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13. ABSTRACT (Maximum 200 Words)

A multidisciplinary project is underway to study the influence of low-level exposure to chemicals to which military personnel had contact during the Persian Gulf War. The research group includes investigator with expertise in neuroscience, molecular biology, enzymology, cardiovascular physiology and neuropharmacology. The overall objectives is to study the effect of chemical exposure from the single cell/gene level to the whole animal and ending with the human condition. Results include but are not limited to: proteomic changes have been identified that are different between pyridostigmine bromide and physostigmine; spectral analysis has showed dramatic alterations in heart rate variability and baroreflex index in mice challenged with stress and/or pyridostigmine; sarin and noise stress and also pyridostigmine bromide and DEET with noise stress induce significant decreases in electron transfer rates compared to noise stress alone, factors have been identified which will increase the reliability of correlations between enzymatic analyses and abnormal or disease states; modest gene expression changes have been determined using Mann- Whitney tests after eliminating those genes with expression levels that appear to represent inactive genes.

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Table of Contents

Cover..... 1

SF 298..... 2

Table of Contents..... 3

Introduction..... 4

Body.....4

Key Research Accomplishments.....115

Reportable Outcomes..... 118

Conclusions.....120

References.....120

Appendices..... 121

INTRODUCTION

A multidisciplinary project is underway to study the influence of low-level exposure to chemicals to which military personnel had contact during the Persian Gulf War. The research group includes investigators with expertise in neuroscience, molecular biology, enzymology, cardiovascular physiology and neuropharmacology. The focus is on testing the effect of low doses of pyridostigmine bromide (PB), DEET, and Sarin with investigations of 1) effect of combined chemical and stress exposure on behavioral, cardiovascular, endocrine and cholinergic function, 2) effect of stress and chemical exposure on auditory brain responses using electrophysiology, energy metabolism using nuclear magnetic resonance (NMR) techniques and mitochondrial function, 3) activity of aldehyde and alcohol dehydrogenases and esterases in human samples with the goal of establishing whether there are alterations in populations characterized as chemically sensitive, and 4) gene expression using a DNA microarray system to test the effect of chemical exposure on a neuronal cell line. The overall objective is to study the effect of chemical exposure from the single cell/gene level to the whole animal and ending with the human condition.

BODY

The details of the research accomplishments will be organized according to the research programs as described in the introduction.

Project 1. The effect of chemical and stress exposure on behavioral, cardiovascular, endocrine and central nervous system function.

Project 2. The effect of stress and chemical exposure on auditory brain responses, energy metabolism and tissue chemical constituents in an animal model.

Project 3. The study of enzymes involved in chemical metabolism, activity of dehydrogenases and esterase in human tissues with the goal of establishing whether there are alterations in populations characterized as chemically sensitive.

Project 4. The study of gene expression using a DNA microarray system to test the effect of chemical on a neuronal cell line.

Considerable progress was made during the third year of the project in terms of research productivity, presentations at national meetings, publications and awards. The program has coalesced into a functional research unit with development of specific program as described above as well as establishment of state-of - the -art facilities for proteomics and genomic analysis, animal physiology (behavior, cardiovascular and endocrine) and biochemical enzymology. It should be noted that the sarin facility was approved for use in the summer of 2003. Thus, we have had one year for the conduct of experiments. It is for this reason that we applied for a no-cost extension in order to finish the work.

Project 1. The effect of chemical and stress exposure on behavioral, cardiovascular, endocrine and central nervous system function.

The investigators are Morris, Lucot, Cool, Grubbs and McDougal. The research personnel are Bernatova, Farah, Dubovicky, Paton, Mach, Mauck, Price, Ropp, Boyd, and Joaquim. There is a general collaborative arrangement between the investigators with the lead person for each of the groups as follows: Behavior (Lucot), Cardiovascular/Endocrine (Morris), Cholinergic Systems (Grubbs), Proteomics and Confocal Microscopy (Cool), Genomics (McDougal).

Experimental Model: The research aims in project 1 focus on stress/chemical interactions, as such, the investigations use the same animal model. This model combines chronic shaker stress with chemical exposure. The group collaborates on the experimental testing with the specific protocols designed for the needs of the individual investigators. The stress model was developed and tested in year 1 (Bernatova et al. and Dubovicky et al.). The basic design uses C57Bl adult male mice exposed to intermittent shaker stress for a 7 day period. The animals are housed in a specialized caging system that is attached to the shaking platform. The timing of the shaking (150 cycles/min with a horizontal excursion of 2.86 cm) is regulated by a computer. The animals are exposed to 45, 2 min shaking sessions/24 hr with an average inter-stress interval of 30 min. The test compounds (saline or pyridostigmine bromide—PB) were administered by osmotic minipump implanted sc under the back of the mouse.

Behavioral Systems
Investigators Jim Lucot, Ph.D., Michal Dubovicky, Ph.D., and Mojmir Mach, M.S.

KEY ACCOMPLISHMENTS:

The major accomplishments were related to studies conducted using sarin. Studies 1) compared the behavioral effects of sarin, physostigmine and pyridostigmine, all inhibitors of AChE 2) determined the combined effect of sarin and stress exposure and 3) determined the behavioral responses to stress in mice lacking the oxytocin gene (model of altered stress reactivity).

1. In contrast to the acute increase in acoustic startle seen with PB and the decrease seen with physostigmine, sarin produced few acute effects despite a larger percent inhibition of AChE than the other two drugs. Further, the onset was a main effect encompassing time points after the initial administration, suggesting a delayed effect.

2. When combined with stress, sarin produced a delayed onset of self-mutilation in several animals. Work is in progress to replicate this finding that could have significant clinical implications (i.e, etiology of Gulf War Syndrome).

3. Results using the oxytocin knock out mice showed that there were alterations in the behavioral response to acute stress. There was a sex specific reduction in locomotor activity after acute shaker stress.

4. Abstracts have been presented at national meetings, papers have been published or are in preparation. M. Mach was recognized for his work at the Experimental Biology. He was asked to present his poster for consideration for an award.
5. Hosted Dr. Servatious and one of his collaborators in a seminar and mini symposium to compare data and to discuss organophosphates, behavior, chemical responses and how these relate to the Gulf War Syndrome.

Publications:
1. Bernatova, I., M. Dubovicky, M. Key, J.B. Lucot, M. Morris. Chronic stress alters cardiovascular and endocrine responses in mice. FASEB J., A 506, 409.1, 2002.

2. Lucot, J.B., M. Dubovicky, J. Wells. Effects of pyridostigmine and chronic shaker stress on acoustic startle response , pre-pulse inhibition and open field behavior of mice. 291.7 2002 Abstracts Viewer/Itinerary Planner, Washington, D.C., Society of Neuroscience, 2002 CD ROM.

3. Mach, M, Morris, M., Lucot, J.B., Behavioral changes after acetylcholinesterase inhibition in C57Bl/6J mice. FASEB. 390.3, 2003.

4. Grubbs, R.D., W.A. Price, B.S. Mauck, M. Mach, J.B. Lucot, S.J. Paton and M. Morris. Effects of exposure to sarin, pyridostigmine bromide and stress on cholinesterase activity in mouse blood and brain. Tox and Risk Assessment Conf. 2003.

5. Dubovicky, M., J.R. Wells, M. Morris, J.B. Lucot. Chronic shaker stress alters day/night behavioral patterns in mice. *Pharmacology Biochemistry and Behavior*, accepted, 2003.

PHYSOSTIGMINE STUDIES

Effect of physostigmine on spatial learning in the Morris water maze

Animals

Male mice C57Bl/6J (25-30 g, n=20)

Water maze protocol

Physostigmine was selected as a positive control for planned experiments with sarin. While it crosses the blood brain barrier like sarin, its non-achei effects are different and would therefore serve to differentiate achei effects of sarin on behavior from non-achei effects. The initial dose was selected based on Symons et al (1988), who reported that this dose improved learning in C57Bl/10J mice. Male C57Bl/6J mice (HARLAN, Chicago, IL; age 2 month) were singly housed in plastic cages with wooden shavings bedding in a temperature controlled room (T=70°F) with 12:12-h light: dark cycle (lights off from 1700). Standard pellet diet and tap water were provided *ad libitum*.

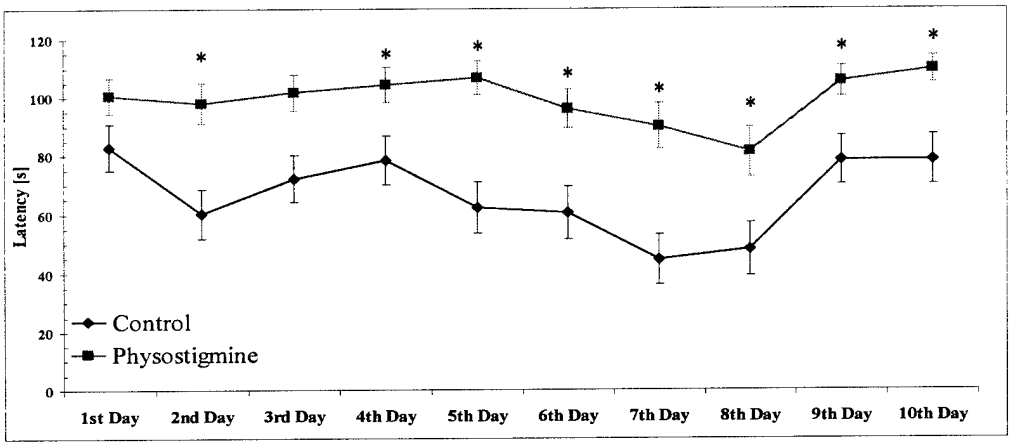
Mice were acclimated to the facility and then handled 3 min per day. On Fri, they were placed in water at 75^0 F colored with white tempera paint and observed for 60 sec to verify that every mouse was able to swim.

On Mon through Fri of the following week, they were injected IP with either saline or 0.1 mg/kg physostigmine 30 min before testing (first through fifth day). Testing consisted of placing the mouse in one of the quadrants facing the wall and permitting it to swim for 2 min. If they failed to find the platform, they were gently guided to the platform and stayed there for 20 sec before the next trial. If they found the platform, they were permitted to remain for 20 sec before the next trial. All mice got four consecutive trials, each randomly started from a different quadrant. The water was cleaned by a scoop, the mouse patted dry and placed in a home cage resting on a heating pad until they were totally dry and then returned to the colony. The following Monday to Wednesday, the platform was moved to NW (sixth through eight day), 1 cm above the water surface and the mice tested using the same protocol like first week (ninth through tenth day). On Thursday and Friday, the platform was hidden in the NW quadrant and testing was conducted.

Results

Administration of physostigmine increased latencies to locate the hidden and the visible platform during testing ($F(1,716)=4.02$, $p<0.00005$) (Fig. 1-1). We found an influence of drug administration on swimming speed ($F(1,712)=124.50$, $p<0.00001$ (Table 1-1), which was probably caused by decreased locomotor activity after administration (Fig 1-3). Circle zone analysis of the water maze revealed a preference for the peripheral circle rather then the center circle ($F(1,168)=26.09$, $p<0.000001$ (Table 1-2), which we confirmed using zone analysis in open field testing.

Fig. 1-1. Latency time of mice in water maze. Days 1-5 and 9-10 were with saline or physostigmine (.1 mg/kg, ip) and measured acquisition of spatial learning. Days 6-8 measured cued learning.



* $p < 0.05$ – significant different compared to control

Table 1-1. Average speed of mice in water maze [cm/s]

Day of testing	Control	Physostigmine (.1 mg/kg, ip)
1st Day	17.31±2.10	8.48±0.99*
2nd Day	15.02±1.38	10.17±3.72
3rd Day	12.47±1.03	6.14±1.24*
4th Day	10.16±1.39	4.44±0.68*
5th Day	13.39±2.51	3.31±0.48*
6th Day	13.76±1.98	3.16±0.42*
7th Day	12.78±1.28	4.20±0.85*
8th Day	14.06±1.89	4.89±0.92*
9th Day	9.74±1.50	4.32±0.62*
10th Day	11.16±1.66	3.41±0.55*

* $p < 0.05$ – significant different compared to control

Table 1-2. Zone analysis of the water maze.

Average % time spent in the ring during days of injection.

Group	Center Ring	Periphery Ring
Control	24.5±1.6	75.5±1.6
Physostigmine (.1 mg/kg, ip)	9.4±1.3*	90.6±1.4*

* $p < 0.05$ – significant different compared to control

Water maze 2nd Experiment: Dose 0.03 mg/kg of physostigmine

Water maze protocol

As the dose of 0.1 mg/kg produced only impairments in performance and speed, a lower dose was tested. On Mon through Fri mice were injected IP with either saline or 0.03 mg/kg physostigmine 30 min before testing. Only the spatial test was conducted.

Results

The dose of 0.03 mg/kg physostigmine did not improve spatial learning and there were no significant differences in latency time (Fig. 1-2). After two days of testing we needed to eliminate two mice from physostigmine group due to bad health status. The average speed in both groups was equal. However, the time spent in the peripheral circle was greater and in the central circle was less ($F(1,294)=9.28$, $p<0.0025$), which became significant on the second day of the testing ($p<0.0101$).

Fig. 1-2. Latency time of mice in water maze (Physostigmine=.03 mg/kg, ip)

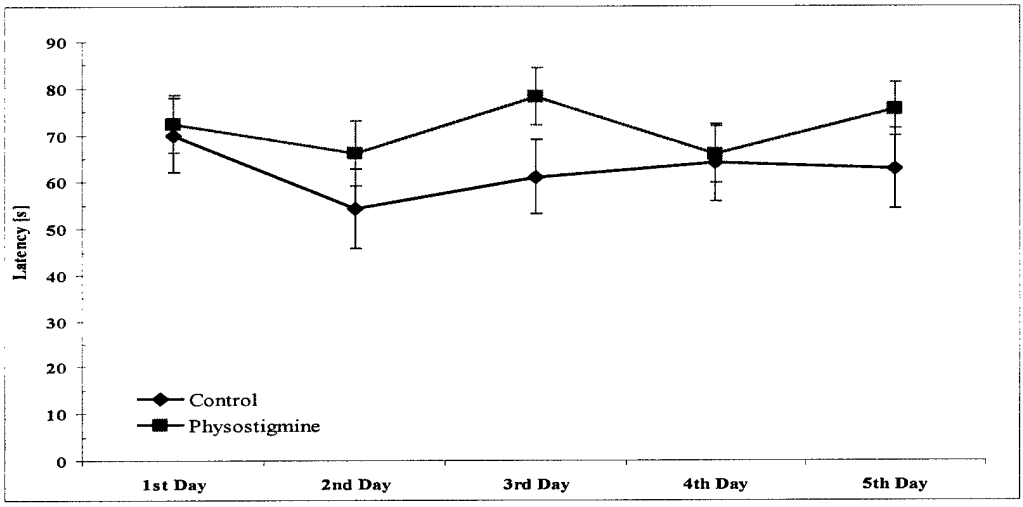


Table 1-3. Average speed of mice in water maze [cm/s]

Day of testing	Control	Physostigmine (.03 mg/kg, ip)
1st Day	8.91±1.06	10.29±1.66
2nd Day	9.75±1.30	8.54±1.36
3rd Day	7.99±1.40	4.55±0.87
4th Day	7.73±1.30	9.51±3.43
5th Day	7.69±1.31	7.21±3.80

Table 1-4. Zone analysis of the water maze

Average % time spent in the ring over the 5 days.

Group	Center Ring*	Periphery Ring*
Control	17.35±2.01	82.65±2.02
Physostigmine	9.35±1.50*	90.65±1.50*

- $p < 0.05$ – significant different compared to control.

