Highlights of the Initial Feasibility Year of the Preclinical Studies

To Identify Mechanisms of the Chronic Neurological Effects of Gulf War Chemicals Against Which to Direct Treatment

Robert W. Haley, M.D.
For the Principal Investigators
University of Texas Southwestern Medical Center
Dallas, Texas

The behavioral dose-finding study by Sinton was supported by the U.S. Army Medical Research and Materiel Command grant number DAMD17-01-1-0741. The rest of the preclinical studies were supported by IDIQ contract VA549-P-0027, awarded and administered by the Department of Veterans Affairs Medical Center, Dallas, TX.

Preclinical Projects

- Mechanistic neuroscience projects each focused on a condition or prior finding of ill Gulf War veterans.
- Preclinical purpose: to discover the mechanism causing the condition or symptom that can be exploited to develop treatment.
- Since projects were high risk (R21), a feasibility year was funded for ≤$300K total cost, with understanding that those with promising findings would be extended for additional years.
- 18 projects submitted October 2006, 10 funded October – December 2008.
- Feasibility year is now ending.

Preclinical Projects

- Development of a Mouse Model of Chronic Neurotoxicity from Gulf War Chemicals
- Effects of Gulf War Chemicals on:
  - The Memory Circuits of the Brain
  - The Autonomic Nervous System
  - Brain Dopamine Turnover
  - Development of Lou Gehrig's Disease (ALS)
  - Development of Brain Cancer
- Possible Mechanisms of these Effects:
  - Neuroinflammation
  - Mitochondrial Damage
  - Intracellular Phosphorylation

"Gulf War Chemicals"

- Chlorpyrifos (CP, Dursban)
  - Most commonly used pesticide in early 1990s, heavy use in GW.
  - Chlorpyrifos (CP) is inert until converted to Chlorpyrifos-oxon (CPO) by P450 in the liver.
- Pyridostigmine Bromide (PB, Mestinon)
  - Medicine used to treat myasthenia gravis
  - Taken by Gulf War soldiers to reduce mortality from soman attack.
- Sarin (GB)
  - Chemical warfare nerve agent present in Kuwaiti Theater
  - DFP (diisopropyl fluorophosphate), surrogate for laboratory study.

Preclinical Projects

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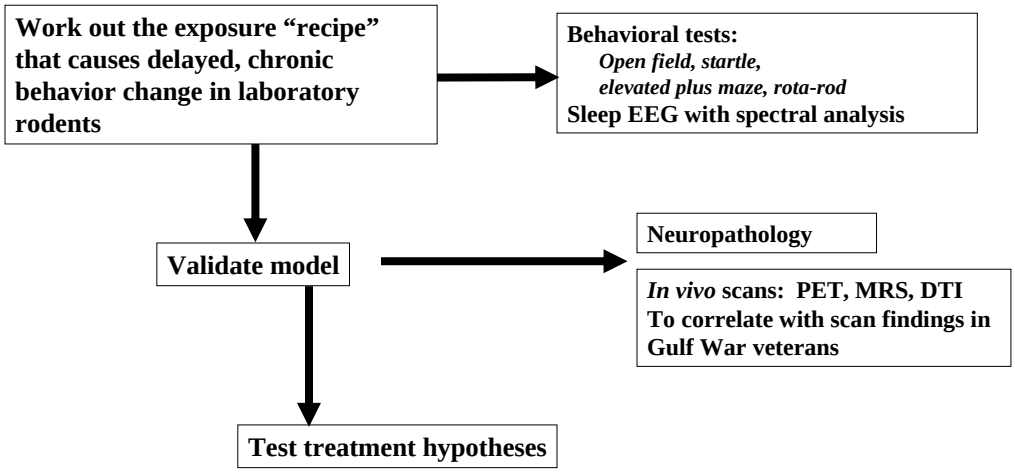
Development of a Mouse Model of Chronic Neurotoxicity from Gulf War Chemicals

Repeated, low-dose exposure of mice to Gulf War chemicals followed by appearance of brain dysfunction lasting ≥3 months

Christopher Sinton, Ph.D.
Department of Internal Medicine
UT Southwestern Medical Center

Goals

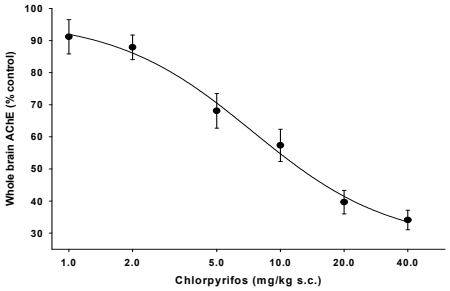
- **Examine the chronic effects of repeated low-dose exposure to agents to which military personnel were potentially exposed in the 1991 Gulf War ("Gulf War chemicals").**
- **Use behavioral testing to detect functional changes in the brain (sensitive to a wide range of changes and can be repeated in same animal over time).**
- **Validate the model with pathologic study and correlate neuroimaging scanning with findings in ill GW veterans.**
- **Use the model to test treatment hypotheses.**



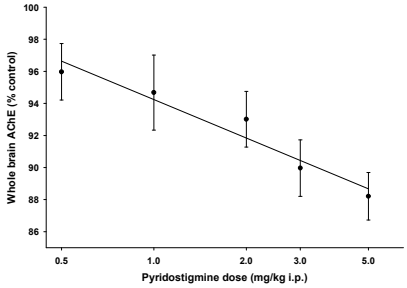
Determine exposure "recipe" that causes delayed, chronic behavior change in laboratory rodents

Establish the brain penetration of a range of doses by parenteral administration.

A. Chlorpyrifos (CP) in the rat



B. Pyridostigmine bromide (PB) in the rat

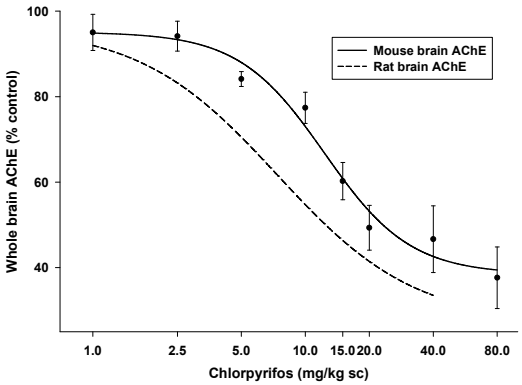


Sinton et al. *Toxicol Appl Pharmacol* 2000; 165:99-105.

Dose-response of the effect of CP (mg/kg s.c. in DMSO) or PB (mg/kg i.p in saline) on whole rat brain AChE activity, expressed as a percentage of control. Determinations were made 10 h after CP administration to allow conversion to CP oxon.

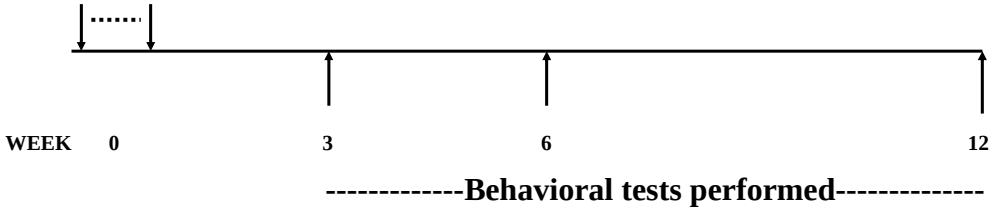
Establish the brain penetration of a range of doses by parenteral administration.

B. Chlopyrifos (CP) in the mouse



Dose-response of the effect of CP (mg/kg s.c. in DMSO) on whole mouse brain AChE activity, expressed as a percentage of control. Determinations were made 10 h after CP administration. For comparison, the data from the corresponding study in rats are also included.

Repetitive treatment with test compound(s)



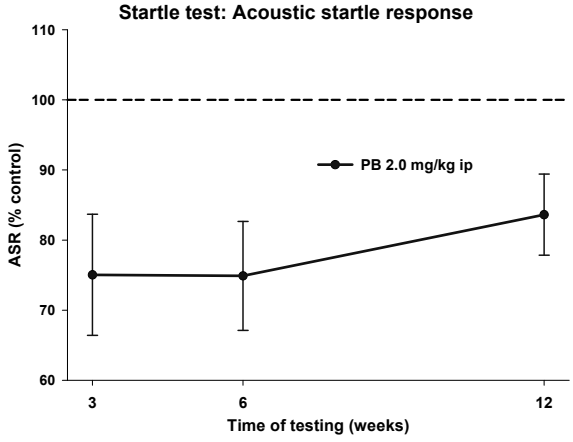
----------Behavioral tests performed----------

Low-dose chemical agent treatment:
Chlopyrifos (CP), Pyridostigmine Bromide (PB)
Once per day for 5 days during week 0
Doses chosen to show no acute symptoms and up to a maximum of the ID_{33} from the dose response curves, i.e. PB up to 2 mg/kg, CP up to 5 mg/kg.

Functional testing:
Behavioral and electrophysiological tests at 3, 6 and 12 weeks after completion of treatment. Results to date are not complete but specific tests show effects of either compound alone and a synergy in one test. The following are examples.

