; MDA = malondialdehyde; α-Syn = α-synuclein protein. CO = Corn Oil (Control); mg tis = mg of tissue; mg prot = mg of protein.

2. PRESENTATION OF RESULTS

2.1 Catecholamines and indolamines

Significant treatment effects were noted on catecholamines (norepinephrine, dopamine and its metabolites DOPAC and HVA) and indolamines (serotonin and its metabolite 5-HIAA).

2.1.1. Dopamine

The mean levels of dopamine were significantly decreased ($p < 0.05$) in adults and in juveniles grown to adults in the low dose mixture treatment (EN 2.15 + PR 15 mg/kg) (Table 2, 3; Figures 14, 15). In the juvenile to adult experiment (Table 3, Figure 15), there was also a significant decrease of dopamine in the mice given the low dose of endosulfan (EN 2.15 mg/kg).

The levels of dopamine in the remaining groups were not significantly altered but graphically in both experiments, the dopamine levels of the treated mice seemed to follow the same patterns of increases and decreases.

2.1.2. DOPAC

DOPAC (3, 4-dihydroxyphenylacetic acid) levels were not significantly altered in any of the treatments in mice dosed only as adults (Table 2, Figure 14). However, DOPAC was significantly increased in mice given the low mixture treatment (EN 2.15 + PR 15 mg/kg) in the animals treated as juveniles and as adults (Table 3, Figure 15).

The increase of DOPAC in these mice corresponds with the higher level of dopamine in mice given EN 2.15 + PR 15 mg/kg as DOPAC is dopamine's metabolite.

2.1.3. HVA

HVA (homovalinic acid), another of the dopamine's metabolites, was significantly increased in the adult mice given the high dose mixture of pesticides (EN 4.3 + PR 150 mg/kg) (Table 2, Figure 14). This appeared to correspond with nonsignificantly elevated levels of dopamine in mice of this same treatment group. In addition, levels of HVA in mice treated only as adults were significantly decreased in the low dose mixture (EN 2.15 + PR 15 mg/kg) and in the low endosulfan treatment groups (EN 2.15 mg/kg). In the latter group of mice,

treatment groups with significantly low levels of HVA corresponded with the treatment groups of mice with significantly low levels of dopamine.

HVA levels were not significantly altered by any of the treatments in mice dosed both as juveniles and as adults (Table 3, Figure 15).

2.1.4. Norepinephrine

Norepinephrine (NE) levels were difficult to measure in mice brains as levels were near the limits of HPLC detection levels. In mice dosed as adults, NE levels were significantly reduced in the permethrin high (PR 150 mg/kg), permethrin low (PR 15 mg/kg) and endosulfan low (EN 2.15 mg/kg) treatment groups. In addition, in mice dosed as juveniles and as adults, treatment with the low dose of endosulfan (EN 2.15 mg/kg) and the low dose of permethrin (PR 15 mg/kg) decreased NE levels (Table 2, Table 3, Figure 14, and Figure 15).

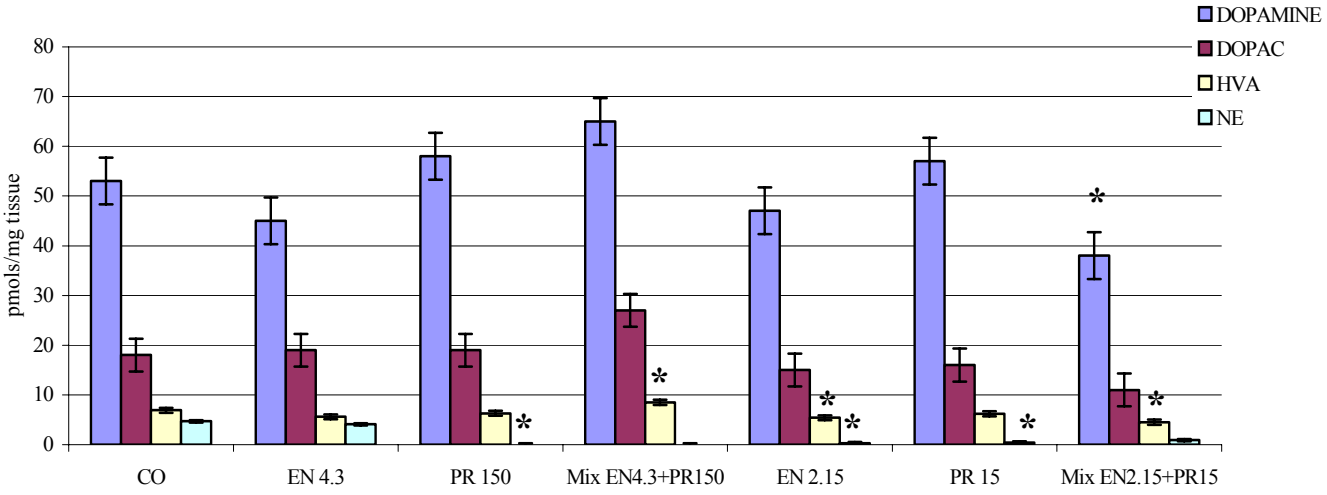


Figure 14: **Catecholamines and metabolite levels in adult mice dosed with pesticides endosulfan and permethrin**.
Levels of dopamine, DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovalinic acid) and NE (norepinephrine) are expressed as mean ± SEM of HPLC results from the striatum of C57Bl/6 adult mice 7-9 months old (n = 6 mice per treatment).
Dosages of EN 4.3, 2.15 mg/ kg; PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally 7 times every other day during a period of two weeks.
EN = endosulfan, PR = permethrin, CO = corn oil (control)
Data are shown as pmols/mg wet tissue.
Asteriks (*) denote significant changes ($p < 0.05$) in the levels of catecholamines when compared with control (CO).

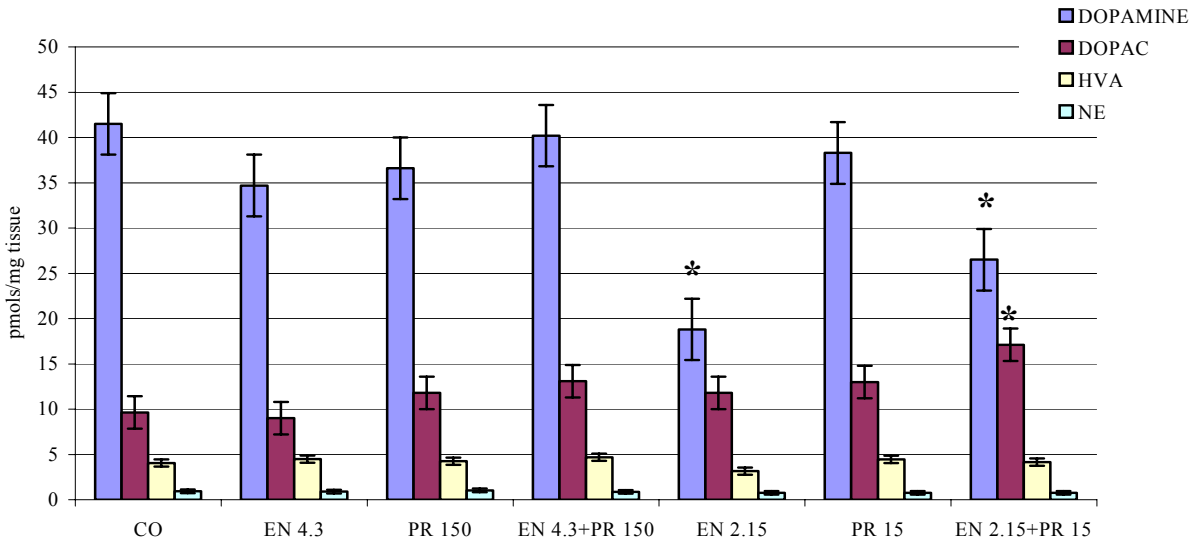


Figure 15: **Catecholamines and metabolite levels in mice dosed as juveniles and as adults with pesticides endosulfan and permethrin.**
Levels of dopamine, DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovalinic acid) and NE (norepinephrine) are expressed as mean ± SEM of HPLC results from the striatum of C57Bl/6 mice (n= 6 mice per treatment).
Dosages of EN 0.7, 1.4 mg/kg, PR 1.5, 15 mg/kg and their mixtures EN 0.7 + PR 1.5 mg/kg and EN 1.4 + PR 15 mg/kg were initially given intraperitoneally daily during a period of two weeks from day 5 to 19.
Re-challenge was performed when mice were 7-9 months old and adult dosages of EN 4.3, 2.15 mg/ kg; PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally 7 times every other day during a period of two weeks.
Only adult final doses are provided in the figure above.
EN = endosulfan, PR = permethrin, CO = corn oil, control.
Data are shown as pmols/mg wet tissue.
Asteriks (*) denote significant changes ($p < 0.05$) in the levels of catecholamines when compared with control = CO.