The effects of physostigmine on open field behavior.

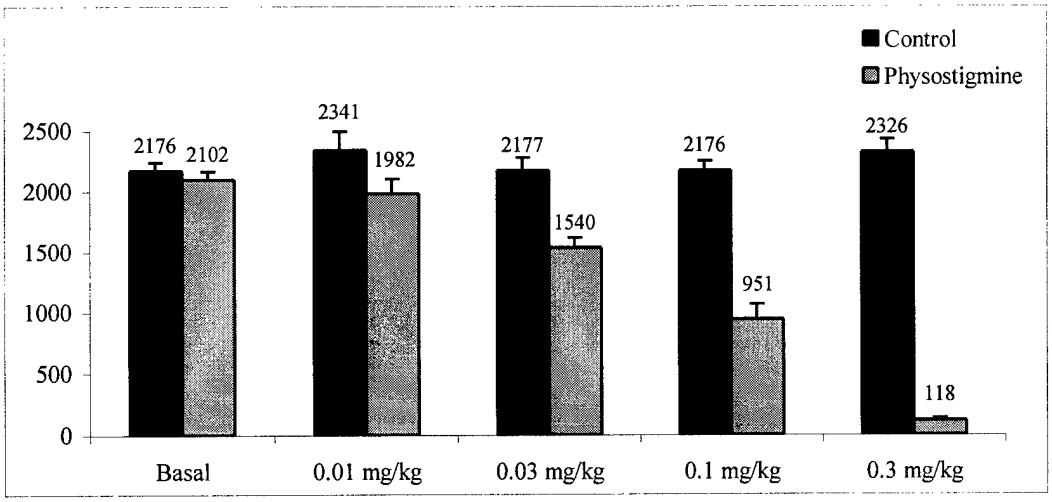
Open field protocol

Physostigmine was administered i.p. 30 min before testing of the doses of doses 0.01, 0.03, 0.1, 0.3 mg/kg. The mouse was placed in the center of the open field arena and the following variables of motor activity were recorded: locomotor activity, fine movement and rearing. Moreover, distance traveled, total time, rest time, number of entries, head pokes and total time spent in individual zones was recorded. Session time was 15 minutes.

Results

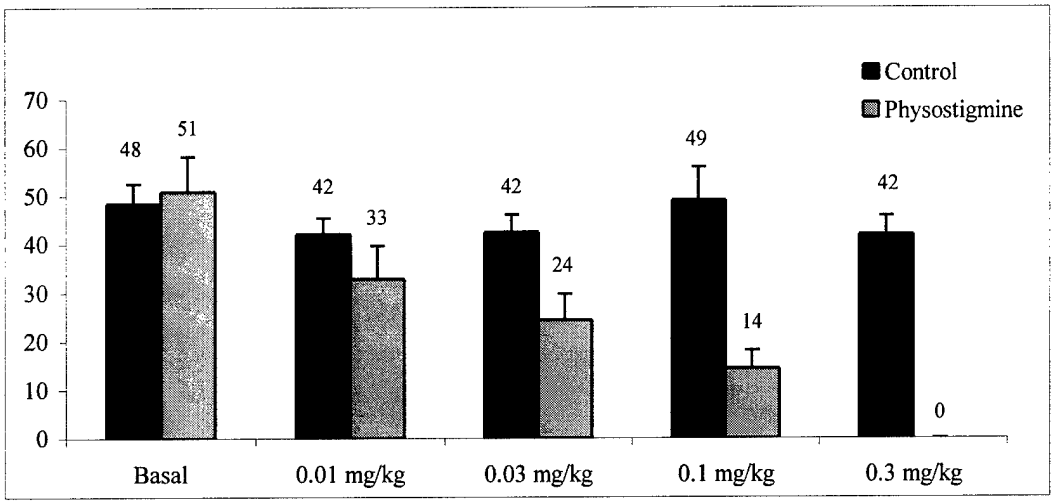
Administration of physostigmine affected motor activity tested in the open-field in a dose-dependent fashion ($F(3,59)=29.84$, $p<0.00001$), with notable impairment in activity at 0.1 mg/kg ($f_{basicmovement}(1,59)=228.03$, $p<0.00001$; $f_{rearing}(1,59)=63.21$, $p<0.00001$) (Fig. 1-3, 1-4). The results also showed that animals at 0.1 mg/kg and higher spent more total time in the peripheral zone rather then in the central zone ($F(1,59)=12.21$, $p<0.0009$) (Table 1-5).

Fig. 1-3. Effect of physostigmine on locomotor activity – Basic movements



* $p < 0.05$ – significant different compared to control

Fig. 1-4. Effect of physostigmine on locomotor activity – Rearing



* $p < 0.05$ – significant different compared to control

Table 1- 5. Zone analysis of the open field (Seconds spent in the ring)

Dose	Peripheral Zone		Central Zone	
	Control	Physostigmine	Control	Physostigmine
Basal	429.73±30.52	474.31±32.16	470.27±30.52	425.69±32.16
0.01 mg/kg	574.72±12.13	658.93±11.79	325.26±34.32	241.07±31.20
0.03 mg/kg	623.17±13.10	672.34±9.42	276.83±37.05	227.66±26.64
0.1 mg/kg	523.23±9.75	714.71±15.87*	376.77±29.25	185.27±47.61*
0.3 mg/kg	613.04±11.74	769.54±31.07*	286.96±35.21	130.42±93.23*

* $p < 0.05$ – significant different compared to control

Effects of physostigmine on acoustic startle response and pre-pulse inhibition

Acoustic startle response and pre-pulse inhibition protocol

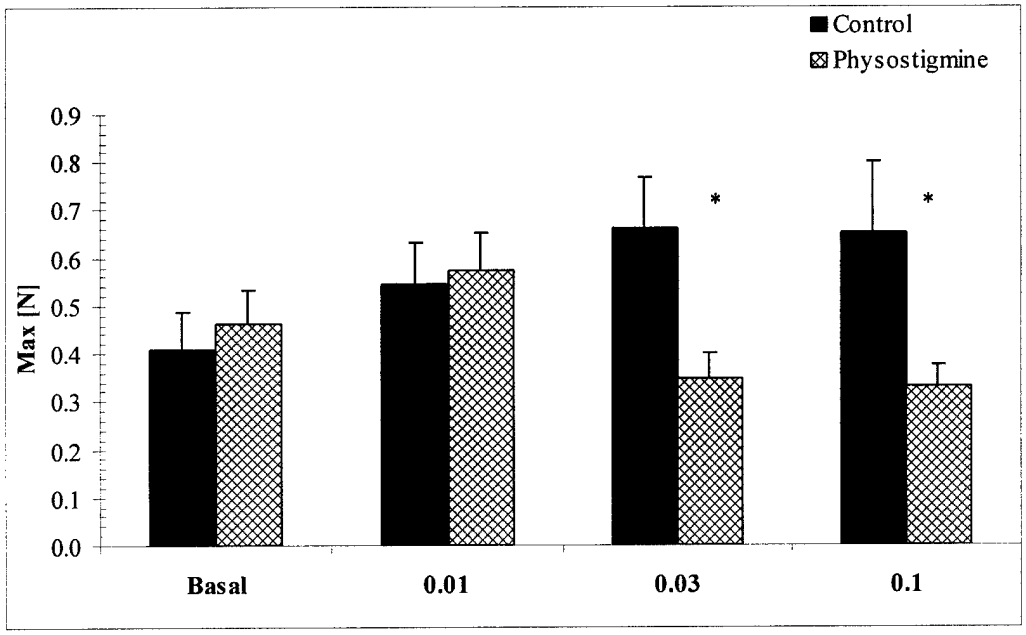
Mice were tested in the SM100 Startle Monitor System Version 4.0 (Hamilton Kinder, 2001, Poway, CA) for acoustic startle response (ASR) and pre-pulse inhibition (PPI). The system was programmed for 6 types of white-noise burst stimulus trials: no stimulus (background, 60 db), pre-pulse (70 db), pulse (100 db and 120 db), pre-pulse plus pulse (70 db+100db and 70 db+120 db). Each trial type was presented 10 times in 10 blocks. Stimuli were presented in random order to avoid order effects and habituation. The inter-trial interval varied from 9 to 16 sec. All animals were regularly handled before individual tests in order to minimize handling-related stress. Mice

were pair matched according to baseline values into the experimental groups using the average of the response to 100 db and 120 db. Tested mice were loosely restrained in holders that were placed on a sensing plate transforming movements of the body (jerks) into an analog signal through an interface. Finally, percentage prepulse inhibition measures were calculated as the difference between the pulse alone and the prepulse + pulse trials, divided by the pulse alone and multiplied by 100. Percentage scores are typically used to minimize the effect of individual variation of startle amplitude on prepulse inhibition. Behavioral tests were conducted 30 min after intraperitoneal administration of PHY of doses 0.0, 0.01, 0.03, 0.1 mg/kg.

Results

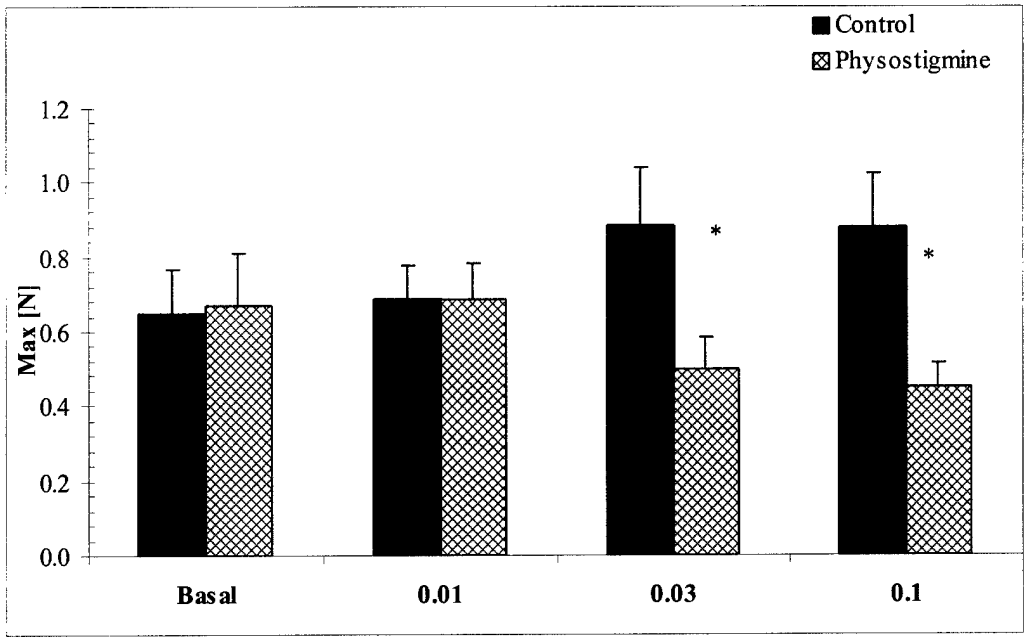
We found that ASR to the 120 db stimulus was significantly decreased at 0.03 [$F(1,13)=5.29$, $p<0.039$] mg/kg and 0.1 mg/kg [$F(1,13)=8.12$, $p<0.014$], and ASR to the 100 db stimulus was also decreased significantly at 0.03 mg/kg [$F(1,13)=7.17$, $p<0.019$] and 0.1 mg/kg [$F(1,13)=4.75$, $p<0.048$] (Figure 1-5, 1-6). The dose 0.01 mg/kg was ineffective. However there were no significant effects on the PPI (data not shown).

Fig. 1-5 - Acoustic Startle Response – 100 db stimulus



* $p < 0.05$ – significant different compared to control

Fig. 1-6 - Acoustic Startle Response – 120 db stimulus



* $p < 0.05$ – significant different compared to control

Conclusion

The water maze data initially appeared to indicate that the dose of 0.03 mg/kg of physostigmine had no effect on spatial learning while the dose of 0.1 mg/kg impaired acquisition and performance of the task, accompanied by a reduced swimming time and enhanced thigmotaxis. Addition of the locomotor activity dose-response data confirm that the dose of 0.1 mg/kg reduces activity and enhances thigmotaxis, two factors which could interfere with finding the platform independent of any effect on learning. The absence of significant effects on swim speed, locomotor activity or thigmotaxis at the dose of 0.03 mg/kg, coupled with no effect on acquisition suggests that physostigmine had no ability to enhance learning on the measure. The robust improvement in learning reported by Symons et al, (1988) was observed in C57BL10J mice. The inability of physostigmine to improve learning in the present study could be due to the use of C57BL6J mice. Physostigmine at the dose 0.03 and 0.1 significantly decreased acoustic startle response. However PPI did not revealed any changes. Even though rodents are sensitive to the acoustic startle response test there are different cholinergic influences on the startle response in different rodent species. In guinea pigs physostigmine enhanced startle response with the maximal effective dose of PHY 0.3 mg/kg (Philippens et al., 1997). Jones and Shannon (2000) studied cholinesterase inhibitors in rats and observed no differences in either the startle response or PPI of 0.01 to 0.1 and 0.3 to 10 mg/kg of PHY, administered subcutaneously. These findings only confirm the finding of Davis (1980) that the role of cholinergic systems in modulating the acoustic startle response is unclear. Possible explanations for differences between guinea pigs, rats and mice are different body weight or direct or indirect interaction of the PHY with other neurotransmitter systems. Other possible explanations could be a different action of the enzyme AChE. There is evidence that guinea pigs are quite different in the detoxification of organophosphorus compounds.

SARIN STUDIES

On February 15, 2002, we received notification that the surety facility was approved on 12/13/01 and received the first shipment on May 15, 2002. However, delays in obtaining the bubblers required for evaluating the effectiveness of the first procedure required postponement of the first experiment until Aug of 2002.

Sarin 0.4 LD_{50} SC (64 µg/kg)

Effects of sarin on locomotor activity

The effects on motor activity of pyridostigmine, stress and their combination was determined in previous experiments. The effects of sarin alone on motor activity was determined to serve as part of the basis for future investigations of the effects of combinations of treatments, such as sarin plus stress, sarin plus pyridostigmine and that combination with stress.

Animals

Male mice C57Bl/6J (25-27 g, n = 20)

Protocols

Mice were injected on 3 consecutive days subcutaneously (SC) with sarin (0.4 $LD_{50;}$ 64 µg/kg). The control group was injected SC with saline. On the first day and at three weeks after administration, blood was collected from a tail cut and analysis of acetylcholinesterase and butyrylcholinesterase activity was performed by Dr. Grubbs lab. Behavioral testing was performed on the 1^{st} day of administration and on the 1^{st}, 7^{th}, 14^{th}, 21^{st} day and 8 weeks after administration. Testing consisted of 15 minutes sessions in the open field.

Results

The three injections produced no gross effects on gait, posture or tearing on any day. Despite being asymptomatic, analysis of the open field data revealed a main effect of treatment on basic movements, rearing and the total time spent in the periphery ($F_{BM}(1,108)=6.083$, $p<0.015$; $f_{rear}(1,108)=3.938$, $p<0.05$; $f_{periohery}(1,108)=4.539$, $p<0.035$). The mice from the sarin group exhibited decreased activity and spent more time in the periphery (Figures 1-10 – 1-11). One day after the last dose, red blood cell AChE activity was inhibited by 73% and remained 64% inhibited at 3 weeks (see Grubbs). After 8 weeks from last sarin administration, the behavior went to normal values and the sarin group was not different from the control.