Evidence of Chronic Brain Effects
Effect of PB on Startle Response in the Rat

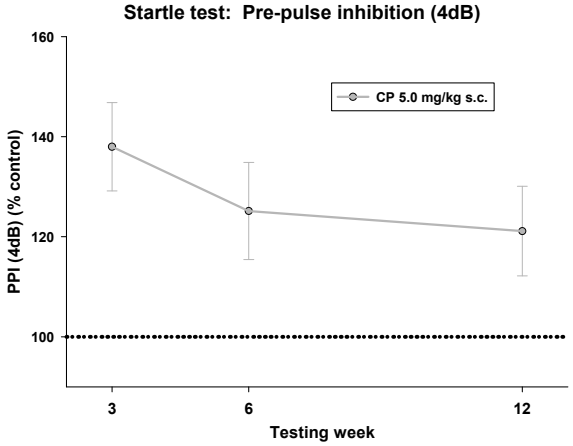
In the rat, an effect of PB at 2 mg/kg was found in the startle test: the magnitude of the acoustic startle response (ASR) was reduced by PB treatment. This effect was present by 3 weeks after PB administration but may have diminished slightly by 12 weeks.



Results expressed as a percentage of the corresponding controls. F (1,46) = 9.19, P = 0.001

Evidence of Chronic Brain Effects
Effect of PB on Pre-Pulse Inhibition of the Startle Response in Mice

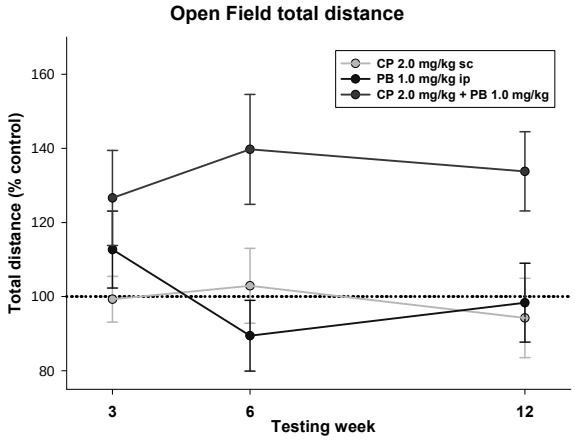
In the mouse, CP at a dose of 5 mg/kg affected pre-pulse inhibition in the startle test. This effect was present at 3 weeks after test compound administration and remained at 12 weeks though reduced in amplitude.



Results expressed as a percentage of the corresponding controls. F (1,30) = 4.32, P = 0.04

Evidence of Chronic Brain Effects
Synergistic Effect of CP and PB on Open Field Activity in Mice

Synergy between CP and PB in the mouse. The combination of CP at 2 mg/kg and PB at 1 mg/kg affected behavior in the open field test, including the total distance traversed during the 120 min test. This effect was not observed when the compounds were administered singly.



Results expressed as a percentage of the corresponding controls. F (1,30) = 7.42, P = 0.01

Provisional Conclusions

- **Low dose repeated exposure to chlorpyrifos (CP) and pyridostigmine bromide (PB) induces pathological CNS effects in the rat and C57 Bl/6 strain of mice, as shown by behavioral differences in the startle and open field tests.**
- **Synergy between CP and PB is confirmed by data from the open field test in the mouse.**
- **The model will subsequently be validated by neuropathology and by neuroimaging techniques showing abnormalities in ill GW veterans, and then will be used to screen for potential treatments.**

Preclinical Projects

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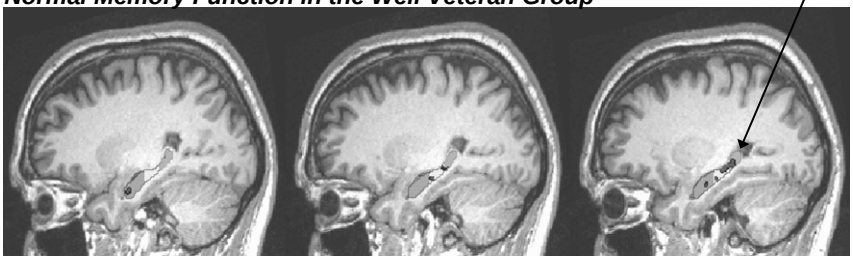
  - Neuroinflammation
  - Mitochondrial Damage
  - Intracellular Phosphorylation

Failure to Activate the Right Hippocampus During fMRI Memory Test

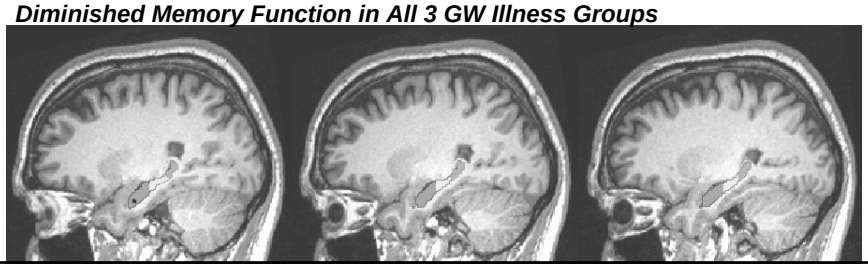
Wendy Ringe, Ph.D., UT Southwestern

***Hippocampus,* the brain's memory center**

Normal Memory Function in the Well Veteran Group



Diminished Memory Function in All 3 GW Illness Groups



Red is brain tissue functioning during memory task.

Detrimental Effects of the Pesticide Chlorpyrifos (CP) on Hippocampal Synaptic Transmission

Haley Speed, PhD, Craig Powell, MD, PhD
UT Southwestern Department of Neurology

Hypotheses:

1. ***Repetitive exposure to low doses of the Chlorpyrifos (CP) would result in impaired hippocampal synaptic transmission, as well as learning and memory deficits.***

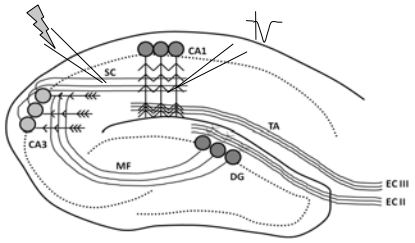
2. ***Short-term and long-term effects of CP exposure on hippocampal function and learning and memory would be very different.***

Methods

10-12 week old C57/Bl6 mice
injected subcutaneously for 5 consecutive days
5mg/kg CP or vehicle (100% DMSO) at 1mg/mL.

Acute: 2 days following end of exposure period, mice were tested for changes in hippocampal synaptic transmission.

Delayed: In separate experiments, 3 months after end of exposure mice were tested for changes in hippocampal synaptic transmission.

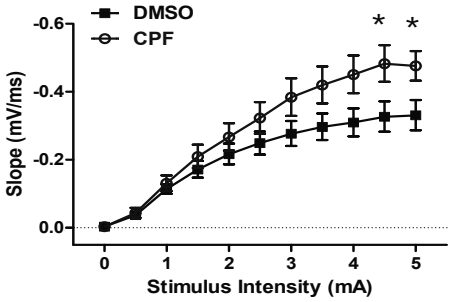


<u>Electrophysiology</u>
Acute transverse hippocampal slices (400mM thick).
Extracellular field recordings in the stratum radiatum
Recordings performed at @ 33ºC.
- ***Input/Output Curves***
- ***Paired-pulse ratio (PPR)***
- ***Strong 100Hz Long-Term Potentiation (LTP)***

<u>Behavior</u>
Social Learning (days 1-4)
Olfactory Control (day 5)
Fear Conditioning (days 11-12)
Morris Water Maze (days 13-25)
Footshock Threshold (day 30)

Acute Effects of CPF on Basal Synaptic Transmission

2 days following end of exposure



CP-treated mice exhibit steeper I/O curve than DMSO-treated mice.

CP-treated mice exhibit a larger maximal fEPSP slope than vehicle-treated mice.

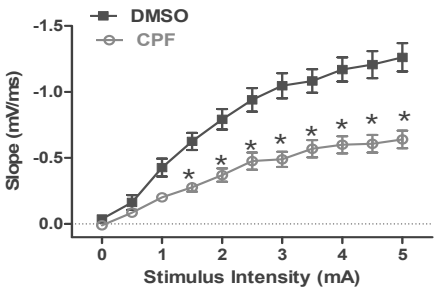
N=8 (DMSO), N=8 (CP); *One-Way ANOVA, P<0.05.

These results suggest that basal synaptic transmission is increased immediately following repetitive, low-dose exposure to CP.

This is consistent with previous studies showing that increased activation of nAChRs following chronic nicotine treatment, similar to levels observed in moderate to heavy smokers (Levin and Rezvani, 2000; Stolerman et al, 2000).

Delayed Effects of CP on Basal Synaptic Transmission

3 months following end of exposure



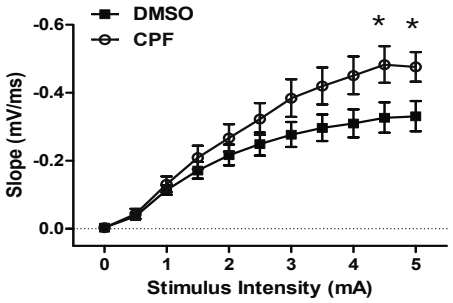
CP-treated mice exhibit more shallow I/O curve than DMSO-treated mice.

