Presentation 8 – Mohamed Abou-Donia

Gene Expression Profiles Following Sarin Exposure

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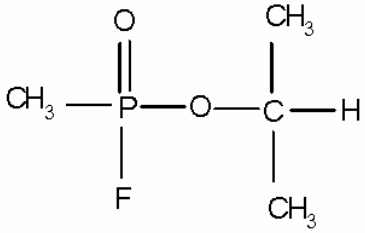
Test Compound



Sarin

Sarin-Induced Neurotoxicity

Sarin causes the following neurotoxic effects:

1. Cholinergic Neurotoxicity
2. Organophosphate-Induced Delayed Neurotoxicity (OPIDN)
3. Organophosphate-Induced Chronic Neurotoxicity (OPICN)
   a. Acute, high-level exposure
   b. Low-level Exposure

Cholinergic System

Neurotransmitter: *Acetylcholine (ACh)*

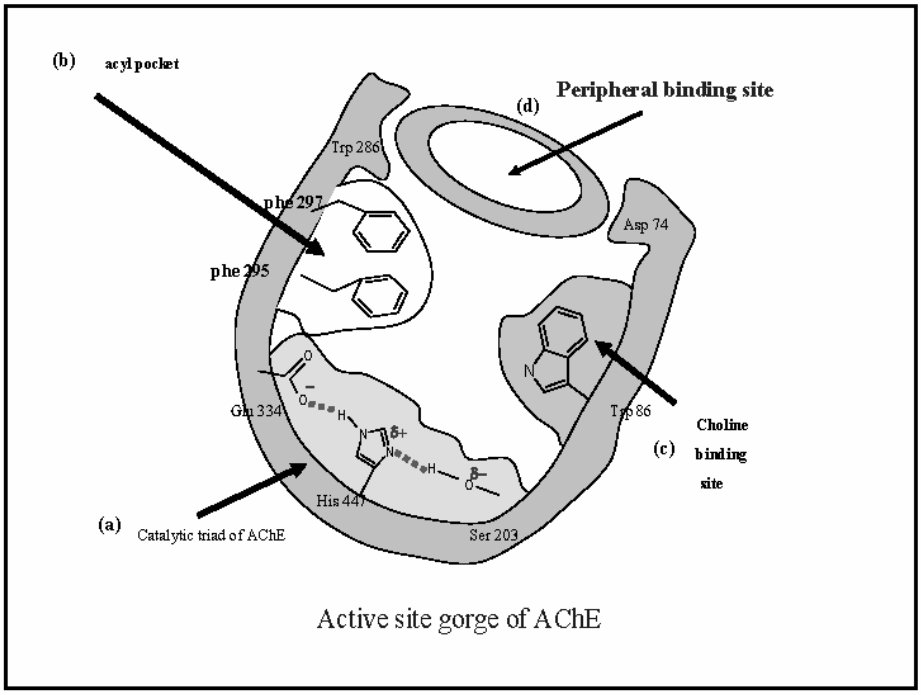
Synthesis: *Choline acetyltransferase (ChAT)*

Choline + AcetylCoA + ChAT → ACh

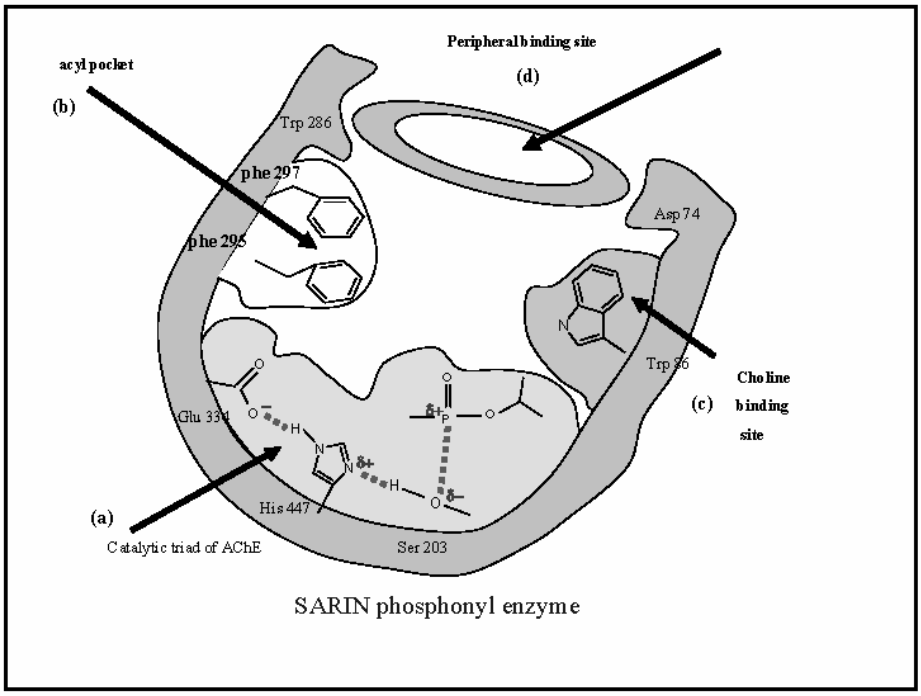
Action: Stimulation of *Muscarinic* and *nicotinic acetylcholine receptors*

