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Novel Neuroprotectants for Sarin plus CBDP Induced Convulsions

Kaushal V. Joshi
Wright State University

Novel Neuroprotectants for Sarin plus CBDP induced convulsions

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By

Kaushal Joshi
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY KAUSHAL V. JOSHI ENTITLED *"NOVEL NEUROPROTECTANTS FOR SARIN PLUS CBDP INDUCED CONVULSIONS"* BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

_____
James B. Lucot, Ph.D.
Thesis Director

_____
Terry Oroszi
Director, Graduate Program

_____
Mariana Morris, Ph.D.
Department Chair

Committee on Final Examination

_____
James B. Lucot, Ph.D.

_____
David R. Cool, Ph.D.

_____
Khalid M. Elased, Ph.D.

_____

Joseph F. Thomas, Jr., Ph.D.

Dean, School of Graduate Studies

ABSTRACT

Joshi, Kaushal .M.S., Department of Pharmacology and Toxicology, Wright State University, 2009.
NOVEL NEUROPROTECTANTS FOR SARIN PLUS CBDP INDUCED CONVULSIONS. [1]

Sarin, also known as Sarin (German agent B) is classified as a weapon of mass destruction. Sarin (O-isopropyl methyl phosphonofluoridate) is a highly toxic nerve agent originally produced for chemical warfare and has been used in terrorist activities. Sarin is an extremely potent acetylcholinesterase inhibitor with high specificity and affinity for the enzyme. High sarin doses causes death due to anoxia resulting from airway obstruction, weakness of the muscles of respiration, respiratory failure and convulsions. Current treatments are still not effective at protecting against long term effects following exposure. A current approach aims to counteract the increased glutamatergic and cholinergic neurotransmission occurring in sarin neurotoxicity. In vitro and in vivo, serotonin (5-HT) 1A agonist prevented toxicity from glutamate. We determined the neuroprotective capabilities of serotonin (5-HT) 1A agonists as novel pharmacological countermeasures to chemical warfare agents. Rodents have higher amount of carboxylesterase enzyme and requires higher doses of sarin than other species. To address this issue we administered 1.5 mg/kg of CBDP (2-/o-cresyl/-4 H-1: 3: 2-benzodioxa-phosphorin-2-oxide), which specifically blocks carboxyl esterase and makes

mouse model comparable to that of human exposure. We determined 1mg/kg dose of serotonin (5-HT) 1A agonists 8-OH-DPAT from dose response curve based on neuroprotection, with toxic challenge of 1.5 mg/kg CBDP and dose of sarin yielding 25-50 % mortality. This mortality rate gave enough number of survivors with seizures and neurodegeneration for reliable baselines. Measurements were mortality, weight loss AChE activity in blood and CNS, functional observational battery (FOB) and histology compared to control and toxic challenge mice. In addition, a time response curve after toxic challenge was determined with 1mg/kg of 8-OH-DPAT at time points of 1, 15, 30, 45, 60 minutes and 2, 4, 6 hours. We observed neuroprotection by 8-OH-DPAT in the dentate gyrus of the hippocampus when administered up to two hours after Sarin. The ability of the combination of serotonin (5-HT) 1A agonist's dose and time after toxic challenge was tested for its ability to reinstate fear potentiated startle (FPS) response. However this test was invalidated by the response of the control group. DPAT like drugs could be useful in treatment of long term effects produced by sarin induced convulsions.

TABLE OF CONTENTS

List of Figures

LIST OF TABLES

Table Page

.

LIST OF ABBREVIATIONS

5-HT_{1A}, 5-hydroxytryptamine1A, serotonin1A

8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin

AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care

ACh, acetylcholine

AChE, acetylcholinesterase

BChE, butrylcholinesterase

CBDP, cresylbenzodioxaphosphorin oxide

ChE, cholinesterase

CNS, central nervous system

CS, conditioned stimulus

CWA, chemical warfare agent

DTNB, Dithionitrobenzoate

GA, Tabun, German agent A

Sarin, Sarin, German agent B

GD, Soman, German agent D

GW, Gulf War

GFAP, glial fibrillary acidic protein

FITC, Fluorescein isothiocyanate

FOB, Functional observation battery

FPS, Fear potentiated startle response

IF, intermediate filament

Iso-OMPA, tetraisoproppyl-pyrophosphoramide

NHS, Normal horse serum

NMDA, N-methyl-D-aspartate

OP, organophosphosphate

PBS, Phosphate Buffer Saline

SC, Subcutaneous

TBI, Traumatic brain injury

TC, Toxic Challenge

TFM, tissue freezing medium

Introduction

Organophosphate nerve agents and its history

The organophosphate (OPs) nerve agents are one of the most toxic chemical warfare agents developed in the 20th century. The first nerve warfare agents were produced by Nazi Germany during World War II. The first was tabun (GA-German agent A) and second was sarin (Sarin-German agent B) known as G-type agents. These nerve agents were weaponized and stocked by Germany, but there have been some debates over use of these weapons in World War II. The first confirmed use of nerve agents in warfare was Iraq's use of tabun and sarin against Iran during the Gulf War(1980-1988). The United Nations delegation confirmed the use of nerve agents and other chemical warfare agents which caused at least 45,000 Iranian casualties.(Newmark, 2004) During the Gulf War (GW-1991), a munitions dump at Khamisiyah, Iraq, was destroyed. Later, in 1996, it was found that the dump might have contained the organophosphate chemical warfare agents, sarin and cyclosarin. (Proctor, et al., 2006) The most recent and significant deliberate use of sarin as nerve agent was two terrorist attacks in Japan. The first nerve gas terrorism occurred at midnight on 27 June, 1994 in quiet residential area of Matsumoto. About 600 people including residents and rescue staff were exposed to sarin gas. Fifty-eight victims were admitted to hospitals and seven died. The second attack was on 20th March 1995 in Tokyo subway during rush –hour., that killed 12 people, 50 severely injured and 5000 people with minor injuries.(Okudera, 2002;Yanagisawa, et al., 2006;Hoffman, et al., 2007) The possibility of terrorists using chemical warfare agents (CWA), particularly sarin is quite real. Nerve agents are well within the capabilities of a good organic chemist who has access to the necessary raw materials and equipment. The nerve agents'

formula and synthetic methods are relatively easily obtained from public information sources, including the internet. For example, Iraqi military scientists used a U.S. patent that was declassified in 1975 to make VX (potent chemical warfare nerve agent). The most popular example is terrorist group Aum-shinrikyo using sarin. (Leikin, et al., 2002) Sarin is colorless, odorless and most volatile of all the nerve agents; it makes sarin most likely to be used in terrorist attack.

Sarin and its properties

Sarin is an organophosphorus compound that contains both carbon and phosphorus atoms. Sarin is basically a derivative of phosphonic (H_3PO_3) acid. The biological action of sarin compounds is related to its phosphorylating abilities. (bou-Donia, 2003b)

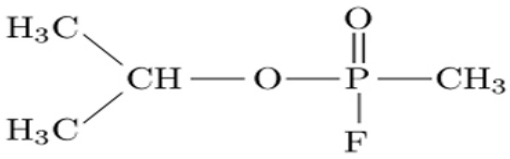

Figure 1. Chemical structure of sarin ($C_4H_{10}FO_2P$). It is also known as isopropyl methylphosphonofluoridate; isopropoxymethylphosphoryl fluoride and Sarin.

Sarin is chemically similar to organophosphate pesticides and all exert their main biological effects by inhibiting acetylcholinesterase. G-type agents (GA, Sarin, and GD) are clear, colorless, and tasteless liquids that are miscible in water and most organic solvents. Nerve agent vapors are heavier than air and odorless. Sarin is the most volatile nerve agent, evaporating at about the same rate as water.

Table 1. Comparison of Physical Properties of Nerve Agents

(Agency for Toxic Substances and Disease Registry., 2003c)

Property	Tabun (GA)	Sarin (Sarin)	Soman (GD)	VX
Description	Clear, colorless and tasteless liquid	Clear, colorless, tasteless and odorless liquid	Pure liquid is Clear, colorless, tasteless and odorless liquid, discolors with aging to dark brown	Ambered colored, tasteless and odorless oily liquid
Molecular Weight	162.3 daltons	140.1 daltons	182.2 daltons	267.4 daltons
Flash point	172.4 °F (78°C)	Nonflammable	249.8°F (121°C)	318.2°F (159°C)
Solubility in water	9.8 g/100 g at 77 °F(25°C)	Miscible	2.1 g/100g at 68 °F (20°C)	3 g/100 g (miscible below 48.9 °F (9.4 °C**)**)
Volatility	490 mg/m^3 at 77 °F (25°C)	22,000 mg/m^3 at 77 °F (25°C)	3,900 mg/m^3 at 77 °F(25°C)	10.5 mg/m^3 at 77 °F (25°C)

Mechanism of cholinesterase inhibition

After exposure, sarin is primarily absorbed from the respiratory tract. Vapors are absorbed through the skin at very high concentrations for G agents and at low concentrations of V agents. The nature and timing of symptoms following dermal contact with liquid nerve agents depend on exposure dose; effects may be delayed for several hours. (Agency for Toxic Substances and Disease Registry., 2003b) Sarin causes neurotoxicity by inhibiting acetylcholinesterase (AChE). AChE is required for degradation of acetylcholine (ACh). ACh is released in response to nerve stimulation and binds to post-synaptic acetylcholine receptors. Its action is rapidly terminated by

hydrolysis with AChE via the serine hydroxyl in the catalytic triad of AChE. AChE very quickly hydrolyzes ACh, which is a continuous process. Sarin inhibits AChE by phosphorylating the serine hydroxyl group at the catalytic triad site. The phosphonic acid ester formed with the enzyme is extremely stable and is hydrolyzed very slowly. The neurotransmitter ACh continues to elicit a response from the post-synaptic nerve, causing acute and chronic toxic effects. (bou-Donia, 2003a;Hoskins, et al., 1986;Wang, et al., 2008)

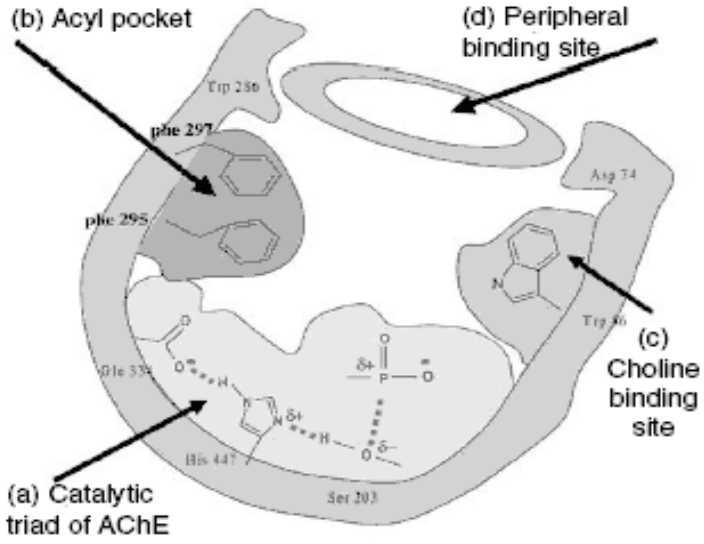

Figure 2. The 3-dimensional structure of AChE

It has an active center located at the base of a narrow gorge about 20 Å in depth. The active center includes the following sites (a) the catalytic triad: Glu 334, His 447, and Ser 203; (b) an acyl pocket Phe 295 and Phe 297; (c) a choline subunit: Trp 86,Glu 202, and Tyr 337; and (d) a peripheral site: Trp 286,Tyr 72, Tyr 124, and Asp 74.

Organophosphorus esters inhibit AChE by phosphorylating the serine hydroxyl group at the catalytic triad site. Phosphorylated AChE undergoes aging—a process that involves the loss of an alkyl group, resulting in a negatively charged monoalkyl enzyme.

Sarin effects and excitotoxicity

Sarin causes rhinorrhea and tightness in the throat or chest within seconds to minutes after exposure. (Agency for Toxic Substances and Disease Registry., 2003a) Sarin causes death due to anoxia, weakness of the muscles of respiration, convulsions and respiratory failure. The main clinical symptoms of acute toxicity by sarin are seizures, tremors and hypothermia. (bu-Qare and bou-Donia, 2002) A study conducted on GW veterans concludes that, sarin and cyclosarin exposure in Iraq was significantly associated with less proficient neurobehavioral functioning on tasks involving fine psychomotor dexterity and visuospatial abilities 4–5 years after exposure. The study also suggested a dose–response association between low-level exposure to sarin and cyclosarin and specific functional central nervous system effects 4–5 years after exposure. (Proctor, et al., 2006) Convulsions are a major sign of OP poisoning of the CNS and OP-induced seizures rapidly leads to structural brain damage. Convulsant doses of soman produce neuropathology in rats and guinea pigs. Understanding the mechanisms underlying the pathophysiology of OP-induced neurotoxicity is a crucial step for the development of effective pharmacological antidotes for long-term effects. (Lemercier, et al., 1983;Filliat, et al., 1999;Taysse, et al., 2006) The regions of the brain where neuronal damage is mostly observed are amygdala, piriform cortex, hippocampus and caudate. Neurodegeneration in the amygdala region of the brain is involved in seizures. (Filliat, et

al., 1999;Lallement, et al., 1991b) Neuropathology is observed within 20-40 minutes after convulsion and leads to irreversible neuronal damage leading to long-term effects. A correlation has been observed between glutamate release in CA1 and CA3 regions of hippocampus and soman induced seizures(Lallement, et al., 1991b;Lallement, et al., 1991a;Miller, 2005).