2.1.5. Serotonin

Mean levels of serotonin in the mice treated only as adults were significantly increased by all treatments except for the high dose of endosulfan (EN 4.3 mg/kg). This dose resulted in a P value of 0.06, close to significant decreased serotonin levels (Table 2, Figure 16). In contrast, in mice dosed both as juveniles and as adults, only endosulfan at the low dose (EN 2.15 mg/kg) and the low dose mixture treatment (EN 2.15 + PR 15 mg/kg) resulted in a significant decrease in mouse brain serotonin levels (Table 3, Figure 17).

2.1.6. 5-HIAA

Levels of 5-HIAA (5-hydroxyindolacetic acid), serotonin's primary metabolite, were significantly decreased by all treatments in all the adult mice except in the ones given the higher endosulfan dose (EN 4.3 mg/kg) (Table 2, Figure 16). However in mice treated as juveniles and as adults, only the high mixture treatment (EN 4.3 + PR150 mg/kg) and the low mixture treatment (EN 2.15 + PR15 mg/kg) resulted in a significant increase of 5-HIAA levels (Table 3, Figure17).

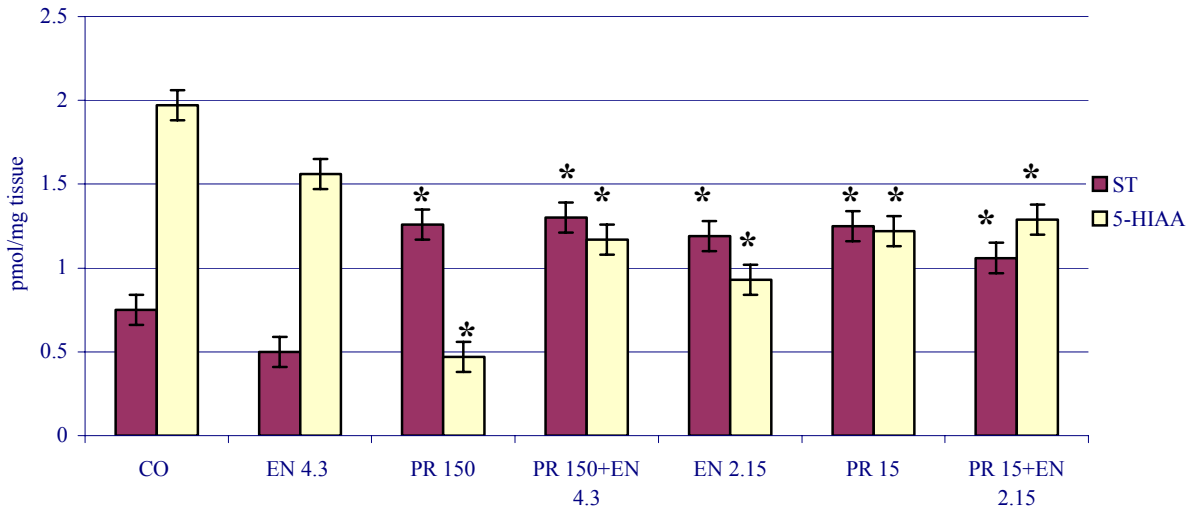


Figure 16: Striatum indolamine levels in adult mice dosed with pesticides endosulfan and permethrin
ST (serotonin) and 5-HIAA (5-hydroxyindolacetic acid) levels expressed as mean ± SEM of HPLC results from the striatum of C57Bl/6 adult mice 7-9 months old (n = 6 mice per treatment).
Dosages of EN 4.3, 2.15 mg/ kg; PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally 7 times every other day during a period of two weeks.
EN = endosulfan, PR = permethrin, CO = corn oil (control)
Data are shown as pmols/mg wet tissue.
Asteriks (*) denote significant changes ($p < 0.05$) in the levels of indolamines when compared with control.

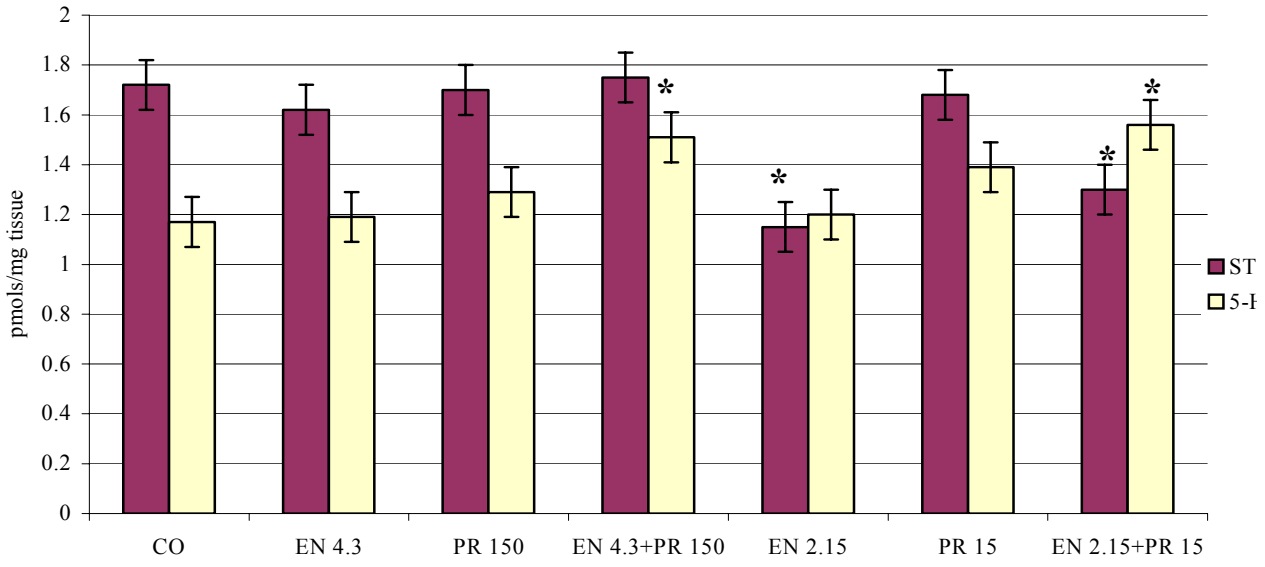


Figure 17: Striatum indolamine levels in mice dosed as juveniles and as adults with pesticides endosulfan and permethrin

ST (serotonin) and 5-HIAA (5- hydroxyindolacetic acid) expressed as mean ± SEM of HPLC results from the striatum of C57Bl/6 mice dosed as juveniles and re-challenged as adults (n = 6 mice per treatment).
Dosages of (EN) 0.7, 1.4 mg/kg, (PR) 1.5, 15 mg/kg and their mixtures EN 0.7 + PR 1.5 mg/kg and EN 1.4 + PR 15 mg/kg were initially given intraperitoneally daily during a period of two weeks from day 5 to 19.
Re-challenge was performed when mice were 7-9 months old and adult dosages of EN 4.3, 2.15 mg/kg; PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally 7 times every other day during a period of two weeks.
Only adult final doses are provided in the figure above.
EN = endosulfan, PR = permethrin, CO = corn oil, control.
Data are shown as pmols/mg wet tissue.
Asteriks (*) denote significant changes ($p < 0.05$) in the levels of indolamines when compared with control.

2.2. Acetylcholinesterase

The activity of acetycholinesterase, the enzyme responsible of acetylcholine degradation, was significantly elevated in adult mice given the following treatments: permethrin high dose (PR 150 mg/kg), endosulfan low dose (EN 2.15 mg/kg) and the low dose mixture treatment (EN 2.15 + PR 15 mg/kg) (Table 2, Figure 18).

Nevertheless, in mice dosed as juveniles and re-challenged as adults, only the higher endosulfan treatment (EN 4.3 mg/kg) caused a significant increase in acetylcholinesterase activity (Table 3, Figure 19).