CP-treated mice exhibit ~50% decrease in the maximal fEPSP slope than vehicle-treated mice.

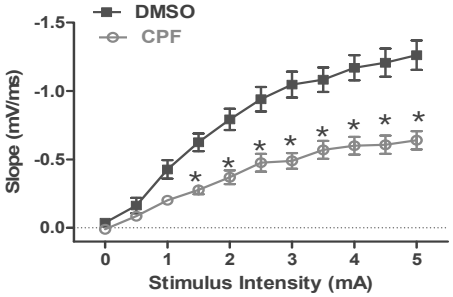
N=12 (DMSO), N=12 (CP); *One-Way ANOVA, P<0.001.

These results suggest that basal synaptic transmission is chronically impaired long after repetitive low-dose exposure to CP.

Summary: Acute and Delayed Effects on Basal Transmission



<u>Acute Effects of CP</u>:
Basal synaptic transmission is increased immediately following repetitive, low-dose exposure to CP.



<u>Delayed Effects of CP</u>:
Basal synaptic strength is chronically decreased following repetitive, low-dose exposure to CP.

Summary of Delayed Effects

Conclusions

- ***Basal synaptic transmission in the CA3-CA1 region of the hippocampus was dramatically decreased with CP treatment compared to control, indicating a decrease in overall excitatory synaptic strength.***
- ***Paired-pulse ratio was not significantly affected by CP treatment at the late time point, indicating that changes in presynaptic function are not likely to contribute to increased excitatory transmission.***
- ***Long-term potentiation was not significantly affected by CP at the late time point, indicating that the increase in excitatory transmission and contextual fear condition is due to some other mechanism.***

Potential Mechanisms Underlying Decreased Excitatory Transmission

- ***Decreased number of synapses due to neuronal cell death.***
- ***Decreased size of postsynaptic densities, reducing synaptic efficacy.***
- ***Decreased neuronal excitability, leading to reduced synaptic efficacy.***
- ***Decreased amount of neurotransmitter released.***

Future Directions

General

- ***Get funding***
- ***CP + PB + Sarin***
- ***Try slightly higher dosage, increase treatment period to 2 weeks***
- ***Observe later time points, 6 months/9 months/1 year***

Electrophysiology

- ***Miniature Excitatory Postsynaptic Currents (mEPSCs)***
- ***Theta-Burst LTP***
- ***Membrane Properties/Firing Properties***

Behavior

- ***Radial arm maze/ T-maze***
- ***Histology/Pathology***
- ***Golgi stain***
- ***Immunohistochemistry with specific markers for excitatory and inhibitory synapses***

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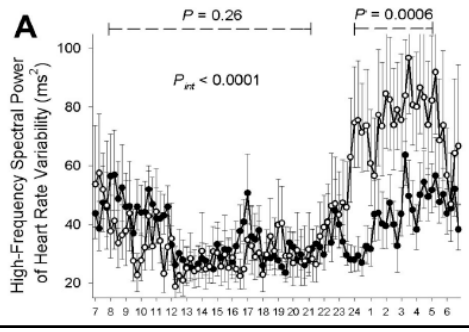
  - Intracellular Phosphorylation

Abnormality of Parasympathetic Autonomic Function in Ill Gulf War Veterans

Embargo Date: September 27, 2004, 5:00 a.m. EST October 1, 2004, The American Journal of Medicine, Volume 117, No. 7

Blunted Circadian Variation in Autonomic Regulation of Sinus Node Function in Veterans with Gulf War Syndrome

Robert W. Haley, MD, Wanpen Vongpatanasin, MD, Gil I. Wolfe, MD, Wilson W. Bryan, MD, Roseanne Armitage, PhD, Robert F. Hoffmann, PhD, Frederick Petty, PhD, MD, Timothy S. Callahan, PhD, Elizabeth Charuvastra, RN, William E. Shell, MD, W. Wesley Marshall, MD, Ronald G. Victor, MD



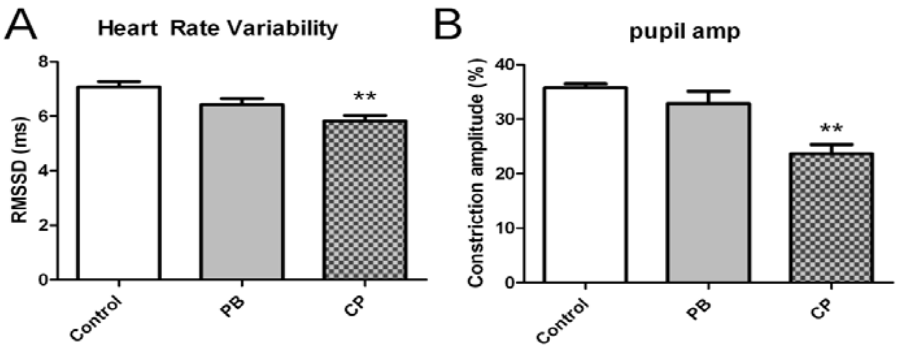
Autonomic Studies

Steven Vernino, MD, PhD
Associate Professor
Director, Autonomic Function Laboratory
Department of Neurology, UT Southwestern

Methods

- C57/Bl6 mice exposed to cholinesterase inhibitors for 5 days (PB at 1 mg/kg/day and/or CP at 5 mg/kg/day)
- Interval before testing: 12 weeks
- Physiological studies of autonomic function:
  - Measurement of Heart Rate Variability (HRV) using ambulatory ECG
  - Quantitation of pupil light reflex
  - Measurement of sympathetic ganglionic transmission
  - Assessment of gastric motility

Results at 12 Weeks Post Exposure



<u>Effect of cholinesterase inhibitors on autonomic function</u>. A) Resting heart rate variability (12 weeks after a one week exposure to drug) was slightly reduced in mice exposed to pyridostigmine (PB) and significantly reduced in mice exposed to chlorpyrifos (CP). This indicates a persistent impairment in cardiovagal *parasympathetic* function. B) The amplitude of pupillary constriction in response to light was impaired in mice 12 weeks after exposure to CP. This indicates a persistent impairment in *parasympathetic* pupillomotor function. **, p<0.01

Conclusions

- In this mouse model, CP and PB enhance autonomic function acutely but impair it chronically.
- Following exposure to these Gulf War chemicals, there are acute changes in synaptic transmission in *sympathetic* autonomic ganglia which recover over time.
- Vagal control of heart rate and pupillary constriction, *parasympathetic* functions, remain chronically impaired.
- *Parasympathetic* function appears to be more persistently affected than *sympathetic* function.

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Abnormal Increase in Brain Dopamine Production in Ill Gulf War Veterans

ORIGINAL CONTRIBUTION

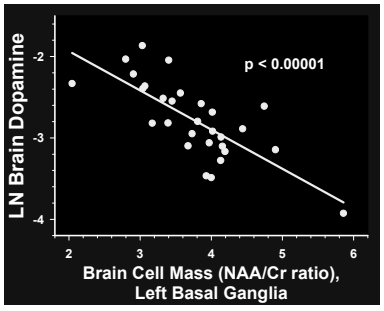
Effect of Basal Ganglia Injury on Central Dopamine Activity in Gulf War Syndrome

ARCHIVES EXPRESS

Correlation of Proton Magnetic Resonance Spectroscopy and Plasma Homovanillic Acid Levels

Robert W. Haley, MD; James L. Fleckenstein, MD; W. Wesley Marshall, MD; George G. McDonald, PhD; Gerald L. Kramer, BS; Frederick Petty, PhD, MD

Arch Neurol. 2000;57:1280-1285



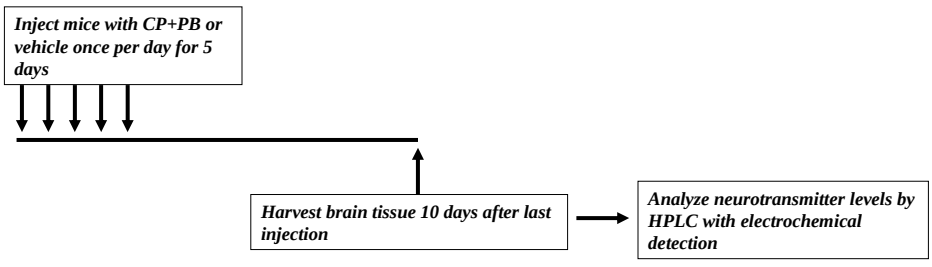
Exposure to Chlorpyrifos and Pyridostigmine Bromide Causes Neurotransmitter Abnormalities

Matthew S. Goldberg, Ph.D.
Xiaodong Ding, Ph.D.
Marian Marvin
UT Southwestern Departments of
Pathology and Neurology

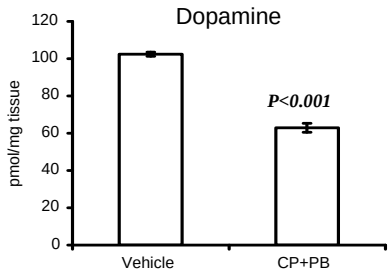
Methods:
Laboratory mice received injections of chlorpyrifos (CP) and pyridostigmine bromide (PB) once per day for **5 consecutive days**. **Chlorpyrifos** was administered **subcutaneously** at a dose of **5 mg/kg** in DMSO. **Pyridostigmine bromide** was administered **intraperitoneally** at a dose of **0.5 mg/kg** in sterile saline. Control mice received vehicles alone.