Sarin exposure leads to a series of events. It causes AChE inhibition and muscarinic receptor over stimulation, which leads to neuronal hyper- excitation and glutamate overflow. Excessive presynaptic release of glutamate causes activation of NMDA postsynaptic receptors, which leads to neurodegeneration through excitotoxicity. Thus, antagonism of the excitotoxic mechanisms may protect the brain from the deleterious effects of OP nerve agents. Ketamine is a noncompetitive NMDA receptor ion-channel blocker with neuroprotective and anti-epileptic properties, used for general anesthesia. It is also. However, it has shown high incidence of psychomimetic side effects. The antitussive drug dextromethorphan and its active metabolite dextrorphan are potent NMDA receptor antagonists. Both compounds were shown to possess anti-excitotoxic and anti-epileptic properties, but individuals seeking its dissociative effects often abuse dextromethorphan. (Miller, 2005) Several other NMDA receptor antagonists have been shown to exert anticonvulsant effects against nerve agent-induced seizures when administered as a pretreatment or as post treatment. This antiepileptic feature of the NMDA receptor antagonists is fully expressed when they are administered concurrently with muscarinic receptor antagonists. Thus, NMDA receptor antagonists do not modify the events responsible for the early phase of the seizure, but block the

subsequent recruitment of glutamate receptor activation and hence the maintenance of seizure activity. It is important to note that NMDA receptor antagonists may have lethal interactive effects on respiratory function in soman-intoxicated subjects in the absence of muscarinic receptor antagonists. Therefore, there is need for other alternatives for treatment of long-term effects. (Solberg and Belkin, 1997)

Fear potentiated startle (FPS) response

There is a correlation between neuropathology and memory impairments in Soman treated rats. (Filliat, et al., 1999) Tokyo subway victims underwent the chronic decline of memory function after poisoning. (Nishiwaki, et al., 2001) Pavlovian fear conditioning procedures are used to assess the behavioral, physiological, genetic and molecular correlates of learning and memory. In the Pavlovian conditioned fear procedure, a neutral stimulus such as tone is paired with foot shock. After few of these pairings the tone conditioned stimulus (CS) elicits a variety of behaviors, one of which is learned fear. One of the prominent of these behaviors is fear-potentiated startle effect, in which conditioned fear is defined as elevated startle amplitude in the presence versus the absence of CS. Fear potentiated startle can be measured after a single CS and shock pairing and can be retained over very long training to test intervals, making it ideal for examining long-term memory. (Falls and W, 1994;McNish, et al., 1997)

Serotonin (5-HT) 1A agonists as novel treatments

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter and utilized in a wide variety of physiological, sensorimotor and behavioral functions. Serotonin signaling is mediated by 14 receptor subtypes with different properties. The 5-HT1A subtype is one of the main mediators of the action of 5-HT which regulates the activity of 5-HT neurons via auto receptors and it regulates the function of several neurotransmitter systems via postsynaptic receptors.(Ogren, et al., 2008) 5-HT1A receptors are required for maintaining normal hippocampal functions. A study of 5-HT1A receptor knockout mice suggests a role for 5-HT1A receptors in hippocampal-related symptoms such as learning and memory. (Sarnyai, et al., 2000) Serotonin1A (5-HT1A) receptors are also localized in the frontal and parietal neocortex, olfactory bulb, cerebral cortex, thalamic and hypothalamic nuclei, raphe nuclei, septum, hippocampus, several nuclei of the brainstem Among these the hippocampus and cortex are susceptible to excitotoxic injury.(Nyakas, et al., 1997;Pompeiano, et al., 1992)

The 5-HT1A receptor agonist repinotan is neuroprotective and attenuates spatial learning deficits following controlled cortical impact injury. This treatment strategy may be beneficial in memory impairments in humans following traumatic brain injury due to exposure to sarin. (Kline, et al., 2001;van der Staay, et al., 2008) The neuroprotection is result of attenuation of the activity of both N-type Ca^{2+} channels and *N*-methyl-D-aspartic acid receptors and MAPK-mediated inhibition of a caspase 3-like enzyme pathway. (Adayev, et al., 1999;Harkany, et al., 2001;van der Staay, et al., 2008)

Another full agonist, 8-OH-DPAT shows neurological protection after traumatic brain injury (TBI). The mechanism for the protective effects of 8-OH-DPAT after TBI is still not clear. (Kline, et al., 2004) 5-HT1A receptor agonist 8-OH-DPAT

has inhibited neuronal cell death in vivo and in vitro. (Adayev, et al., 1999;Oosterink, et al., 2003) The OPs nerve agent exposure causes seizures that induce release of glutamate. Glutamate causes excitotoxicity and leads to neurotoxicity. One of the hypothesized mechanisms is inhibition of glutamate release by activation of 5-HT1A receptors. (McDonough, Jr. and Shih, 1993;Srkalovic, et al., 1994) Additionally, several full and partial 5-HT1A receptor agonists have been developed, generally with lower selectivity than 8-OH-DPAT. A current approach aims to counteract the increased glutamatergic and cholinergic neurotransmission occurring in sarin neurotoxicity. The physiological mechanisms underlying the neuromodulatory functions of 5-HT1A receptors are presently not well characterized. We tested 8-OH-DPAT as a neuroprotectant against sarin poisoning. (Ogren, et al., 2008)

Use of CBDP

Rodents have a high amount of carboxylesterase enzyme compared to humans. Carboxylesterase is a chemical scavenger that inactivates organophosphate nerve agents among other things. This makes rodents relatively insensitive to the toxic effects of organophosphorus compounds. There will be differences in the neurotoxicity produced by single dose of sarin in rodents in comparison to humans. So we propose to use CBDP (2-/o-cresyl/4H: 1: 3: 2-benzodioxaphosphorin-2-oxide) which is a carboxylesterase inhibitor. Subcutaneous CBDP has reduced LD 50 range of soman across species from 22.8 to 125 µg/kg to 11.8 to 15.6 µg/kg. (Clement and Erhardt, 1990;Maxwell, et al., 1987) Doses of CBDP between 1.0 to 2.0mg/kg should prove useful as pretreatment in OPs induced neurotoxicity in rat, it makes mouse model comparable to actual human

exposure. (Jimmerson, et al., 1989) Preliminary work has shown that an optimum dose of 1.5 mg/kg CBDP specifically blocks carboxylesterase, and potentiates the response of C57BL/6 mice to sarin. This dose of CBDP does not inhibit AChE and produce toxic effects of its own.

Hypothesis

We tested the hypothesis that the 5-HT1A agonist 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino) tetralin) will work as a neuroprotectant against sarin poisoning. The toxic challenge (TC) was the dose of sarin with CBDP yielding 25-50% mortality in the group. That gave enough number of survivors with seizure and neurodegeneration for reliable baselines. We determined the neuroprotective dose response curve for 8-OH-DPAT against the toxic challenge. Then, we determined time response curve of the best dose of 5-HT1A agonist based on neuroprotection. Finally, we tested the correlary hypothesis that a selected dose will prevent from memory deficits.

Specific Aim 1

Test the hypothesis that best dose of 8-OH-DPAT can act as a neuroprotectant for neurotoxicity produced by sarin induced convulsions in a dose dependant manner. Measurements were mortality, weight loss and FOB compared to control and toxic challenge mice. This was used to select best dose for subsequent studies.

Specific Aim 2

Test the hypothesis that best dose of 8-OH-DPAT can act as a neuroprotectant for neurotoxicity produced by sarin induced convulsions in a time dependant manner. Measurement were mortality, weight loss, FOB, ChE activity and histology compared to control and toxic challenge mice

Specific Aim 3

The ability of the combination of 8-OH-DPAT agonist dose of best dose and selected time after toxic challenge was tested for its ability to reinstate Fear potentiated startle (FPS) response.

Research design

Animals

The subjects were C57BL/6 male mice at 20-25 g body weight. They were housed in an AAALAC (Association for the Assessment and Accreditation of Laboratory Animal care) approved facility on a 12hr light/dark cycle with free access to food and water. All of our previous studies have been with this strain. Before administration of drugs animals were kept 1 week in house cage so they could acclimate to the environment and handled for 3 days to eliminate signs of stress. Studies in mice have demonstrated interstrain differences both in neurochemical measures and in cognitive abilities. A C57BL/6J mouse has good learning abilities and memory trace retention (at 10 days) in a simplified Morris maze. (Bel'nik, et al., 2009)

Drugs

We used diluted sarin (Aberdeen Proving Ground, MD) from the stock of 1.9 mg/ml saline solution. It was injected in a volume of 1 ml/200g weight for each dose. Mice received 0.5ml/a00g body weight, for a dose of 1.5 mg/kg CBDP (a generous gift of R.A. Donald M.Maxwell, Pharmacology Division, United States Army Medical Institute of Chemical Defense, Aberdeen Proving Ground, Maryland 21010-5425, USA). We diluted CBDP to 0.3mg/ml in a 10% ethanol and propylene glycol solution for the dose of 1.5mg/kg. Sarin and CBDP injections are subcutaneous. Our previous work established 1.5mg/kg CBDP as the optimum dose for carboxylesterase inhibition and it does not interfere with cholinesterase activity. Sarin was injected 1 hour after CBDP injection to

allow inhibition of carboxylesterase. 8-OH-DPAT doses of 0.3,1, 1.7 and 3 mg/ml were selected for dose response curve. 8-OH-DPAT was dissolved and diluted in saline for volume of 1ml/200g weight for each dose. Doses of 8-OH-DPAT were given 1 minute after Sarin injection for dose response curve. For the time response curve best selected dose (1 mg/kg) was given at different time points 1,15,30,45,60 min and 2,4,6 hours after toxic challenge.

Weight Loss

There was observed correlation between organophosphate nerve agent produced neurotoxicity and weight loss in mice. (Filliat, et al., 2007) In our previous work, we observed that toxic challenge dose causes weight loss in mice. We measured percentage body weight loss of mice on the first and third day after the injections.

Functional Observational Battery (FOB)

FOB contains end points that provides information of general health of mice. It also provides data needed for analysis of neurotoxicity produced by neurotoxicants. (Ross J F, 2000) We used a modified FOB which was used for ChE inhibitor pesticides.(Moser, 1995;Shih and Romano, 1988) Mice were observed for a Functional Observational Battery for 1-hour post Sarin dosing. The FOB scores were taken 15, 30, 45 and 60 min after the sarin dose. The FOB is based on 5 criteria with a total low score of 6 and a high score of 21. It includes measures of posture, motor behavior, gait, breathing and eye closure. Data is presented as percent of maximum score.