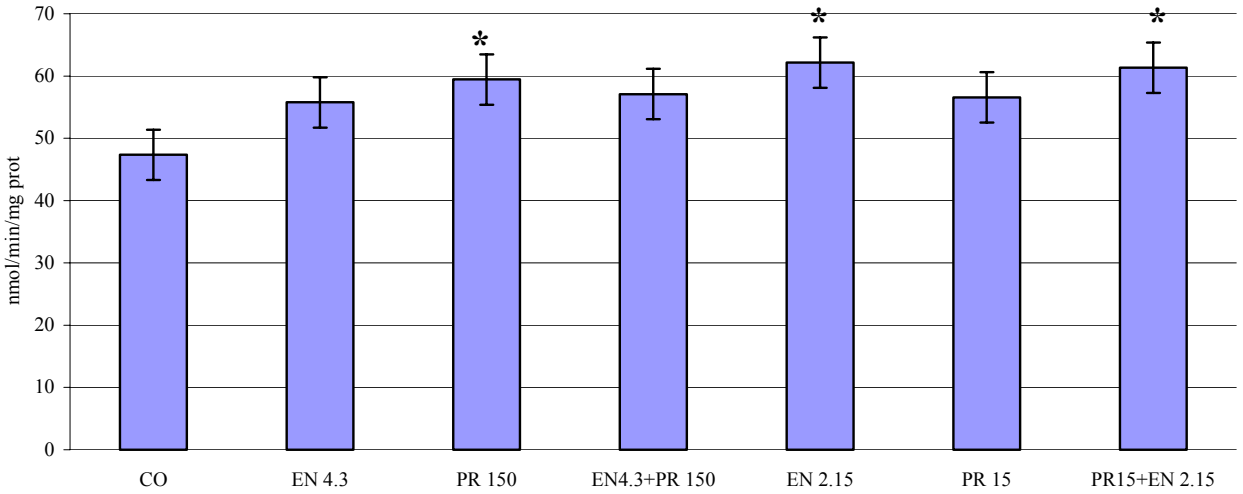


Figure 18: Acetylcholinesterase activity levels in adult mice dosed with pesticides endosulfan and permethrin.
Acetylcholinesterase activity was measured by spectrophotometry and expressed as mean ± SEM of results obtained from the cortex of C57BL/6 adult mice 7-9 months old (n = 6 mice per treatment).
Dosages of EN 4.3, 2.15 mg/ kg; PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally 7 times every other day during a period of two weeks.
EN = endosulfan, PR = permethrin, CO = corn oil (control)
Data are shown as nmols/min/mg protein.
Asteriks (*) denote significant changes ($p < 0.05$) in the levels of acetylcholinesterase when compared with control.

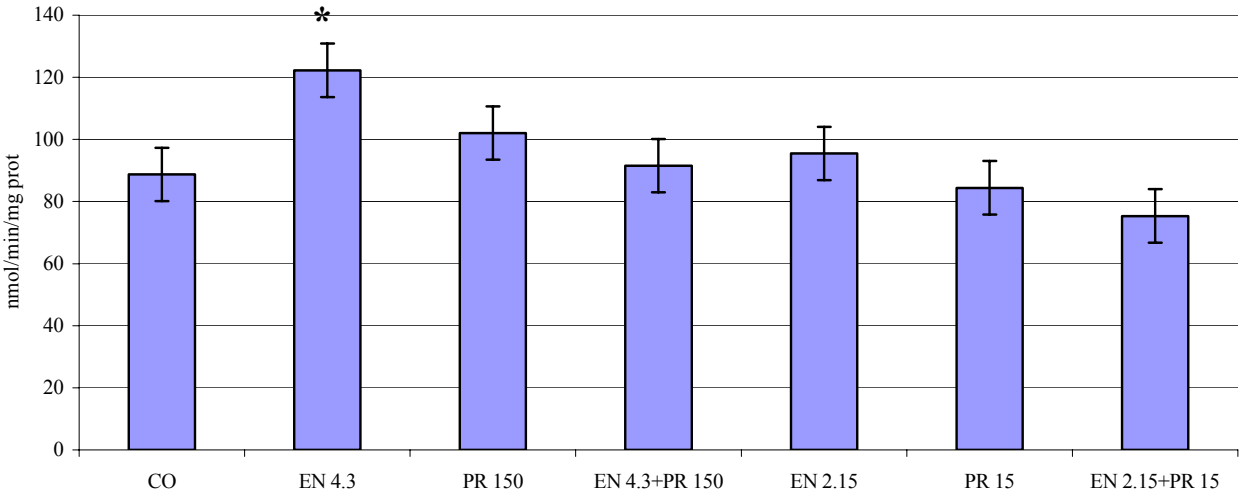


Figure 19: Acetylcholinesterase activity levels in mice dosed as juveniles and as adults with pesticides endosulfan and permethrin.
Acetylcholinesterase activity was measured by spectrophotometry and expressed as mean ± SEM of results obtained from the cortex of C57BL/6 mice treated as juveniles and re-challenged as adults (n = 6 mice per treatment).
Dosages of (EN) 0.7, 1.4 mg/kg, (PR) 1.5, 15 mg/kg and their mixtures EN 0.7 + PR 1.5 mg/kg and EN 1.4 + PR 15 mg/kg were initially given intraperitoneally daily during a period of two weeks from day 5 to 19.
Re-challenge was performed when mice were 7-9 months old and adult dosages of EN 4.3, 2.15mg/ kg; PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally 7 times every other day during a period of two weeks.
Only adult final doses are provided in the figure above.
EN = endosulfan, PR = permethrin, CO = corn oil, control.
Data are shown as nmols/min/mg protein.
Asteriks (*) denote significant changes ($p < 0.05$) in the levels of acetylcholinesterase when compared with control.

2.3. TBARS

Determination of thiobarbituric acid reactive species, (TBARS) indicated significantly increased lipid peroxidation in adult mice given the high dose of endosulfan and permethrin mixture (EN 4.3 + PR150 mg/kg) and in treatment groups receiving low permethrin (PR 15 mg/kg) and low endosulfan (EN2.15 mg/kg) doses (Table 2, Figure 20). In mice dosed as juveniles and re-challenged as adults there were no statistical significances among the treatment groups (Table 3, Figure 21). However, the metabolism of dopamine generates free radicals, and graphically we can observe a pattern between the levels of dopamine and DOPAC and the increases and decreases in the levels of thiobarbituric acid reactive substances. This occurred in mice treated as adults and in mice dosed as juveniles and re-challenged as adults (Table 2, Table 3).

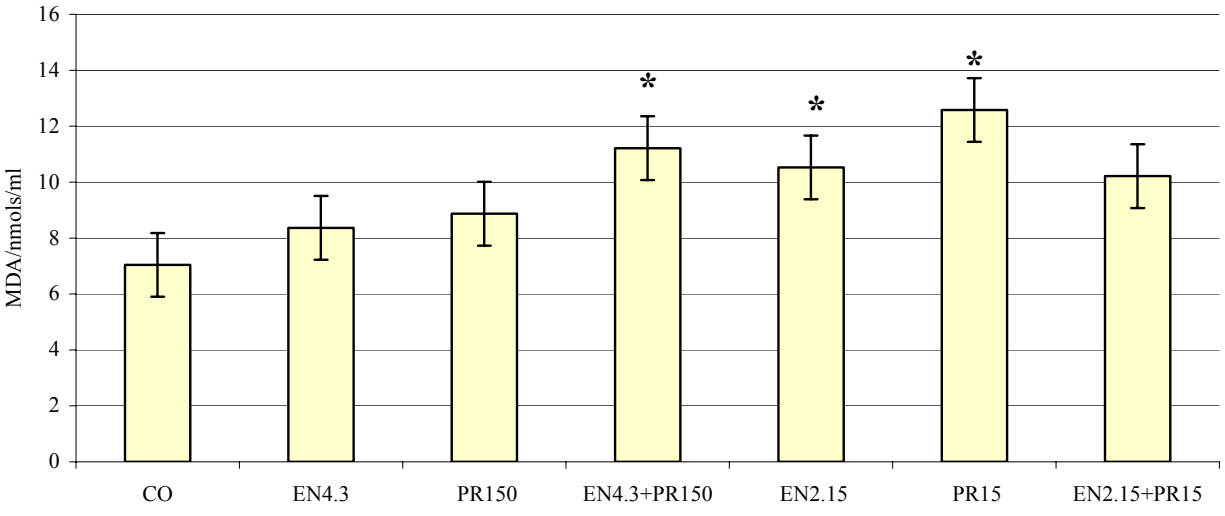


Figure 20: TBARS (thiobarbituric reactive substances) levels in adult mice dosed with pesticides endosulfan and permethrin.
TBARS levels were measured by spectrophotometry and expressed as mean ± SEM of results obtained from the cortex of C57BL/6 adult mice 7-9 months old (n = 6 mice per treatment).
Dosages of EN 4.3, 2.15 mg/ kg; PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally 7 times every other day during a period of two weeks.
EN = endosulfan, PR = permethrin, CO = corn oil (control)
Data are shown as nmol MDA/ml. MDA = Malondialdehyde
Asteriks (*) denote significant changes ($p < 0.05$) in the levels in lipid peroxidation when compared with control.