Hydrolysis: *Acetylcholinesterase (AChE)*

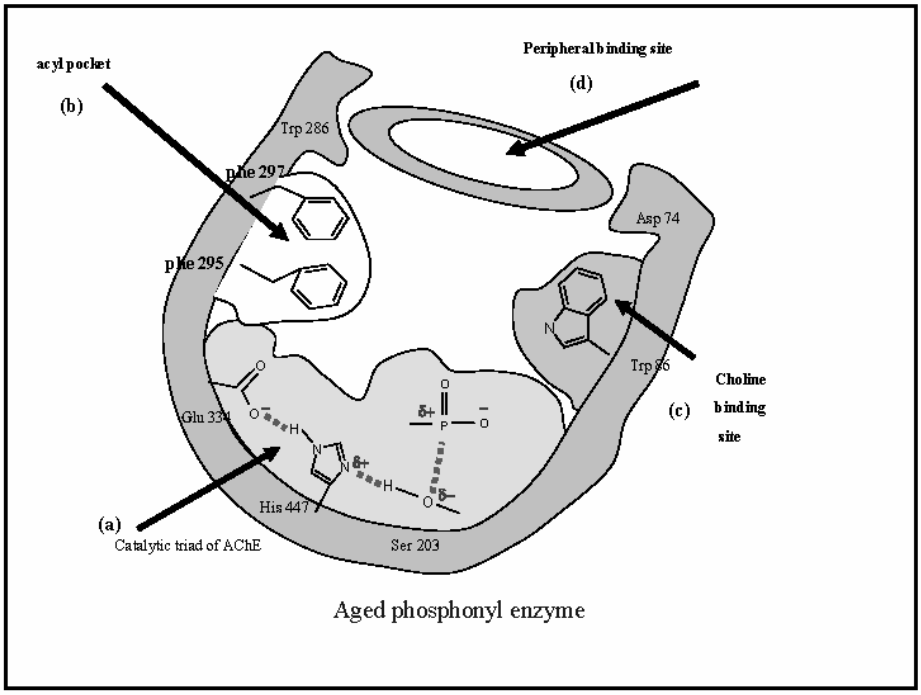
ACh + AChE → Choline + Acetic acid



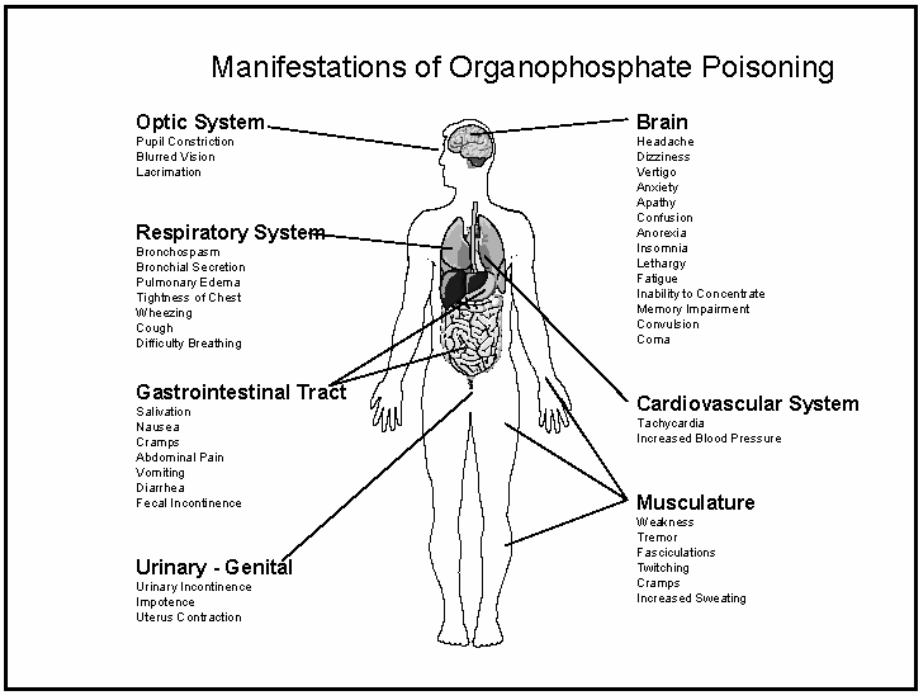
Active site gorge of AChE



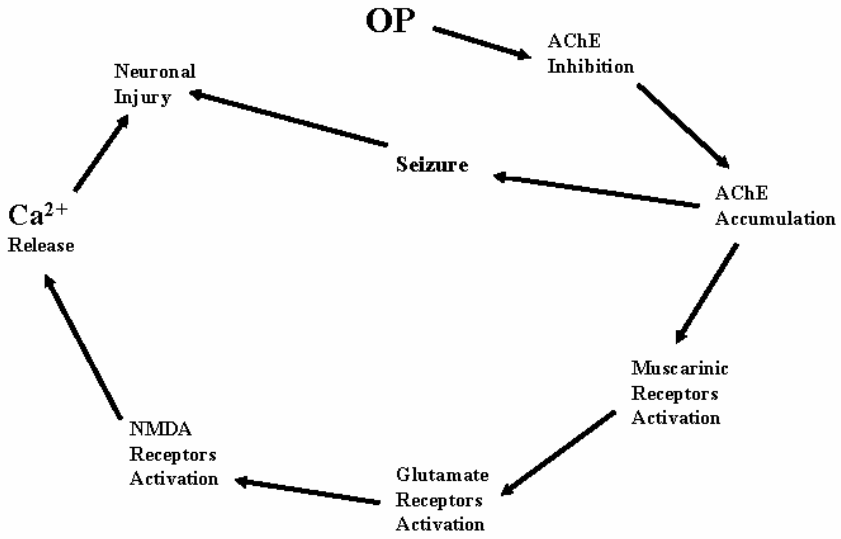
SARIN phosphonyl enzyme



Aged phosphonyl enzyme



Cholinergic cascade following a large toxic dose of organophosphates (OP)



Human Exposure to Sarin

1. **<u>High-level (Acute) Exposure:</u>**
   a) Matsumoto City, Japan
   At midnight of June 27, 1994
   b) Tokyo subway trains
   At 8:05 AM, on March 20, 1995
2. **<u>Low-level Exposure:</u>**
   In 1991during the Persian Gulf War
   U.S. military personnel were exposed to low-level sarin during the destruction of Iraqi munition-containing sarin at Khamisiyah

Matsumoto (Cholinergic)

1. Sarin release was at midnight on June 27, 1994
2. About 600 persons were exposed
3. Fifty eight were admitted to hospitals
4. Seven died
5. Miosis was the most common symptom
6. Severe cases developed CNS symptoms and cardiomyopathy
7. A few victims complained of arrhythmia and showed cardiac contraction.

Tokyo (Cholinergic)

Patients: 58 , (Zuzuki, et al. 1999)

Severe Poisoning:

Reduced consciousness, miosis, flushing, respiratory distress, *fasciculation, tachycardia, high blood pressure* (nicotinic responses), and flaccid paralysis. Plasma AChE activity: 35% of normal. (Hospital)

Mild poisoning:

Headaches, dizziness, nausea, chest discomfort, abdominal cramps, marked miosis.

Tokyo (Cholinergic)

Route of exposure:

Absence of bradycardia, excessive secretions, which are common in dermal or ingestion exposure, may be related to exposure to gas.

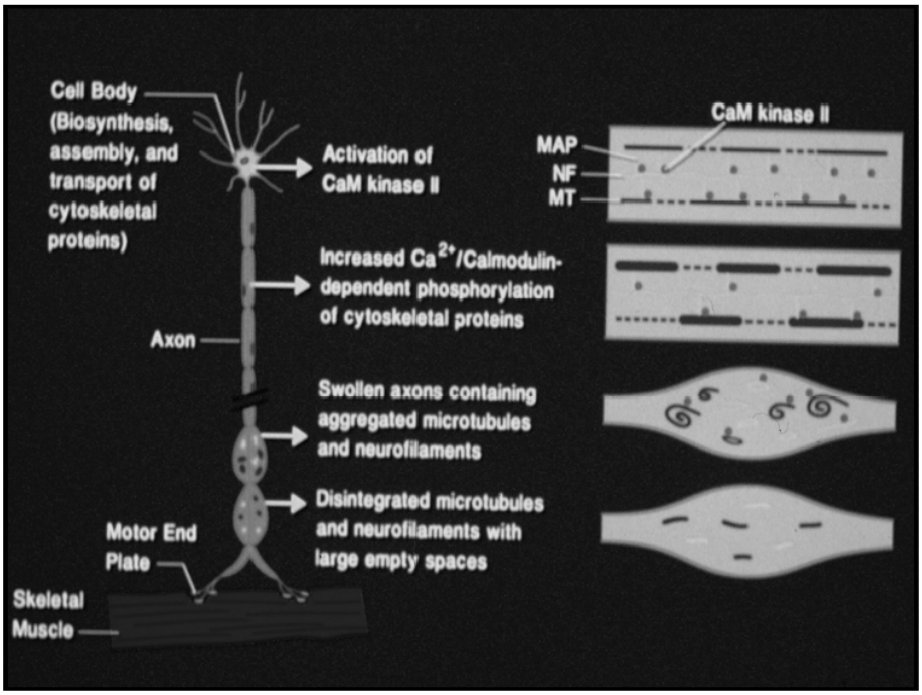
Treatments:

1. Atropine eye drops for marked miosis.
2. Pralidoxime Iodide (2-PAM)

OPIDN

Characteristics of OPIDN:

1. A latent period, ranging between 6 and 14 days.
2. Neuropathological lesions are in the medulla of the brain, spinal cord, and sciatic nerve.
3. Primary degeneration of the axon, followed secondary degeneration of myelin (Wallerian).
4. Species and age sensitivity.
5. Inhibition of neurotoxicity target esterase (NTE).



Sarin-Induced OPIDN

The patient: A 51-year man inhaled sarin in the Tokyo subway, died 15 Months later.

neuropathologic examination:

marked *nerve decreased* in the sural nerve, moderate *nerve fiber loss* in the sciatic nerve, unremarkable dorsal root ganglia, dorsal roots and posterior column of the spinal cord.

Conclusion:

Pathology is consistent with OPIDN.

Sarin-Induced Chronic Neurotoxicity (OPICN)

1. Results from direct action of sarin on brain neuronal and glial cells.
2. Leads to neurologial dysfunctions characterized by:
   a) Cognition impairment.
   b) Locomotor and sensory deficits.
   c) Body weakness and incoordination.
   d) Behavioral abnormalities.

OPICN

1. Six to eight months after Tokyo poisoning, some victims showed delayed effects on psychomotor performance, visual nervous system, and the vestibulo-cerebellar system, (Yokoyama et al., 1998).
2. Females were more sensitive than males in exhibiting delayed effect on the vestibulo-cerebellar system.

OPICN

1. **Three years and nine months** after the **Tokyo** attack, some victims and rescue workers complained of chronic decline of memory (Nishiwaki et al, 2001).
2. **Three years** after the **Matsumoto** attack, some victims complained of fatigue, shoulder stiffness, weakness, blurred vision (Nakajima et al., 1999)
3. Others complained of insomnia, had bad dreams, husky voice, slight fever, and palpitation.

OPICN

"Chronic decline of psychomotor function and memory still exist in Tokyo subway workers, 7 years after the sarin exposure"

K. Miyaki et al., J. Occup. Health 47:,299-304 (2005)

The Gulf War Syndrome

1. Between the invasion of Kuwait by Iraq on August 2, 1990, and March 1991, the U.S. had 697,000 military personnel in the Persian Gulf region.
2. Since their return, several thousands, complained of the following chronic symptoms:
   headache, loss of memory, fatigue, muscle and joint pain, ataxia, skin rash, respiratory difficulties, and gastrointestinal difficulties.
3. A recent report showed that PGW veterans are twice more likely to develop amyotrophic lateral Sclerosis (ALS) than other military personnel.

HYPOTHESIS

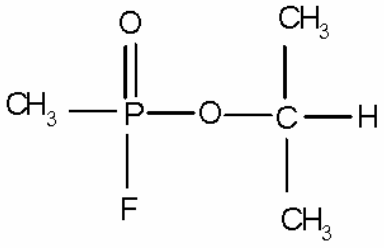
Exposure to low level sarin, alone or in combination with other chemicals and/or stress was involved in the development of the Persian Golf War Veterans' illnesses.

Specific Aims

To investigate the neurological deficits in the adult male rat, following exposure to sarin:

1. Clinical signs
2. Brain AChE and Plasma BChE
3. M2 muscarinic receptors
4. Integrity of the blood blood barrier (BBB)
5. Brain neuropathological alterations

Test Compound



Sarin

The stock solution of Sarin (1.9 mg/ml) in saline was stored frozen at –80^0 C prior to use.

Experimental

Treatment: A dose-response, time course study

Male Sprague-Dawley rats (225 g) were given a single i.m injection of :

1. Saline, 0.1 ml/kg (Control)
2. Sarin, 1.0 x LD_{50} (100 µg/kg)
3. Sarin, 0.5 x LD_{50} (50 µg/kg)
4. Sarin, 0.1 x LD_{50} (10 µg/kg)
5. Sarin, 0.01 x LD_{50} (1 µg/kg)
6. Groups of 15 animals from each treatment and control were sacrificed at: 24 hour, 7 days, 30 days, and 10 months.

Clinical Condition

Sarin at 1 x LD50:

Severe tumors, seizures, and salivation within 3 – 5 min. of treatment. Half of the animals died within 15 minutes.

Sarin at 0.5 x LD50:

Tremors by 15- 30 min. no animals died.

Sarin at 0.1 and 0.01 x LD50:

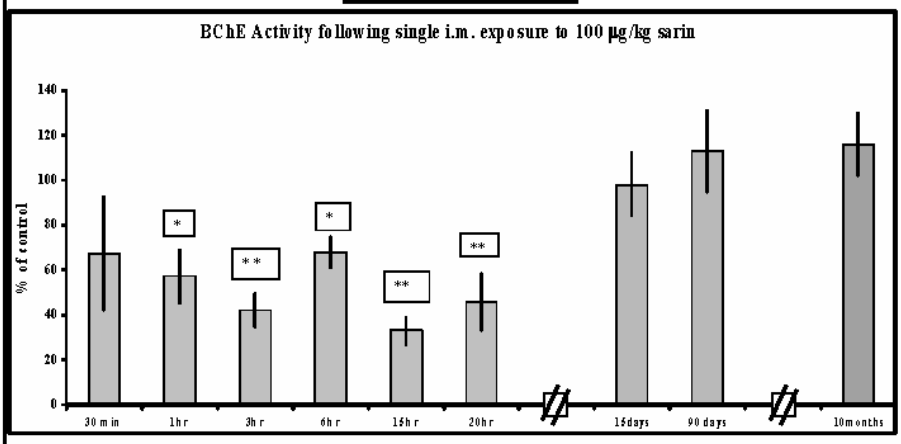
No toxicity signs.