Table 2. Functional observation battery scoring

Posture	□ normal	□ flattened	□prostration	□ side/contorted
Motor behavior	□ normal	□ tail twitching □ hypo-loco	□ tremors	□ convulsions
Gait score	□normal	□ staggering □ splayed	□ severe staggering	□ unable to move
Eyelid	□open	□ half closed	□ slight protrusion	□ severe protrusion
Breathing	□ normal	□ fast shallow	□ slow labored	

Blood and brain cholinesterase (ChE) activity

We used modified version of the colorimetric assay of acetyl cholinesterase on Packard Fusion^{TM} Microplate Analyzer. (ELLMAN, et al., 1961) The mice were decapitated after 14 days of Sarin injection and blood were collected in heparin tubes. Two eppendorf tubes were prepared for each blood sample inhibited for inhibition of butrylcholinesterase (BChE) activity and uninhibited for total cholinesterase activity. For inhibited tubes 5ul 10mM iso-OMPA (tetraisoproppyl-pyrophosphoramide) and for uninhibited 5ul of $NaPO_4$ pH 7.4 are incubated for 45 minutes on ice. That allows for inhibition of BChE activity in inhibited tubes. Following incubation 245 µl 0.1 M $NaPO_4$ pH 7.4 buffers is added in inhibited and uninhibited tubes and mixed by inverting tubes. Than 96 well plate is used with triplicate sample. Total 6 wells are used for each sample, with 140 µl 0.1 M $NaPO_4$ pH 8.0, 20ul sample, 20 µl 10 mM DTNB (Dithionitrobenzoate) and 20µl 10mM ATCh (Acetyl choline). The plate was transferred to the plate analyser as quickly as

possible. BChE activity is then calculated by subtracting AChE activity from total ChE activity. Brain cholinesterase activity determined as described above. Brain samples are sonicated in 0.1 M $NaPO_4$ pH 7.4 buffer containing 0.5 % Tween 20 with a probe sonicator for 5 seconds for 2-3 times. We used Tween 20 rather than Triton X-100, which inhibits BChE activity. (Li, et al., 2000) Samples were centrifuged at 13000 rpm for 5 minutes at 4ºC. For inhibited sample, 1µl of iso-OMPA is added to 99µl sample supernatant. Protein content of the supernatant was determined using Bradford method (BioRad, Inc)

Neuropathology

Fourteen days post sarin treatment, mice were lightly anesthetized with CO_2 ,and sacrificed by decapitation and brain tissue collected for histology. Brains were quickly removed and quickly frozen into isopentane for 10-12 seconds and then stored at -80°C until sectioning. Frozen coronal sections of 10 µm were collected using cryostat (Thermo Shandon series, Pittsburg, PA) and TFM (TBS, INC., Durham, NC) as freezing medium). The section were collected on Fisher plus slides and stored in -20°C until fixed for staining. We performed immunohistochemistry using Glial fibrillary acidic protein (GFAP) as a marker for neurodegeneration. GFAP is a intermediate filament (IF) protein that is found in astrocytes in CNS. There is correlation between neurodegeneration and improper GFAP regulation. Neurodegenerative condition leads to higher expression of GFAP that leads to glial scarring. (Liedtke, et al., 1996) On the first day of staining, the slides were taken out and let dry about 30 minutes at room temperature. A boundary was

made around sections with a liquid repellent PAP pen (Daido Sangyo, Tokyo, Japan) and let dry about 10-15 min. Then slides were transferred to a humid chamber which is large petri dish with filtered paper saturated with distill water in a plastic box covered with lid on top. The slides were washed with 3 times for 5 minutes and 1 time for 10 minutes with 0.01 M phosphate buffer solution containing 0.1 % triton (0.01 M PBS/0.1 %TX). The last wash was rinsed and allowed to dry. The slides were blocked with 10 % normal horse serum (10 % NHS is made by using 500µl normal horse serum in 4.5 ml of 20 mM TBS (Tris buffered saline) for 30 minutes. Drain off NHS and allow PAP ring to dry. Primary antibody solution was prepared at 1:150 dilution of rabbit anti-GFAP (Zymed, South San Francisco, CA) in 0.01 M PBS/0.1 %TX to block antibody. The slides were kept overnight at room temperature. Next day primary antibody was drained off from the slides. Then the slides were washed with 3 times for 5 minutes and 1 time for 10 minutes with 0.01 M PBS/0.1 %TX. Following this step, all steps were performed in the dark. Fluorescent probe-conjugated secondary antibody solution horse anti-rabbit FITC (Amersham, Pittsburg, PA) at a 1:50 dilution in 0.01 M PBS/0.1 % TX were prepared and added to slides which are kept for 2-4 hours. Then the slides were washed with 3 times for 5 minutes and 1 time for 10 minutes with 0.01 M PBS. We allowed slides to dry and coverslip with gel –mounting medium (Bio-meda, Foster city, CA). Slides were stored at 4°C. We examined slides using a Leicia microscope fitted with an Optronics camera. Total cell count was done specified area. Images will be analyzed using ImageJ (1.41O) software.

Procedure for FPS testing

FPS consists of pre-training, training and post training sessions. Mice are tested in SM100 startle monitor system version 6.12. (Kinder Scientific, 2001, Poway, CA) In the fear-potentiated startle procedure, conditioned fear is operationally defined as elevated startle amplitude in the presence versus absence of the stimulus that was previously paired with shock. This assumes that the CS does not increase or decrease startle amplitude prior to Pavlovian fear conditioning. If it does then these unconditioned effects on startle could exaggerate or mask subsequent conditioned fear. For these reason the FPS test is administered prior to Pavlovian fear conditioning and is referred to as the pre-test. After a 5 min acclimation period, they are presented with 32 startle stimuli at two white noise intensities in 50ms pulses at 75 & 80 db (8 of each), both in the presence and absence of the 30 sec, 12kHz pure tone of 70dB CS. The inter interval between trial is 1 min. The training session (Pavlovian fear conditioning) consists of 5 min. acclimation, 20 tone (CS of 29.5 sec, 12 kHz pure tone of 70dB) and 0.5 sec of electric shock of 0.4 mA. Pavlovian fear conditioning is repeated for two days for total of 40 trials. The inter interval between trial ranges from 1 min to 3.5 min. The post-training test is same as pre training test and is conducted 24 hour after Pavlovian fear conditioning. Finally, the percent fear-potentiated startle is computed by dividing the difference score by the amplitude of startle on noise burst alone trails and multiplying by 100 (FPS= [(tone + noise burst alone- noise burst alone)/ noise burst alone] X100. (Falls, 2002)

Statistical analysis

We used using Statistica software (version 7) for statistical analysis. One-way ANOVA was used to test for differences among two or more independent groups. Fisher Least significant difference test was used to identify individual group differences. In all groups *=$p<0.05$, significant, different from toxic challenge.

Results

Mortality rates from dose response curve for 8-OH-DPAT after TC

We measured the mortality rate in different groups to find if there is any effects of DPAT on mortality. The lower dose DPAT group showed lower mortality rate. The highest dose of DPAT (3 mg/kg) increased the lethality of the TC (Table 3).

Dose	N	deaths	Percent
TC	9	3	33.33%
0.3mg/kg	8	2	25.00%
1mg/kg	12	3	25.00%
1.7mg/kg	9	4	44.44%
3mg/kg	6	5	83.33%

Table 3 Mortality rates comparison of different doses of 8-OH-DPAT given 1 min. after toxic challenge (TC). TC was 1.5 mg/kg of CBDP and 32 µg/kg dose of sarin.

Weight loss from dose response curve for 8-OH-DPAT after TC

To find out correlation of neurotoxicity and weigh loss we measured weight loss up to 3 days post sarin injections. Mean percentage weight loss at day1 and day3 after injection, comparison of different doses of 8-OH-DPAT given 1 minute. after toxic challenge (TC) was measured. TC was 1.5 mg/kg of CBDP and 32 µg/kg dose of sarin.

At day 1, there was greater weight loss in 0.3 and 1.7 mg/kg dose of 8-OH-DPAT group compared to TC group. The weight loss of 1 mg/kg dose group was lower than TC group. ($F_{3, 27}$ =7.2907, $p<0.05$) A one way ANOVA showed significant difference of weight loss of 0.3 and 1.7 mg/kg dose of 8-OH-DPAT group compared to TC group. At day 3, there was no significant difference in the weight loss of any of the group, but 1 mg/kg dose group did not lose weight as much as TC group. The two doses 0.3 and 1.7 mg/kg showed almost similar weight loss compared to toxic challenge. Based on weight loss and mortality rates we selected 1mg/kg dose for subsequent studies. (Figure. 3)

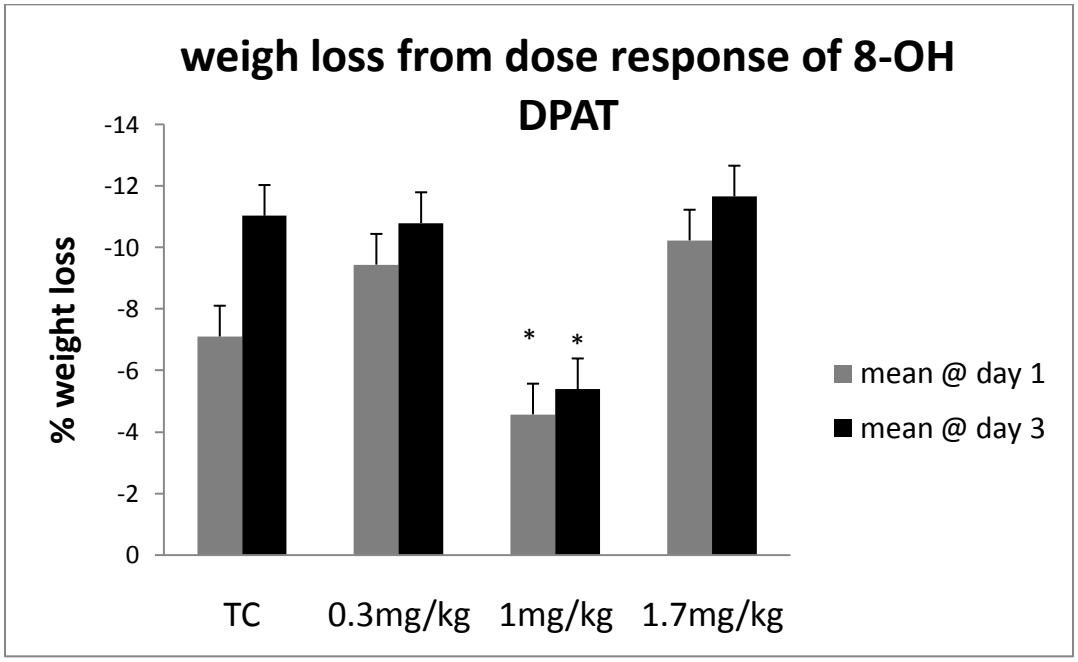

Figure. 3 mean percentage weight loss on day 1 and 3 of of different doses of DPAT groups compared to toxic challenge group.

Mortality rates from time response curve for 8-OH-DPAT

The mortality rate of the 1 mg/kg DPAT group was lower than toxic challenge. The weight loss at day 1 and 3 was also lower than TC group. Based on these results we chose 1mg/kg dose of DPAT for time response curve. The mortality rate of 1, 15 and 30 min 8-OH-DPAT group is not higher than TC group, but 45 and 60 min 8-OH-DPATgroup is higher than TC group. (Table 4) The 2 hour and 4 hour DPAT groups' toxic challenge was 1.5mg/kg of CBDP and 1 hour later 40ug/kg dose of sarin. The 4 hour DPAT group mortality rate was higher than toxic challenge group. (Table 5) The 6 DPAT group toxic challenges were 1.5mg/kg of CBDP and 1 hour later 42ug/kg of sarin. The 6 hour DPAT group mortality rate was higher than toxic challenge group. DPAT did not have any effects on mortality rates as some of the mice died before DPAT injections. (Table 6)

Dose	**N**	**deaths**	**Percent**
TC	9	3	33.33%
1 min DPAT	12	3	25.00%
15 min DPAT	10	2	20.00%
30 min DPAT	6	2	33.33%
45 min DPAT	8	3	37.50%
60 min DPAT	12	7	58.33%

Table 4. Mortality rates of 1 mg/kg DPAT at various time points post toxic challenge (TC) injections compared to TC.

Dose	N	deaths	Percent
TC	6	2	33.33%
2hr DPAT	9	3	33.33%
4hr DPAT	10	6	60.00%

Table 5. Mortality rates of 2 and 4 hour DPAT groups post toxic challenge (TC) injections compared to TC.

Dose	N	deaths	Percent
TC	5	2	40.00%
6hr DPAT	11	5	45.45%

Table 6. Mortality rates of 6 hour DPAT group post toxic challenge (TC) injections compared to TC

Weight loss from time response curve for 8-OH-DPAT

Mean percentage weight loss at day1 and day 3 comparisons of vehicle control, DPAT control, toxic challenge (TC) and 1mg/kg of 8-OH-DPAT given after TC at different time points was performed. The control group received propylene glycol and 1-hour later saline. DPAT control group received 1 mg/kg dose of 8-OH-DPAT. At day 1, there was higher weight loss in 8-OH-DPAT group of 15, 30, 45 and 60 min than toxic challenge group. There was lower weight loss in 1 min 8-OH-DPAT group. There was

no significant difference of weight loss between TC and 8-OH-DPAT group at different time points at day 1. One-way ANOVA showed significant difference between control group and TC, 15, 30, 45, 60-minute 8-OH-DPAT group. ($F_{7, 53}$=6.2234, p<0.0001) At day 3, there was higher weight loss in 60 min 8-OH-DPAT group than toxic challenge group. At day 3, there was lower weight loss in 8-OH-DPAT group 1, 15, 30 and 45 min compared to TC group. There was no significant difference between of weight loss between TC and 8-OH-DPAT group at different time points at day 3. One way ANOVA showed significant difference between control group and TC, 15, 45, 60 minute 8-OH-DPAT group. ($F_{7, 51}$=5.2500, p<0.001) (Figure 4) There is higher weight loss on day 1 and 3 of 2 hour DPAT group compared to toxic challenge. (Figure 5) There is lower weight loss in 6 hour DPAT group compared to toxic challenge at both day 1 and 3. (Figure 6)