Fig. 1-10. Open field – Basic Movements

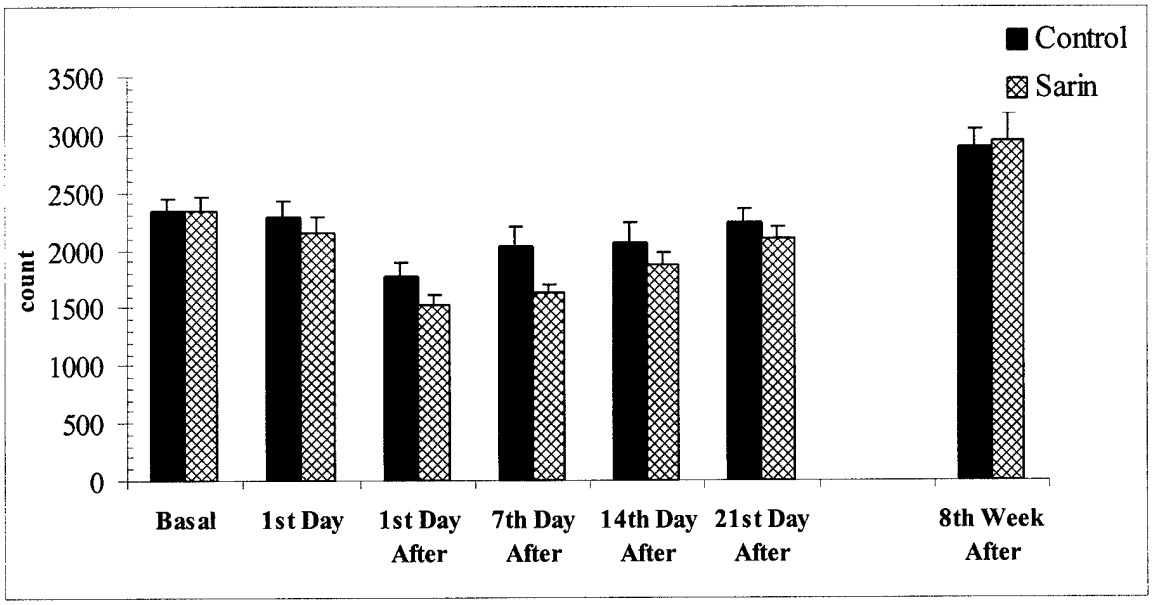
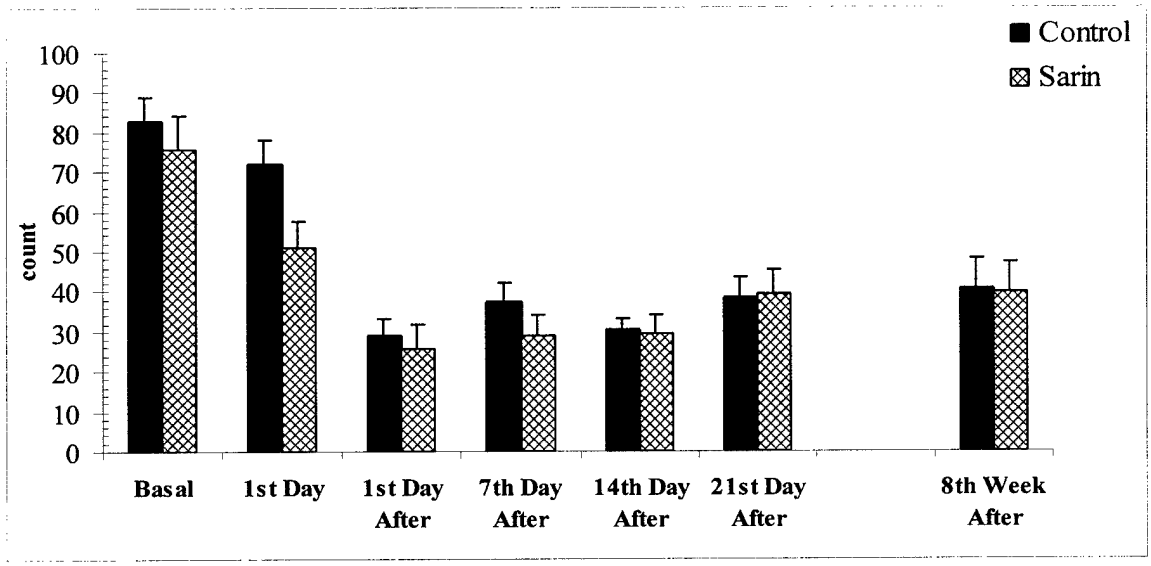


Fig. 1-11. Open field – Rearing



Effects of sarin on acoustic startle response

Animals

Male mice C57Bl/6J (25-27 g, n = 20)

Acoustic startle response and pre-pulse inhibition protocol

Mice were tested in the SM100 Startle Monitor System Version 4.0 (Hamilton Kinder, 2001, Poway, CA) for acoustic startle response (ASR) and pre-pulse inhibition (PPI). The system was set up for 6 types of white-noise burst stimulus trials: no stimulus (background, 60 db), pre-pulse (70 db), pulse (100 db and 120 db), pre-pulse plus pulse (70 db+100db and 70 db+120 db). Each trial type was presented 10 times in 10 blocks. Stimuli were presented in random order to avoid order effects and habituation. The inter-trial interval varied from 9 to 16 sec. All animals were regularly handled before individual tests in order to minimize handling-related stress. Tested mice were loosely restrained in holders that were placed on a sensing plate transforming movements of the body (jerks) into an analog signal through an interface. The behavioral tests were provided on 1st day after administration and then every week once until 49th day.

Results

Repeated ANOVA did not revealed any effect on the response to startle stimuli of 100db and 120db or 100 db PPI. However, there were slight changes in 120 db PPI that did not become significant after repeated ANOVA measures (Fig. 1-12-1-15). This slight inhibition of PPI in the sarin group could be result of a delayed effect of exposure to sarin, in the next experiment we will combine exposure to sarin and chronic stress, what we suggest to be a model of battlefield and exposure to chemical warfare agents.

Fig. 1-12. Acoustic Startle Response – 100 db stimulus

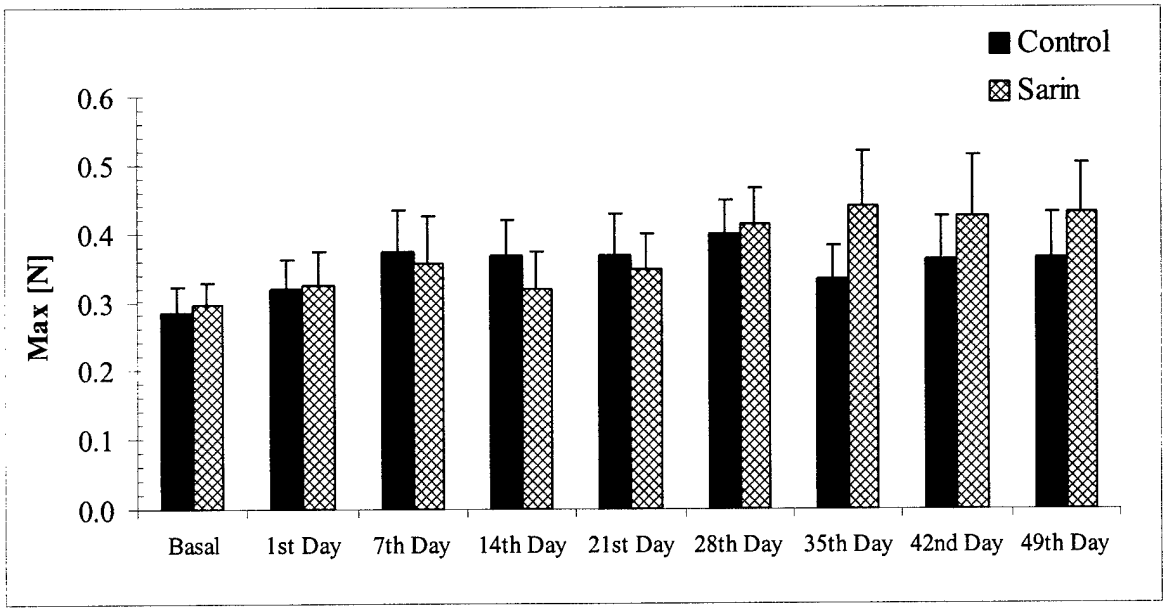


Fig. 1-13. Acoustic Startle Response – 120 db stimulus

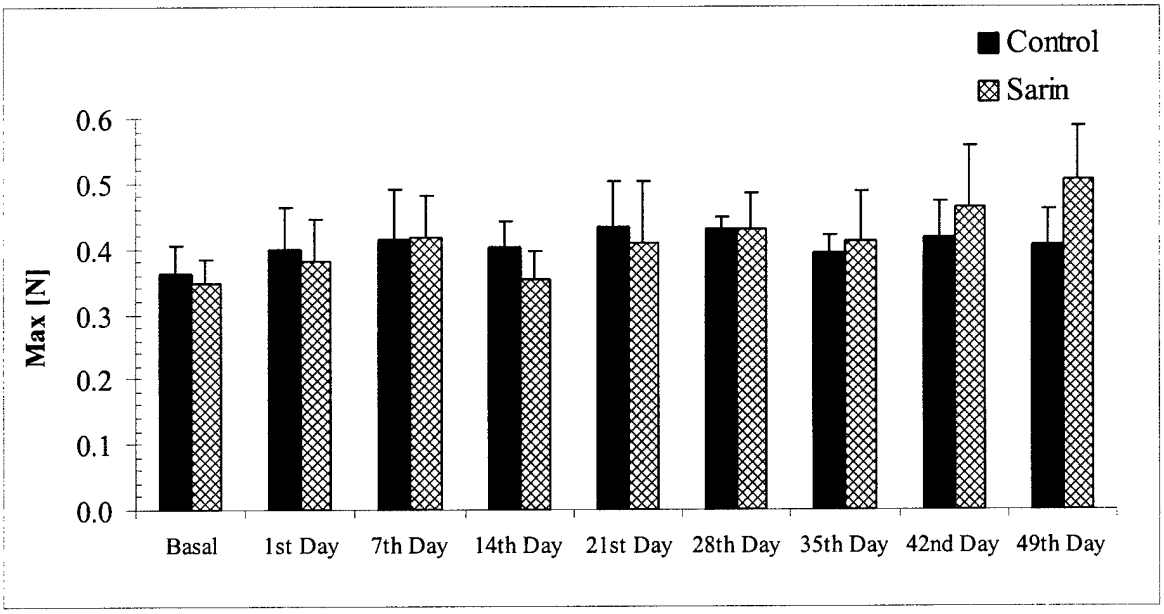


Fig. 1-14. Acoustic Startle Response – PPI of 100 db stimulus

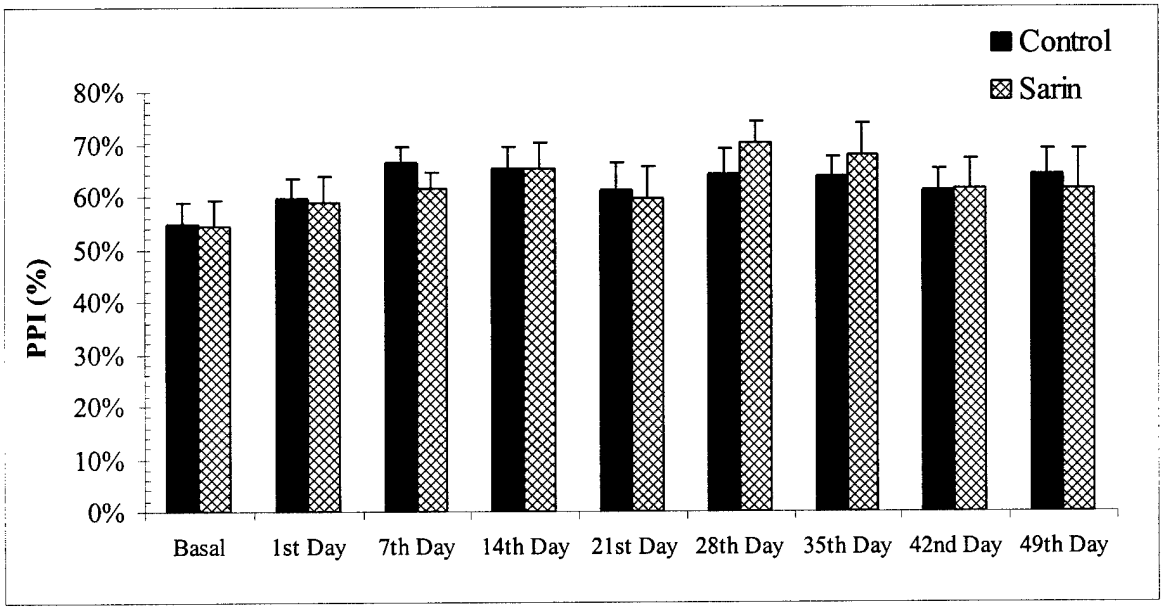
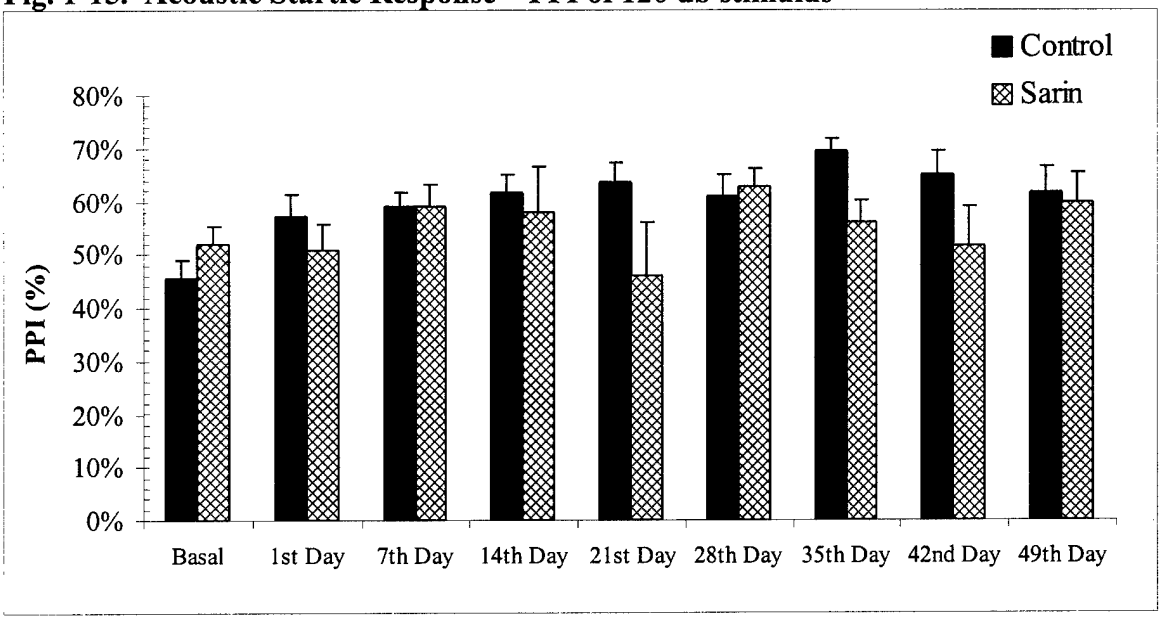


Fig. 1-15. Acoustic Startle Response – PPI of 120 db stimulus



Effects of sarin combined with chronic shaking stress on acoustic startle response

Animals

Male mice C57Bl/6J (25-27 g, n = 20)

Shaker

Animals were kept in their home cages fixed to a cage rack with automated watering system that was mounted on the shaker. Standard pellet diet and tap water were provided ad lib. Mice were exposed to intermittent shaker stress for 90 min per day over seven days. This total time was distributed into 45, two min shaking periods separated by randomized still periods with a mean duration of 30 min (13 – 44 min).