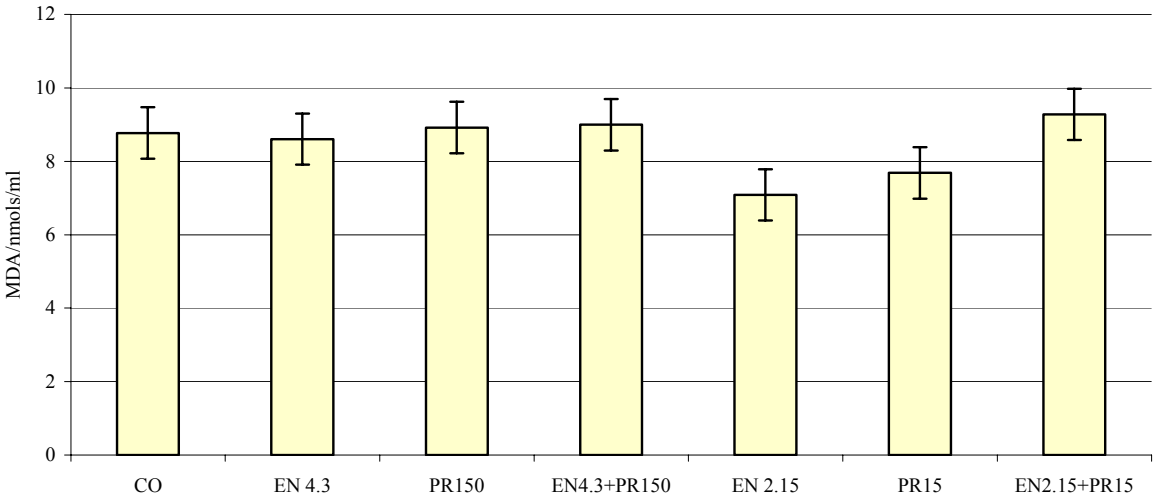


Figure 21: TBARS (thiobarbituric reactive substances) levels in mice dosed as juveniles and as adults with pesticides endosulfan and permethrin.
TBARS levels were measured by spectrophotometry and expressed as mean ± SEM of results obtained from the cortex of C57BL/6 mice treated as juveniles and re-challenged as adults. (n = 6 mice per treatment).
Dosages of (EN) 0.7, 1.4 mg/kg, (PR) 1.5, 15 mg/kg and their mixtures EN 0.7 + PR 1.5 mg/kg and EN 1.4 + PR 15 mg/kg were initially given intraperitoneally daily during a period of two weeks from day 5 to 19.
Re-challenge was performed when mice were 7-9 month old and adult dosages of EN 4.3, 2.15 mg/ kg; PR 150, 15 mg/kg and their mixtures, EN 4.3 + PR 150 and EN 2.15 + PR 15 mg/kg were given intraperitoneally 7 times every other day during a period of two weeks.
Only adult final doses are provided in the figure above.
EN = endosulfan, PR = permethrin, CO = corn oil, control.
Data are shown as nmol MDA/ml. MDA = Malondialdehyde

2.4. α-Synuclein

α-Synuclein bands were visualized, photographed and analyzed using the Kodak image station 440 CF.

Mice dosed as adults

Normal α-synuclein was detected as a 16-17 kDa band in the cerebral cortex of all mice in all the treatment groups, including control. Rat cerebrum positive control also showed this

band. These 16-17 kDa bands corresponded to regular non-aggregated α-synuclein. There were no statistical differences on the amount of non-aggregated protein (Table 4).

Additionally, we observed several double bands of different intensities in samples from all treatment groups except for the control which double bands were almost imperceptible. More intense double bands were detected in samples obtained from adult mice dosed with the high mixture of pesticides (EN 4.3 + PR 150 mg/kg) and with the low permethrin dose (PR 15 mg/kg) (Figure 22). These bands corresponded to molecular weights of approximately 25 kDa. In the high mixture treatment group (EN 4.3 + PR 150 mg/kg) we could observe a third band at the range of 45 kDa as well. Band net intensity of this treatment (EN 4.3 + PR 150 mg/kg) showed significant differences ($p < 0.05$) when analyzed statistically (Table 4). Results were also expressed as the proportion of α-synuclein aggregated (Table 4, Figure 22).

α-Synuclein aggregated proportion *= aggregated protein / (non-aggregated protein + aggregated protein)*

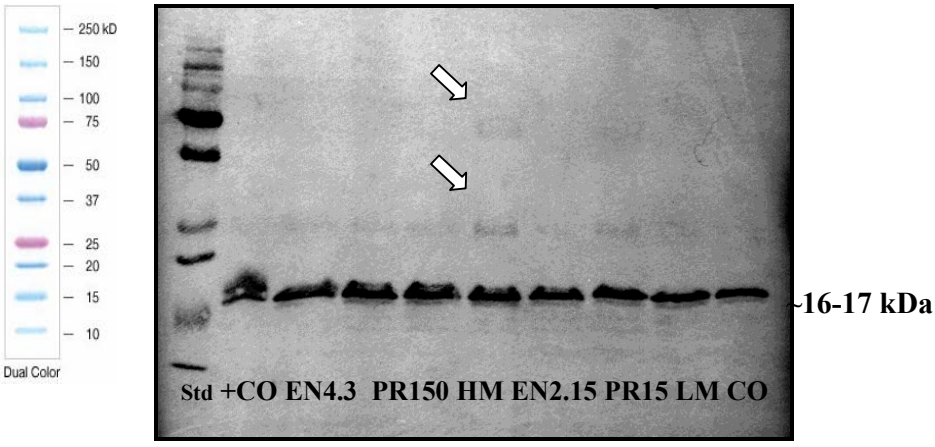


Figure 22: Synuclein western blotting in brain of adult mice treated with endosulfan and permethrin. Although the molecular mass is 14 kDa, synuclein has an irregular electrophoretic mobility that leads to an apparent molecular mass of 17–18 kDa (Fasano et al 2003)
Std= Standard,
+ =Positive control (Rat cerebrum)
CO = Corn oil (Negative control)
EN = Endosulfan
PR = Permethrin
HM = High dose mixture (EN 4.3+PR 150 mg/kg)
LM = Low mixture (EN 2.15+PR 15 mg/kg)
Arrows denote statistical significances in the aggregated proportion of α-synuclein

ADULTS	TRT	NONAGG1	NONAGG2	BAND1.1	BAND1.2	BAND 2.1	BAND 2.2
	CO	88975.9	137385				
	EN 4.3	113874.4	186358.3	4134.2	6472.4		
	PR 150	95162.6	175909	5117	18386		
	EN 4.3+PR 150	89944.6	171221	**2844.5**	**17524**	**2868.3**	**7868.8**
	EN 2.15	56525.8	130737	1927.6	5292.2		
	PR 150	73420.3	144927	2120.9	10262.0		
	EN 2.15+ PR 15	105654.4	118648.4	2015.3	6955.4		

Table 4: α-Synuclein net intensity values for the bands obtained by western blot from cortex of C57BL/6 adult mice treated with endosulfan (EN) and permenthrin (PR).
Data obtained using the Kodak image station 440 CF.
This table represents the data obtained from 2 western blots.

NONAGG1 and NONAGG2 are the values for the net intensity of the bands of two western blots that correspond to normal α-synuclein or the non-aggregated protein. There were no significant differences in the values of the non-aggregated α-synuclein

BAND 1.1 and BAND 1.2 are the values for the net intensity of the double bands of two western blots that correspond to aggregated α-synuclein.

BAND 2.1 and BAND 2.2 are the values for the net intensity of the triple bands of two western blots that correspond to aggregated α-synuclein.

Doses were: EN 4.3, 2.15: PR 150, 15 and mixtures EN 2.15 + PR15 and EN 4.3 + PR150 mg/kg.
Treatment (TRT) in bold was statistically significant ($p < 0.05$) when corresponded to control and represents the proportion of α-synuclein aggregated.

Mice dosed as juveniles and re-challenged as adults

Normal α-synuclein was detected as a 16-17 kDa band in the cerebral cortex of all mice in all the treatment groups, including control. Rat cerebrum positive control also showed this band. These 16-17 kDa bands corresponded to regular non-aggregated α-synuclein. There were no statistical differences in the amount of non-aggregated protein. In addition, double bands were visible for all the remaining treatments except for the control, in which bands were almost imperceptible.

Mice dosed with the low mixture dose of endosulfan and permethrin (EN 2.15 + PR 15) mg/kg showed a more intense double band at approximately 25 kDa that was statistically significant and represented the proportion of α-synuclein aggregated (Table 5, Figure 23).