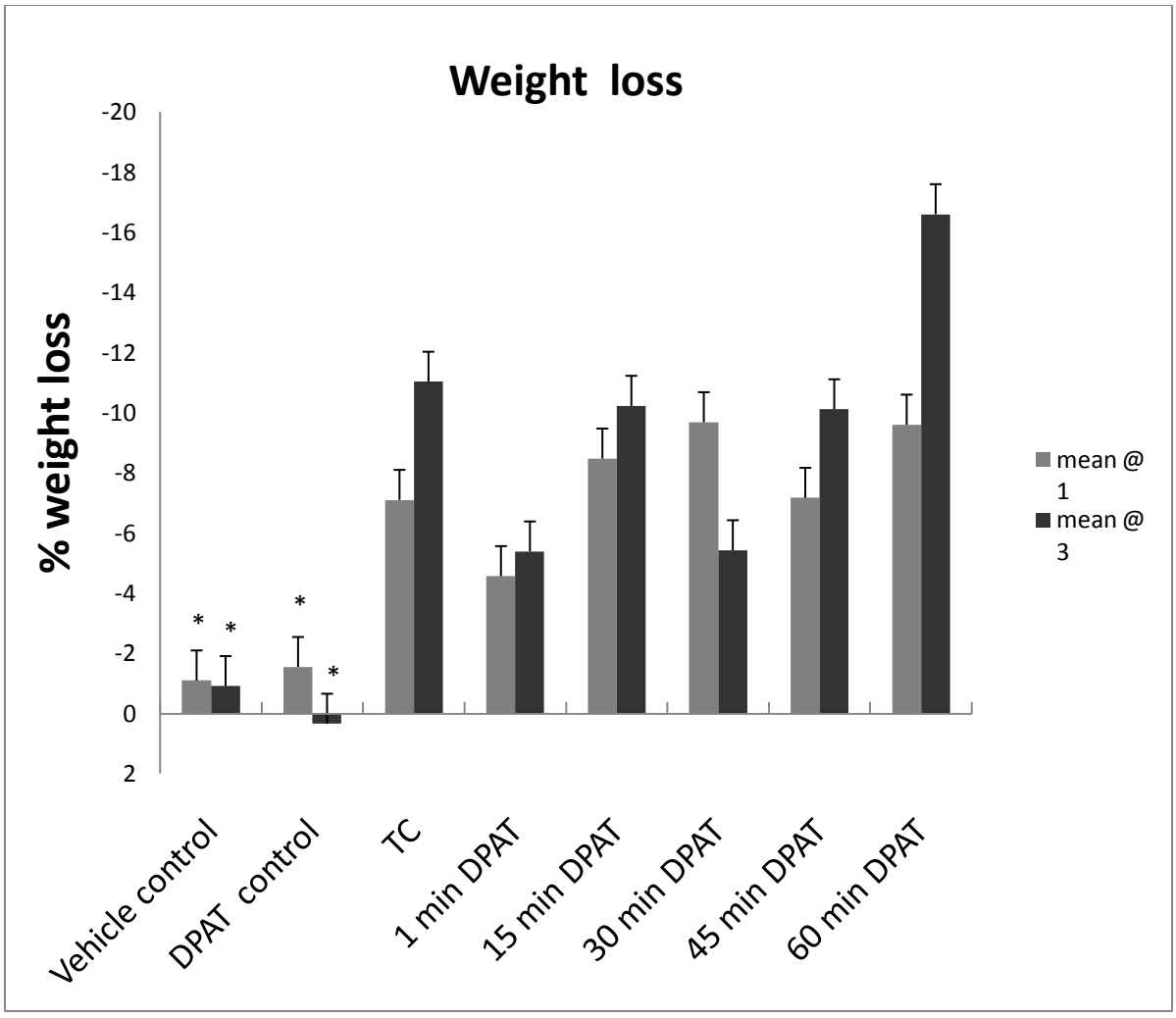

Figure 4. Mean percentage weight loss on day 1 and 3 of DPAT group compared to toxic challenge group.

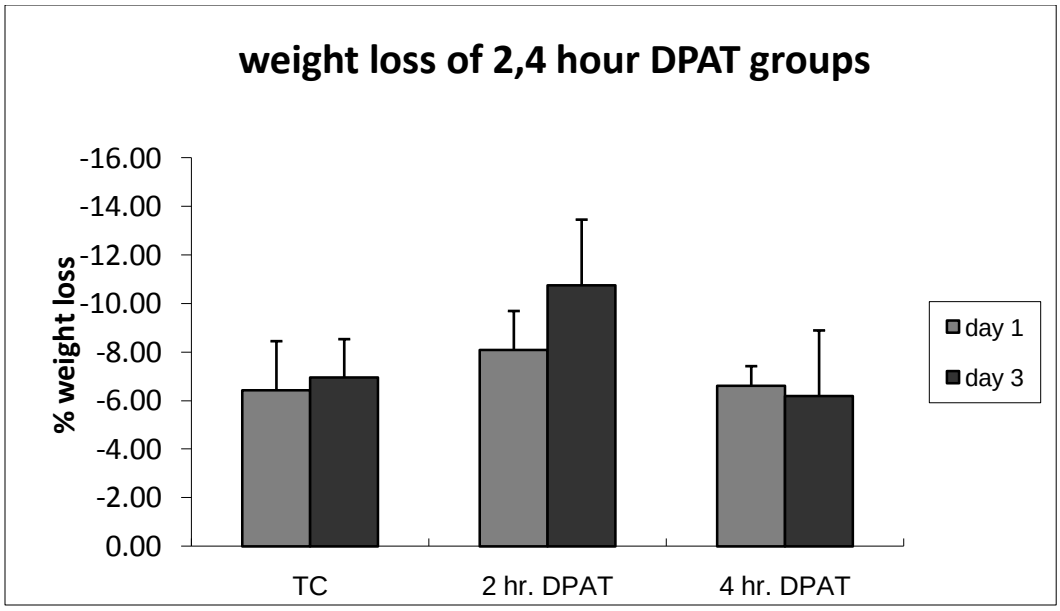

Figure 5. Mean percentage weight loss on day 1 and 3 of 2 and 4 hour DPAT groups compared to toxic challenge group.

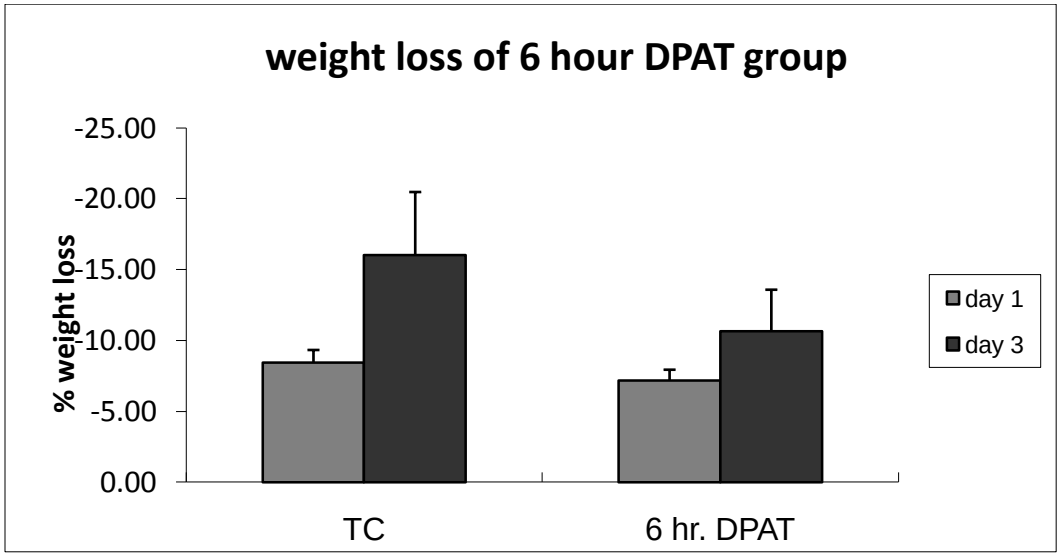

Figure 6. Mean percentage weight loss on day 1 and 3 of 6 4 hour DPAT group compared to toxic challenge group.

Functional observational battery scores from time response curve for 8-OH-DPAT

All groups injected with Sarin showed signs of poisoning and mortality. This is required in groups for brain pathology. Functional Observational Battery scores were calculated for animals injected with the vehicle control, DPAT control, TC and TC+ DPAT at different time points thereafter. A one way ANOVA performed on percentage of maximum score showed significant differences between control and Sarin treated groups. ($F_{7, 65}$=54.520, p<0.0001) (Figure 7) For 2, 4 and 6 hour DPAT groups FOB scores were measured at longer time points until 24 hours. At 24 hours 2 and 4 hours FOB scores are lower than toxic challenge group. (Figure 8) FOB score of 6 hour DPAT group is higher than toxic challenge at 24 hours. (Figure 9)

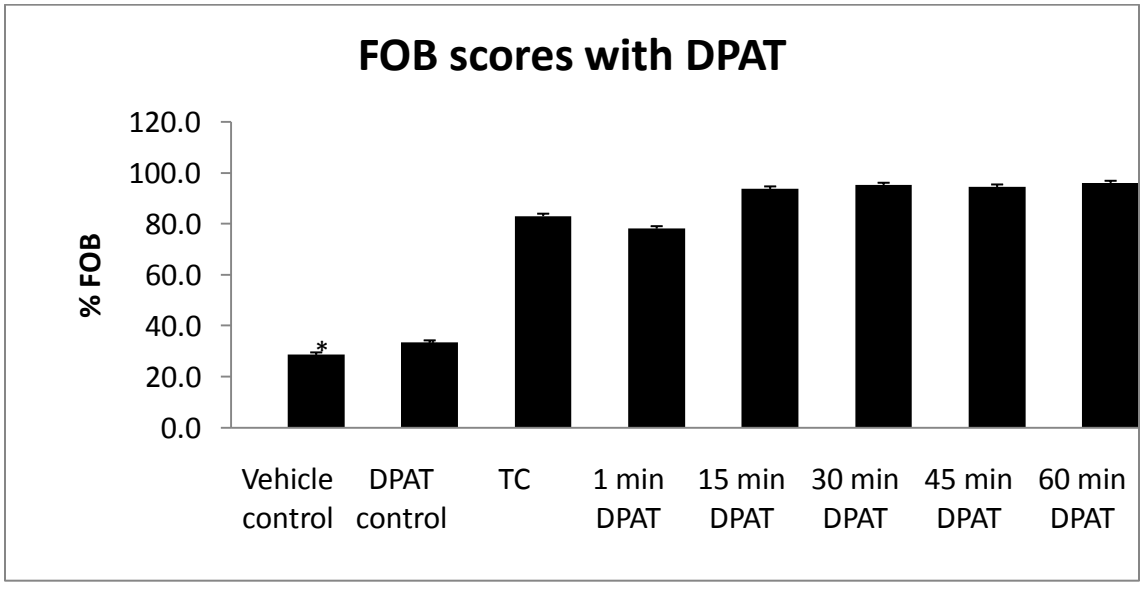

Figure 7. Percentage FOB scores of DPAT, vehicle control groups to toxic challenge group.

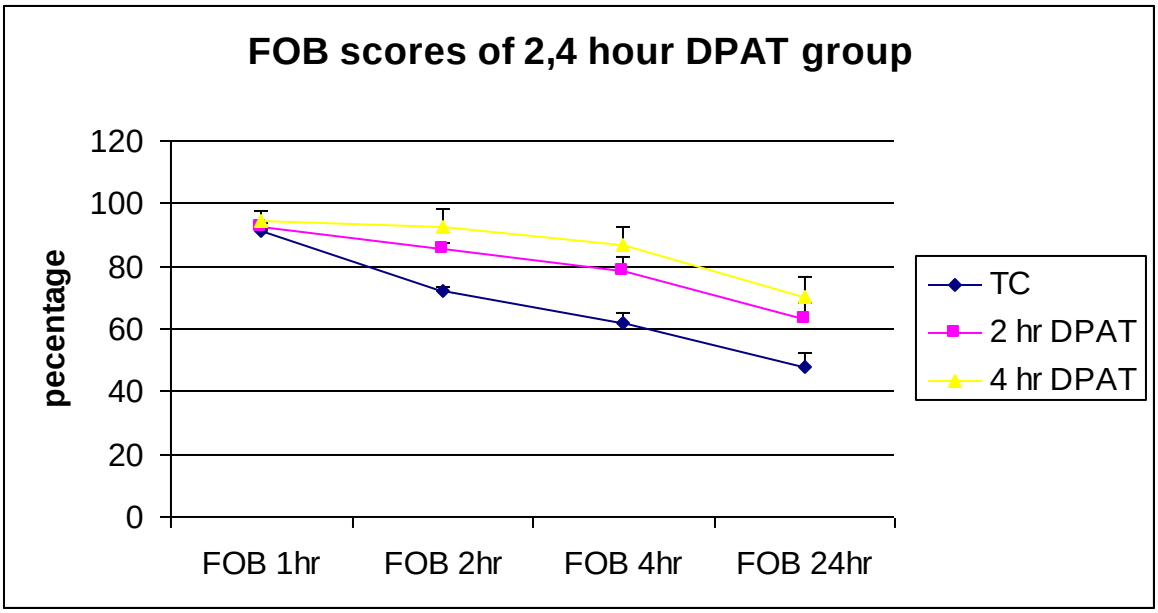

Figure 8. Mean percentage FOB scores of 2, 4 hour DPAT groups compared to toxic challenge at different time points.