Administration procedure on the 1st day

During the administration (9:00 – 11:30) of drugs shaker machine was setup for shaking interval 30 min between each 2-min shaking. Acoustic startle response was tested 30 min after injections.

Administration procedure on the 2nd and 3rd day

During the administration (9:00 – 11:00) of drugs shaker machine was setup for shaking interval 30 min between each 2-min shaking. Injections were provided 20 min after 2-min shaking and next shaking was 10 min after injections

Results

After 7-day shaking stress combine with sarin exposure (3 x 0.4 LD50) there were no differences between groups (Fig. 1-16-1-19). During this long term experiment one mouse died and three mice exhibit self mutilation behavior. All animals were from the sarin group.

Fig. 1-16. Acoustic Startle Response – 100 db stimulus

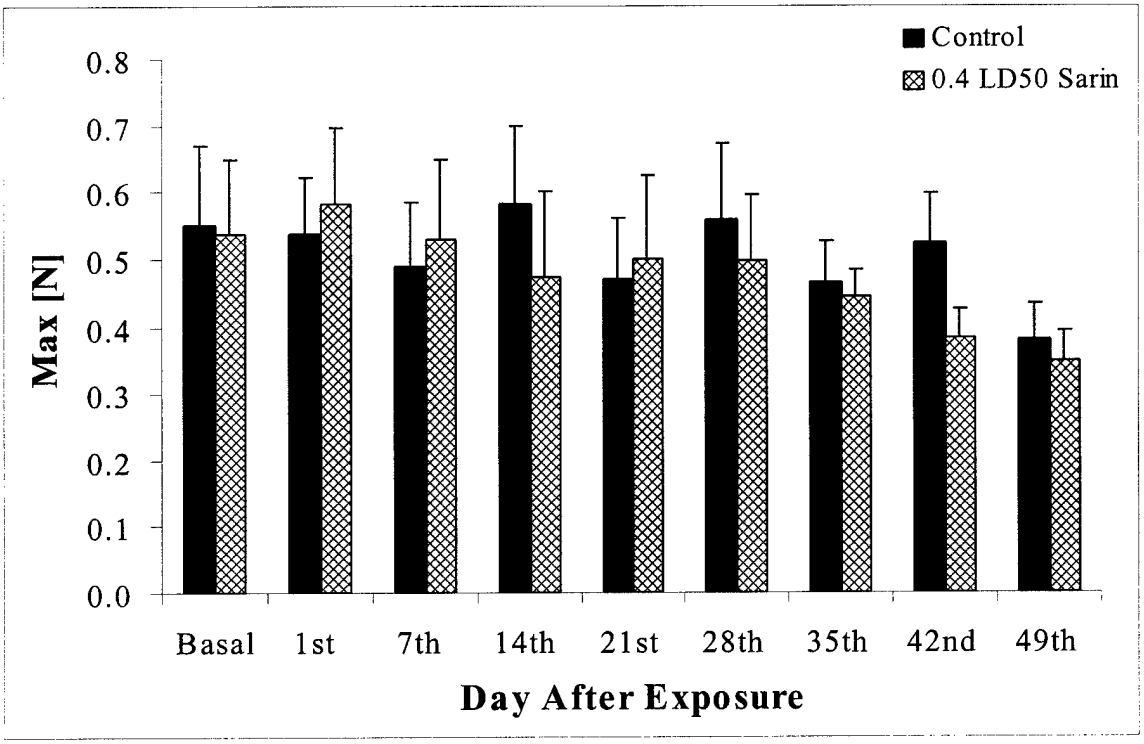


Fig. 1-17. Acoustic Startle Response – 120 db stimulus

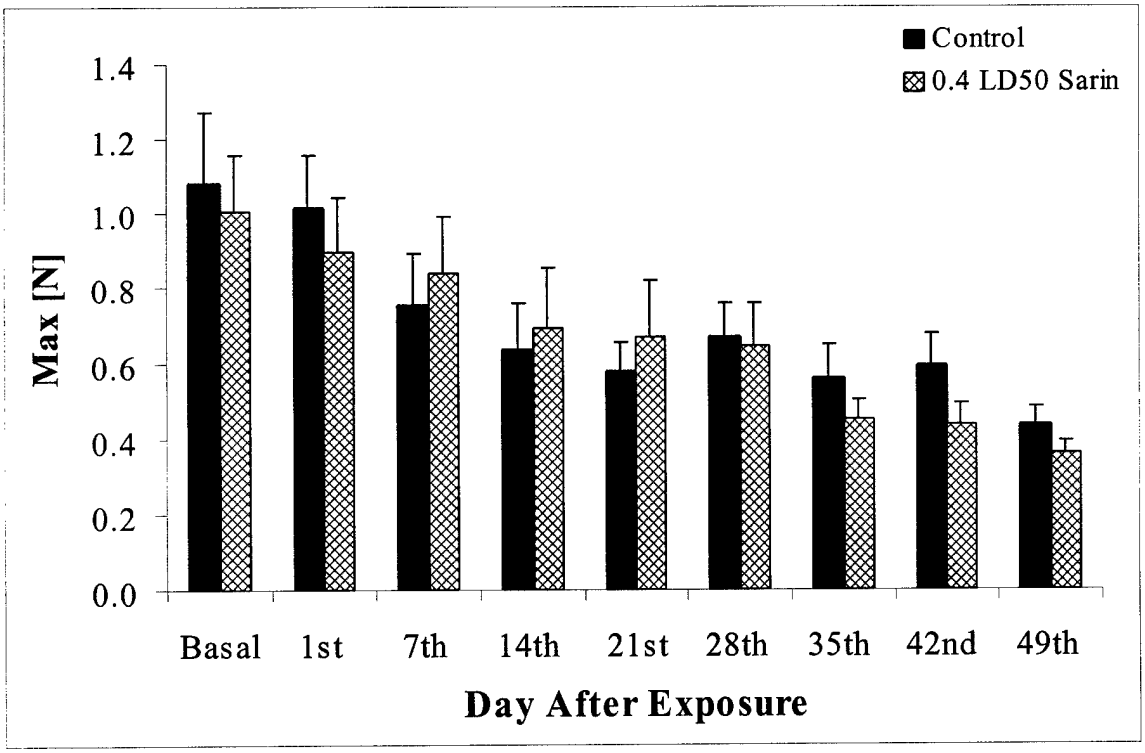


Fig. 1-18. Acoustic Startle Response – PPI of 100 db stimulus

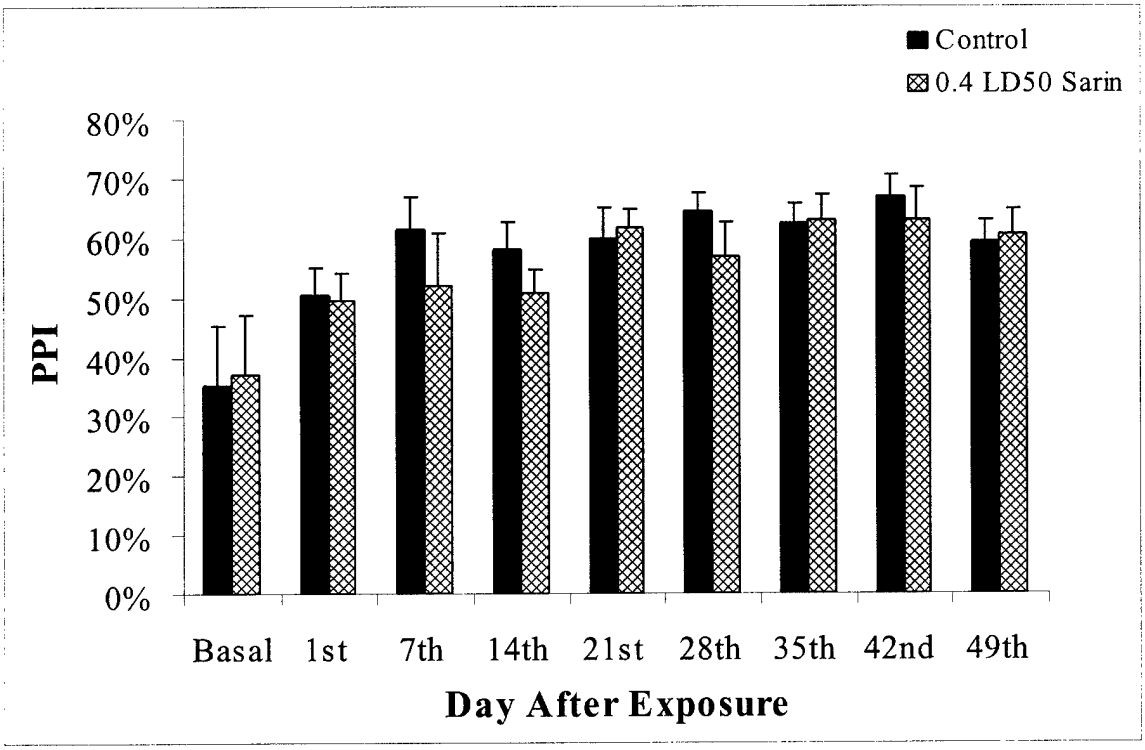
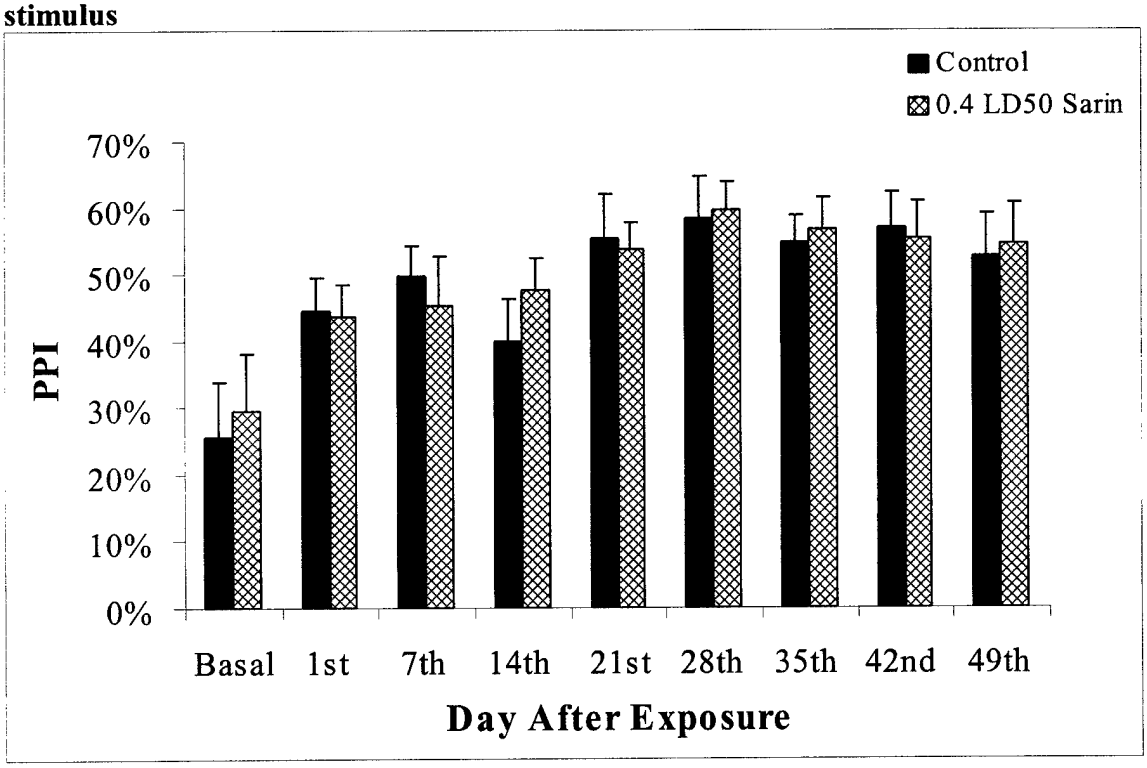


Fig. 1-19. Acoustic Startle Response – PPI of 120 db stimulus



Conclusions

Sarin seems to be less potent inhibitor than PHY of locomotor activity and ASR at the dose 3 x 0.4 LD_{50}. However, Dr. Grubbs lab data showed that we achieved up to 80% inhibition of red blood cell acetylcholinesterase on the 1st day after administration. The behavioral data shows a delayed effect of treatment with sarin rather than an acute effect, which is in contrast with the PB experiment that showed an acute effect rather than delay effect. Further, previous studies found that PB increased startle responding, presumably by potentiating ach at the neuromuscular junction, while PHY decreased the response. The CNS action of PHY was sufficient to overcome its peripheral effects, which would mimic PB. It is not clear why sarin produced no large acute effects, since the inhibition of AChE was greater than with either PB or PHY.

OXYTOCIN (OT) KNOCKOUT MICE (OTKO)

Preliminary experiment

Baseline testing was initiated in the OTKO mice to form the basis for planned experiments with sarin, stress and sarin plus stress. The mice were tested at ages ranging from 8 to16 weeks. To date, we have tested 7 male OTKO, 5 male wild types, 6 female OTKO and 3 female wild types.

Elevated Plus Maze

The elevated plus maze (Hamilton-Kinder, Poway, CA) was used in the presence of 300 lux illumination. The mice were tested in 5 minutes sessions without previous handling. There were significant changes in total time spent in the closed arms and open arms, but only in the females. OT +/+ females spent more time in the open arms and less in the closed arms. ($F_{CA}(1,7)=12.18$, $p<0.01013$; $F_{OA}(1,7)=17.877$, $p<0.003897$) (Table 1-8)

Table 1-8. Elevated Plus Maze: Mean ± S.E.M

		N	Movement (Beam Breaks)		
OT -/-	Female	6	538.3333	±	25.08740
OT +/+	Female	3	559.0000	±	38.93584
OT -/-	Male	7	508.7143	±	40.67860
OT +/+	Male	5	550.4000	±	18.05159
			Closed Arms (Total Time)		
OT -/-	Female	6	227.5167*	±	5.11426
OT +/+	Female	3	167.6333	±	18.36503
OT -/-	Male	7	197.6571	±	19.37884
OT +/+	Male	5	187.8600	±	8.92360
			Open Arms (Total Time)		
OT -/-	Female	6	50.1833**	±	6.78107
OT +/+	Female	3	103.9000	±	17.96923
OT -/-	Male	7	78.9000	±	21.35171
OT +/+	Male	5	58.8200	±	12.81395

Open Field

The mice were tested on 2 days to evaluate habituation and motor activity during the 15 minute sessions. The open field was 40.6 x 40.6 cm that was divided into central (20.3 x 20.3 cm) and intermediate and peripheral (both 5.1 cm wide) zones. The mouse was placed in the center of the open field and the following variables of motor activity were recorded: locomotor activity, fine movements (grooming) and rearing. Moreover, distance traveled, total time, rest time, number of entries and head pokes in individual zones were recorded.

After two days of testing, there were no differences in motor activity between OT +/+ and OT -/- in either males or females (Figures 1-20 and 1-21).