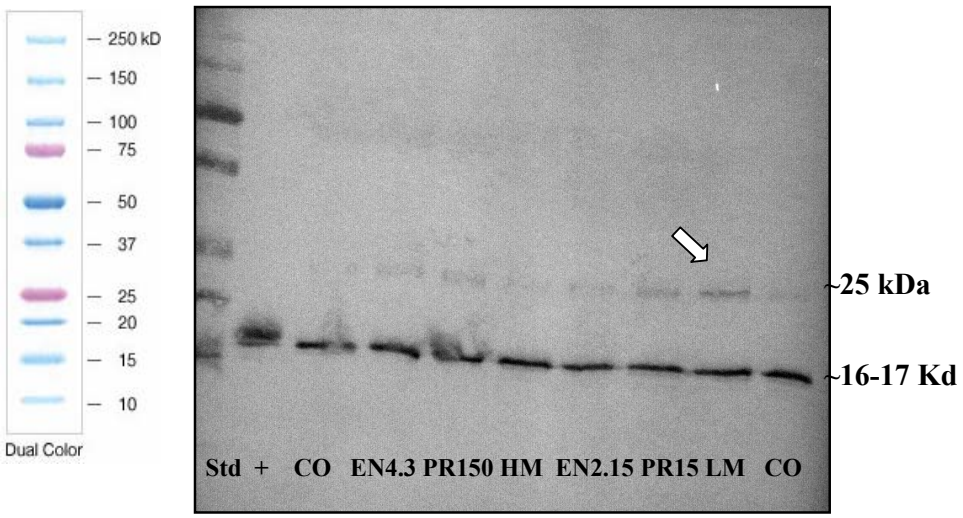


Figure 23: Synuclein western blotting of mice dosed with endosulfan and permethrin as juveniles and re-challenged as adults. Although the molecular mass is 14 kDa, synuclein has an irregular electrophoretic mobility that leads to an apparent molecular mass of 17–18 kDa (Fasano et al 2003).
Std = Standard,
+ = Positive control (Rat cerebrum)
CO = Corn oil (Negative control)
EN = Endosulfan
PR = Permethrin
HM = High dose mixture (EN 4.3 + PR 150 mg/kg)
LM = Low mixture (EN 2.15 + PR 15 mg/kg)
Arrows denote statistical significances in the aggregated proportion of α-synuclein.

JUVENILES	TRT	NONAGG1	NONAGG2	BAND11	BAND12
	CO	35058.1	14378.5		
	EN 4.3	33290.7	20331.5	4536.35	1837.9
	PR 150	24852.6	16520	1358.24	3004
	EN 4.3+PR 150	34988.2	20238.4	4822.4	969.7
	EN 2.15	29087.7	15598.4	2424.4	1192..2
	PR 150	39382.7	13672	1284.12	1909
	EN 2.15+ PR 15	33497	15257	**4689.6**	**4034.9**

Table 5: α-Synuclein net intensity values for the bands obtained by western blot from cortex of C57BL/6 mice dosed as juveniles and re-challenged as adults with endosulfan (EN) and permethrin (PR).
Data obtained using the Kodak image station 440 CF.

NONAGG1 and NONAGG2 are the values for the net intensity of the bands of two western blots that correspond to normal α-synuclein or the non-aggregated protein. There were no significant differences in the values of the non-aggregated α-synuclein

BAND 1.1 and BAND 1.2 are the values for the net intensity of the double bands of two western blots that correspond to aggregated α-synuclein.

Doses as juveniles were: EN 0.7, 1.4 mg/kg, PR 1.5, 15 mg/kg and their mixtures EN 0.7 + PR 1.5 mg/kg and EN 1.4 + PR 15 mg/kg. Re-challenged as adults consisted of: EN 4.3, 2.15: PR 150, 15 and mixtures EN 2.15 + PR15 and EN 4.3 + PR150 mg/kg.
Treatment (TRT) in bold was statistically significant ($p < 0.05$) when compared to control and represents the proportion of α-synuclein aggregated.
There were no triple bands see in this experiment.

2.5. Pesticide interactions

Interactions between permethrin and endosulfan were assessed for all the parameters studied. However we have only graphed the significant values of these interactions for each parameter. The presence of endosulfan in mice given permethrin resulted in a larger number of significant interactions in adult mice than in mice dosed as juveniles and re-challenged as adults when compared to controls.

Mice dosed as adults

2.5.1. Effects of endosulfan on homovalinic acid (HVA) levels in permethrin and vehicle treated mice. Interaction at the high mixture treatment dose (EN 4.3 + PR 150 mg/kg).

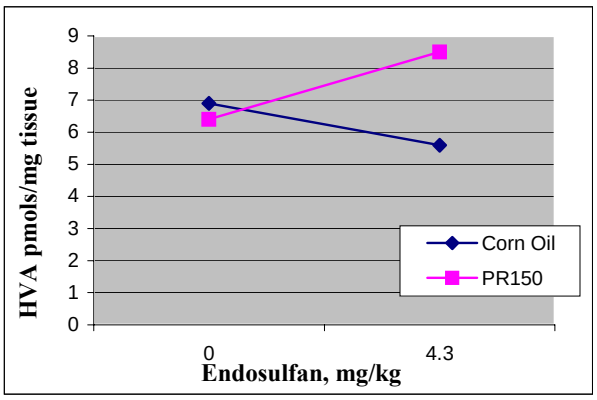


Figure 24: Homovalinic acid (HVA) interaction after high doses of permethrin and endosulfan. Endosulfan (EN) 4.3 mg/kg increased the difference between mice given corn oil and permethrin (PR) 150 mg/kg.

2.5.2. Effects of endosulfan on serotonin levels in permethrin and vehicle treated mice. Interaction at the low mixture treatment dose (EN 2.15 + PR 15 mg/kg)

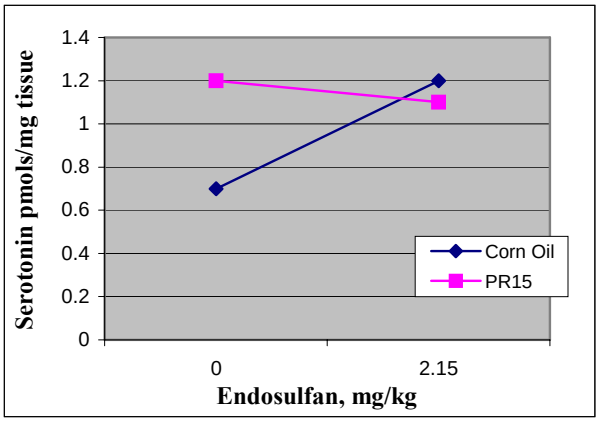


Figure 25: Serotonin interaction after low doses of permethrin and endosulfan.
Endosulfan (EN) 2.15 mg/kg decreased the difference between mice given corn oil and permethrin (PR) 15 mg/kg.

2.5.3. Effects of endosulfan on 5-hydroxyindolacetic acid (5-HIAA) levels in permethrin and vehicle treated mice. Interaction at the low mixture treatment dose (EN 2.15 + PR 15 mg/kg).

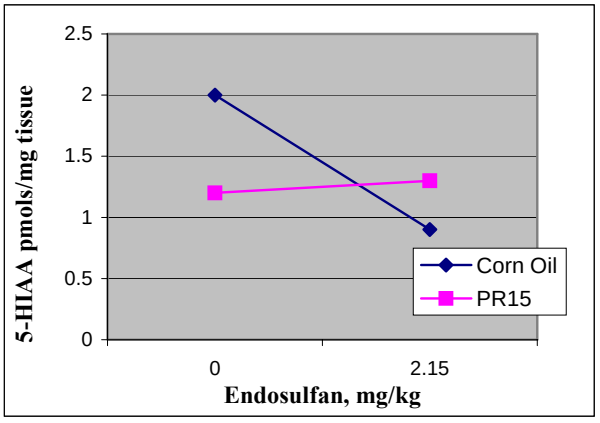


Figure 26: 5-Hydroxyindolacetic acid (5-HIAA) interaction after low doses of permethrin and endosulfan.
Endosulfan (EN) 2.15 mg/kg decreased the difference between the mice given corn oil and permethrin (PR) 15 mg/kg.

2.5.4. Effects of endosulfan on 5-hydroxyindolacetic acid (5-HIAA) levels in permethrin and vehicle treated mice. Interaction at the high mixture treatment dose (EN 4.3 + PR 150 mg/kg).

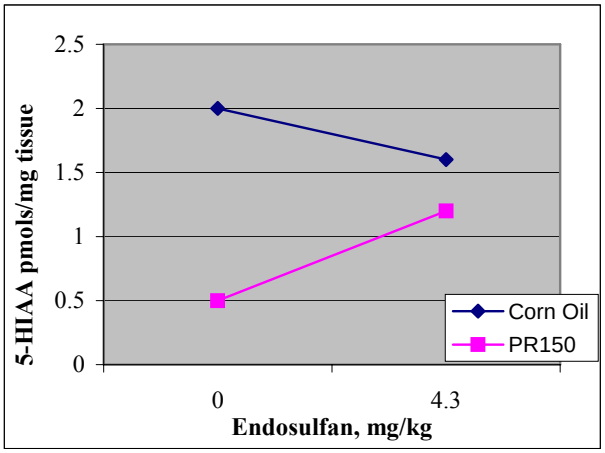


Figure 27: 5-Hydroxyindolacetic acid (5-HIAA) interaction after high doses of permethrin and endosulfan.
Endosulfan (EN) 4.3 mg/kg decreased the difference between mice given corn oil and permethrin (PR) 150 mg/kg.