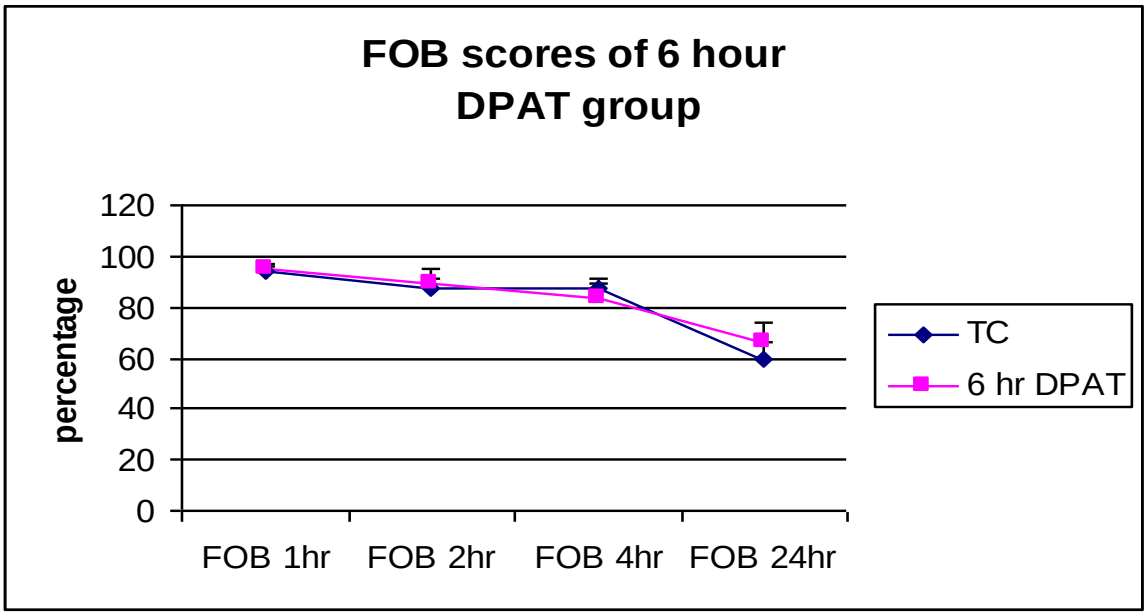

Figure 9. Mean percentage FOB scores of 6 DPAT group compared to toxic challenge at different time points.

Blood ChE and AChE activity

The blood ChE and AChE activity was measured 14 days post injection. The ChE activity was measured on trunk blood collected by decapitation. There is no significant difference of ChE activity between any of the groups. There is also no significant difference between AChE activities between any of the groups (Figure 10)

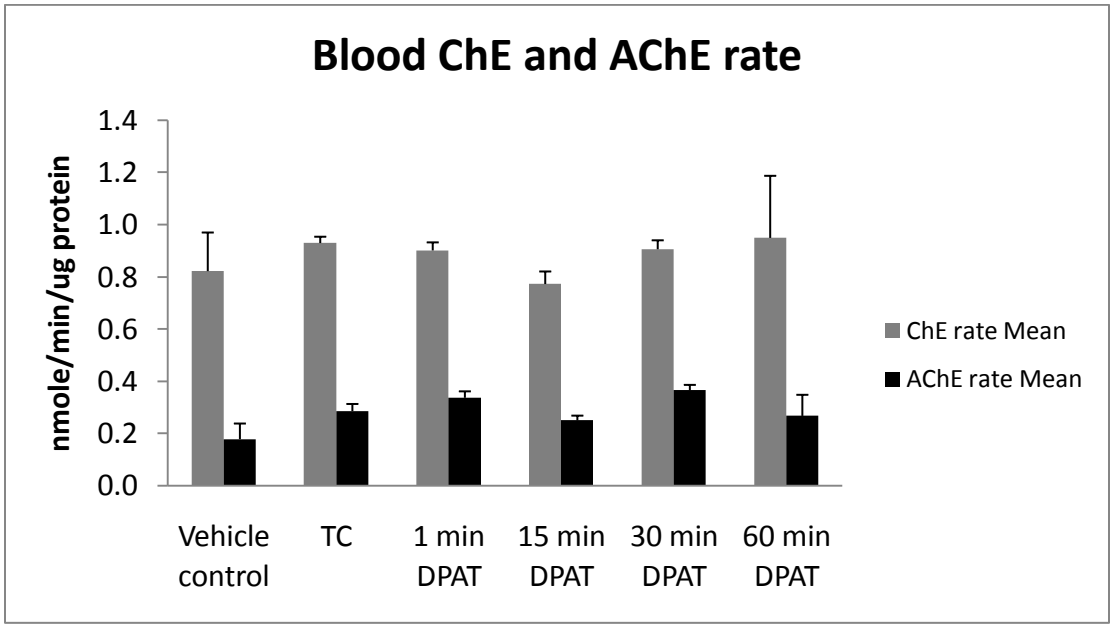

Figure 10. Blood ChE activity of different groups 14 days post Sarin injection.

Frontal cortex AChE activity

The frontal cortex ChE and AChE activity was measured 14 days after injection. Mice were euthanized using CO_2 and decapitated. The ChE activity in all 8-OH-DPAT groups after15 min was lower than the toxic challenge group. (Figure 11)

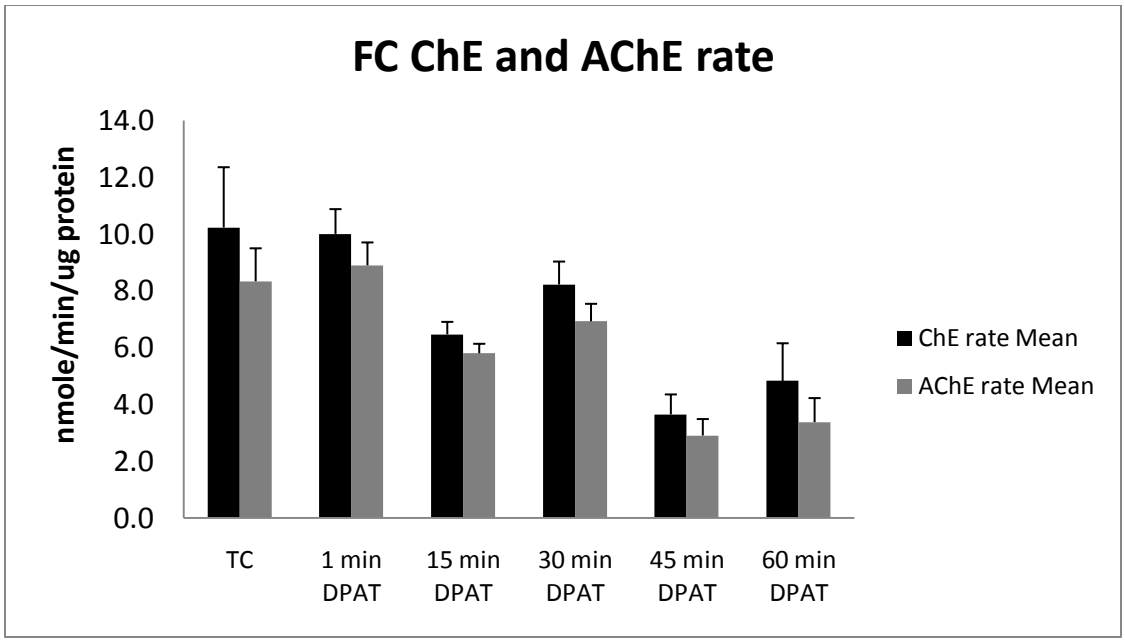

Figure 11. Frontal cortex ChE and AChE activity in DPAT groups compared to TC group

GFAP cells counts in dentate gyrus from time response curve for 8-OH-DPAT

Glial fibrillary acidic protein (GFAP) is a marker for neurodegeneration, which is highly responsive to neuronal damage. An increase in GFAP is an indication of trauma to these cells. GFAP cell counts was conducted in dentate gyrus of hippocampus for animals injected with the vehicle control, DPAT control, TC and 1 mg/kg DPAT at various time points post TC injections at 14 days post injection. We observed significant differences of GFAP cells in dentate gyrus of hippocampus in up to 2 hour time points compared to TC group, with the values the same as for background. ($F_{7, 37}$=7.0575, $p<0.0001$) (Figure 12, 13)

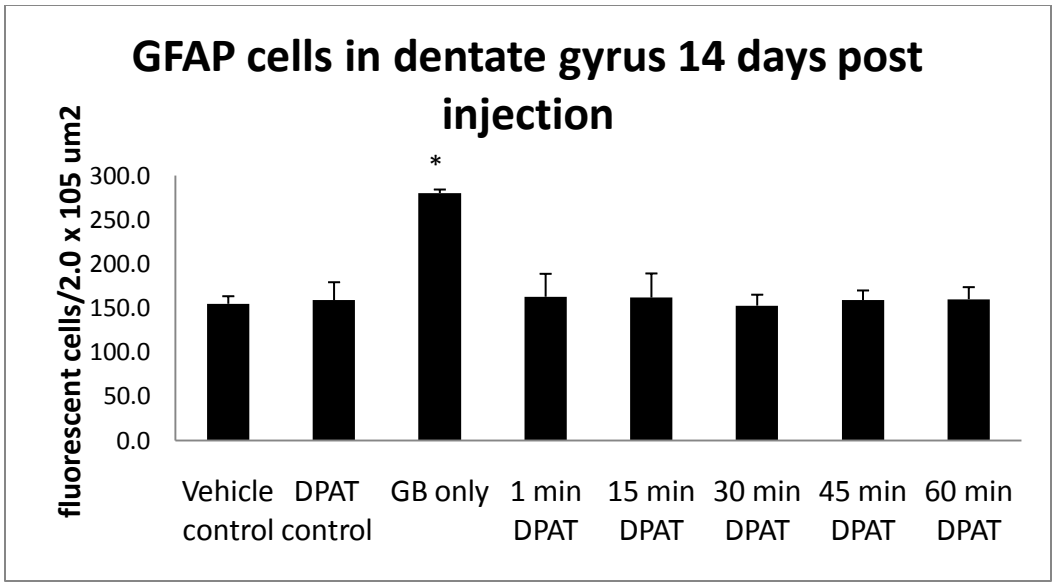

Figure 12. GFAP cells in dentate gyrus of hippocampus in different groups .

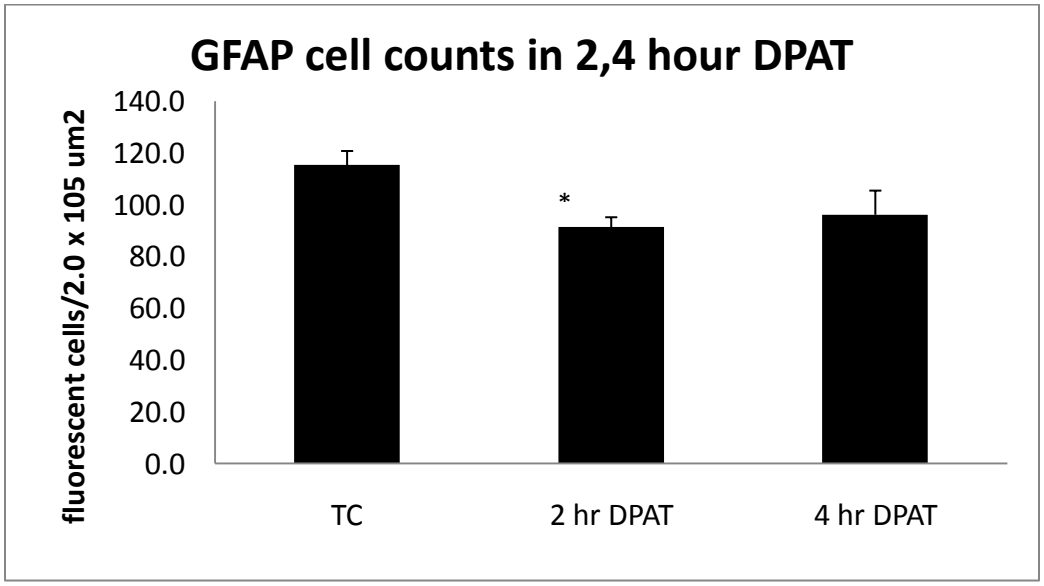

Figure 13. GFAP cells in dentate gyrus of hippocampus in 2 and 4 hour DPAT groups

Images of Dentate Gyrus of Hippocampus of different groups stained for GFAP

Below are images of the dentate gyrus of the hippocampus of different groups stained for GFAP. (Figure 14,15) All the picture were taken on 20x magnification using optronics camera using a Leica microscope. We euthanized all the groups two weeks after injections. All the injections were SC. route. The control group received propylene glycol and 1 hour later saline. The DPAT control group received propylene glycol, 1 hour later saline and 1 minute after 1 mg/kg of 8-OH-DPAT. The toxic challenge group received 1.5 mg/kg of CBDP and 1 hour later 32 ug/kg of Sarin injection. The DPAT groups received toxic callenge and 1mg/kg of 8-OH-DPAT at time points according to groups.