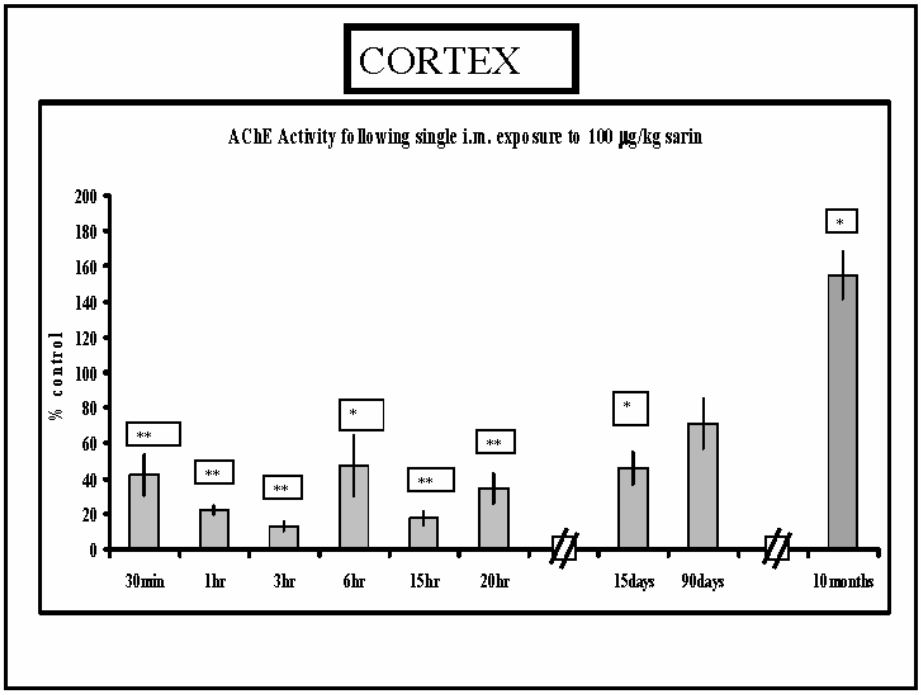
Effect on enzymatic activity

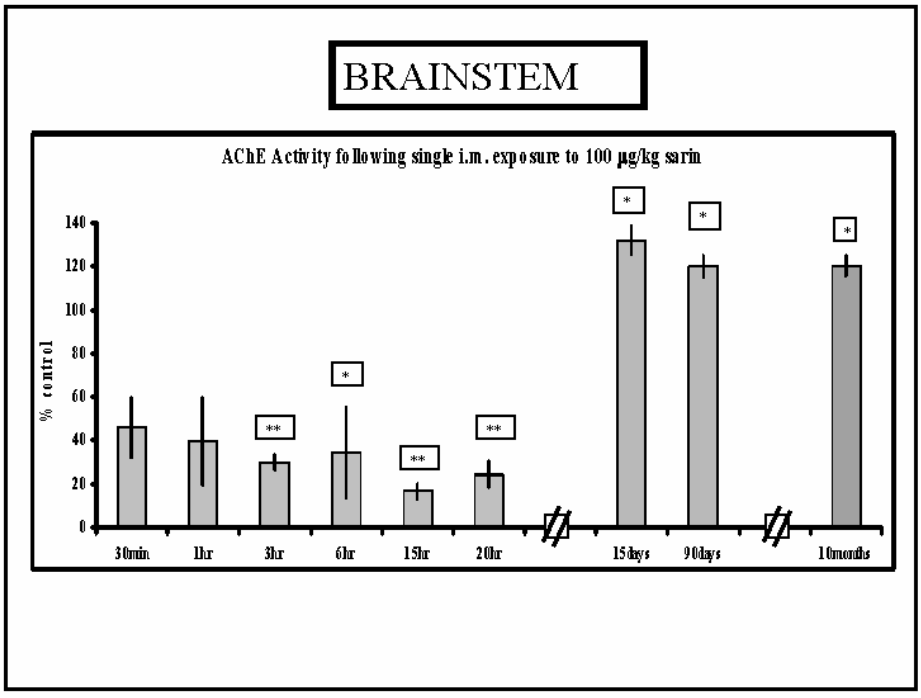
The effect of sarin exposure was determined on the following enzymes:

1. Plasma butyrylcholinesterase (BChE)
2. Brain acetylcholineasterase (AChE)
3. Brain Choline acetyltransferase (ChAT)

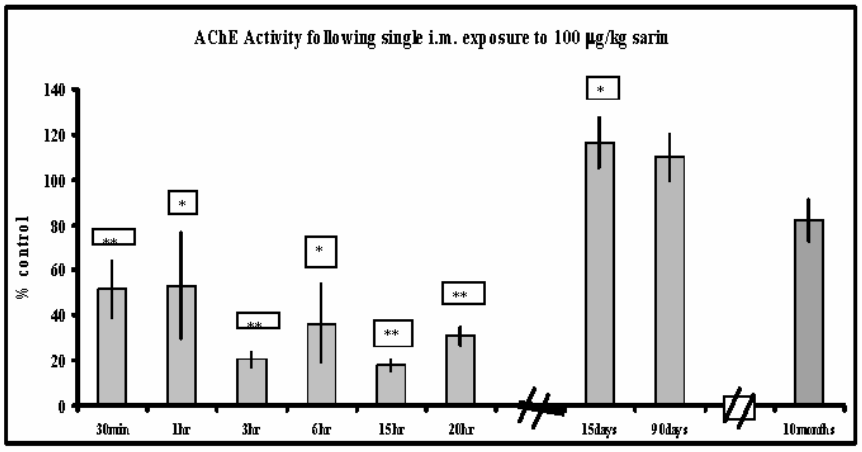
PLASMA



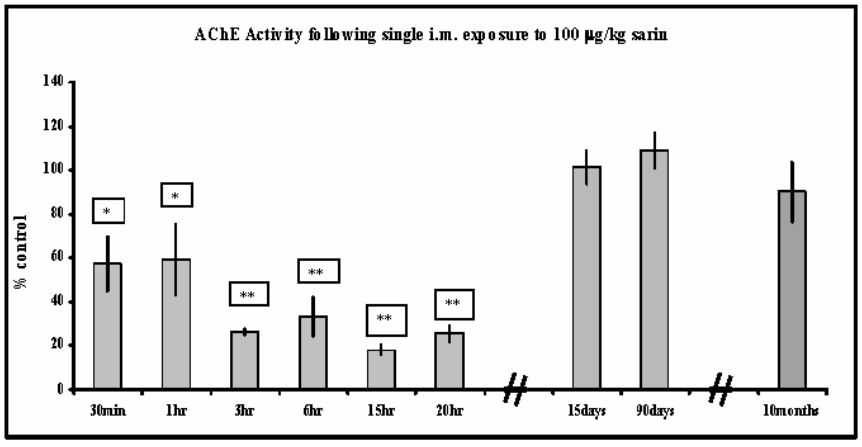

CORTEX
AChE Activity following single i.m. exposure to 100 µg/kg sarin
% control
200
180
160
140
120
100
80
60
40
20
0
**
**
**
*
**
**
*
*
30min
1hr
3hr
6hr
15hr
20hr
15days
90days
10 months


BRAINSTEM
AChE Activity following single i.m. exposure to 100 µg/kg sarin
% control
140
120
100
80
60
40
20
0
**
**
**
*
*
*
30min
1hr
3hr
6hr
15hr
20hr
15days
90days
10months