2.5.5. Effects of endosulfan on thiobarbituric reactive substances (TBARS) levels in permethrin and vehicle treated mice. Interaction at the low mixture treatment dose (EN 2.15 + PR 15 mg/kg).

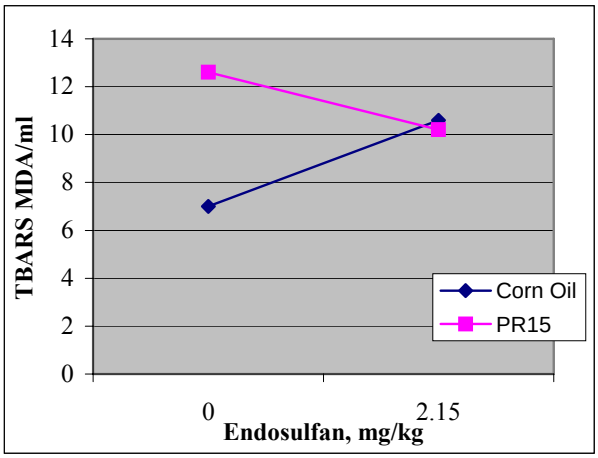


Figure 28: Thiobarbituric reactive substances (TBARS) interaction after low doses of permethrin and endosulfan.
Endosulfan (EN) 2.15 mg/kg decreased the difference between mice given corn oil and permthrin (PR) 15 mg/kg.

Mice dosed as juveniles and re-challenged as adults.

2.5.6. Effects of endosulfan on acetylcholinesterase levels (AChE) in permethrin and vehicle treated mice. Interaction at the high mixture treatment dose (EN 4.3 + PR 150 mg/kg).

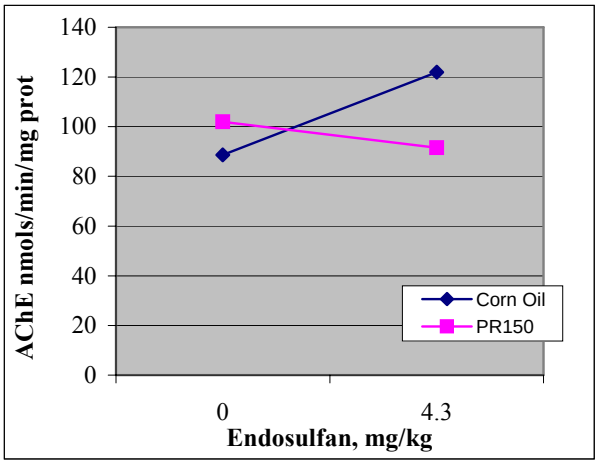


Figure 29: Acetylcholinesterase (AChE) interaction after high doses of permethrin and endosulfan. Endosulfan (EN) 4.3 mg/kg increased the difference between mice given corn oil and permethrin (PR) 150 mg/kg.

PART 5: DISCUSSION

The present research examined the hypothesis that exposure to the pyrethroid permethrin and the organochlorine endosulfan, either alone or in combination, could contribute to the disruption of the dopaminergic or cholinergic pathways in C57BL/6 mice. It was hypothesized that the exposure to these pesticides during early life could result in an augmented susceptibility to subsequent environmental exposures, potentially unmasking a silent neurotoxicity later in life.

Exposure of mice to the pesticides permethrin, endosulfan and their mixture was predicted to result in lower levels of catecholamines, indolamines and acetylcholine in mice dosed as juveniles and re-challenged as adults than just in adults. In addition, exposure of mice to the pesticides permethrin, endosulfan and their mixture was predicted to result in higher levels of TBARS and α-synuclein expression in animals dosed as juveniles and re-challenged as adults than just in adults. However, results of these studies revealed that mice exposed to permethrin, endosulfan and their combination as juveniles and re-challenged later in life were not more affected than naïve adult mice when compared to controls. In fact, the mice re-challenged later in life with these two pesticides appeared to be less susceptible to altering the endpoints examined.

1. CATECHOLAMINES AND INDOLAMINES

Mice dosed only as adults

As in our studies, previous studies by Bloomquist and colleagues (Karen et al 2001) showed that low i.p doses of the pyrethroid permethrin (1.5 mg/kg) in adult C57Bl/6 mice had little effect on dopamine levels. Instead, these authors noted a 34% up-regulation of the levels of the dopamine transporter (DAT) over that of the control value. This would be an expected

response to increased dopamine levels, as DAT is responsible for DA re-uptake into presynaptic neurons. Thus, Bloomquist and colleagues assumed that the observed increase in dopamine uptake was compensating for increased free levels of synaptic dopamine. In contrast, their studies demonstrated that higher doses of permethrin (200 mg/kg) decreased dopamine uptake by 50% even though no effects on striatal dopamine levels were noted.

However, another study from the same institution showed that low levels of permethrin (3 mg/kg) reduced the amount of dopamine transporter inmunoreactive protein in the caudate and putamen (Pittman et al 2003). In contrast, the high dose of permethrin did not produce a significant change in dopamine transporter in their studies. These investigators explained this result by saying that the areas of the brain chosen for the study were extremely restricted to the caudate and the putamen rather than other portions of the brain that could be more resistant to neurodegenerative effects of dopamine depletion. Therefore, both studies agree in that low levels of permethrin regulate the dopamine transporter to a greater degree than high levels of permethrin. Additionally, both failed to detect permethrin-induced changes on the levels of catecholamines titers such as striatal dopamine.

In the present study, the total levels of catecholamines in the corpora striata of adult mice were not significantly changed when permethrin was dosed alone. This could be due to compensatory mechanisms in the basal ganglia that balance the amount of dopamine levels until there is a damage of 70-80% of the dopamine terminals (Bezard et al 1998). In our study, mice were treated just for a period of two weeks, which may be too short a time to observe dramatic changes on the levels of dopamine and its metabolites.

The other pesticide used in these studies, endosulfan, has been reported to activate cholinergic (Anand et al 1986), dopaminergic (Anand et al 1985) and serotoninergic (Seth et al 1986, Paul et al 1994) metabolic pathways. Several studies with mesencephalic neuron cultures have shown that application of organochlorines such as dieldrin caused cytotoxicity in

dopaminergic neurons more than GABAergic neurons, suggesting some specificity of action (Sanchez Ramos et al 1998). Kirby et al (1996) observed that the organochlorine heptachlor up-regulated dopamine transport in striatal synaptosomes at relatively low doses. Again it seems that in both cases lower doses of these pesticides are more likely to cause an effect than high doses. In the present investigation, the low dose alone of endosulfan didn't cause depletion of striatal dopamine. Alternatively, we observed a significant decrease in dopamine levels in adult mice when they were given the low dose mixture of permethrin and endosulfan (EN 2.15 + PR 15 mg/kg) when compared with control. There is evidence that organochlorines and pyerthroid insecticides affect the dopamine transporter (DAT) and dopamine release in the striatum (Kirby et al 1999, 2001; Karen et al 2001), but in this case we observed a decrease in the levels of the dopamine. Bloomquist and colleagues (Bloomquist et al 2002; Kirby et al 2002, Karen et al 2001, Pittman et al 2003) instead, failed to detect any changes in the titers of catecholamines in these studies. Since endosulfan (LD_{50} = 43 mg/kg) is more toxic than heptachlor (LD_{50} = 100 mg/kg) and in our case mice were treated 7 times instead of 3 times during a 2-week period it is possible that the interaction of these two pesticides at these concentrations could contribute to the marked reduction of dopamine levels. Feeding studies with another organochlorine, dieldrin, have shown that it causes depletion of whole-brain dopamine in adult ducks (Sharma 1973), rats (Wagner and Greene 1974) and Ring doves (Heinz et al 1980). In addition, in the adult mice given both endosulfan and permethrin in combination (EN 2.15 + PR 15 mg/kg), the levels of HVA (homovalinic acid), a dopamine metabolite, were also significantly reduced when compared with control.

This is expected since the levels of dopamine are reduced. The levels of HVA were also decreased in mice given the low dose of endosulfan (EN 2.15 mg/kg), suggesting that this pesticide contributed to low levels of dopamine or a lower metabolism of this neurotransmitter. In contrast, HVA levels were increased in adult mice given the high mixture treatment (EN 4.3

+ PR 150 mg/kg). Although the levels of dopamine were not significantly higher when compared with control, significantly higher levels of HVA imply higher dopamine metabolism.