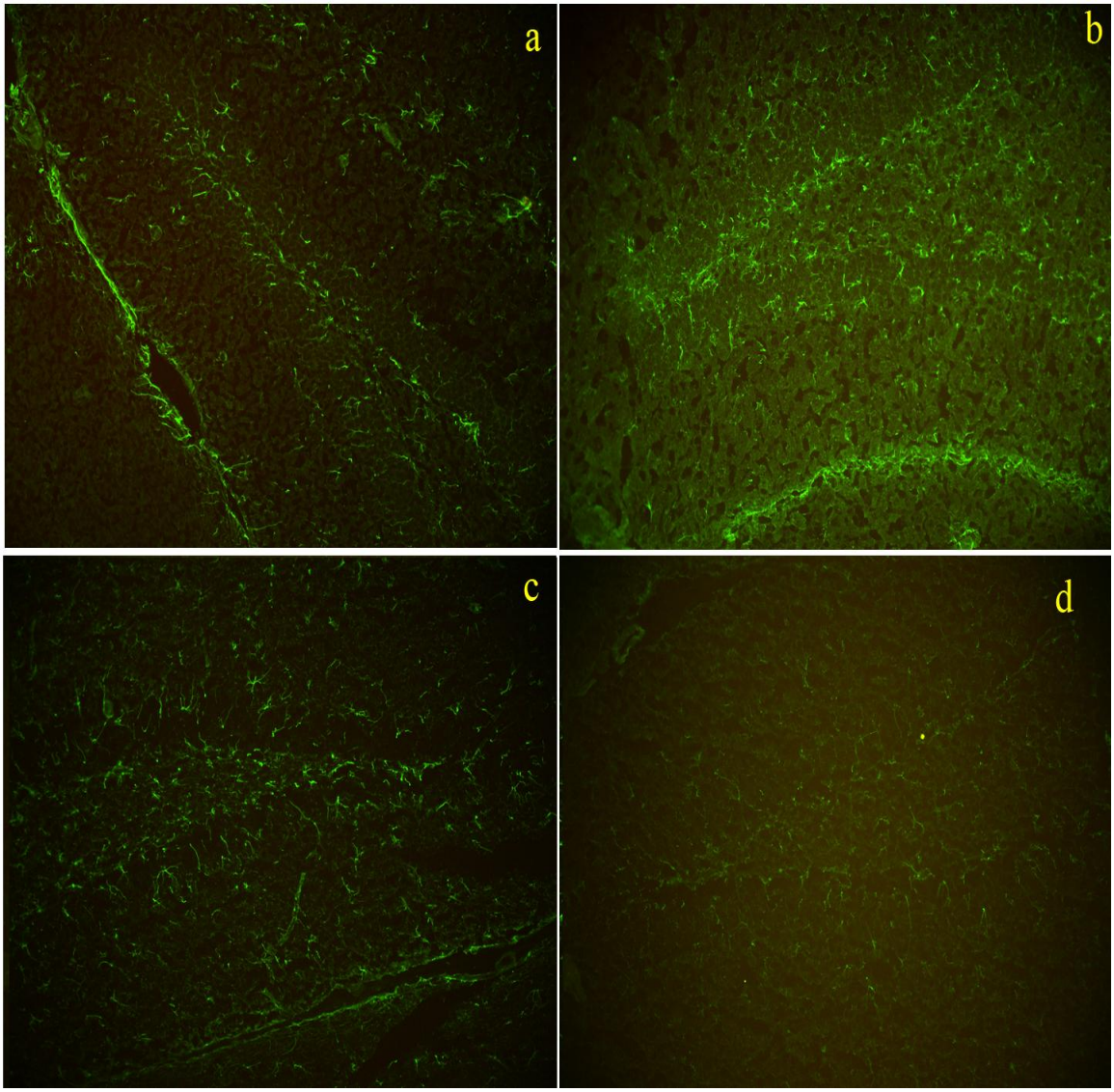

Figure 14. Dentate gyrus of hippocampus of different groups ,mouse euthanized 14 days after injection (a) control group dose with propylene glycol and saline (b) toxic challenge group dosed with 1.5mg/kg CBDP and 32ug/kg Sarin (c) DPAT control group dosed with propylene glycol, saline and 1mg/kg 8-OH-DPAT (d) 1 min DPAT group dosed with toxic challenge and 1 minute later 1m/kg 8-OH-DPAT

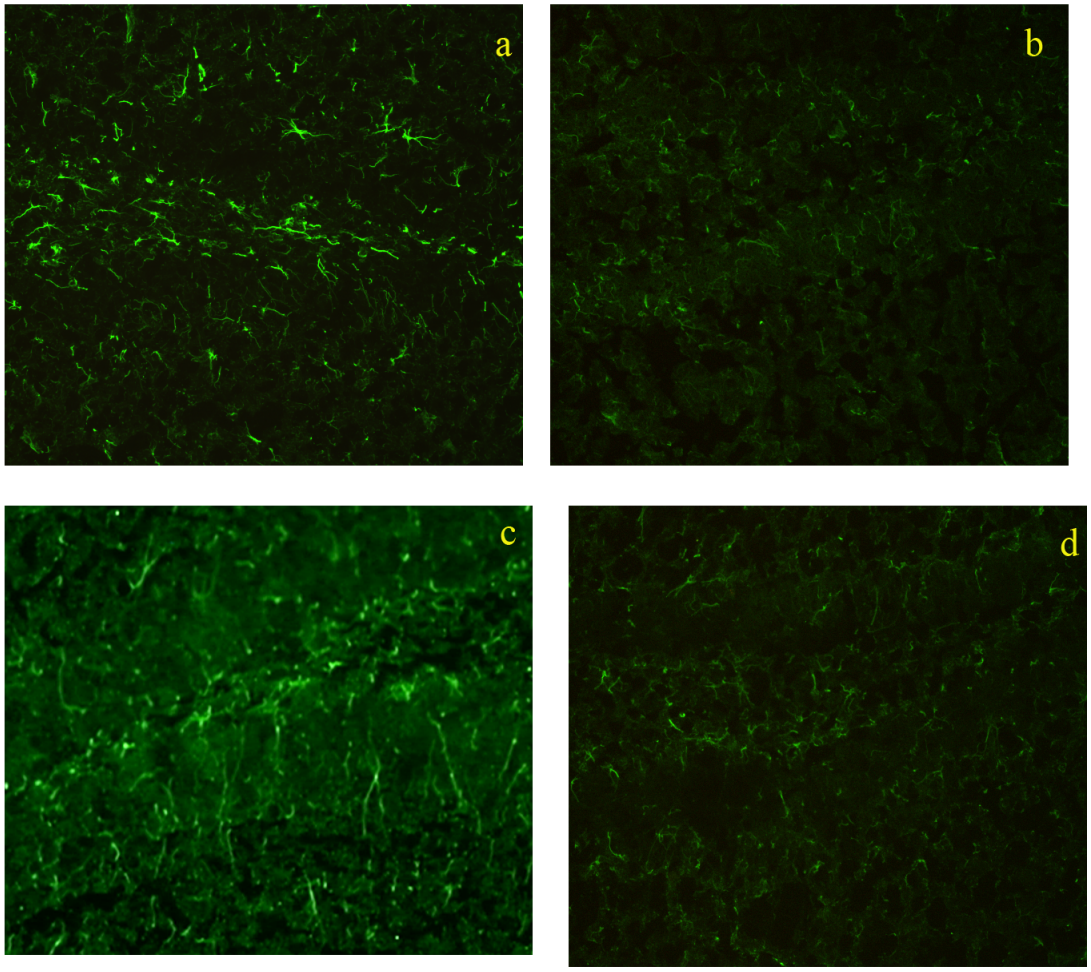

Figure 15 Dentate gyrus of hippocampus of time points of DPAT groups, 1mg/kg DPAT given after toxic challenge at (a) 15 min (b) 30 min (c) 45 min (d) 60 min

Fear potentiated startle (FPS) response

We performed fear potentiated startle response test on three groups control, toxic challenge and 45 min DPAT group. Mice were assigned into groups according to averages of their startle amplitude. All injections were S.C. route. The control group received propylene glycol and 1-hour later saline. The toxic challenge group received 1.5 mg/kg CBDP and 1-hour later 42ug/kg of Sarin. The DPAT group received toxic challenge and 45 minute later 1mg/kg of 8-OH-DPAT. Three weeks after injections mice were fear conditioned for Pavlovian fear conditioning the training session which consists of 5 min. acclimation, 20 tone (CS of 29.5 sec, 12 kHz pure tone of 70dB) and 0.5 sec of electric shock of 0.4 mA. Pavlovian fear conditioning was repeated for two days for 40 trials. 24 hours later mice were tested for posttest, which is same as pre test. At 75, 80 db white noise and average FPS TC group learned the conditioning. We previously preformed three weeks FPS studies on asymptomatic doses of Sarin (0.4 LD50) and the Sarin group did not learn. In our experiment the symptomatic dose of Sarin group learned and left our test inconclusive.

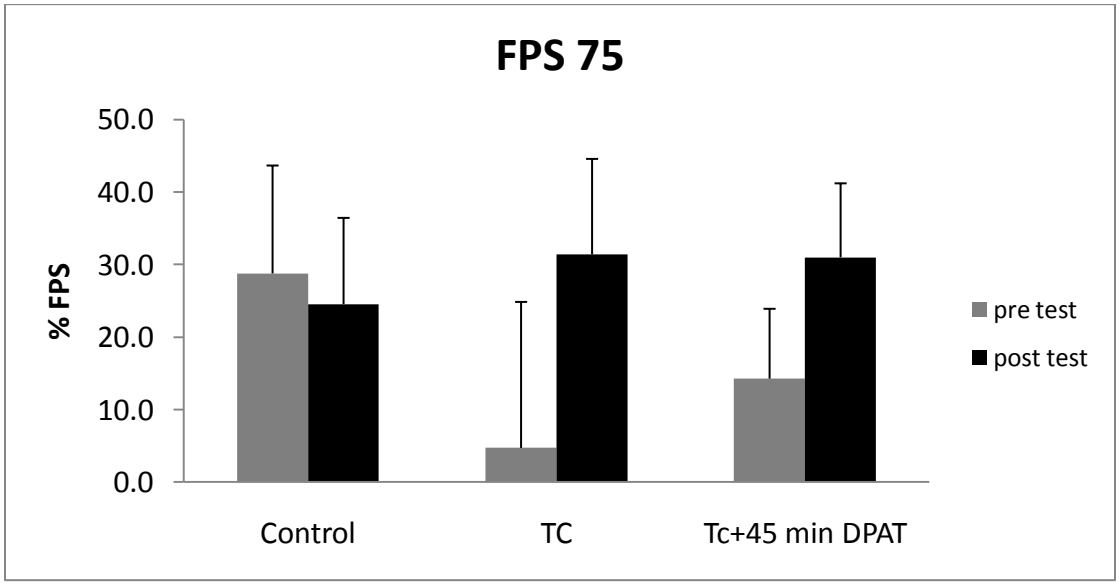

Figure 16. Fear potentiated startle at 75 db white noise

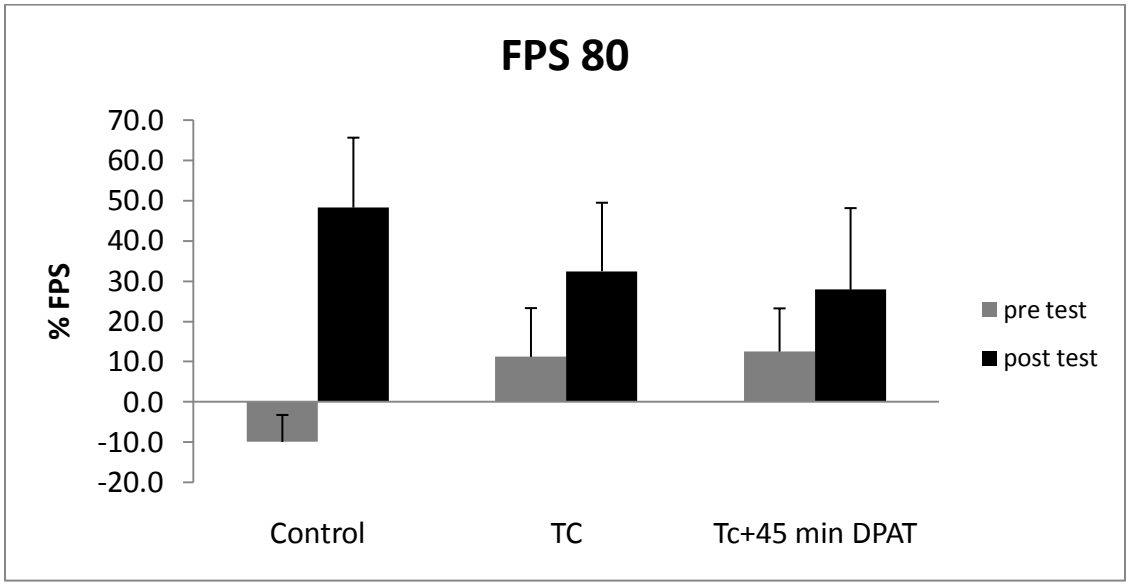

Figure 17. Fear potentiated startle at 80 db white noise of different groups.