Brain tissues were harvested for analysis **10 days** after the last injection was analyzed by High Performance Liquid Chromotography (HPLC) and levels of neurotransmitters were quantified by electrochemical detection.

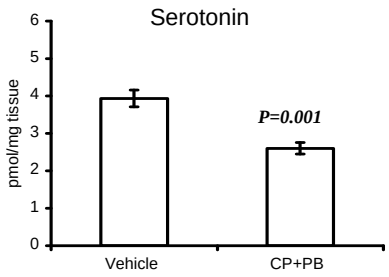
Studies of effects 3 months after last injection are pending.



Decreased Dopamine and Serotonin in Striatum



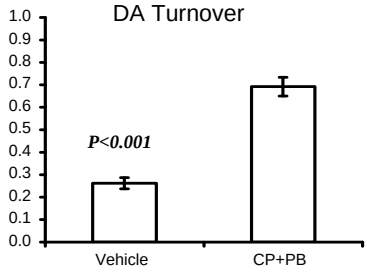
N= 4 mice per group



N= 4 mice per group

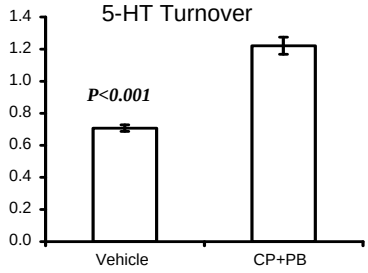
10 days after the last injection, 3 month followup pending.

Increased Turnover of Dopamine and Serotonin



N= 4 mice per group

Turnover = ratio of metabolites to dopamine (DA)



N= 4 mice per group

Turnover = ratio of metabolite to serotonin (5-HT)

10 days after the last injection, followup pending.

Preclinical Projects

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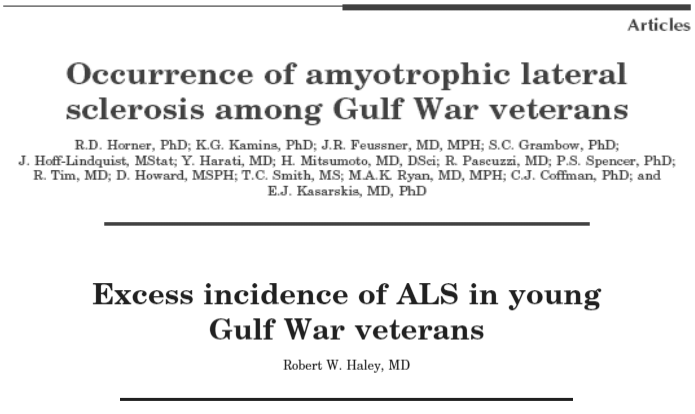
Significant Increase in ALS in GW Veterans Demonstrated by Two Studies



Articles

Occurrence of amyotrophic lateral sclerosis among Gulf War veterans

R.D. Horner, PhD; K.G. Kamins, PhD; J.R. Feussner, MD, MPH; S.C. Grambow, PhD; J. Hoff-Lindquist, MStat; Y. Harati, MD; H. Mitsumoto, MD, DSci; R. Pascuzzi, MD; P.S. Spencer, PhD; R. Tim, MD; D. Howard, MSPH; T.C. Smith, MS; M.A.K. Ryan, MD, MPH; C.J. Coffman, PhD; and E.J. Kasarskis, MD, PhD

Excess incidence of ALS in young Gulf War veterans

Robert W. Haley, MD

***Neurology* 2003;71:742-749, 750-756**

Effects of Chlorpyrifos and Pyridostigmine on Mouse Models of Lou Gehrig's Disease (ALS): *In Vitro* and *In Vivo* Studies

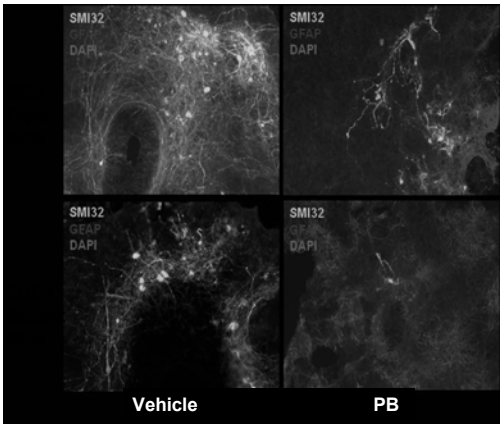
Krishna Puttaparthi, Christina Luther,
Jeffrey L. Elliott

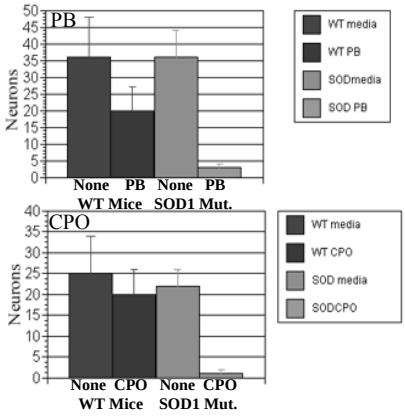
Department of Neurology, University of Texas Southwestern
Medical Center, Dallas, Texas 75390

Methods of *In Vitro* Study

- Spinal cord slices removed from:
  - Normal ("Wild Type") Mice and
  - Transgenic "ALS" Mice (Human G93A SOD1 gene).
- Treated *in vitro* for 4 weeks with:
  - Chlorpyrifos oxon (CPO, 50 μm or 1 μm)
  - Pyridostigmine (PB, 10 μm or 2.5 μm)
  - Vehicle
- Immunohistochemical staining to quantify the numbers of neuronal cell bodies (SMI-32 antibody).

In Vitro Effect of PB and CPO on Neurons of Spinal Cords from Wild Type vs SOD1-Mutant Mice

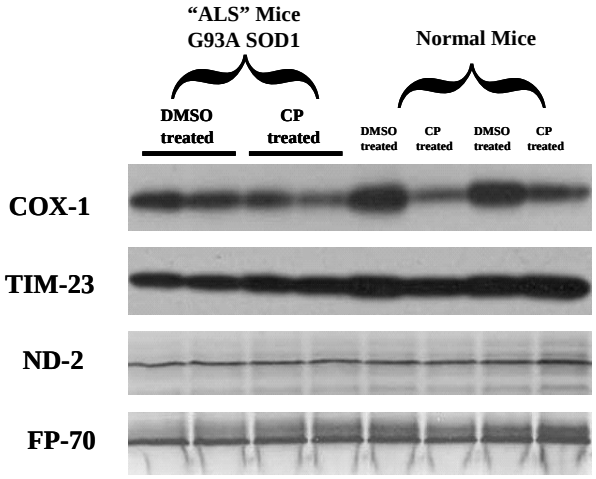




Methods of *In Vivo* Study

- Five month old mice:
  - Normal ("Wild Type") Mice and
  - Transgenic "ALS" Mice (Human G93A SOD1 gene).
- Injected i.p. *in vivo* for 2 of 3 weeks with:
  - Chlorpyrifos (CP, 5 mg/kg)
  - DMSO Vehicle
- Spinal cords harvested 30 days and 60 days after last dose.
- Mitochondrial membranes isolated from dissected spinal cords.
- Probed with antibodies for complexes of the electron transport chain:
  - Complex IV (COX-1)
  - Complex II (Fp70)
  - Complex I (ND2)
  - TIM23

In Vivo Effect of Chlorpyrifos (CP) on Complex IV* of the Mitochondrial Electron Transfer Chain



*Cytochrome c oxidase, subunit 1 (COX-1) = Complex IV

Preclinical Projects

- Development a Mouse Model of Chronic Neurotoxicity from Gulf War Chemicals
- **Effects of Gulf War Chemicals on:**
  - The Memory Circuits of the Brain
  - The Autonomic Nervous System
  - Brain Dopamine Turnover
  - Development of Lou Gehrig's Disease (ALS)
  - **Development of Brain Cancer**
- Possible Mechanisms of these Effects:
  - Neuroinflammation
  - Mitochondrial Damage
  - Intracellular Phosphorylation

Increased Risk of Brain Cancer in Gulf War Veterans Exposed to Khamisiyah CW Release

Mortality in US Army Gulf War Veterans Exposed to 1991 Khamisiyah Chemical Munitions Destruction

Tim A. Bullman, MA, Clare M. Mahan, PhD, Han K. Kang, DrPH, William F. Page, PhD

Results. The risks of most disease-related mortality were similar for exposed and unexposed veterans. However, exposed veterans had an increased risk of brain cancer deaths (relative risk [RR] = 1.94; 95% confidence interval [CI] = 1.12, 3.34). The risk of brain cancer death was larger among those exposed 2 or more days than those exposed 1 day when both were compared separately to all unexposed veterans (RR = 3.26; 95% CI = 1.33, 7.96; RR = 1.72; 95% CI = 0.95,3.10, respectively).