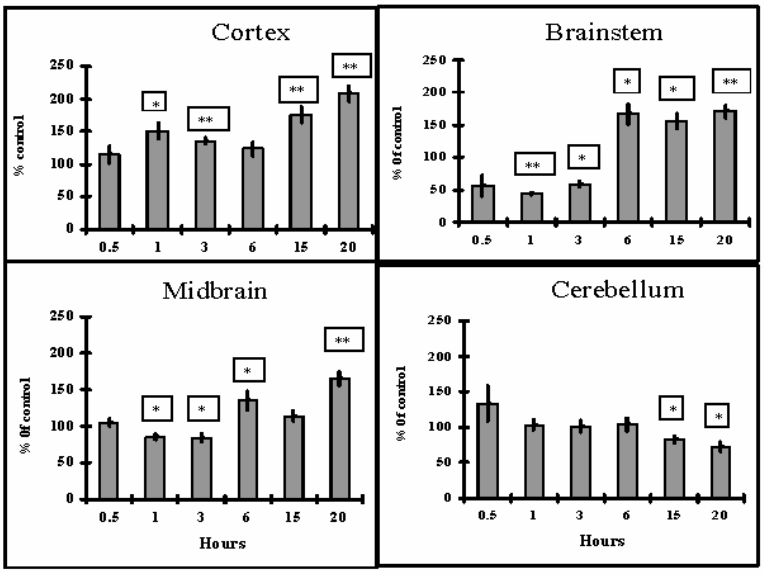
MIDBRAIN



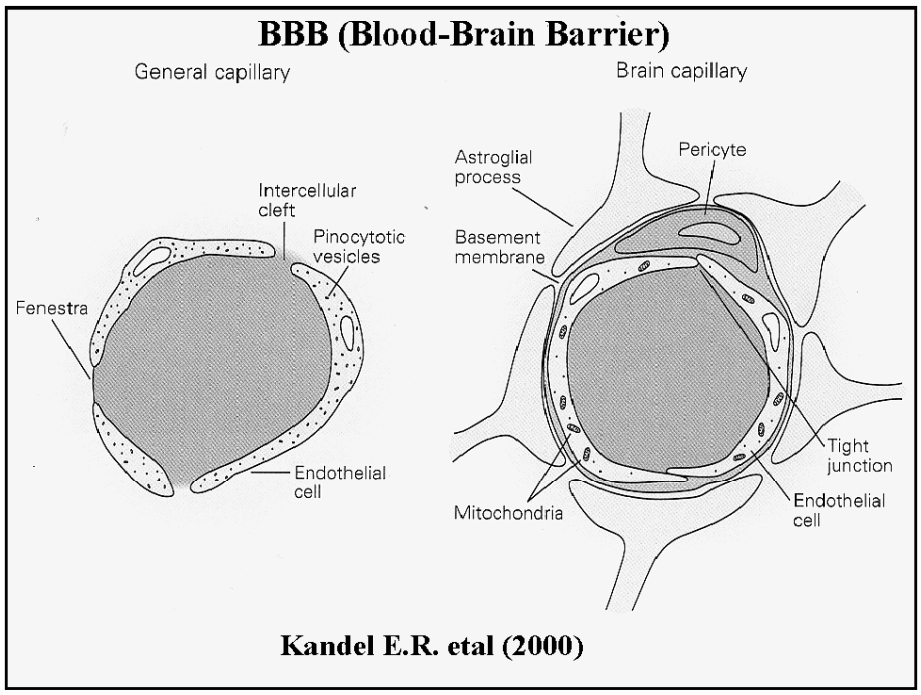
CEREBELLUM



ChAT Activity following single i.m. exposure to 100 μg/kg sarin



Effect of Sarin on the Blood Brain Barrier

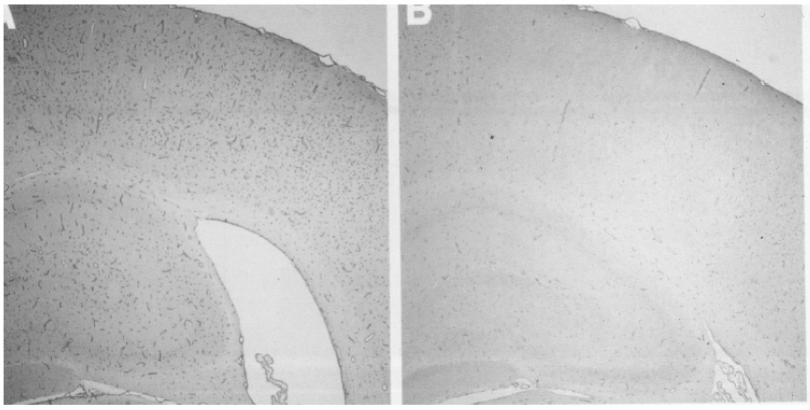


Blood Brain Barrier studies

Alterations in the permeability of the BBB were assessed by immunohistchemical staining of *endothelial barrier antigen* (EBA) (using SMI-71 antibody).

This staining visualizes BBB protein in brain capillaries and in smaller vessels invading the brain parenchyma.

EBA Immunostaining



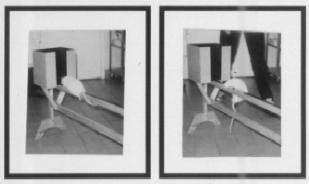
Control **1.0 x LD50 sarin**

Effect of Sarin on Sensorimotor Performance

Control and treated animals were evaluated for sensorimotor performance using the following tests:

1. Beam walk performance and time
2. Incline plane performance
3. Forepaw grip time

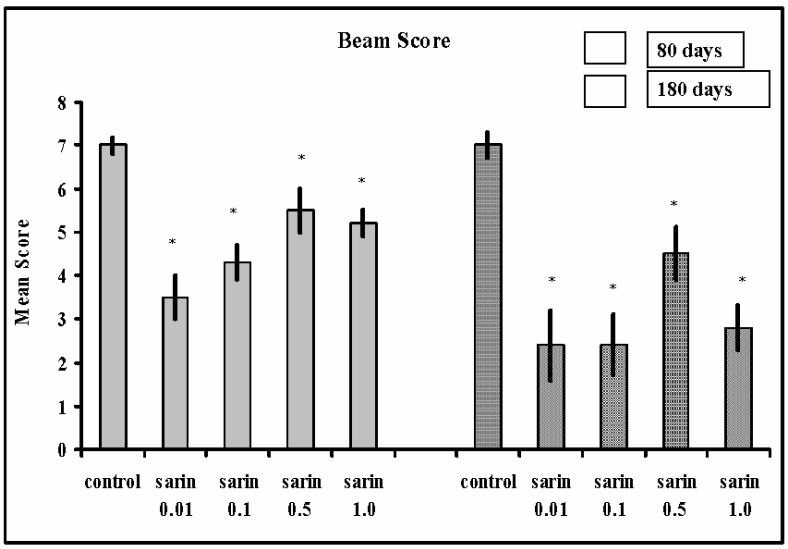
Beam Walk

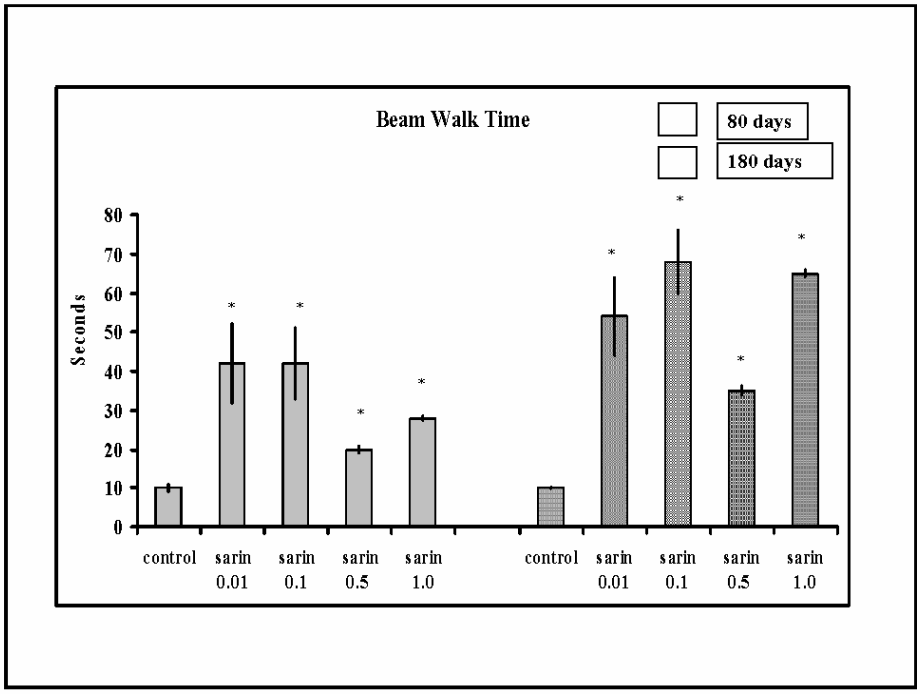


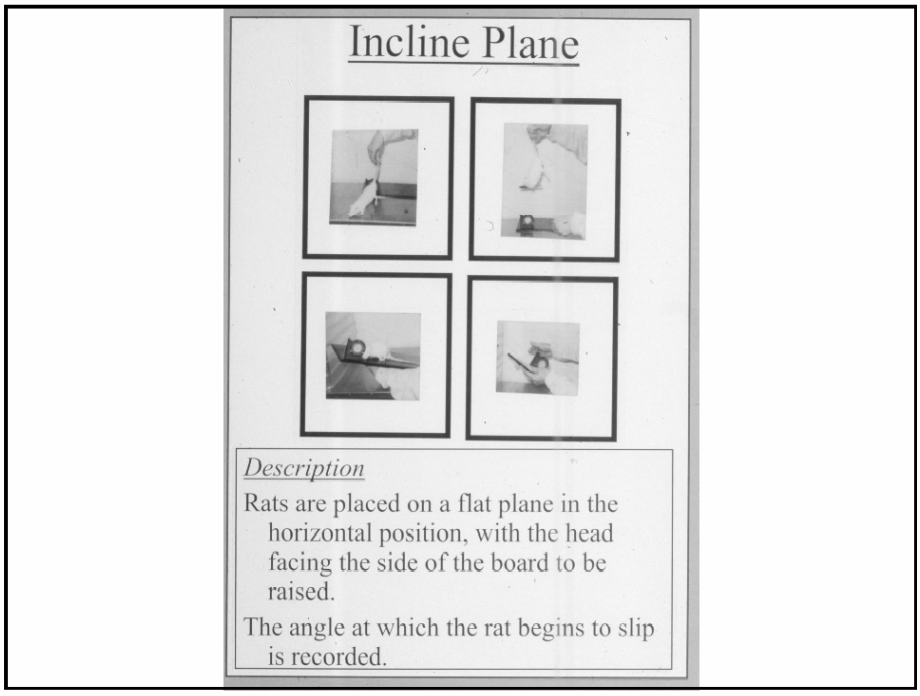
The apparatus consists of an elevated wooden beam, a goal box with an opening located at the end of the beam, and a light source.

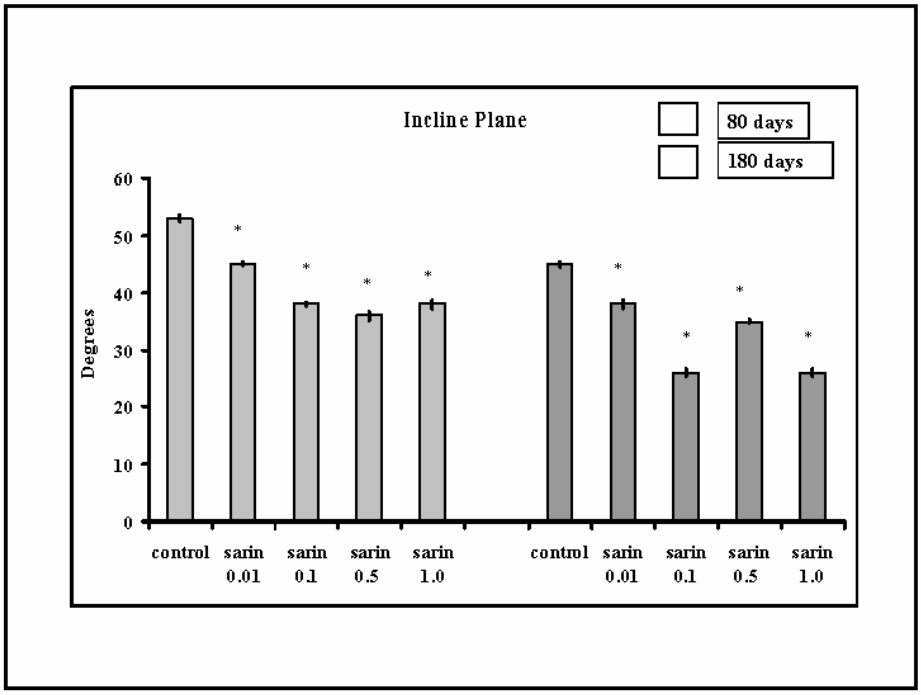
BW Time: The time until the animal's nose entered the box (up to 90 sec.).

BW Score: A 7-point scoring system for the the use of the hind paw to aid locomotion.




Beam Walk Time
80 days
180 days
Seconds
0
10
20
30
40
50
60
70
80
control
sarin 0.01
sarin 0.1
sarin 0.5
sarin 1.0
control
sarin 0.01
sarin 0.1
sarin 0.5
sarin 1.0


Incline Plane
Description
Rats are placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised.
The angle at which the rat begins to slip is recorded.