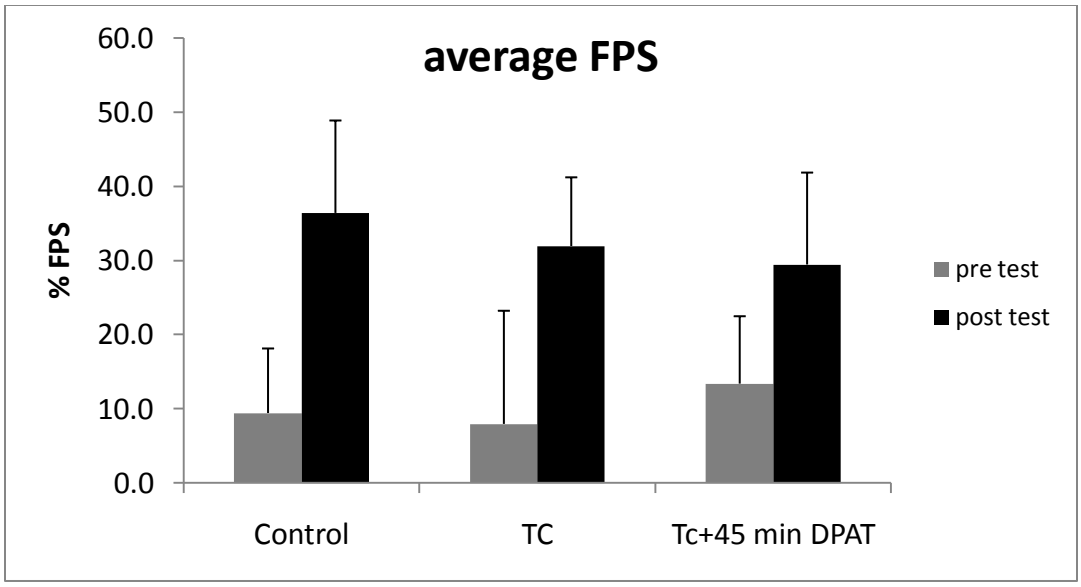

Figure 18. Average Fear potentiated startle of 75 and 80db white noise of different groups.

Immunohistochemistry of FPS group

The FPS control mice were treated with propylene glycol and saline. The TC group received 1.5mg/kg CBDP and 42ug/kg Sarin. The DPAT group was injected 1.5mg/kg CBDP and 42ug/kg Sarin and 45 minutes later 1 mg/kg of 8-OH-DPAT. Mice were euthanized 4 weeks after injection. Immunohistochemistry was performed on frozen sections and GFAP cells in dentate gyrus region were calculated. GFAP cell were significantly lower in dentate gyrus of hippocampus in control and 45 min DPAT group compared to TC group. ($F_{2, 24}$=82.174, $p<0.0001$) (Figure 19)

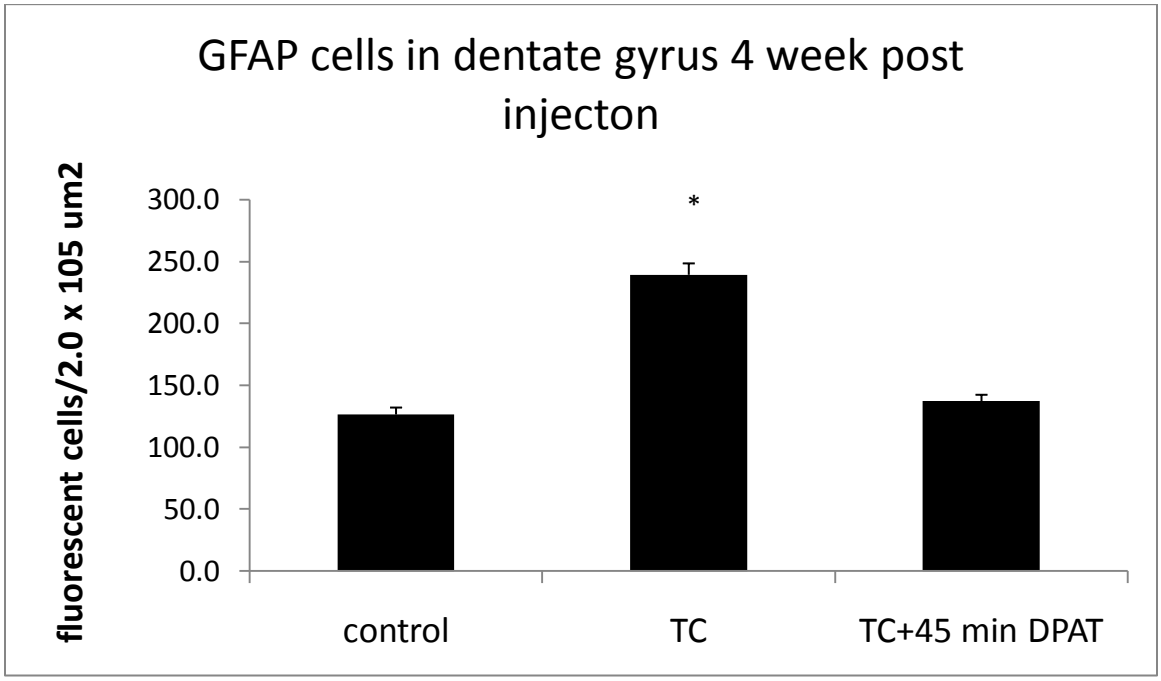

Figure 19. GFAP cells in dentate gyrus of hippocampus in different groups.

Discussion

The main objective of the study was to look for novel neuroprotectants for treatment of long-term effects produced by sarin induced convulsions. This required identification of the dose of sarin that will produce a mortality rate with enough survivors for reliable baseline. We also wanted to make our model comparable to actual human exposure. We used CBDP with sarin to inhibit the high levels of carboxylesterase seen in rodents to reduce the total sarin dose and thus to make the mouse model closer to humans. We tested the 5-HT-1A agonist 8-OH-DPAT for treatment of sarin and CBDP induced convulsions. We performed dose response and time response curves for 8-OH-DPAT and looked at the parameters of mortality rates, weight loss, FOB, blood and brain cholinesterase activity, immunohistochemistry for degenerating neurons and FPS. We observed neuroprotection by DPAT in the dentate gyrus of hippocampus up to 2 hours after the administration of the toxin. We also tested other 5-HT-1A agonists in our laboratory but they were ineffective. The mechanism of the neuroprotection produced by DPAT is still not clear. We observed that 8-OH-DPAT influenced on the pattern, intensity, and duration of sarin-induced seizures using ECoG; this mechanism could underlie the neuroprotection produced by 8-OH-DPAT in dentate gyrus of hippocampus.

Use of Sarin and CBDP

Carboxylesterase are important in the detoxification of a number of organophosphorus insecticides. Humans have low carboxyl esterase in blood and tissue in comparison of rodents. (Pope, et al., 2005) Rodents will require higher doses of sarin to produce its effects. Administration of CBDP significantly reduced the dose of sarin necessary to produce symptoms. Administration of CBDP potentiated Sarin almost five

times from 200ug/kg to 42ug/kg to produce 25-50 % mortality rates. Carboxylesterase is important detoxifier of sarin. The administration of this dose of CBDP specifically blocks CaE; therefore less Sarin dose was required to produce its effects. The CBDP and Sarin injection were separated by 1 hour. At the time of Sarin administration carboxyl esterase was blocked by CBDP. The lower dose of Sarin irreversibly blocked cholinesterase and produced toxic effects. CBDP administration reduced our Sarin dose from a range of 160-200 ug/kg to 32- 42 ug/kg., while even repeated low level sarin exposure of 64 ug/kg is asymptomatic. (Jimmerson, et al., 1989) CBDP dose of 2 mg/kg or higher significantly lowers cholinesterase activity in blood and brain.(Mach, et al., 2008) We used 1.5 mg/kg of CBDP which did not interfere with cholinesterase activity, making our model closer to actual human exposure.

Dose response curve of 8-OH-DPAT

We determined the neuroprotective dose response curve for the 5-HT-1A agonist 8-OH-DPAT against the toxic challenge of 1.5mg/kg of CBDP and 32ug/kg of Sarin over the range from of 0.3, 1, 1.7 and 3 mg/kg of 8-OH-DPAT. The 5-HT1A receptor is the most studied and best characterized 5-HT receptor subtype to date. 5-HT1A receptors are abundantly expressed in cortical and hippocampal (CA1/CA3 and dentate gyrus) regions that are critically involved in learning and memory and susceptible to neuronal damage. (Kline, et al., 2007) 5-HT1A receptor involvement in modulation of the major neurotransmitters ACh, DA, and glutamate, the serotonin (5-HT) system, is considered a significant pharmacological target for the treatment of various central nervous system (CNS) diseases. Administration of 5-HT1A receptor agonists before or after focal

cerebral ischemia provides neuroprotection as evidenced by decreased histopathology. Single administration 5-HT1A receptor agonist of 8-OH-DPAT decreases cortical lesion volume and hippocampal neuron survival. Single systemic administration of the 5-HT1A receptor agonist 8-OH-DPAT enhances behavioral recovery after experimental traumatic brain injury (TBI). Intraperitoneal administration of 0.5 mg/kg dose of 8-OH-DPAT confers neurological protection after TBI in Hippocampal CA1/CA3 region. (Cheng, et al., 2008; Kline, et al., 2004) 8-OH-DPAT is much more potent when injected subcutaneously than when injected Intraperitoneal, the potency difference being approximately 17-fold. (Fuller and Snoddy, 1987) The important influence of route of administration on the potency of 8-OH-DPAT must be considered in interpreting the various functional effects that have been reported with it. After oral administration, 8-OH-DPAT goes first-pass metabolism and shows low oral bioavailability of, rather than poor absorption from the GI tract. (Mason, et al., 1995) We selected doses of in range of 0.3 to 1.7 mg/kg of 8-OH-DPAT for subcutaneous administration for our dose response curve. The mortality rate for our toxic challenge group is 33.33 %. The lower dose group 0.3 and 1 mg/kg of 8-OH-DPAT showed less mortality than toxic challenge but mortality increased with higher doses of 1.7 and 3 mg/kg. 8-OH-DPAT is not a rescue drug for treatment of nerve agent exposure therefore; we did not see any significant effects on mortality rate. The reason for higher mortality rate in higher doses of DPAT groups is still not clear. There could be unknown interaction between higher doses of DPAT with CBDP or Sarin causing higher mortality.

We measured total body weight loss on mice up to 3 days post sarin injections. Body weight is related to general health status, periodic measurement can

indicate if animals are recovering or still in poor health. Knowledge of body weight is crucial for neurotoxicity testing. Body weight loss can be an indicator of neurotoxin effects on CNS. Rats treated with artelinic acid showed signs of neurotoxicity and significantly lost weight. (Si, et al., 2007) At day, 1 0.3 and 1.7 mg/kg DPAT group showed higher weight loss compared to toxic challenge group. Only one mouse survived in 3mg/kg group after injection. We did not include that group in our results due to low number in the group. At both day 1 and 3, 1 mg/kg the DPAT group weight loss was lower than the toxic challenge group, while at day 3 other doses had higher weight loss. We selected as the best dose 1mg/kg of 8-OH-DPAT for the time response curve with toxic challenge based on lower mortality and lower weight loss data.

Time response of 1 mg/kg of 8-OH-DPAT with toxic challenge

We performed a time response of 8-OH-DPAT with the 1mg/kg selected dose from the dose response curve. We gave DPAT at 1, 15, 30, 45, 60 minutes 2, 4 and 6 hour after toxic challenge. Weight loss of mice treated with DPAT 1 and 15 min after toxic challenge was lower than TC group. At later time points in DPAT group the morality was similar or higher than toxic challenge group. Since DPAT is not a rescue drug it does not reduce the mortality rate in the Sarin treated animals. That there were no significant differences in lethality between DPAT groups suggests that it was without major effect. The small difference in the mortality rate could be due to inter individual differences in susceptibility of animals to Sarin treatment. We measured total body weight loss up to 3 days in all DPAT groups. Only the 1-minute DPAT group had lower weight loss than toxic challenge at both 1 and 3 days. None of the DPAT group has

significantly lower weight loss than toxic challenge group. Lower food and water intake could be the reason of weight loss after Sarin injection after 3 days.