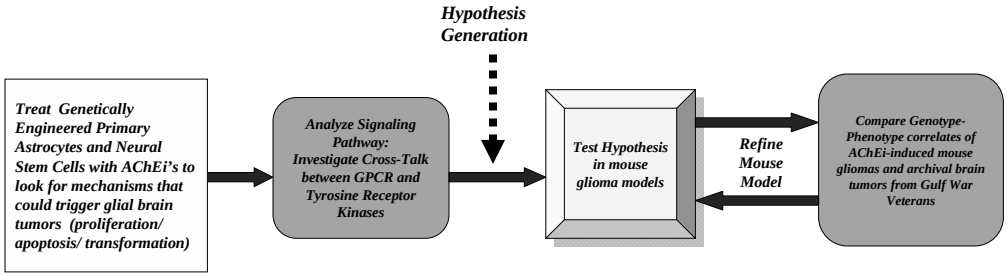
Conclusions. Exposure to chemical munitions at Khamisiyah may be associated with an increased risk of brain cancer death. Additional research is required to confirm this finding. (*Am J Public Health.* 2005;95:1382–1388. doi:10.2105/AJPH.2004.045799)

Can Gulf War Chemical Agents Trigger Glial Brain Tumors?

T Mashimo, V Vemireddy, S Sirasanagandla,
S Nannepaga, X Yang, R Bachoo

Annette G. Strauss Center for Neuro-Oncology,
Simmons Cancer Center and Department of Neurology

Gulf-War and Glioblastoma Overview

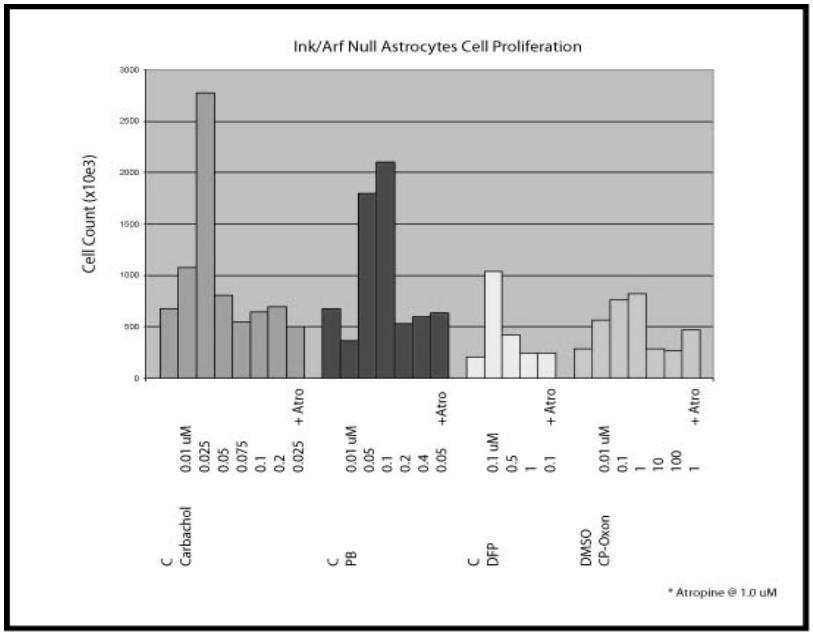


Schematic Project Overview

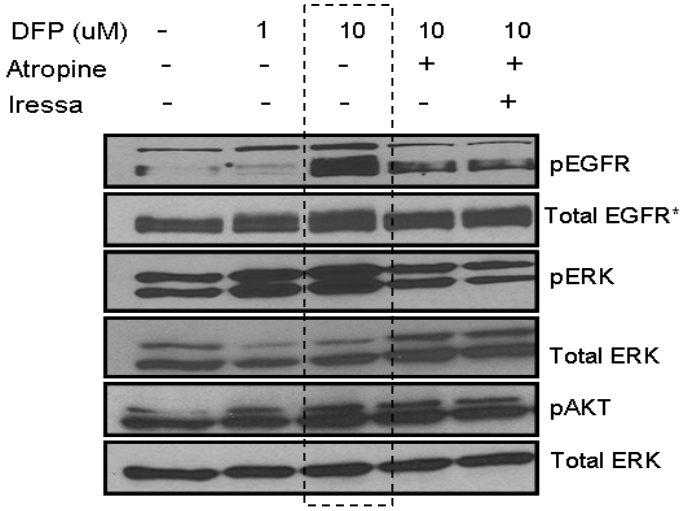
Acetylcholinesterase inhibitors (AChEi) used in this study:

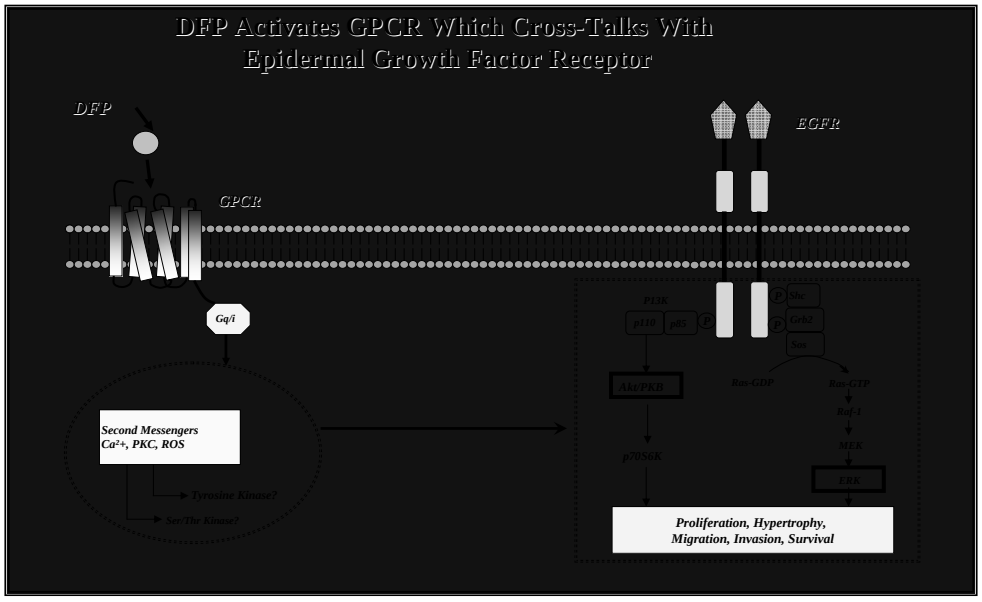
DFP (diisopropyl-fluro-phosphate, a structural analog of sarin

Pyridostigmine Bromide (PB)



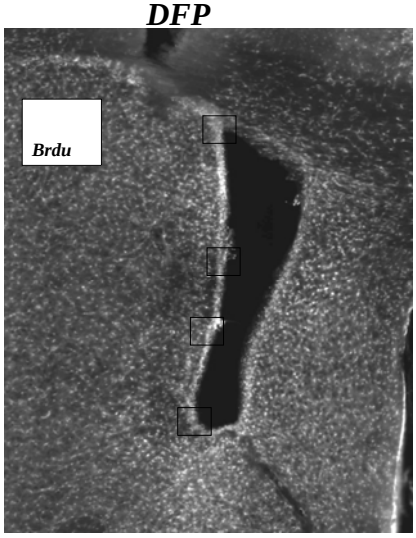
DFP Activates a Muscarinic Receptor Which Cross-Talks With Epidermal Growth Factor Receptor



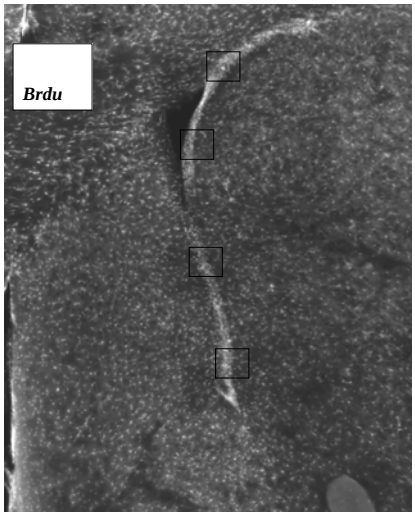


DFP Increases Proliferation of Neural Stem Cells In Vivo in the Adult Mouse Brain Subventricular Zone

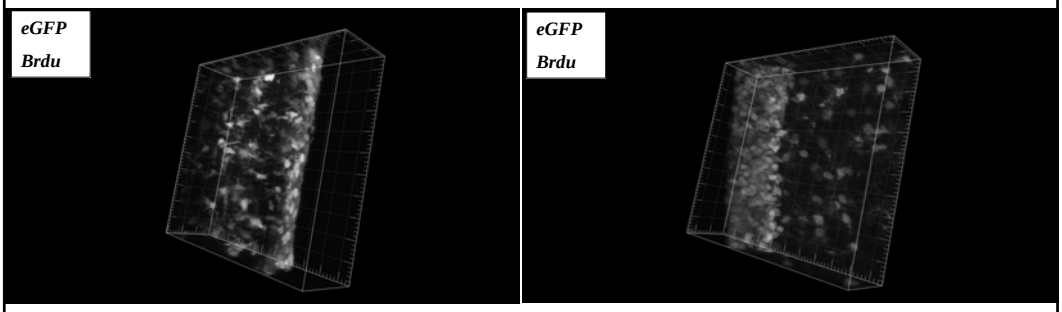
DFP



Control



DFP stimulates Neural Stem Cell Proliferation In the Adult Mouse Brain



DFP ***Control***

38 / 209 = 18.2 % ***VS*** ***4 / 185 = 2.1 %***

Conclusions

- Using primary cultures of astrocytes and neural progenitor cells derived from both mouse and humans—presumptive cell types that give rise to gliomas—DFP and PB can transactivate classic oncogenic signaling pathways by directly activating muscarinic receptors.
- In-vitro, DFP and PB exposure can induce proliferation of astrocytes.
- In-vivo, mice treated with DFP show a marked increase in proliferation of stem/progenitors cells in the subventricular zone and in the hippocampus as well as a diffuse astrogliosis extending to cortical, subcortical and white matter tracks.