Fig. 1-20. Locomotor activity of OTKO and wild type males and females

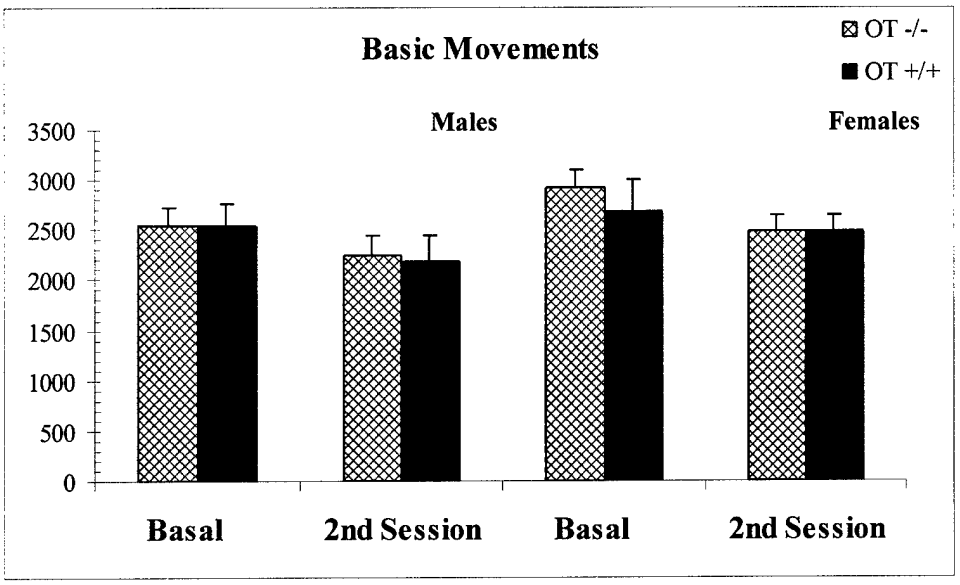
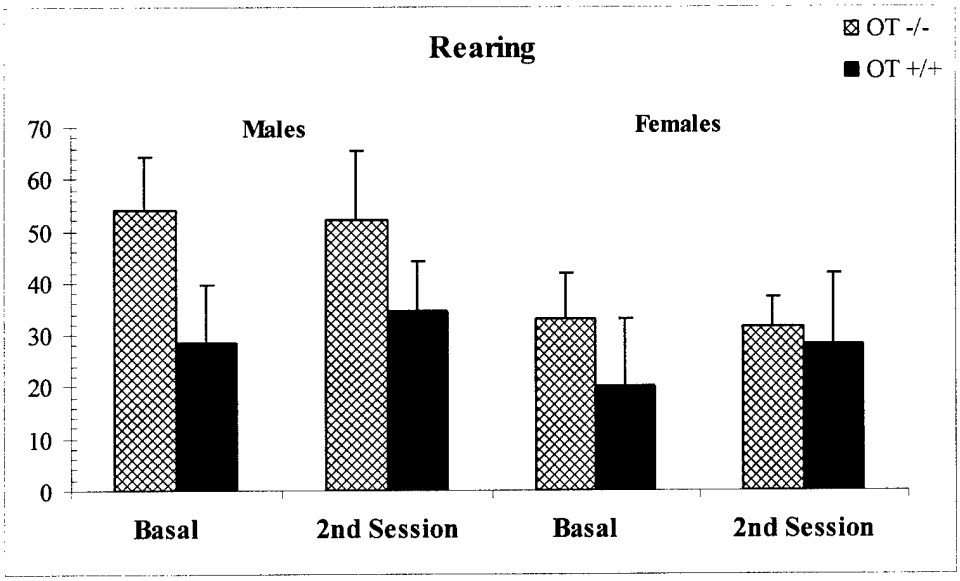


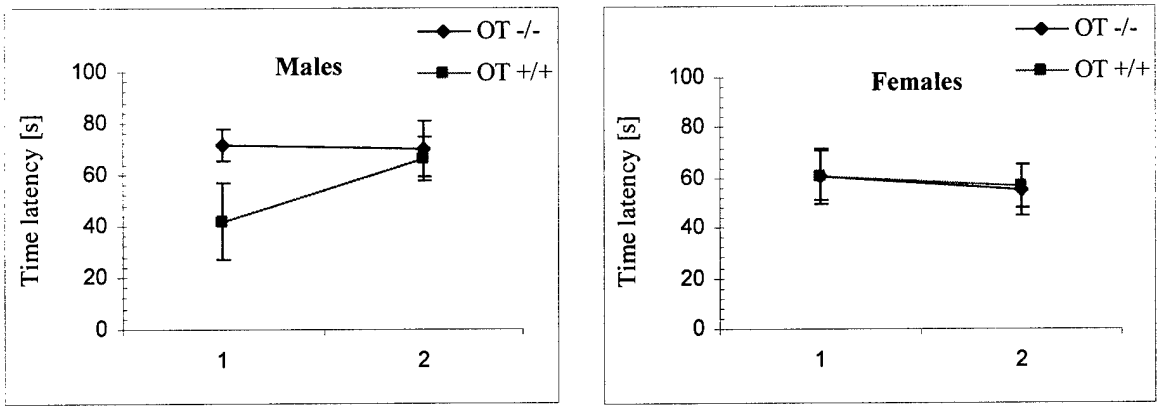
Fig. 1-21. Rearing of OTKO and wild type males and females



Water Maze

Spatial memory was tested using a modified Morris water maze. Each mouse was placed in one of the quadrants facing the wall and permitted to swim for 90 sec. Mice that sank were removed. If they failed to find the platform, they were gently guided to the platform and given 20 sec before the next trial. If they found the platform, they were permitted to remain for 20 sec before the next trial. All mice got four consequential trials, each randomly started from a different quadrant. The water was cleaned by scoop, the mouse toweled dry and placed in a home cage resting on a heating pad until they were totally dry and than returned to the colony. There were no significant differences between OT +/+ and OT -/- in either males or females. (Fig. 1-22)

Fig. 1-22. Time to reach the platform in a water maze of OTKO and wild type males and females

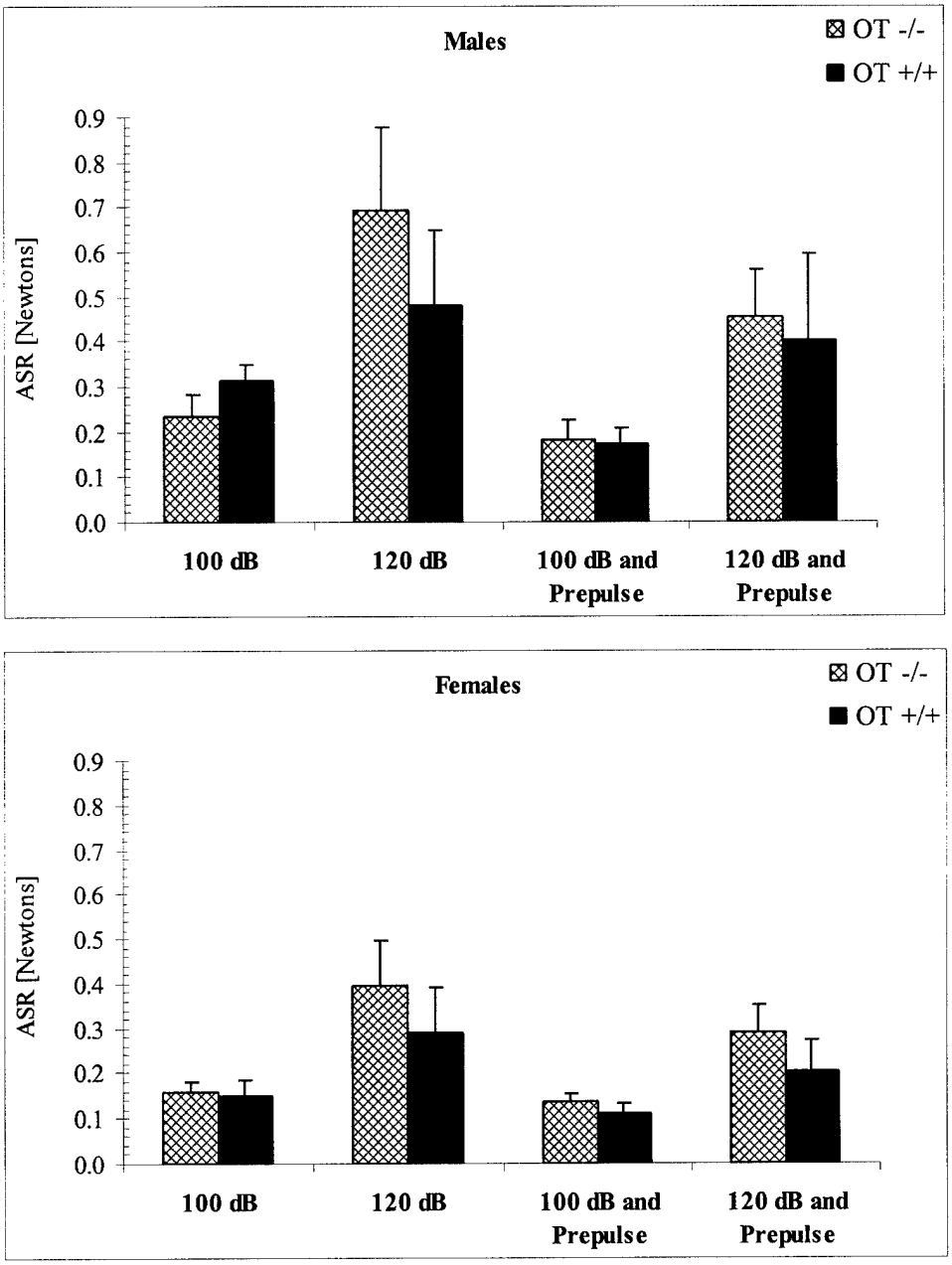


Acoustic startle

The system was set up for 6 types of white-noise burst stimulus trials: no stimulus (background, 60 db), pre-pulse (70 db), pulse (100 db and 120 db), pre-pulse plus pulse (70 db+100db and 70 db+120 db). Each trial type was presented 10 times in 10 blocks. Stimuli were presented in random order to avoid order effects and habituation. The inter-trial interval varied from 9 to 16 sec. All animals were regularly handled before individual tests in order to minimize handling-related stress. Tested mice were loosely restrained in holders that were placed on a sensing plate transforming movements of the body (jerks) into an analog signal through an interface.

There were no significant differences in acoustic startle response between OT +/+ and OT -/- in either males or females. (Fig. 1-23)

Fig. 1-23. ASR and PPI in OTKO and wild type males and females



The preliminary experiment showed slight changes in the elevated plus maze which confirmed findings of authors Mantella and Amico (FEMALE OXYTOCIN DEFICIENT MICE DISPLAY ANXIETY - LIKE BEHAVIOR). Therefore we continued with more OTKO animal studies of the behavioral differences between knock-out and wild type mice following acute shaker stress.

Behavioral differences after acute stress in OTKO mice

Animals
Males: 129SV OT k.o. (n=20); 129SV wild (n=12)
Females: 129SV OT k.o. (n=24); 129SV wild (n=12)

Behavioral tests:
Experiment I (n=24) – without stress
Elevated Plus Maze (without previous handling)
Handling (3 min/Day) for 3 consecutive days
Open Field (15 min Session)
Acoustic Startle Response
1 week interval between the above tests

Experiment II (n=44) following stress
Elevated Plus Maze (without previous handling)
Handling (3 min/Day) for 3 consecutive days
Open Field (15 min Session)
Acoustic Startle Response
1 week interval between the above tests

Experimental Protocols
The two experiments used 48 mice, 24 in each. In the first experiment animals were tested without previous stress conditions. In the second experiment, animals were exposed to 15 min. Shaker stress and immediately after that evaluated by the following basic behavioral tests.

Elevated Plus Maze (see above)
Open Field (see above)
Acoustic Startle Response (see above)

Statistical Analysis
The data were analyzed by means of 2-way ANOVA with two factors, condition (stress/non-stress) and genotype (OT +/+ vs. OT -/-). Males and females were tested separately without further comparison of gender differences, because the variable of phase of hormone cycle was not measured. Software using the program was STATISTICA, 6.1 (statsoft, Inc., Tulsa, OK). The results are presented as a means ± S.E.M. ($p \leq 0.05$).

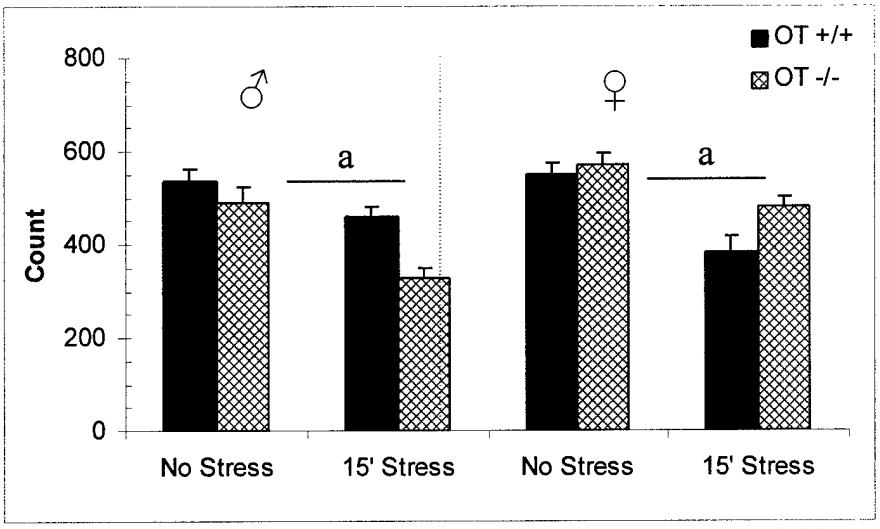
Results

Elevated Plus Maze
We didn't find any statistical changes between knock-out and wild type animals, however there was a significant main effect of condition (stress/non-stress) in basic movements and spending time in the closed arms (Table 1-9, Fig. 1-24, 1-25) in both genders. The ANOVA also revealed a significant main effect of group in the basic movements of males ($F(1,28)=10.836$, $p<0.0027$).