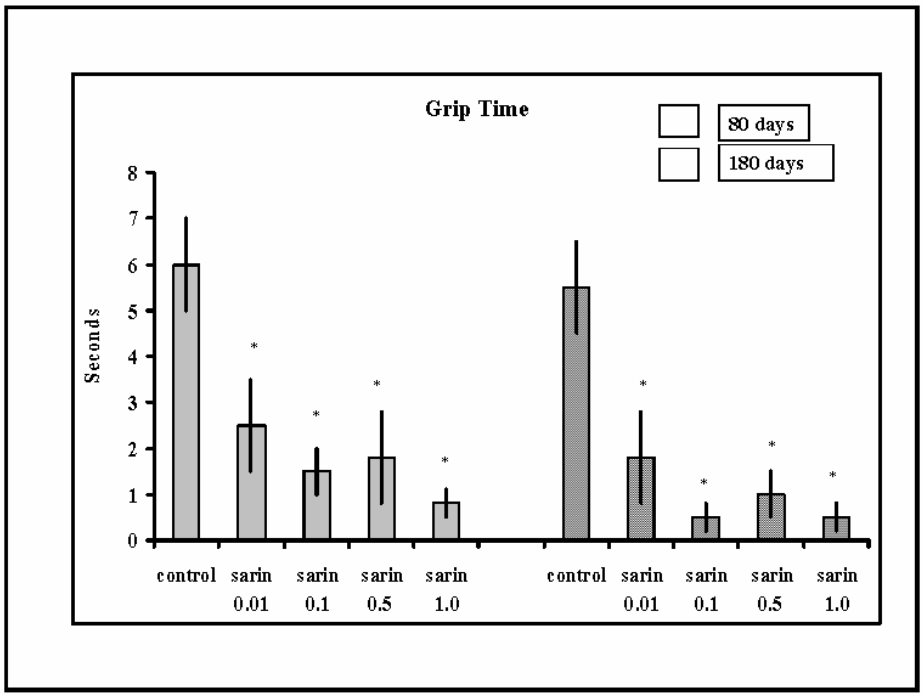


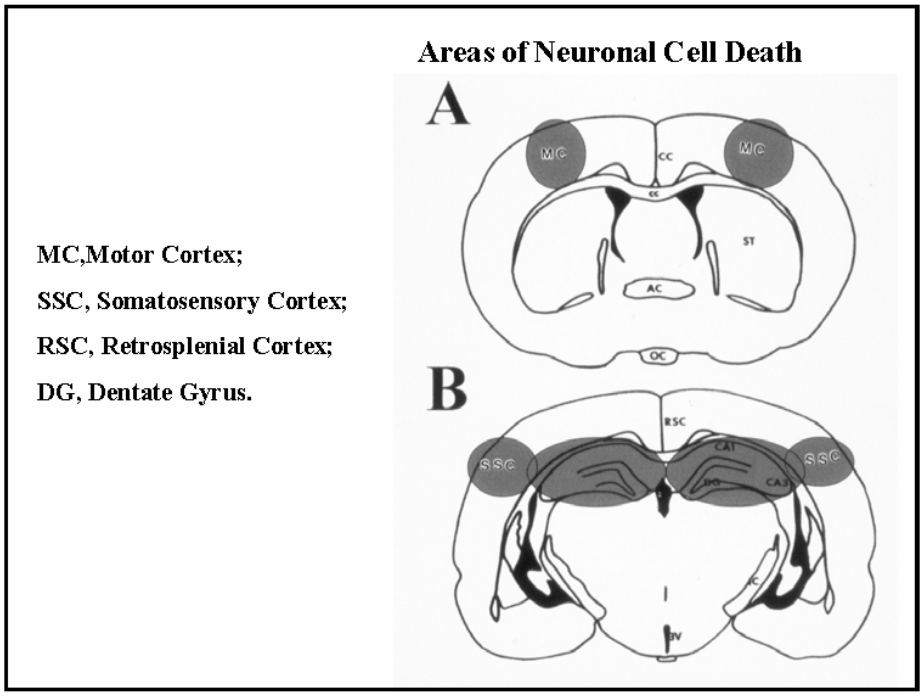
GRIP TIME

PURPOSE: To assess forepaw grip strength

PROCEDURE:

1. Have the rats grip a 5-mm diameter wood dowel
2. Time to release grip is recorded in seconds.


Grip Time
80 days
180 days
Seconds
0
1
2
3
4
5
6
7
8
control
sarin 0.01
sarin 0.1
sarin 0.5
sarin 1.0
control
sarin 0.01
sarin 0.1
sarin 0.5
sarin 1.0


Areas of Neuronal Cell Death
MC,Motor Cortex;
SSC, Somatosensory Cortex;
RSC, Retrosplenial Cortex;
DG, Dentate Gyrus.
A
MC
MC
CC
ST
AC
OC
B
RSC
SSC
SSC
CA1
CA3
DG
3V

Neuropathological Studies

NEUROPATHOLOGICAL STUDIES INCLUDED:

1. Cerebral cortex
2. Hippocampus
3. Cerebellum

AChE-Induced NeuronalCell Death

1. An increased AChE protein, in Alzheimer disease causes aggregation of β amyloid peptide, causing neuronal cell death (Inestrosa et al., 1996; Calderon et al., 1988).
2. Over expression of AChE activates caspases, leading to apoptosis.

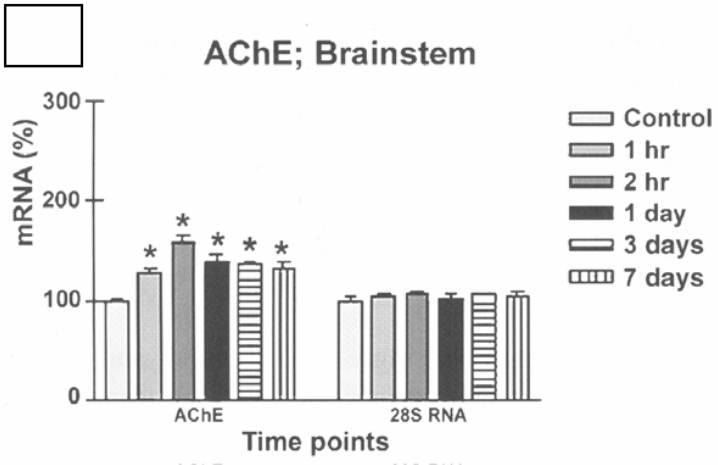
Sarin-Induced Expression of mRNA Coding of Acetylcholinesterase

Dose: 0.5 x LD_{50} im sarin

Time points: 1 and 2 hours, 1, 3, and 7 days.

Results:

Sarin produced immediate and persistent induction of AChE mRNA levels in various regions of the brain.