Conclusions

- Using genetically defined in-vitro and in-vivo model systems, we have identified cooperating links between organophosphate exposure and mechanisms which could trigger brain tumors.
- Continuance of this work may lead to early detection and identification of potential targets for preventive or therapeutic intervention.

Preclinical Projects

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  - Development of Brain Cancer
- **Possible Mechanisms of these Effects:**
  - **Neuroinflammation**
  - Mitochondrial Damage
  - Intracellular Phosphorylation

Neuroinflammation and GWI Cytotoxic Signature

Bioluminescence Imaging
Day 2 to Week 12 post
repeated CP/PB exposure

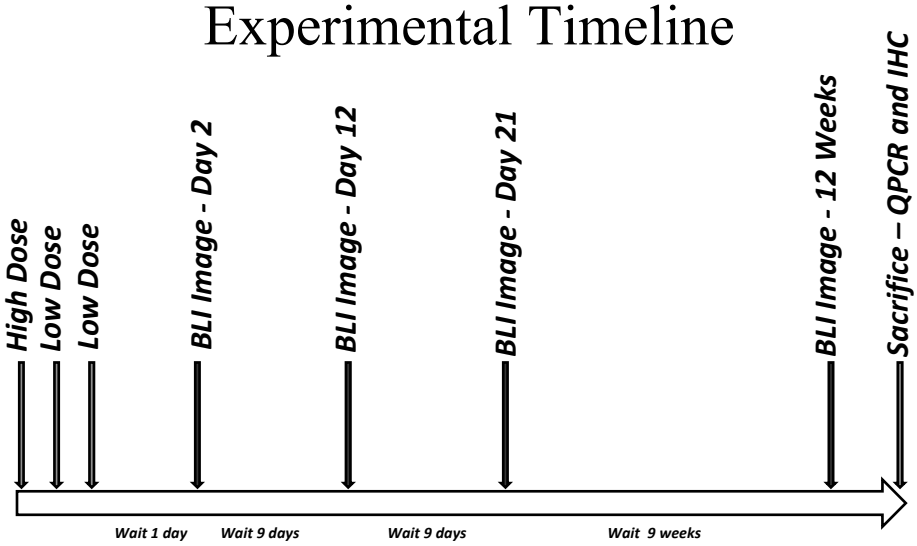
Malu Tansey, Ph.D.
Department of Physiology, UT Southwestern

- Objective: To investigate if repeated organophosphate exposure induces acute, chronic, delayed or sustained neuroinflammation in mice.
- Experimental Approach:
  - TGFβ (SBE)-Luciferase promoter in brain microglia and other immune cells will emit light in response to inflammatory stimuli
  - Use SBE-Luc reporter mice for longitudinal whole-body bioluminescent imaging (BLI) studies to identify optimal time-window for cellular and molecular analyses of brain tissue. Dissect out brain at peak response and localize inflammatory response (immunohistochemistry) and obtain gene expression signature for GWI neurototoxicity (real-time QPCR).

Experimental Design

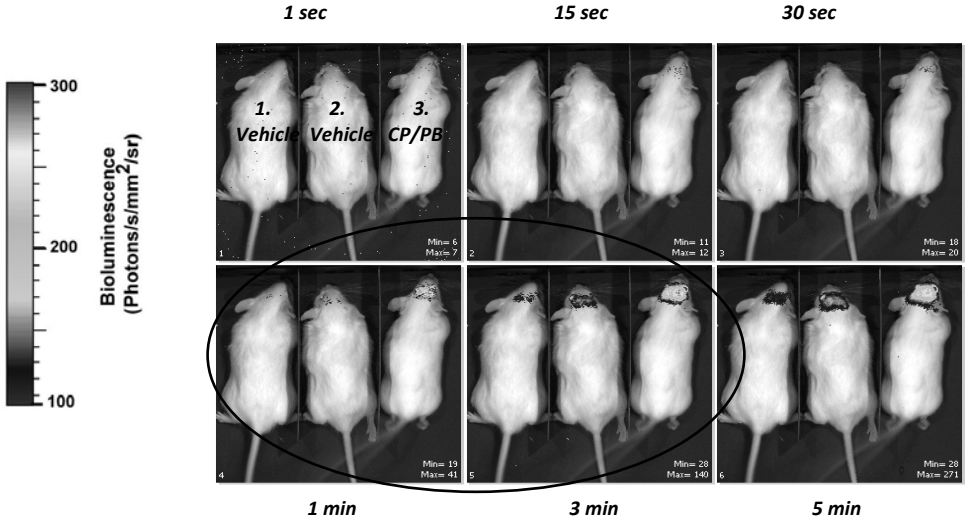
- All SBE-Luciferase mice were between 1-2 months old
- Dosing regimens
  - Vehicle (saline/DMSO) or
  - High dose (PB 2mg/kg s.c. and CP 5 mg/kg i.p) for 1 day then low dose (PB 1mg/kg and CP 5 mg/kg) for 2 days
- For Bioluminescent Imaging, animals were anesthetized with 0.1 ml ketamine cocktail. If a booster was needed, 0.05 ml of ketamine was used.
- Animals were weighed to determine amount of luciferin to be given subcutaneously
- 10 minutes prior to imaging, a 450mg/kg dose of 30mg/ml luciferin/saline mixture was injected (s.c.)
- After imaging, all mice were placed in a heating pad to recover and returned to their home cages.

Experimental Timeline



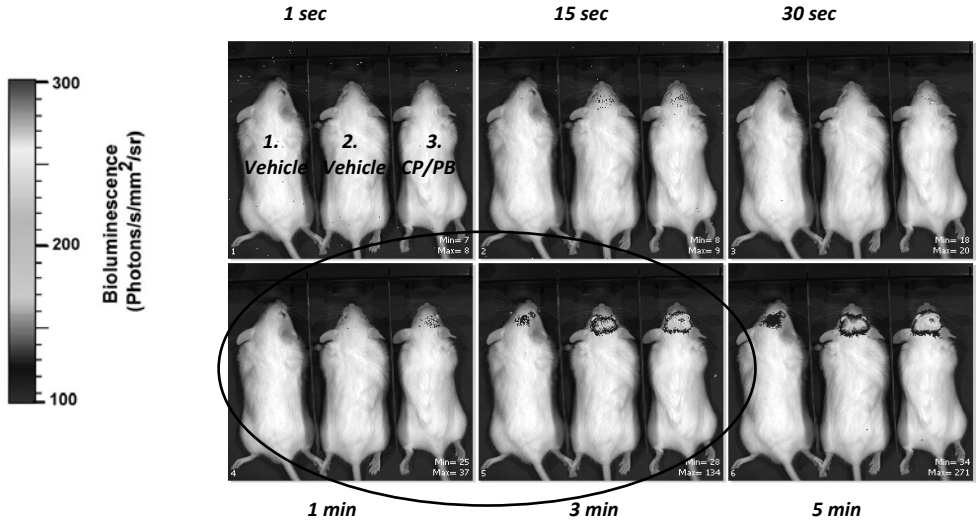
- *Dosing regimen:*
  - *Vehicle (saline/DMSO) or*
  - *High dose (PB 2mg/kg s.c. and CP 5 mg/kg i.p) for 1 day then low dose (PB 1 mg/kg and CP 5 mg/kg) for 2 days*

SBE-Luc Imaging at Day 2 - GWI Project



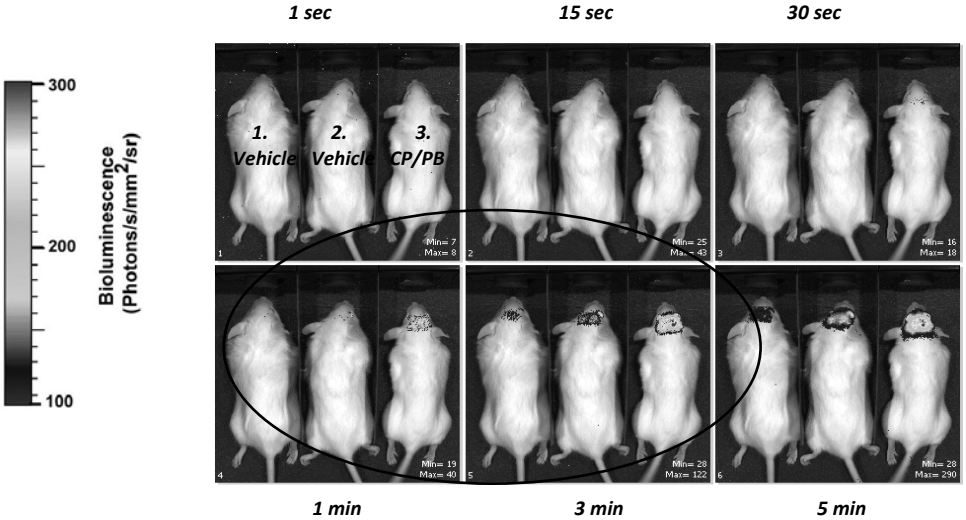
The top 3 panels are images taken at 1, 15 and 30 second exposures. The bottom 3 panels are images taken at 1, 3 and 5 minutes (max exposure). Increase in intensity (blue to red) indicates increased inflammation.