Table 1-9. Plus Maze - ANOVA results between non-stress and stress condition

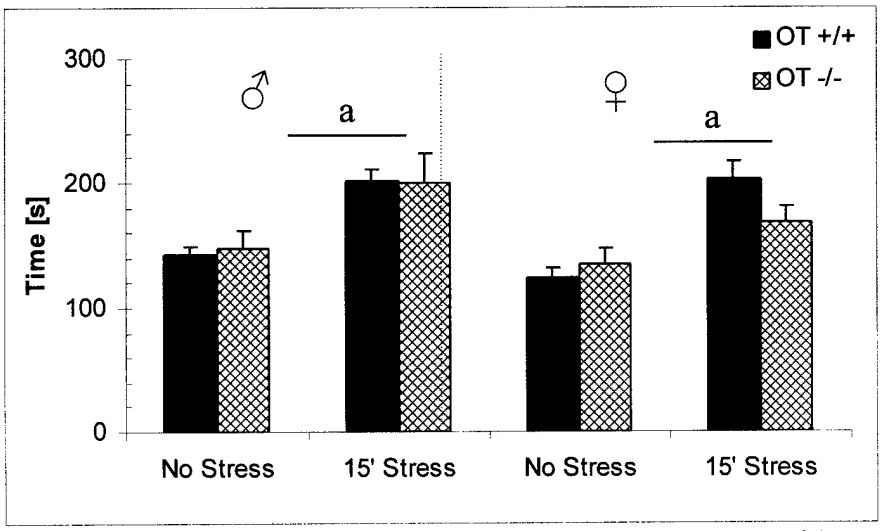
	Males	Females
Basic Movements	F(1,28)=19.897, **p<0.0001**	F(1,32)=15.648, **p<0.0004**
Closed Arms Tot. Time	F(1,28)=9.609, **p<0.0044**	F(1,32)=14.024, **p<0.0007**

Fig. 1-24. Elevated Plus Maze – Basic Movements



a – significantly different from non-stress conditions, p<0.01

Fig. 1-25. Elevated Plus Maze – Closed Arms (Tot. Time)



a – significantly different from non-stress conditions, p<0.01

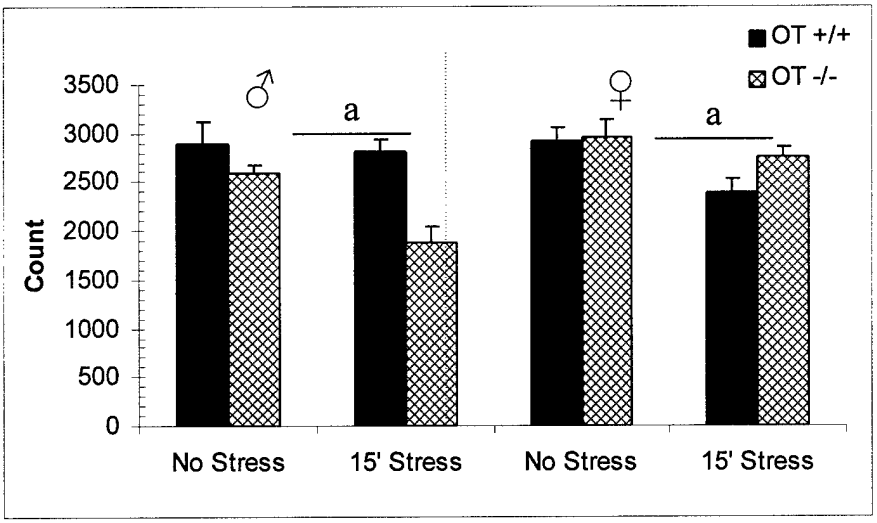
Open Field

Acute shaker stress (AS) significantly changed behavior (basic movements, periphery total time, rearing) in both genders and groups. The main effect of condition (stress/non-stress) was significant on all measures (Table 1-10). The main effect of group (OT+/+ vs. OT-/-) was significant only in males for basic and fine movements ($F_{BM}(1,28)=16.216$, $p<0.00039$; $F_{FM}(1,28)=14.905$, $p<0.0006$), however, periphery total time was not quite significant ($F(1,28)=3.019$, $p<0.09$) (Fig. 1-29). The interaction was close to significance for basic movements ($F(1,28)=4.155$, $p<0.051$; Fig. 1-26) and fine movements ($F(1,28)=3.077$, $p<0.09$). Rearing was decreased after stress in males the same way in both groups and therefore the effect of group and interaction were not significant (Fig. 1-27). Using 3 minutes intervals during open field data analyzing we observed significant differences in habituation after stress (Fig. 1-29).

Table 1-10. Open Field - ANOVA results between non-stress and stress condition

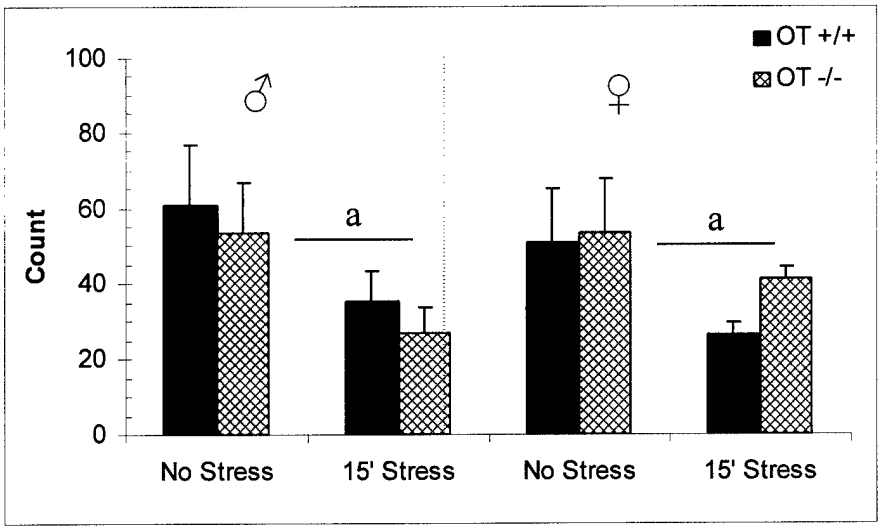
	Males	Females
Basic Movements	F(1,28)=6.541, **p<0.016**	F(1,34)=6.017, **p<0.019**
Rearing	F(1,28)=6.032, **p<0.02**	F(1,34)=6.041, **p<0.019**
Periphery Tot. Time	F(1,28)=31.97, **p<0.000005**	F(1,34)=16.749, **p<0.0002**

Fig. 1-26. Open field – Basic Movements



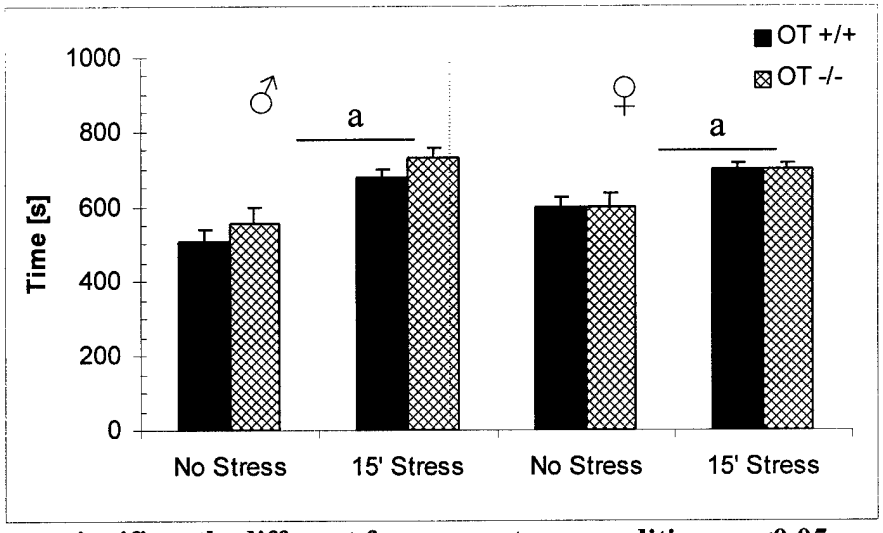
a – significantly different from non-stress conditions, p<0.05

Fig. 1-27. Open field – Rearing



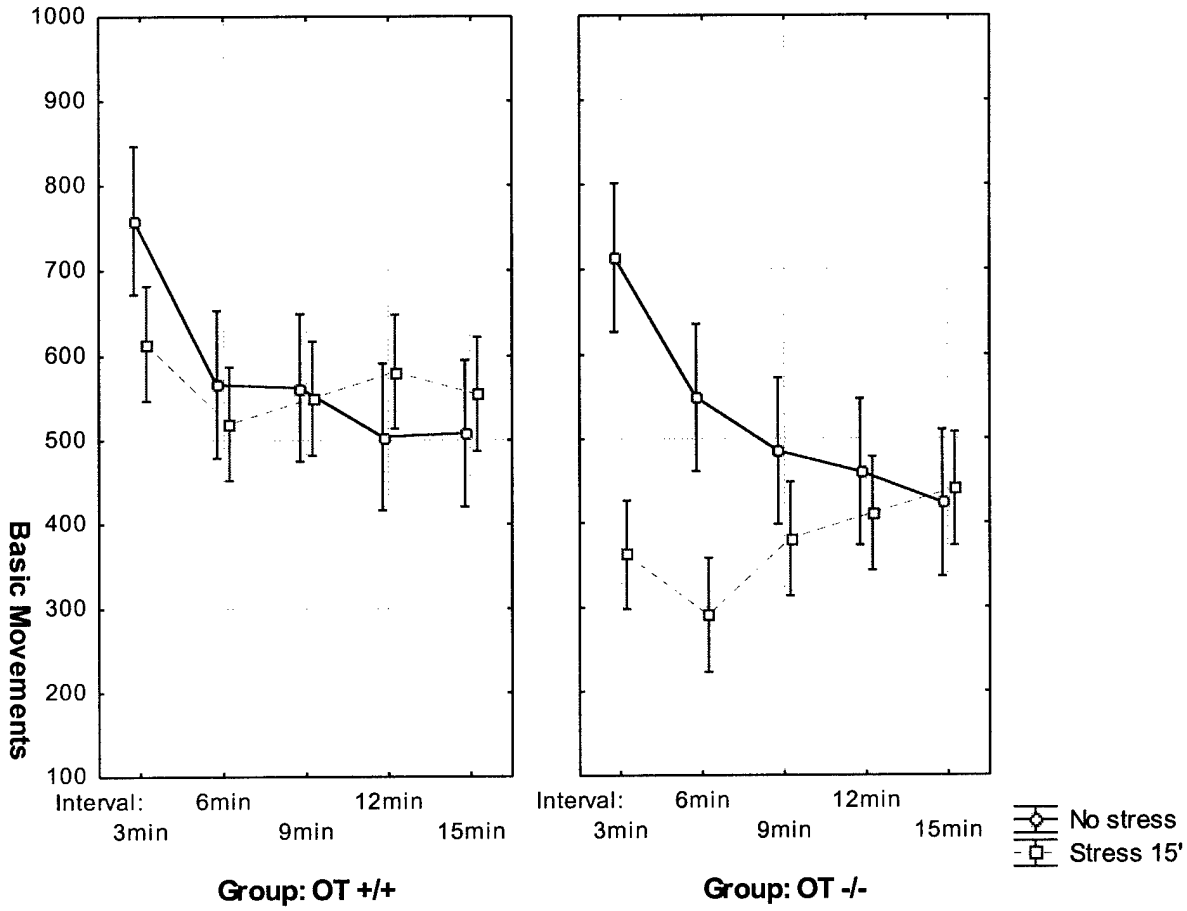
a – significantly different from non-stress conditions, p<0.05

Fig. 1-28. Open field – Periphery Total Time



a – significantly different from non-stress conditions, p<0.05

Fig. 1-29. Open field – 3 min. Basic movements interval



Acoustic Startle Response

This test revealed that stress did not change the startle response to the 100 and 120 db stimulus. Pre-pulse inhibition was also not changed. (Fig. 1-30-1-33)

Fig. 1-30. Acoustic Startle Response – 100 db stimulus

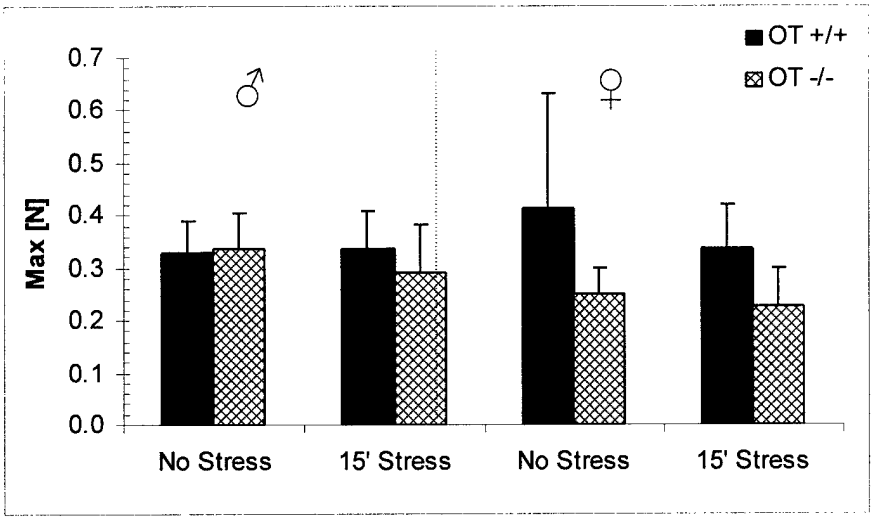


Fig. 1-31. Acoustic Startle Response – PPI of 100 db stimulus

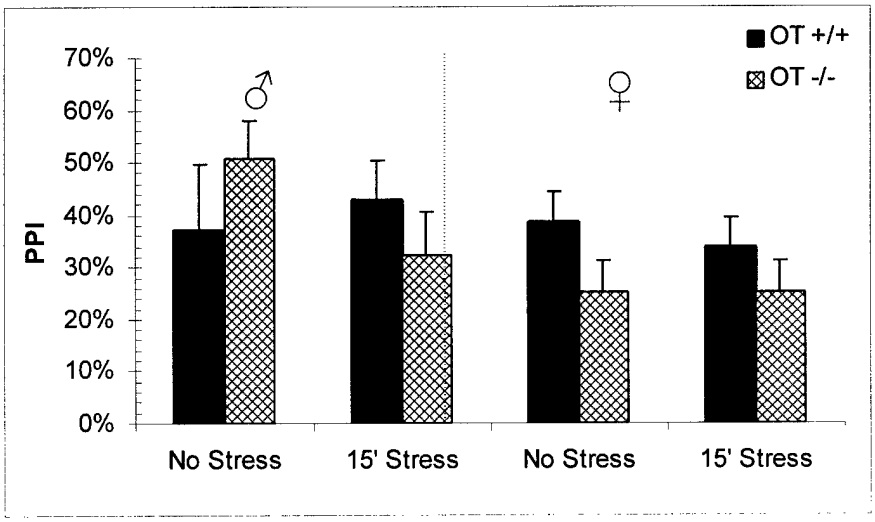


Fig. 1-32. Acoustic Startle Response – 120 db stimulus

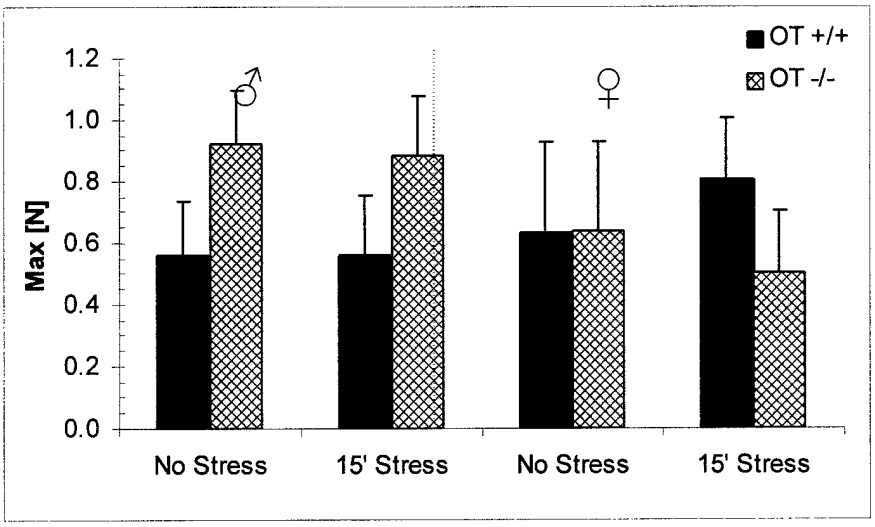
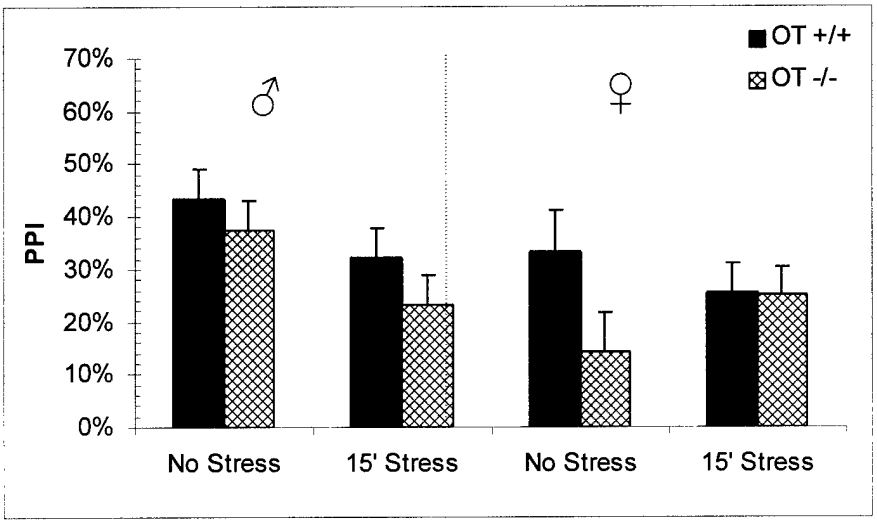


Fig. 1-33. Acoustic Startle Response – PPI of 120 db stimulus



Conclusion

In general the acute shaker stress changed the behavior of mice, as expected, while we also expected differences between OT +/+ and OT -/-. These differences we found only in males, especially in the locomotor activity. Females responded to stress the same way in both genotypes. The acoustic startle response did not revealed any changes in the response to the stimuli after acute stress. We conclude 1) there is a gender specific role for OT in the behavioral response to stress and 2) OT attenuates the locomotor response to stress in males.