Mice were observed using a Functional Observational Battery for 1-hour post Sarin dosing. As neurobehavioral assessments during the preliminary stages of chemical testing are important, it is critical that the screening procedures utilized be valid indicators of neurobehavioral function and that they be sensitive, specific, and reliable. In many instances, the effects observed with the FOB may be predictive of symptomology in humans and comparisons can be made between effects detected with the FOB and other methods of measuring neurotoxicity. (Moser, 1990;Si, et al., 2007) We observed higher FOB scores in all Sarin treated groups. There were no significant differences between any of the Sarin treated groups. We observed a significant difference in FOB scores between vehicle control and Sarin treated groups. We only measured 1 hour time point FOB score post injection and observed no difference in groups, so we concluded that animals do not recover from acute symptoms if they are not treated with rescue therapy. We decided to monitor FOB scores for 24 hours in 2, 4 and 6-hour DPAT groups. We did not observe any significant difference in FOB score between toxic challenge and DPAT groups in any time points. It confirms DPAT is not a rescue drug and it does not have any effects on acute effects produce by neurotoxicity of sarin dose.

We also measured blood and brain cholinesterase activity 14 days post injections in groups. Acute symptoms of sarin are produced by irreversible inhibition of the cholinesterase enzymes. CBDP alone does not produce any effects on total ChE levels. We have previously determined in our laboratory that toxic challenge doses of

Sarin significantly reduce blood cholinesterase activity compared to control group animals 4 days post injections. We observed no significant difference in blood ChE and AChE in any of the groups 14 days after treatment. However, new red blood cell production rapidly restores blood AChE levels and the BuChE in plasma is available from the liver. Frontal cortex ChE activity was lower in DPAT groups than the toxic challenge group. It is not clear why this occurred as DPAT neither inhibits nor regenerates AChE (Mason, 2000) Thus, DPAT is not providing neuroprotection by reducing in acute symptoms, restoring the animal's general health more rapidly or by regenerating AChE.

Neuroprotection by 8-OH-DPAT

Sarin exposure leads to neuronal damage, which includes cell degeneration in specific areas like the hippocampus, the piriform cortex and the thalamus. This damage might expand with time to include additional brain areas. Soman exposure causes cell death in mice, in which damaged neurons continue to be present in hippocampal CA1 up to 90 days post soman exposure. GFAP staining was evident in dentate gyrus of soman exposed mice, the GFAP staining consistently increased until 15^{th} days post exposure. The cascade of events induces long-term effects. Inhalation of sarin vapors induced impaired memory processes seen at 1-month post exposure with no recovery of function during the 6 months follow-up period. Similarly, long-term follow-up of victims of the sarin attacks in Japan demonstrated neurological as well as emotional and cognitive changes up to 7 years post exposure. (Collombet, et al., 2007;Grauer, et al., 2008) Long-term impaired neurobehavioral functioning effects were also recently reported following

low-dose inhalation exposure to sarin and cyclosarin during the 1991 Gulf War. An appropriate treatment might halt the damaging processes and may even allow for functional recovery. Current treatment therapies include atropine and 2-PAM, which are rescue therapy for acute toxicity. Current therapy still does not protect from long term effects. We found neuroprotection in dentate gyrus of hippocampus with 1mg/kg dose of 8-OH-DPAT. The neuroprotection was significant up to 2 hour DPAT injection after exposure to sarin. The groups were euthanized 14 days post injections it shows that toxic challenge mice groups are still showing signs of neurodegeneration. The four-hour DPAT group GFAP cell count is lower than toxic challenge but is not significant that shows DPAT is not effective post 2 hour as treatment. (Collombet, et al., 2007;Grauer, et al., 2008) Initiation and early expression of the seizures are cholinergic phenomenon; anticholinergics readily terminate seizures at this stage and no neuropathology is evident. However, if not treated, a transition phase occurs during which the neuronal excitation leads to neuropathology, where Control with anticholinergics becomes ineffective. With prolonged epileptic form activity the seizure enters a predominantly non-cholinergic phase, 5-HT-1A may work as a neuroprotectant. Since DPAT is neuroprotective up to 2 hour it can be given to a patient who does not receive timely rescue therapy to prevent long term effects. (McDonough, Jr. and Shih, 1993) In another study in our laboratory, we tested the hypothesis that the neuroprotective effects of 8-OH-DPAT against sarin-induced neuropathology resulted from alteration of the seizure pattern. Swiss Webster mice were implanted subcutaneously (sc) with transmitters and cortical electrodes for telemetry recordings. Recordings of baseline electroencephalography (EEG) were obtained and mice were then injected with 200 µg/kg (sc) of Sarin and EEG recordings

were taken for 4 hours unless death resulted. A second group was dosed with 200 µg/kg of Sarin and 1 min later DPAT (1.0 mg/kg) (sc). All of the sarin dosed mice exhibited behavioral signs of seizure activity. The recordings substantiated that seizures occurred and they continued increasing in severity of spikes. There was a significant difference in the seizure activity of mice dosed with sarin alone compared to the mice with sarin+DPAT. The EEG of the sarin dosed mice indicated both high frequency and amplitude seizure patterns. The seizure pattern of the sarin+DPAT dosed mice revealed seizure patterns with greater intervals between spikes, lower spike amplitudes, and fewer spike trains. (Table 6) (Figure 20, 21) These studies demonstrate that 8-OH-DPAT influences the pattern, intensity, and duration of sarin-induced seizures in mice. (unpublished versions of work done by Belinda Sims)

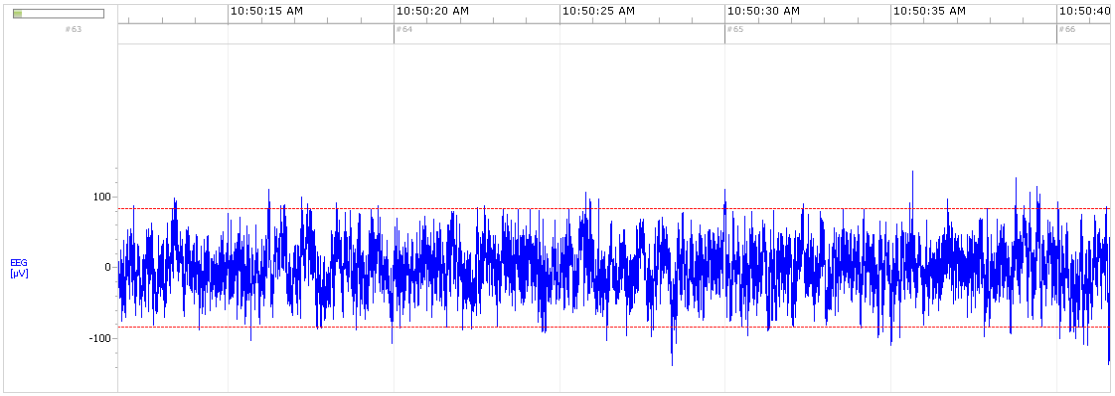

Figure20: 30 second baseline recording of cortical EEG of control Swiss Webster mouse.

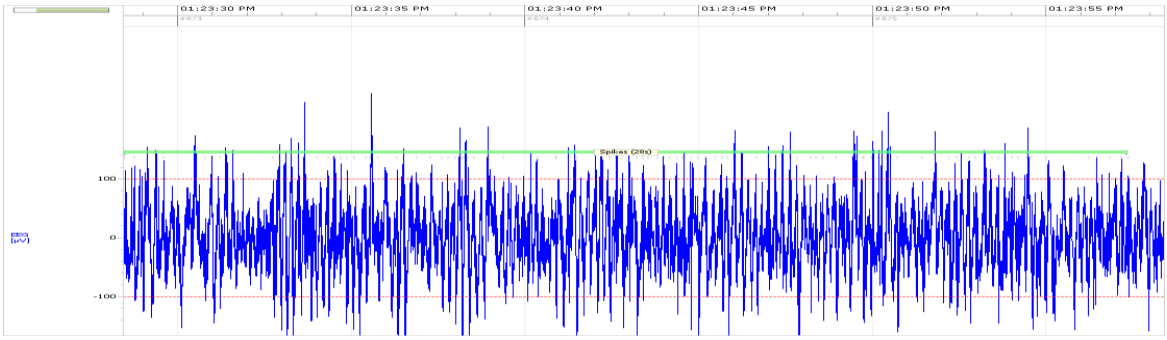

Figure 21. 30 second seizure recording of cortical EEG. Swiss Webster mouse dosed with 200µg/kg of Sarin, 3 hrs post injection. Green marker shows 28 second spike train and the red dotted lines indicate baseline amplitude threshold.

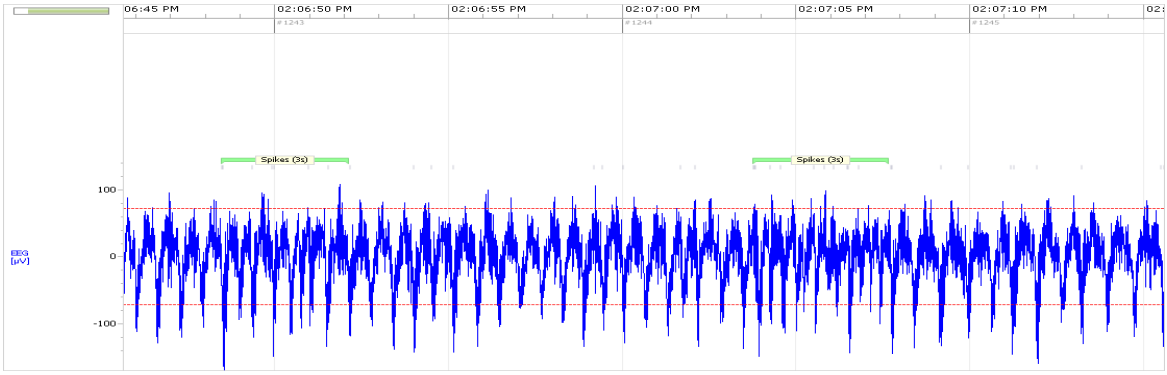

Figure 22. 30 second seizure recording of cortical EEG. Swiss Webster mouse dosed with 200µg/kg of Sarin and 1.0 mg/kg DPAT (1 min post Sarin), 3 hrs post injection. Green markers show 3 second spike trains, red dotted lines indicate baseline amplitude threshold.

Group	3 hr, Mean Spike Train Duration, (seconds)	Total number of spike trains	Inter Spike Train Mean (seconds)	Amplitude 3 hrs post injection (µV)
Sarin ALONE	3.7±1.4	785±293	0.26±0.03	0.91± 0.24
Sarin + DPAT	3.4±0.3	185±25	0.43±0.06	0.21 ± 0.08

Table 6. EEG data of Sarin group compared to DPAT group.

8-OH-DPAT and emotional learning

Sarin exposure causes memory impairment in rodents and humans. We tested the ability of the 8-OH-DPAT agonist dose of 1mg/kg and 45 minute after toxic challenge for its ability to reinstate Fear potentiated startle (FPS) response. We have previously determined in our laboratory that two low doses sarin to mice produces impairment of acquisition of fear-potentiated startle response. We assumed that our doses Sarin with CBDP would produce impairment in acquisition of startle response. On the contrary, our toxic challenge group learned the conditioning. This result negated the ability of the study to demonstrate the ability of 8-OH-DPAT to reverse learning deficits. The vehicle control group learned the conditioning but the difference between pre and posttest was not significant, as we have previously determined. The results show that our dose was on the breaking point between interference at low doses and noninterference at higher doses.

Conclusion

We were able to utilize CBDP rodents to more accurately model actual human exposure. CBDP reduced the dose of Sarin which produces 25-50 % mortality in mice. This model helps to reduce variability produce by single actual dose of Sarin. We also observed significant neurodegeneration in dentate gyrus of hippocampus in toxic challenge group. These results are consistent, repeatable and similar to our previous laboratory experiments. We were able to produce neuroprotection with the 1mg/kg dose of the 5-HT-1A agonist 8-OH-DPAT. 8-OH-DPAT was able to produce neuroprotection

up to 2 hours post toxic challenge. DPAT did not produce any effects on mortality rate, weight loss or ChE activity. 8-OH-DPAT does not produce any effects on cholinesterase inhibition. Our laboratory observed that 8-OH-DPAT influences the pattern, intensity, and duration of sarin-induced seizures in a fashion that could account for the observed neuroprotection in the dentate gyrus of hippocampus. We suggest that the efficacy of 8-OH-DPAT treatment lies in its ability to attenuate glutamate release, thereby putting an end to seizure activity due to NMDA receptor stimulation. (Srkalovic, et al., 1994;McDonough, Jr. and Shih, 1993) We have also tested other 5-HT-1A agonists in our laboratory post toxic challenge injections, including buspirone and S-14506. Both these compounds increased the mortality rate compare to toxic challenge group but did not have any effects on weight loss and FOB. The mechanism neuroprotection of 8-OH-DPAT is still not definitively determined. Further work should be done to identify the mechanism of DPAT action. Sarin exposure leads to long-term effects if current rescue therapy atropine and 2-PAM is not given to stop initial seizure activity. Since DPAT is effective up to 2 hours after sarin, it may be an effective therapy for neuroprotection from the long-term effects produced by sarin induced convulsion. Since DPAT does not protect from acute symptoms, it is not useful as rescue agent. 8-OH-DPAT may be used as addition to current rescue treatment of atropine and 2-PAM.

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