Introduction

Gene profiling studies have the promise to delineate global alterations in molecular expression as toxic effects and mechanisms of action of chemicals with multitude effects, such as sarin GB:

(*O*-isopropyl methylphosphonofluoridate)

Specific Aim

To study sarin-induced global expression profiles at:

1. **15 min; after 0.5 x LD_{50} sarin**
2. **30 min; after 1.0 x LD_{50} sarin**

(Intramuscular LD_{50} = 100 µg/kg)

Using Affymetrix : Rat Neurobiology U34 Chips in male Sprague-Dawley rats,

Results: Clinical condition

Rats given 1 X LD_{50} exhibited:

1. Excessive salivation, severe tremors, seizures, and convulsions within 5-10 min.
2. Prolonged convulsion ensued for 3 hr.
3. One half of the animals died within 3 hr.
4. The remaining animals survived the 3-month experiment.

RNA Isolation

1. At each time-point, animals were euthanized, brain dissected out, and separated into the cortex, cerebellum, midbrain and brainstem.
2. RNA was extracted using Trizol solution
3. About 200-250 µg/µl of total RNA was applied on RNA chip and analyzed on the Agilent Bioanalyzer 2100.

Chips

1. Rat Neurobiology U34 array of the Affymetrix gene chip was used.
2. It allowed monitoring the relative abundance of more than 1200 mRNA transcripts.
3. It contains genes representing different cell types, signaling pathways, and other functional and structural groups relevant to the nervous system.

Affymetrix chip hybridization

1. The double-stranded cDNA from total RNA was synthesized and isolated from the rat tissue.
2. Biotin-labeled cRNA was generated by *in vitro* transcription from the DNA.
3. The cRNAs were hybridized to the oligonucleotide probes on the probe arrays for a 16 h incubation at 45 C.
4. The DNA chips were scanned with the Affymetrix gene chip scanner.

Data Analysis

Duke University Bioinformatics Shared Resources Consortium

1. Affymetrix Micrtoarray Software Solutions were used to identify the list of genes showing statistically significant levels of alterations.
2. Partek clustering and treeview analysis program was used to identify clustering of genes that showed alteration.

RESULTS

Number of genes showed predominated alterations:

1. At 15 minutes (0.5 X LD_{50}) a total of 65 genes
2. At 3 months (1.0 X LD_{50}) a total of 36

RESULTS: 15 Minutes

At 15 minutes (0.5 X LD_{50}) the following classes of altered genes predominated:

1. Ion channel and Cell adhesion molecule (8 genes).
2. Cytoskeletal proteins (8 genes).
3. Neuropeptides and their receptors (5 genes each).

RESULTS : 15 Minutes

The following categories had 2 genes each:

1. Cholinergic signaling
2. Energy metabolism
3. GABAnergic signaling
4. Glutamergic and aspartate signaling
5. Mitochondria associated proteins
6. Myelin proteins
7. Neurotransmission and related transporters
8. Serotonergic signaling,and
9. Tyrosine phosphorylation molecule

RESULTS : 15 Minutes

The following categories had 1 gene each:

1. ATPases and ATP-based transporters
2. Catecholaminergic signaling
3. Cyclic nucleotide signaling
4. Mitochondria associated proteins
5. Nitric Oxide signaling
6. TNF beta family, and
7. Transcription factors

RESULTS : 15 Minutes

Other altered genes at 15 minutes (0.5 X LD_{50}) were:

1. Cholinergic signaling
2. Calcium channels
3. Calcium binding proteins
4. Transporters
5. Chemokines
6. GABAnergic
7. Glutamatergic
8. Aspartate
9. Catecholaminergic
10. Nitric oxide synthase
11. Purinergic
12. Serotonergic signaling molecules

Results: 15 Minutes (% of Control)

Receptors

- Nicotinic ACh receptor (150 ± 9)
- Muscarinic ACh receptor (214 ± 4)
- Glutamate receptor (177 ± 2)
- NMDA receptor-like long variant (142 ± 2%)
- GABA-A receptor α-subunit (130 ± 5)
- Dopamine receptor (225 ± 3)
- A1 adenosine receptor (201 ± 1)
- Purinergic receptor (193 ± 2)
- Tyrosine Kinase receptor (238 ± 3)

Results: Clinical condition

Rats given 0.5 X LD_{50} exhibited:

1. Did not develop any of the signs seen in animals given.
2. They were inactive 1 X LD_{50}.
3. All animals survived the experimental period

Results: 15 Minutes

Down-regulated genes (4 out of 27) ranging from 46 ± 11% - 38%

1. Cyclic nucleotide signaling
2. Detoxification molecules
3. Mitochondria associated proteins
4. Neurotransmission and neurotransmitter transporters

Results: 15 Minutes

1. Metabolism Enzyme:
   Cytochrome P-450 (65% ± 4%)

2. Detoxification Enzyme:
   Glutathione *S*-transferase (71% ± 2)

Results: 15 Minutes

Mitochondrial Associated Proteins:

Bax apoptosis exposure (75 ± 5)

Bcl-2-related ovarian killer protein (BOK, 70 ± 7).

Nitric oxide signaling:

Nitric oxide synthase (Nos-2, 167% ±1)

Results: 3 Months

- A total of 38 genes were altered
- An equal number of gene showed up-regulation and down regulation (50%)

Results: 3 Months

Calcium/calmodulin Protein Kinase II

Brain Region	% of control
Brainstem	140 ± 5
Cerebellum	182 ± 2
Cortex	35 ± 3
Midbrain	62 ± 6

Results: 3 Months

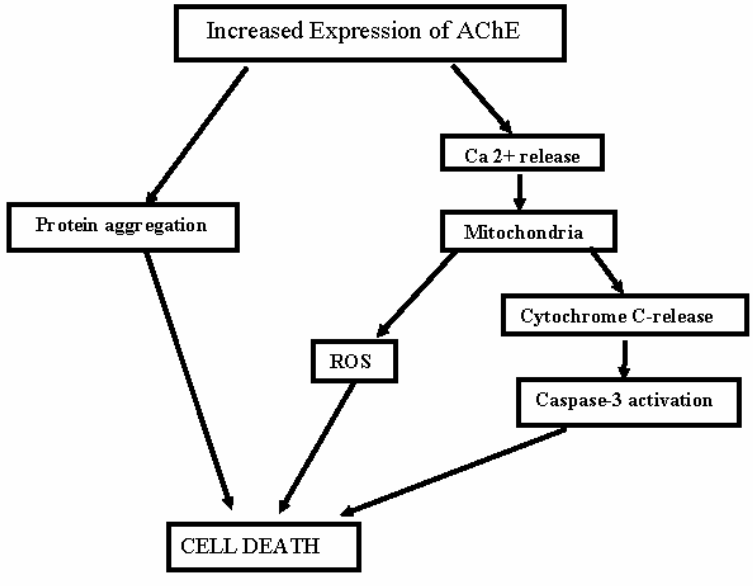
Receptors

1. GABA A receptor (166 ± 2)
2. Glutamate receptor, AMPA subtype (236 ± 1).

RESULTS : 3 Months

At 3 months (1.0 X LD_{50}) the following classes of altered genes predominated:

1. Calcium channels
2. Calcium binding proteins
3. Cytoskeletal proteins
4. Cell adhesion molecule
5. GABAnergic signaling molecules



Proposed mechanism of neuronal cell death by increased expression of AChE