CHOLINERGIC SYSTEMS
Investigators: Robert Grubbs, Ph.D., Brena Mauck, M.S. and William Price, B.S.

KEY RESEARCH ACCOMPLISHMENTS:

- Completed validation of the shaker stress/mini-pump pyridostigmine bromide (PB) exposure model in mice from the perspective of effects on cholinesterase activity in blood and several brain regions.
- Completed assessment of effect of PB and stress on apoptosis in brain.
- Completed assessment of effect of stress and PB on muscarinic receptor density in brain.
- Began the assessment of sarin effects on blood and brain cholinesterase and brain muscarinic receptor density.

REPORTABLE OUTCOMES:

- Grubbs, R.D., W.A. Price, S.J. Paton, and M. Morris (2003) "Effects of exposure to sarin, pyridostigmine bromide, and stress on cholinesterase activity in mouse blood and brain", 2003 Toxicology and Risk Assessment Conference, April 28-May 1 Fairborn OH.

- Mauck, B. S., Morris, M., and Grubbs, R. D. Effect of pyridostigmine and stress on brain cholinergic receptor density in C57Bl mice. FASEB J, 17: A622, 2003.

Validation of the model - We have shown that administering PB via an Alzet™ minipump implanted subcutaneously induces a reproducible inhibition of blood cholinesterase activity as predicted. To achieve our targeted 30-40% inhibition of blood cholinesterase activity, we determined that a dose of 10 mg/kg PB was needed. While this dose is considerably higher than what we were expecting to use, it apparently reflects the absorption kinetics of this route of administration, since this same dose was lethal to mice when given iv. Shaker stress alone produced no changes in blood or brain CHE activity.

The effect of PB on brain cholinesterase activity appears to depend on the specific brain area assayed, and more importantly, on the proximity of the area to circumventricular organs, where the blood-brain barrier is absent. For example, the frontal cortex appears to be insensitive to PB exposure while in the brain stem PB induced a small but statistically significant ($P \leq 0.02$) inhibition of CHE activity. In contrast, analysis of hypothalamus from animals exposed to 10 mg/kg PB revealed a significant reduction in ChE activity regardless of whether the animal had been stressed. These observations are of particular interest in view of the recent work conducted with AChE knockout mice indicating a previously unrecognized and potentially important role for BChE in the brain [1] [2].

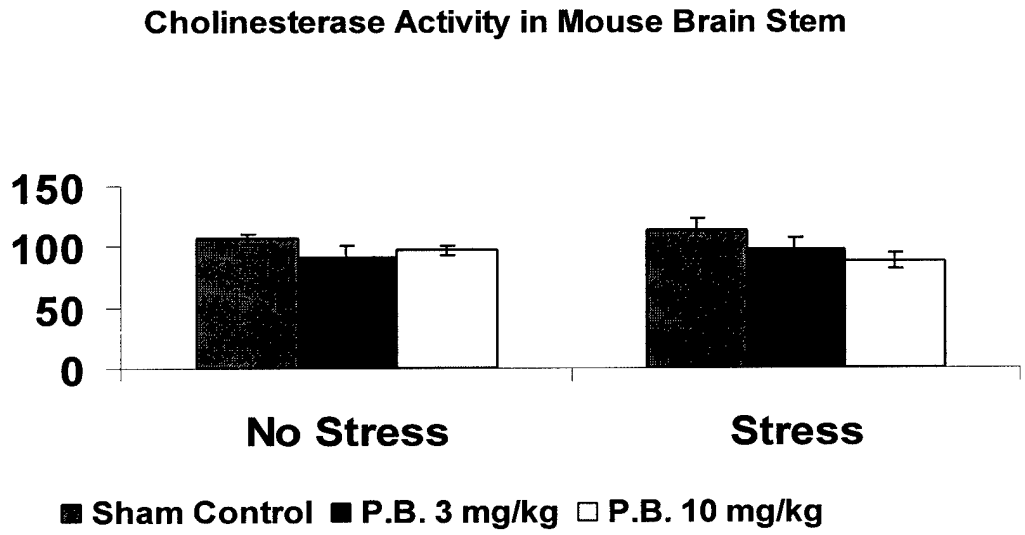


Figure 1-34. Effects of stress and PB on cholinesterase activity in mouse brain stem. Animals were exposed to PB and/or stress for 7 days. Sham denotes animal underwent surgery with no pump implanted. PB treatment (both doses) differs significantly ($p \leq 0.002$) from sham.

Brain Apoptosis Studies:
Assessment of apoptosis in brain areas following stress and/or exposure to PB has been uniformly negative. This analysis was conducted at several times points (day 7-35 at one week intervals for caudate and hippocampus and day 7 & 37 for all other areas). The brain areas that were analyzed include frontal cortex, caudate nucleus, hypothalamus, hippocampus, cerebellum, nucleus basalis, substantia nigra, pedunculopontine nucleus, and the horizontal and vertical diagonal bands. These studies examined time points beginning 7 days after initial exposure to PB and extending out in time to several weeks. To ensure further that we had not missed an early apoptotic event, we performed one additional control study this quarter in which earlier time points (8, 24, and 48 hr after PB exposure) were examined for signs of apoptosis. These studies again were negative, strengthening our conclusion that PB exposure does not induce neuronal apoptosis in these animals.

Given the negative data obtained from these studies it was necessary to produce positive control data to validate our findings. We utilized the neurotoxin, MPTP, which selectively targets dopaminergic neurons in the substantia nigra, as our positive control on the basis of several published studies in C57 mice showing that it induced apoptosis in this area. We observed an appropriate level of apoptosis (~10%), confirming that our methodology was working well. To the best of our knowledge, no one else looked at the effects of PB and stress on apoptosis in mice, so we are writing this work up for publication. These results contradict the published findings of Li et al., but their study was done in rats and seems to have lacked appropriate controls.

Brain Cholinergic Receptor Autoradiography:

The first phase of the autoradiographic studies consisted of experiments in which various brain areas were examined for changes in muscarinic and nicotinic receptor density following PB exposure and/or stress. The second phase of this work consisted of experiments in which brain sections have been subjected to an autoradiographic protocol designed to distinguish between three different subtypes of muscarinic receptors.

The pseudocolor images shown in Figure (1-35) illustrate the specificity of binding (inhibition by atropine) and the inherent regional differences in muscarinic receptor density. The highest densities are seen in the caudate followed by the cerebral cortex. Receptor density was quantified by densitometric analysis and expressed as femtomoles of ligand bound/ mg protein. Differences between treatment groups were analyzed by ANOVA.

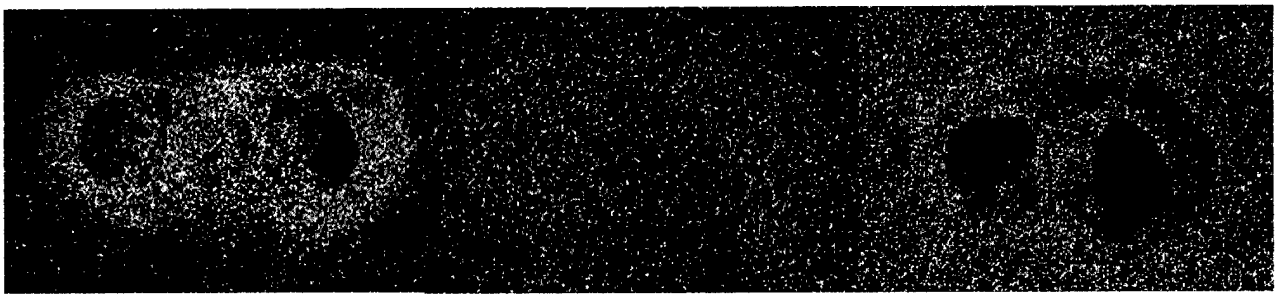


Figure 1-35. Autoradiographic images of mouse brain sections labeled with [^{3}H] N-methyl-scopolamine with (center) and without (side panels) 1 um atropine.

The results from phase one of this study identified brain areas in which muscarinic and nicotinic receptor densities were altered by stress and by exposure to PB or to physostigmine, our positive control which is known to be freely accessible to the brain. These results are shown in Table 1-11 below.

Table 1-11. Muscarinic and nicotinic receptor densities.

Day 7	Muscarinic				Nicotinic		
	Stress	Treatment	S+T		Stress	Treatment	S+T
Cpu(Cpu)		Y+			+	Y+	
Cortex (Cpu)	+	Y+			+	PB-	
VDB (Cpu)	+				-		YS+
HDB (Cpu)	+		PB-		-		Y-,YS+
PN	+	Y+	Y+, YS+			Y+	
SN		Y+			-	Y+	YS+
Hypo (CVO)						Y-	
Thalamic Nuclei		Y+	PB-, Y+			Y-	YS-
Cortex (HH)		PB+, Y+			+		YS-
CA1 (Hippo)							
CA3 (Hippo)		Y+	PB-				
DG (Hippo)			PB-				
ML (Hippo)			PB-			Y-	
NB		Y-				Y-	YS-
Cpu (NB)		Y-			+	PB-, Y-	YS-
PPN						Y+	

Statistically significant increases (+) and decreases (-) in receptor density with respect to sham controls are indicated. Significant differences observed following treatment with either physostigmine (Y) or pyridostigmine (PB) are indicated. Many other brain areas were examined but showed no significant changes. We used these data to direct our focus for the next part of the study in which we used a protocol that permits subtype selective labeling of muscarinic receptors to determine which subtypes changed following exposure of the animals to stress, PB or physostigmine. Knowing where the cell bodies for cholinergic neurons are located and where their associated axonal fields' project, we were able to map subtype specific changes in muscarinic Ach receptor densities and correlate these changes with the functions ascribed to these areas.

Significant changes were seen in cholinergic receptor densities primarily in cholinergic nuclei and their projection fields. Stress alone and physostigmine alone produced changes in diagonal band nuclei and in the projection fields, amygdala, limbic cortex, and the hippocampus at the end of a 7-day exposure. PB produced changes in diagonal band nuclei at day 7 but changes in the amygdala and limbic cortex were not seen until day 37. This delayed effect is important in that soldiers did not experience symptoms until months after they had been exposed to PB. The combination of stress and drug did not increase the magnitude of density changes as expected. In fact, stress and physostigmine interacted in an antagonistic manner with the combination producing a change in receptor density of smaller magnitude than either component alone. This neuroprotective effect of stress is supported by studies found in the literature. The regions showing altered receptor densities in this study are known to be involved in memory and learning, areas where veterans experience problems.

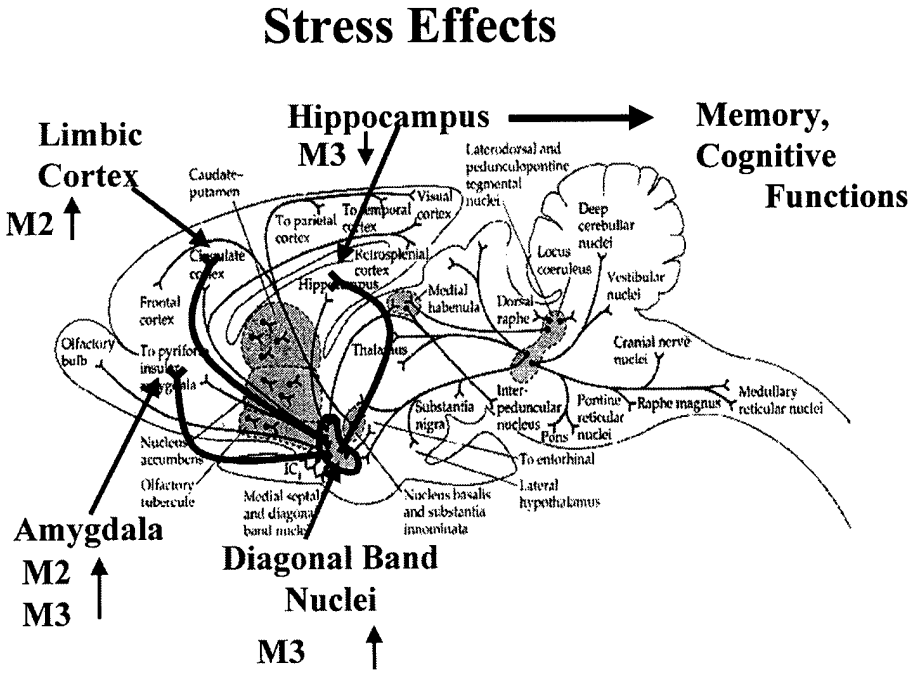


Figure 1-36. Summary of muscarinic receptor density changes produced by stress. M1, M2, and M3 denote muscarinic receptor subtypes; arrows indicate whether receptor density increased or decreased.

Drug Effects - Physostigmine

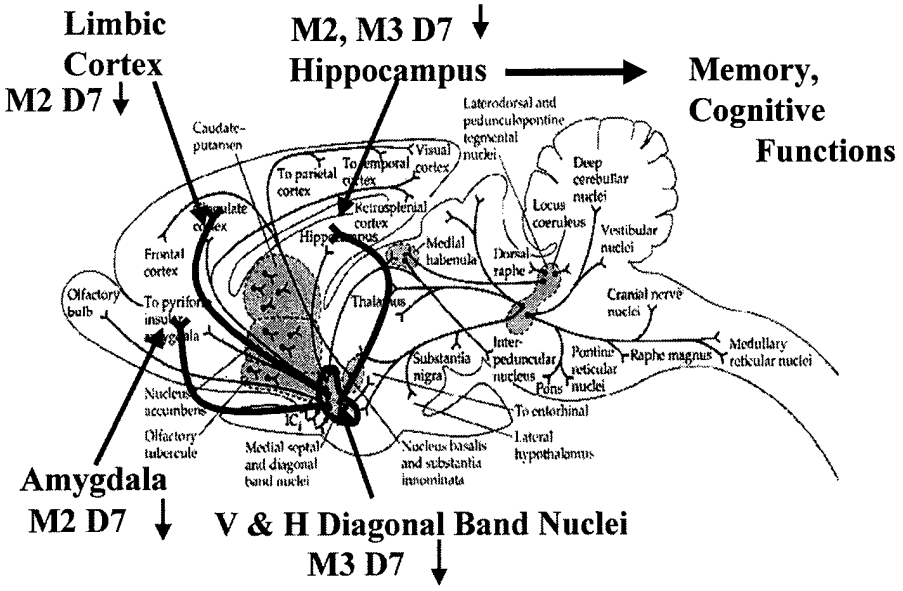


Figure 1-37. Summary of muscarinic receptor density changes produced by physostigmine. M1, M2, and M3 denote muscarinic receptor subtypes; arrows indicate whether receptor density increased or decreased. D7 denotes the day of the effect.