Levels of NE were also decreased in most of the treatment groups of this short-term study performed in adults. Since DA and NE are both synthesized from tyrosine, and DA is the precursor of NE in this process, it is expected that a decrease in DA should result in a decrease in NE. This occurred in adult mice given the low mixture of endosulfan and permethrin (EN 2.15 + PR 15 mg/kg). In other treatment groups in which NE levels were decreased, there were no significant changes in the levels of dopamine. Although we measured NE in this study, NE levels in corpora striata are not high enough to be easily detected because NE is mainly synthesized and located in the locus coeruleus (Levitt et al 1979).

In addition to catecholamines (DA and its metabolites), indolamines (serotonin and its metabolite 5-HIAA) were measured in adult mice. Anand et al (1985) demonstrated an increase in the levels of serotonin when adult rats were treated with 3 mg/kg of endosulfan intraperitoneally during 10 subsequent days. Another study that treated immature male rats with 2 mg/kg of endosulfan showed increased serotonin concentrations in the cerebrum and midbrain regions (Vanaja et al 1994). In our studies in adult mice, we also observed a significant increase in the levels of serotonin in all pesticide treatments groups except those given high doses of endosulfan (EN 4.3 mg/kg).

Additionally, in our studies in adult mice, the serotonin metabolite 5-hydroxyindolacetic acid (5-HIAA) was significantly reduced in all the treatments except in mice given the endosulfan high dose. Alteration of serotonin metabolism is found primary in depression and many cases of Parkinson's disease have been associated to depression (see literature review, Parkinson's disease). Thus, as in our study, many studies show that lower levels of 5-hydroxyindoleacetic acid could be related to depression associated with cases of Parkinson's and Alzheimer's disease (Mayeux et al 1984).

Mice dosed as juveniles and re-challenged as adults

To interpret the results obtained for the study performed in mice dosed as juveniles and re-challenged as adults we compare our studies to several previous studies that assessed the toxicity of pesticides in juvenile rats. For example, Medepalli et al (1994) dosed rat pups orally with 6 mg/kg of endosulfan and measured the levels of NE, DA and serotonin at days 10 and 25 after dosing. In their studies, DA levels were decreased at both 10 and 25 days. They also noted that serotonin levels were increased at 10 days but decreased at 25 days, and NE levels varied although they were somewhat increased the entire time of study.

In our experiment in which mice were treated as juveniles and then re-challenged as adults, the dramatic changes hypothesized for the levels of catecholamines and indolamines were not observed. Dopamine levels in this study were decreased in the low mixture treatment group (EN 2.15 + PR 15 mg/kg) as they were in the mice dosed only as adults. In addition, the low endosulfan treatment group (EN 2.15 mg/kg) showed significantly decreased levels of dopamine. Also, one of the main DA metabolites, DOPAC (3,4-dihydroxyphenylacetic acid), was significantly increased in the low mixture treatment group (EN 2.15 + PR 15 mg/kg), suggesting a high dopamine metabolism and activity of the metabolizing enzymes MAO (monoamine oxidase) and COMT (catechol-O-methyltransferase). There was no change in the levels of HVA. Again, these findings may be attributed to the suggestion that low doses of permethrin and endosulfan exert a greater effect on the regulation of the dopamine transporter (DAT) and dopamine release than higher doses of these compounds (Bloomquist et al 2002; Kirby et al 2002, Karen et al 2001, Pittman et al 2003).

Regarding the levels of serotonin and NE, in our long-term study, NE was significantly decreased. This corresponded to the decreased levels of dopamine at the low dose treatments. As in the short-term study performed in adults, as DA is the precursor of NE, if DA is decreased, NE should be decreased. Although in the low mixture treatment (EN 2.15 + PR 15

mg/kg), NE was not significantly decreased, the P value for this group was P=0.09. Again, NE is not the main neurotransmitter of the basal ganglia and its detection is difficult.

Serotonin levels were significantly decreased corresponding to the decreased levels of dopamine at the low dose treatments, suggesting that these pesticides are able to alter both serotoninergic and dopaminergic systems at these concentrations. In contrast, the levels of the serotonin metabolite, 5-HIAA, were increased in the low dose mixture of pesticides (EN 2.15 + PR 15 mg/kg) and high dose mixture (EN 4.3 + PR 150 mg/kg) treatments groups, suggesting high serotonin metabolism. These findings support the results of the investigation of Medepalli et al (1994), who also saw diminution of serotonin levels at 25 days. However, in our study, mice were not used for the experiment as juveniles and, therefore, we cannot say at what point in the development of these juveniles that these changes occurred. Mice used in the Medepalli study were juveniles assessed as juveniles whereas in our study, mice were kept until adulthood (7-9 months). Therefore, changes occurring in mice of our study could have occurred at any point between the original dosing and re-challenge. Inclusion of mice assessed only as juveniles right after exposure to pesticides could have answered these questions. Other experiments have been performed with juveniles that were grown to adults but the studies were done with pesticides other than those used for the present experiment.

For example, Thiruchelvam et al (2002) carried out this kind of experiment in C57BL/6 mice with the pesticides maneb (MB) and paraquat (PQ). Levels of striatal DA were decreased by 37% following developmental exposure with MB + PQ only, but following adult re-challenge, the levels of dopamine were reduced by 62% compared to control.

Although we were expecting to find more dramatic changes on the levels of catecholamines and indolamines in this long term study, we found significantly decreased levels of dopamine in the low endosulfan treatment group (EN 2.15 mg/kg) and in the low mixture treatment group (EN 2.15 + PR 15 mg/kg). The use of more specific techniques such

as the study of receptors or changes in the dopamine and serotonin transporters may provide conclusive information to interpret these results.

2. ACETYLCHOLINESTERASE

Acetylcholine is the major neurotrasmitter in Alzheimer's disease. Because there has been much controversy about permethrin and endosulfan reducing or increasing the amount of acetylcholine and contributing to neurodegenerative diseases (see literature review), we wanted to examine this neurotransmitter by measuring the activity of acetylcholinesterase (AChE) in the cortex of mice treated only as adults and mice treated as juveniles and re-challenged as adults.

In the study with adult mice, the levels of acetylcholinesterase were significantly increased in the low mixture (EN 2.15 + PR 15 mg/kg), low endosulfan (EN 2.15 mg/kg) and high permethrin (PR 150 mg/kg) treatment groups. This differs from results obtained when mice were dosed as juveniles and re-challenged as adults. Under the latter pesticide treatment regimen, AChE was only increased in the high endosulfan (EN 4.3 mg/kg) treatment group. Previous studies both with endosulfan and pyrethroids such as permethin reported no alteration (Medepalli et al 1994) or decreased levels (Valeswara et al 1995) of AChE. In both of our studies we found higher levels of AChEsterase when compared with control. It has been proposed that dopamine controls the neurotransmission of acetylcholine in a reciprocally symmetric manner through stimulatory D_1 and inhibitory D_2 receptors (Di Chiara et al 1994). Therefore to fully understand these results we would have to look at the interaction between these receptors in a separate study.

3. TBARS AND α-SYNUCLEIN

Measurement of TBARS (thiobarbituric acid reactive species) detects lipid peroxidation produced by free radicals or oxygen reactive species. The metabolism of dopamine and norepinephrine in the brain occurs via oxidation or metabolic breakdown by monoamine oxidase to produce reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals (Cadet and Brannock 1998). α-Synuclein is a major component of Lewy bodies. The α-synuclein fibrillation process has been associated with the pathogenesis of neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Lee et al 2002). This aggregation process has been linked to the action and production of free radicals and the use of pesticides (Smith et al 2003, Manning-Bog et al 2001). In the present investigation we observed that levels of TBARS followed a pattern similar to the increases and decreases of the levels of dopamine, especially DOPAC (dopamine's main metabolite) among the groups of mice included in the study. In general, treatment groups that seemed to have higher amounts of dopamine or DOPAC also seemed to have higher amount of TBARS or peroxidation levels. Therefore, there seems to be a relationship between the levels of peroxidation and the levels of dopamine and its metabolites in pesticide-treated mice. Supporting our studies, Berman et al (1996) noted that reactive oxygen species and possibly dopamine quinones can modify dopamine transport function. In their study they observed, in rat striatal synaptosomes, that oxidation products of DA are able to inhibit the DAT function leading to increased levels of extra-cellular DA. In addition, other studies detected that midbrain and basal ganglia of Parkinsonian brains contain increased levels of DA peroxidizing activity when compared to normal brains. Furthermore, increased DA peroxidizing activity in Parkinson's patients generates the toxic compound dopaminochrome that may play a role in the pathogenesis of this disease (De Iuliis et al 2002). In general, because the substantia nigra is rich in dopamine,

Parkinson's disease patients show evidence of oxidative stress by the findings of increased lipid peroxidation and decreased reduced glutathione (Fahn et al 1992).