SBE-Luc Imaging at Day 12 - GWI Project



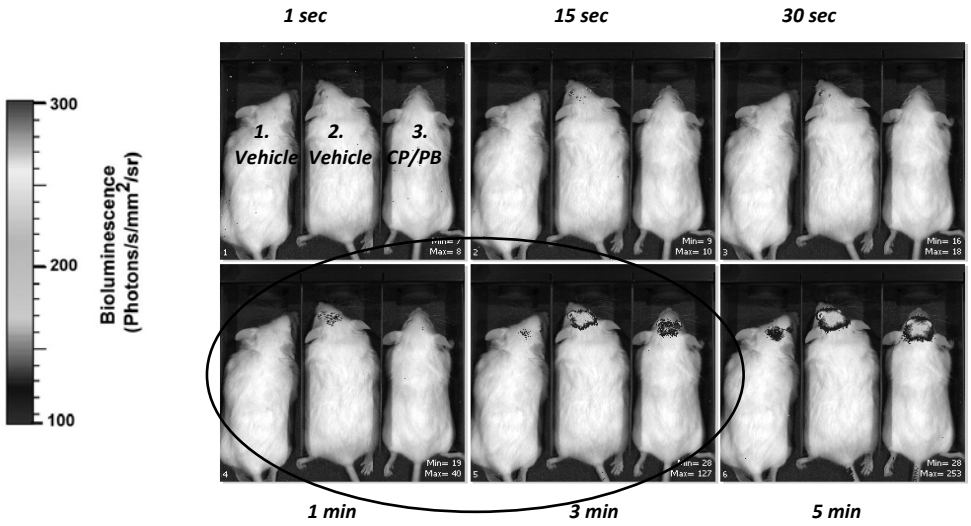
The top 3 panels are images taken at 1, 15 and 30 second exposures. The bottom 3 panels are images taken at 1, 3 and 5 minutes (max exposure). Increase in intensity (blue to red) indicates increased inflammation.

SBE-Luc Imaging at Day 21 - GWI Project



The top 3 panels are images taken at 1, 15 and 30 second exposures. The bottom 3 panels are images taken at 1, 3 and 5 minutes (max exposure). Increase in intensity (blue to red) indicates increased inflammation.

SBE-Luc Imaging at Week 12- GWI Project



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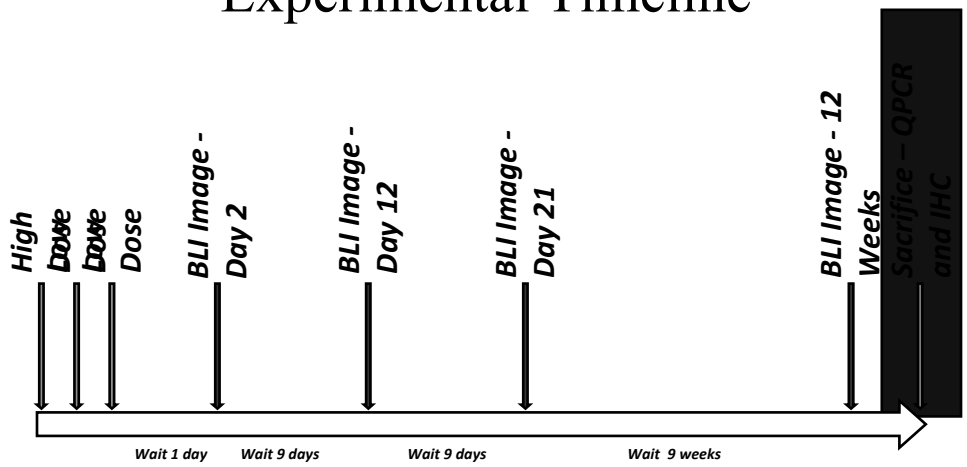
Experimental Timeline



Next Step: Study will be repeated with second cohort of mice to be sacrificed at Day 12 for molecular and cellular analyses of neuroinflammation

Preliminary Summary

- We observed a good deal of variability in the neuroinflammatory responses among the mice which may not be dissimilar to the variability of symptoms reported among vet populations who were exposed to GWI toxins.
- Based on the imaging data, the peak of neuroinflammation in the responder mice occurs between Day 12 and Day 21 post CP/PB exposure, making this the optimal time window for detailed cellular and molecular analyses of the neuroinflammation response (Subsequent Projects). Therefore, we are repeating this study to confirm these findings and to sacrifice mice at Day 12 for molecular and cellular analyses of neuroinflammation.
- The objectives of Subsequent Projects are to harvest brain tissue to obtain a GWI signature of inflammatory gene expression by QPCR and to establish the spatial pattern of microglia activation by fluorescence immuhistochemistry.

Preclinical Projects

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- Effects of Gulf War Chemicals on:
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  - The Autonomic Nervous System
  - Brain Dopamine Turnover
  - Development of Lou Gehrig's Disease (ALS)
  - Development of Brain Cancer
- **Possible Mechanisms of these Effects:**
  - Neuroinflammation
  - **Mitochondrial Damage**
  - Intracellular Phosphorylation

Do Gulf War Chemicals Damage Mitochondria?

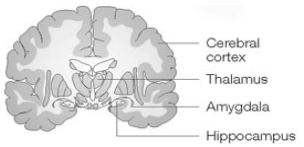
Jun Wu and Ilya Bezprozvanny
Department of Physiology, UT Southwestern

Hypothesis: GW chemicals damage neuronal mitochondria and make them susceptible to secondary stressors such as glutamate or aging.

Step 1: Develop a <u>cellular</u> model of Gulf War Illness (GWI)
Step 2: Test mitochondria damage by GWI agents
Step 3: Evaluate CoQ10 as GWI therapeutic agent

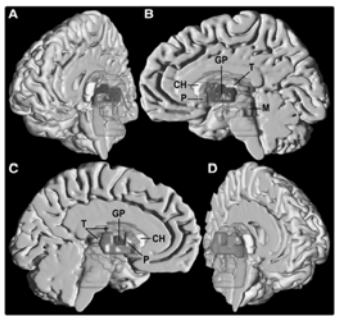
Neuronal Cell Cultures Exposed to GW Chemicals

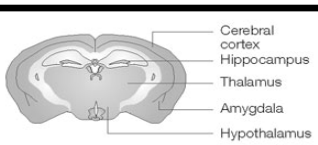
Human brain



***Nature* 2005**

1991 Gulf War Veterans





Mouse brain

Primary neuronal culture

Exposure to GWI agents (with/without other stress)

IsoCyte platform (high throughput scanning)

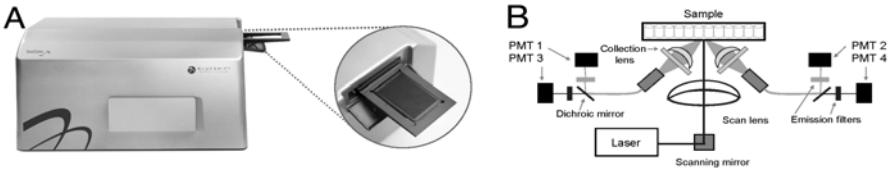
R.W. Haley et al., *Psychiatry Research: Neuroimaging* 2009

Gulf War Chemicals Tested Thus Far

GWI agent	Category	Solvent
Carbaryl (Sevin)	Carbamate pesticide	DMSO
Chlorpyrifos (Dursban) & Chlorpyrifos oxon	Organophosphorous pesticide	DMSO
DEET	Insect repellant	DMSO
DFP (in lieu of sarin)	Nerve agent surrogate	anhydrous isopropanol
Permethrin	Pyrethroid insecticide	DMSO
Pyridostigmine bromide	Carbamate medication	DMSO

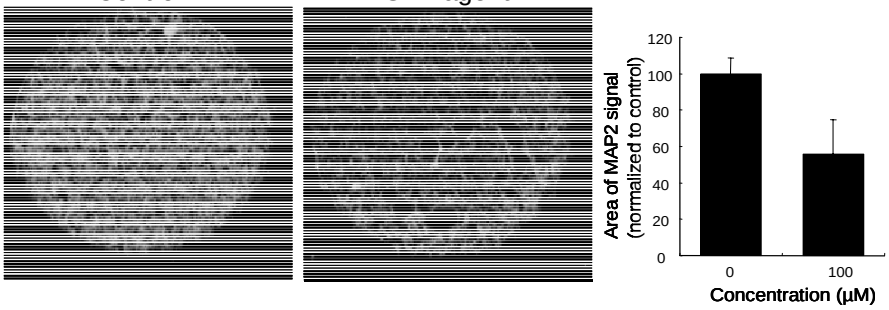
Quantitative Measurement of Neuronal Cell Death