In our studies, high levels of TBARS, dopamine and DOPAC seemed to be related to the existence of more intense double protein bands in the western blots for α-synuclein. If this is correct, a possible mechanism for α-synuclein aggregation could be associated with the changes in the levels of catecholamines and peroxidation induced by pesticides. In fact, as in our studies, the investigation of Gillette et al (2003) reported that permethrin was able to modulate the dopaminergic system at low doses in a persistant manner. However they failed to view double bands of aggregated α-synuclein.

Mice dosed as adults

In our adult mice study, levels of TBARS were significantly elevated in the high mixture treatment group (EN 4.3 + PR 150 mg/kg), the low dose of endosulfan (EN 2.15 mg/kg) and the low dose of permethrin (PR 15 mg/kg). The treatment groups with high levels of dopamine or DOPAC had high levels of TBARS. Additionally, samples from these treatment groups also produced more intense double bands of α-synuclein depicted on western blots. Thus, western blot net intensity of the α-synuclein aggregated proportion was significant in the high mixture (EN 4.3 + PR150 mg/kg) treatment group. This treatment group showed 2 more double bands (~25 and ~45 kDa) in addition to the normal α-synuclein (~17 kDa), indicating aggregation of this protein in mice given this treatment. Other investigators have observed aggregation of α-synuclein (Hashimoto et al 1999; El-Agnaf et al 2002; Campbell et al 2001, Fasano et al 2003). As in our adult study, aggregates of α-synuclein in these studies migrated at an apparent molecular mass ranging between 19-45 kDa. These studies indicated as well that dopamine autoxidation and oxidative stress could be possible mechanisms for the aggregation of this protein.

Mice dosed as juveniles and re-challenged as adults

When mice were dosed as juveniles and re-challenged as adults, there were no significant differences in the levels of TBARS. However when represented graphically, the pattern seemed to correspond to the levels of DOPAC. Statistical analysis of the α-synuclein aggregated proportion indicated differences from control when these mice were given the low mixture of permethrin and endosulfan (EN 2.15 + PR 15 mg/kg). Samples from mice in this treatment group demonstrated a very intense double band (~25 kDa) visible in the western blot. Again, this appeared to correspond with the highest amount of DOPAC at the same treatment. In this case there were no triple bands visible but only this extra band (~25 kDa) in addition to the one for normal α-synuclein (~17 kDa) was observed.

In general, levels of catecholamines seem to be related to the levels of lipid peroxidation in the brains of the mice dosed with pesticides. Levels of lipid peroxidation seem to exert an effect on the levels of α-synuclein aggregation. Oligomerisation of this protein seems to correspond more to higher levels of dopamine and its metabolites than depletion of this neurotrasmitter. Mice dosed as juveniles and re-challenged as adults did not appear to be more affected than mice only dosed as adults when compared with control. Furthermore, mice dosed as juveniles and re-challenged as adults seem to develop an adaptation mechanism that could potentially protect them from subsequent exposure to pesticides. Future studies should include more specific techniques to determine the relationship among the factors studied.

4. PESTICIDE INTERACTIONS

Pesticide interactions were presented only when statistically significant (HVA, serotonin, 5-HIAA, TBARS and acetylcholinesterase).

Endosulfan with permethrin resulted in more interactions in adult mice than in mice dosed as juveniles and re-challenged as adults when comapared to controls. Only the

acetylcholinesterase interaction was siginificant for the latter experiment. Both high mixture (EN 4.3 + PR 150 mg/kg) and low mixture (EN 2.15 + PR 15 mg/kg) treatment groups were involved in interactions in adult mice. In this adult study, addition of endosulfan reduced the difference between corn oil and permethrin treated mice for serotonin, 5-HIAA and TBARS. Incorporation of endosulfan increased the difference between mice given corn oil and mice given permethrin for HVA. It is difficult to explain mechanisms associated with the significant interactions seen in our study. The literature contains no references that examined neurotoxic effects of endosulfan and permethrin in combination.

As in our studies, Costa (1988) observed that organoclorines and pyrethroids were able to alter neurotransmission by interacting with energy metabolism, sodium channels or ATPases. Many studies agree in that pyrethroids such permethrin and organochlorines such heptachlor induce changes in the levels of neurotransmitters and their transporters at relatively low doses (Bloomquist et al 2002, Kirby et al 1996). In this study, low doses of endosulfan seem to decrease the amount of serotonin in the presence of low doses of permethrin. This may correspond with an increase in the levels of the primary serotonin metabolite (5-HIAA) when the mixture is present at the same concentrations. Additionally, in our study, acetylcholinesterase levels were decreased when mice were given both endosulfan and permethrin. This could be related to the observations of Rao and Rao (1995) who demonstrated that permethrin inhibited AChE activity in rat brain. More studies about interactions are necessary to fully understand the effect in combinations of these two pesticides. Therefore, precise mechanisms responsible for these interactions remain to be defined.

PART 6: CONCLUSIONS

The present investigation studied the levels of neurotransmitters, oxidative status and aggregation of α-synuclein in C57BLl/6 male mice exposed to different concentrations of the pesticides permethrin, endosulfan and its mixtures at different ages.

It was hypothesized that the exposure to these pesticides during early life could result in an augmented susceptibility to subsequent environmental exposures, potentially unmasking a silent neurotoxicity later in life. However, instead, mice dosed as juveniles and re-challenged as adults seemed to develop an adaptation response to the exposure of these pesticides.

In general:

- Low doses of these pesticides and its low dose combination mixture seem to exert a greater effect on the levels of catecholamines and indolamines than high doses of the same pesticides. Therefore, there seems to be an inverse dose-response for some of the paramenters examined. These findings correspond to the results obtained by Bloomquist and colleagues (Bloomquist et al 2002; Kirby et al 2002, Karen et al 2001, Pittman et al 2003).

- Acetylcholinesterase levels were increased in some of the treatment groups possibly due to the fact that dopamine seems to be able to control the neurotransmission of acetylcholine in a reciprocally symmetric manner through stimulatory D_1 and inhibitory D_2 receptors (Di Chiara et al 1994). More studies are needed to fully explain this observation.

- High dopamine and DOPAC levels in the striatum seem to correspond to high levels of TBARS in the cortex and, therefore, oxidative stress. Actions produced on the corpora striata seem to have an effect on the cortex through the thalamocortical pathway. Threfore, increased peroxidation derived from the auto-oxidation of dopamine into several toxic metabolites and free radicals in the basal ganglia seems to have an effect in cortical areas possibly affecting α-synuclein aggregation among other structures or areas.

- α-Synuclein aggregation seems to be related to the levels of dopamine and its metabolites and to the levels of lipid peroxides. Because aggregation of α-synuclein seems to be increased in all treatment groups except in the control, pesticides appear to contribute somehow to this protein aggregation. α-Synuclein is a common protein in normal brains but it is found in higher amounts as aggregates from patients with neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.

Taking these facts into account, exposure to pesticides such as endosulfan and permethrin could make a contribution towards the initiation or aggravation of such diseases.

PART 7: FUTURE STUDIES

Future research should focus on the study of the dopamine and serotonin transporters and their relationship with α-synuclein since this will determine if the aggregations of this protein is more specifically related to changes occurring in the thalamocortical pathaway. Changes in transporters can occur without changes in neurotransmitters (Gillette et al 2003). In addition, dopamine receptors 1 and 2 (D_1 and D_2) should be examined to fully understand their possible effect on the control of acetylcholine release in the striatum and cortex. Receptor studies will also provide information about the possible pharmacodynamic adaptation mechanisms that could take place in these mice within the basal ganglia. Furthermore, to better assess this adaptation response of juvenile mice re-challenged as adults, liver tissues should be analyzed for changes in the metabolic pathways contributing to bioavailability of pesticides, as pharmacokinetics may contribute to adaptation.

A larger animal population should also be considered in order to improve statistical strength. More tissue samples would also permit measurment of all the endpoints proposed both in cortex and striatum.

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VITA

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Right after graduation, she got an ISEP (International Student Exchange Program) scholarship to pursue graduate studies in the United States of America. In August 2001, she arrived at Virginia Tech as an undergraduate student. After one year taking undergraduate courses and undergraduate research at the Virginia-Maryland Regional College of Veterinary Medicine, Dr. Hara P. Misra proposed that she stay and enroll in a Master's program at the same institution. Here, she continued her academic responsibilities under the supervison of Dr. Hara P. Misra, Dr. Marion Ehrich and Dr. Virginia Buechner-Maxwell.

After graduation, Carolina will continue her professional formation in Barcelona, Spain, working at Dr. Manuela Martinez's foundation for the Zellweger syndrome.