IsoCyte laser scanner cytometer

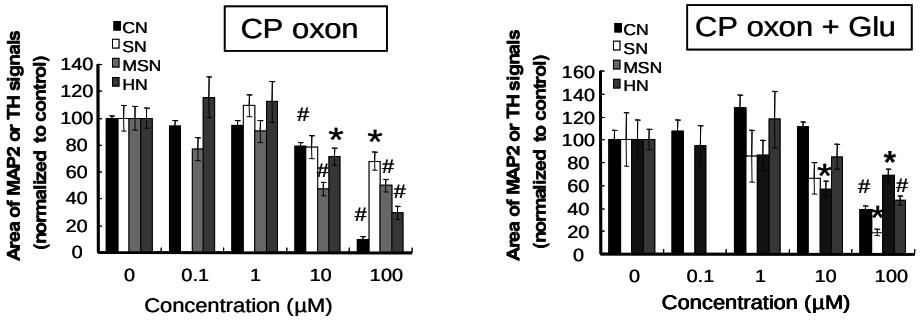


Quantification of cell death (MAP2 staining)

Control + GWI agent



Initial Results: Toxic Effects of GW Chemicals on Neurons



	GWI agents (μM)	*GWI agents (μM) + Glutamate*
Medium spiny neurons	CP oxon (10), DEET (100)	CP oxon (10), DEET (10), PB (100)
Hippocampal neurons	CP oxon (10)	Carbaryl (10), Permethrin (10)
Cortical neurons	Carbaryl (100), CP oxon (10)	Carbaryl (100), CP oxon (100)
Substantia nigra neurons	CP oxon (10)	Carbaryl (100), CP oxon (100)
Thalamic neurons	Carbaryl (100), CP oxon (100)	Carbaryl (100), CP oxon (10)

Provisional Conclusions

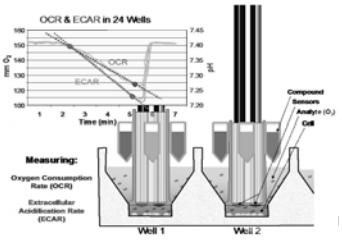
Toxic effects of Gulf War chemicals are exacerbated in the context of glutamate excitotoxicity such as:

- DEET and PB to Medium Spiny Neurons
- CP oxon to Thalamic Neurons
- Carbaryl and Permethrin to Hippocampal Neurons

These results suggest that the agents damage neuronal mitochondria so that excitotoxic stress of glutamate accelerates cell death.

Future Direction

Directly determine damage to mitochondria induced by exposure to GWI agents



Method:
Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) with Seahorse XF24 Extracellular Flux Analyzer to quantify mitochondrial function in neuronal cultures.

Evaluate potential neuroprotective agents such as mitochondrial supplement CoQ10 to treat or prevent GWI in the future. Use cellular model of GWI as readout.

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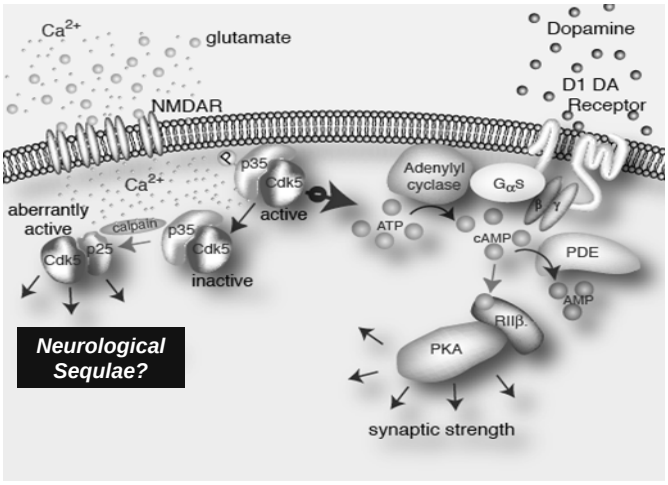
Altered Intracellular Phosphorylation by Exposure to Gulf War Chemicals

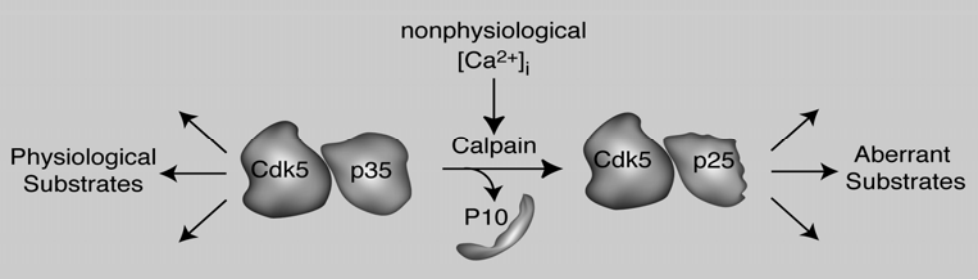
James A. Bibb, Ph.D.
Department of Psychiatry
The University of Texas Southwestern
Medical Center

Dysregulation of Cdk5 and hyper-activation of dopamine receptors in the limbic circuitry of the brain may contribute to the neurological symptoms of GWI



Further studies would investigate the mechanisms by which these neurotoxins induce dysregulation of these critical signaling pathways both during and after exposure and target them for the development of novel treatment strategies.

The neuronal protein kinase Cdk5 controls important functions in the brain, but, when dysregulated, causes neural injury and neurodegeneration



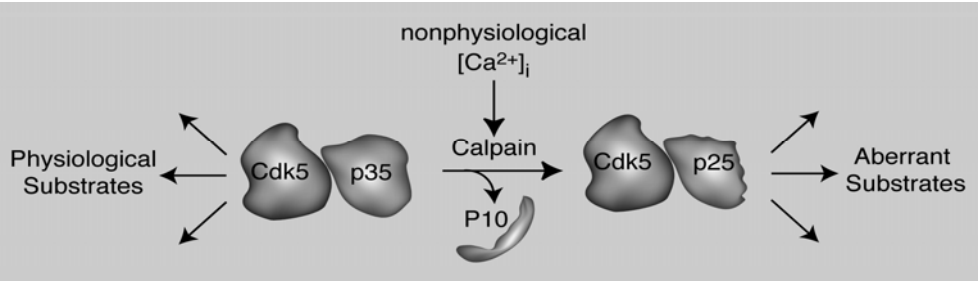
Dysregulation of Cdk5 occurs when its activating cofactor p35 is cleaved by calpain to produce p25.

The resulting Cdk5/p25 complex is neurotoxic and phosphorylates substrates that lead to neuronal death.

<u>Dysregulation of Cdk5 is associated with:</u>

Stroke and ischemic injury
Traumatic brain injury
Cerebral Palsy
Neurotoxicity
Alzheimer's Disease
Huntington's Disease
Parkinson's Disease

Question: Does exposure to Gulf War Chemicals cause an increase in the amount of p25? If so, it suggests that this mechanism may be involved in neurotoxic effects of these chemicals.



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Traumatic brain injury
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Neurotoxicity
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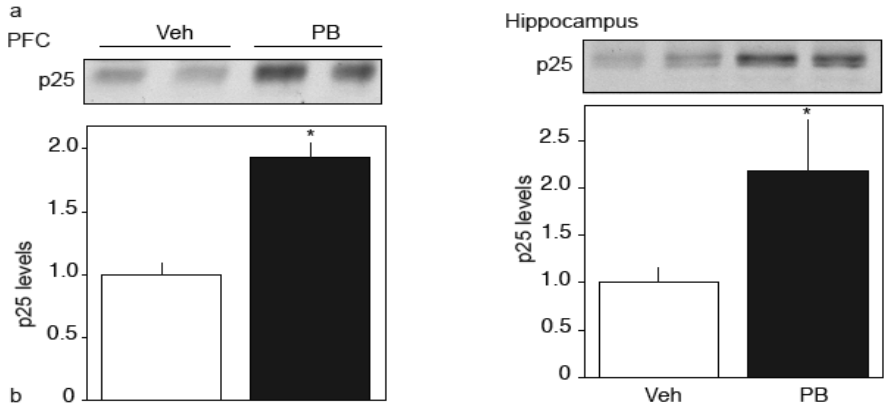
Parkinson's Disease

Aberrant Cdk5 (p25) is generated in mouse cortex and hippocampus by 10 daily doses of pyridostigmine bromide (1 mg/kg) followed by a 4 week delay period



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Principal Investigators of the Preclinical Projects

Steve Vernino, MD, PhD
Malu Tansey, PhD
Christopher Sinton, ph.D.
Craig Powell, MD, PhD
Amyn Habib, MD
Matthew Goldberg, PhD
Jeffrey Elliott, MD
Donald Cooper, PhD
James Bibb, PhD
Ilya Bezprozvanny, PhD
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