Drug Effects – Pyridostigmine Bromide

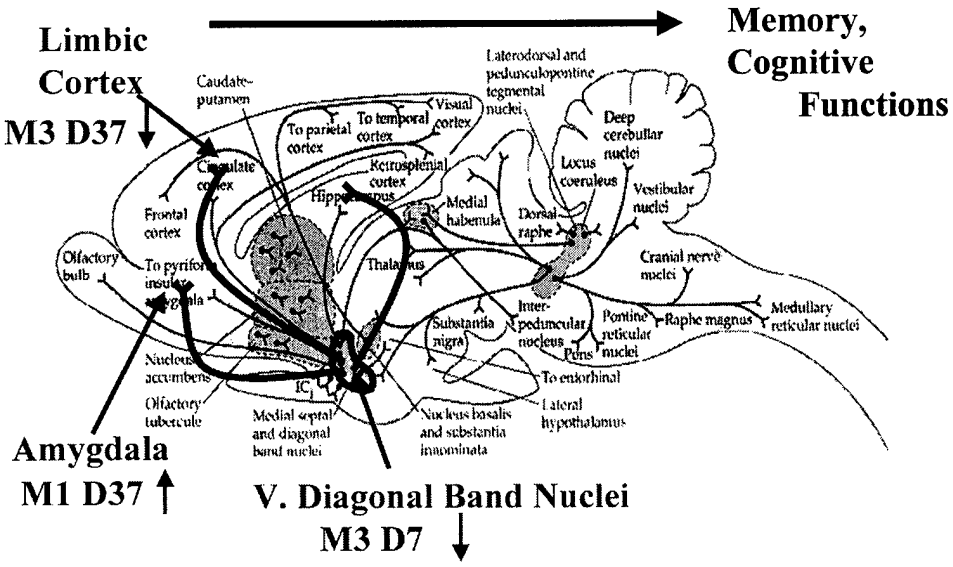


Figure 1-38. Summary of muscarinic receptor density changes produced by pyridostigmine. M1, M2, and M3 denote muscarinic receptor subtypes; arrows indicate whether receptor density increased or decreased. D37 denotes the day of the effect.

Sarin:
We have now conducted several experiments in which mice were exposed to sarin via repeated subcutaneous injections and the ChE activity measured in whole blood and brain at various times after the last injection. These data are important for establishing the magnitude of the primary effect of the agent and as a point of reference for interpreting other data sets (see Lucot). We chose to examine two doses of sarin, 0.05 and 0.4 X LD_{50}. Exposure to 0.05 X LD_{50} sarin on three successive days produced essentially no change in ChE activity in either whole blood or prefrontal cortex of mice in two experiments. In contrast, exposure to 0.4 X LD_{50} sarin on three successive days produced a significant decrease (66%) in AChE activity in whole blood after 24 hr (Figure 1-39). A 40% decrease in blood BChE activity was also observed at this dose.

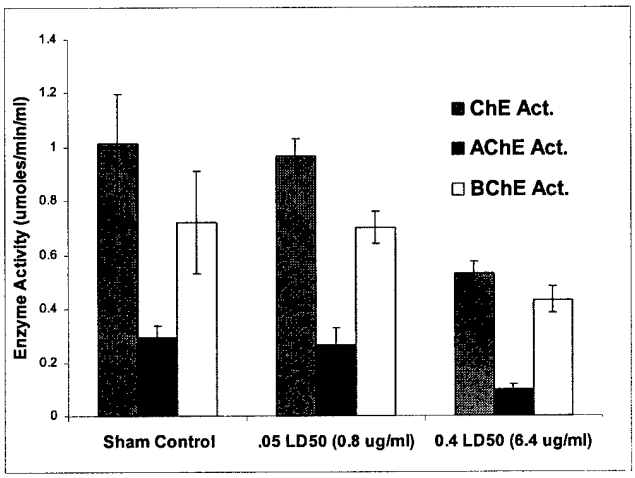


Figure 1-39. Effect of sarin on blood cholinesterase activity 24 hr after third exposure.

In another experiment, exposure to 0.4 X LD_{50} sarin on three successive days produced a substantial decrease (88%) in AChE activity in whole blood 24 hr after the last sarin injection, while BChE activity decreased by 45% (Figure 1-40). By day 23 after the last sarin injection, AChE activity had recovered substantially (71% of sham control) but not completely, while BChE activity also approached normal (87% of control). By 55 days after the last injection, full recovery of cholinesterase function was apparent.

In another experiment we established the early (0.5 -72 hr) time course of sarin inhibition after single injections of 0.4 X LD_{50} (Figure 1-41). Again this information is essential to interpreting other ongoing studies looking at whether the animals respond to sarin exposure by up-regulating production of AChE and BChE. We found that blood AChE and BChE activities were inhibited by 85% and 65%, respectively, within 30 min, but recovered to basal levels within 24 hr. Interestingly, when we measured ChE activity in the prefrontal cortex and the hypothalami of these animals, we found no detectable inhibition by sarin. We hypothesize that the lack of effect in the CNS in this experiment can be explained by the effective scavenging of sarin by peripheral carboxyesterases and BChE following a single exposure. In the earlier experiments utilizing repeated sarin exposures, the additional exposures would have overwhelmed this "protective" capacity of the blood and allowed sarin to inhibit AChE and BChE in the CNS. We are currently testing this hypothesis in animals exposed twice to this dose of sarin.

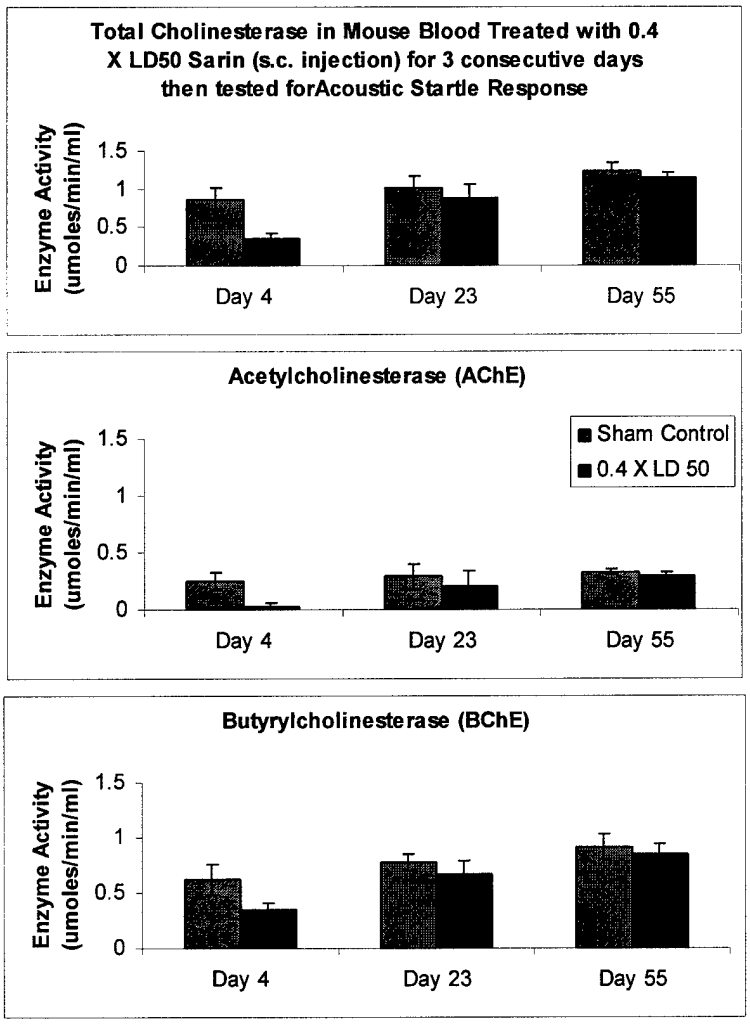


Figure 1-40. Time course of cholinesterase recovery following exposure to sarin.

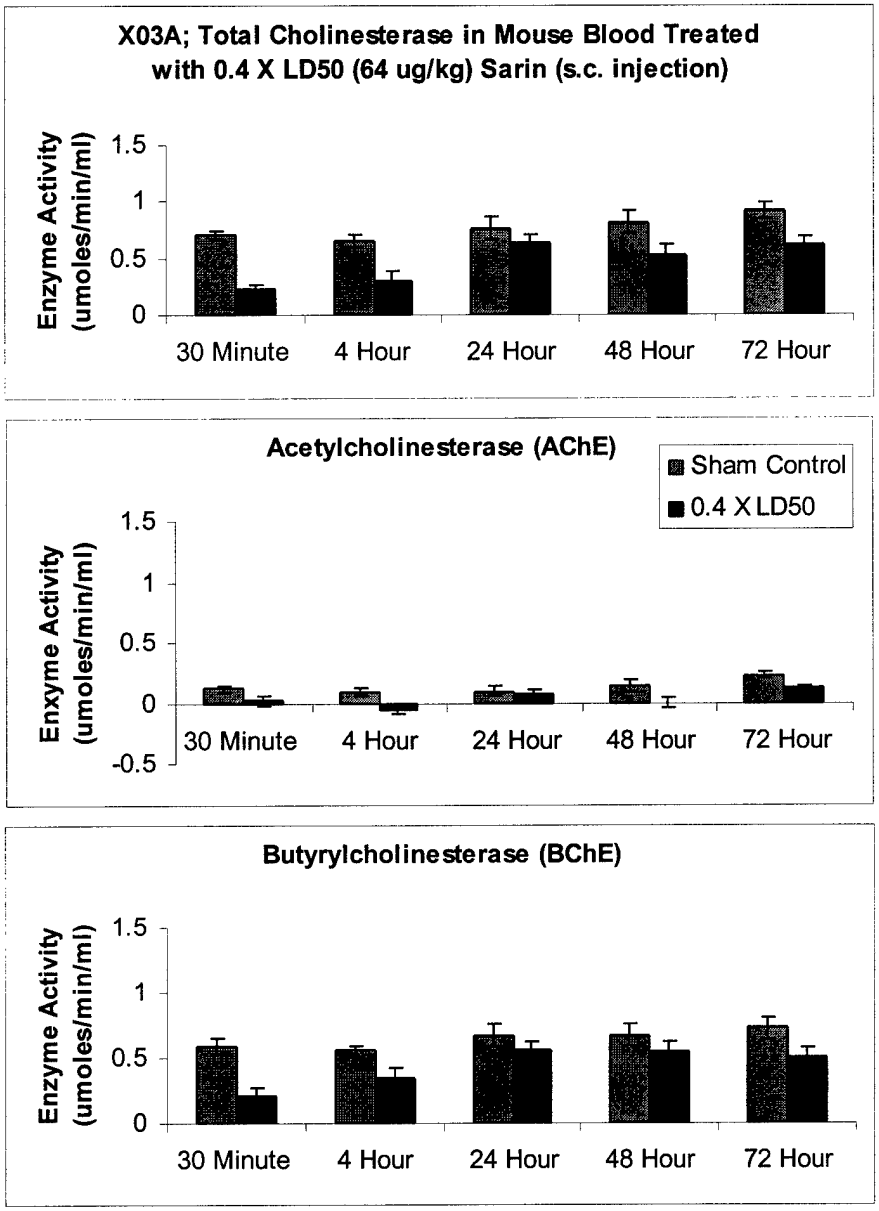


Figure 1-41. Effect of a single exposure to 0.4 X LD_{50} sarin on blood ChE activity over 72-hr.

1. Li, B., *et al.*, *Abundant tissue butyrylcholinesterase and it possible function in the acetylcholinesterase knockout mouse.* Journal of Neurochemistry, 2000. 75: p. 1320-1331.
2. Mesulam, M.-M., *et al.*, *Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine.* Neuroscience, 2002. 110(4): p. 627-639.

Progress Report- Year 3- Cool

Objectives- To study the effect of pyridostigmine Br and sarin on peptide hormones of the hypothalamic-pituitary axis using proteomic tools: SELDI-TOF mass spectroscopy, Western blotting, acetylcholinesterase enzyme assays.

Key Research Accomplishments-

1. Analyzed hypothalamic tissue from six experiments for pyridostigmine, physostigmine or sarin's effects on acetylcholinesterase activity
2. Analyzed all hypothalamic and frontal cortex tissue by Western blot for acetylcholinesterase protein levels.
3. Analyzing hypothalamus, anterior and posterior pituitary tissue from 2 experiments for pyridostigmine and physostigmine effects on the proteome.
4. Analyzed testes from sarin treated mice for acetylcholinesterase activity.
5. Developed a method for sequencing peptides "on proteinchip" using carboxypeptidase Y enzyme to remove amino acids from the C-terminus of peptides and proteins.

Peer Reviewed Publications and Abstracts

- **Cool, D.R.** and Debrosse, D., Extraction of oxytocin and arginine-vasopressin from serum and plasma for radioimmunoassay and SELDI-TOF MS. (2003) J. Chromatography B, 792: 375-380.
- Ropp, S. A., Paton, S.J., Morris, M. And **Cool, D.R.**, *Effects of subacute pyridostigmine and stress on hypothalamic and cerebral cortex acetylcholinesterase and proteomic profiles.* FASEB Apr 2003, Abs# 388.13.

Reportable Outcomes

A) During the period of the last year, six experiments were completed in which hypothalamic tissue was collected and analyzed for acetylcholinesterase activity. Peptide sequencing was also initiated during this period to further analyze proteomic data. A summary of the experiments follows.

1) X02A- Mice were treated with 0.4 (64 μg/kg) or 0.05 (8 μg/kg) of the LD50 dose of sarin by subcutaneous injection for 3 days (3X 0.4 or 3X 0.05 LD50 sarin).
2) X02C-.Time Course for Sarin treatment on AChE activity (2X0.6 LD50 9.6 μg/kg)
3) X02D- Long term effects of sarin on AChE Activity.
4) X03A- Time Course II for Sarin treatment on AChE activity (1X0.4 LD50).
5) PY03A- Mice (10 per group) were implanted with mini-pumps containing 10 mg/kg/day pyridostigmine, 2.9 mg/kg/day physostigmine or saline for 7 days. The animals were sacrificed at the end of the experiment and tissues harvested.
6) PY03A- Mice (10 per group) were injected subcutaneously with pyridostigmine (40 mg/kg) or physostigmine (11 mg/kg) or saline (Sham) for 15 min. The animals were sacrificed at the end of the experiment and tissues harvested.
7) Development of C-terminal peptide hormone sequencing by SELDI-TOF MS.

Results:

A) Sarin Experiments- X02A, X02C, X02D and X03A

Hypothalamic lysates from the shams and sarin-treated mice were analyzed using the acetylcholinesterase (AChE) assay and Western blots for AChE protein.

Multiple Injection Effects- Kinetic analysis revealed that the AChE activity in the hypothalamus was not affected by sarin (0.4 or 0.05 LD50-64 or 8 μg/kg) in multiple injection experiments (Figure 1-42). In contrast, a drastic change was seen when a larger dose of sarin was given (0.6 LD50-9.6 μg/kg) as an injection on day one, followed 24 hours later by a second injection after which the mice were sacrificed at 30 min- 4 hour time points (Figure 1-43). It was clear that the second injection of sarin caused a precipitous decrease in acetylcholinesterase activity at the 30 min, 1 hr, and 2 hr time points. By 4 hours after the second sarin injection, AChE activity was beginning to increase (Figure 1-43). When AChE protein was analyzed by Western blots, the AChE protein levels were increased only in the mice that received 3 injections of sarin (0.4 LD50-64μg/kg) (Figure 1-42). No changes in AChE protein were observed in mice receiving 0.05 LD50 (8 μg/kg) injections.

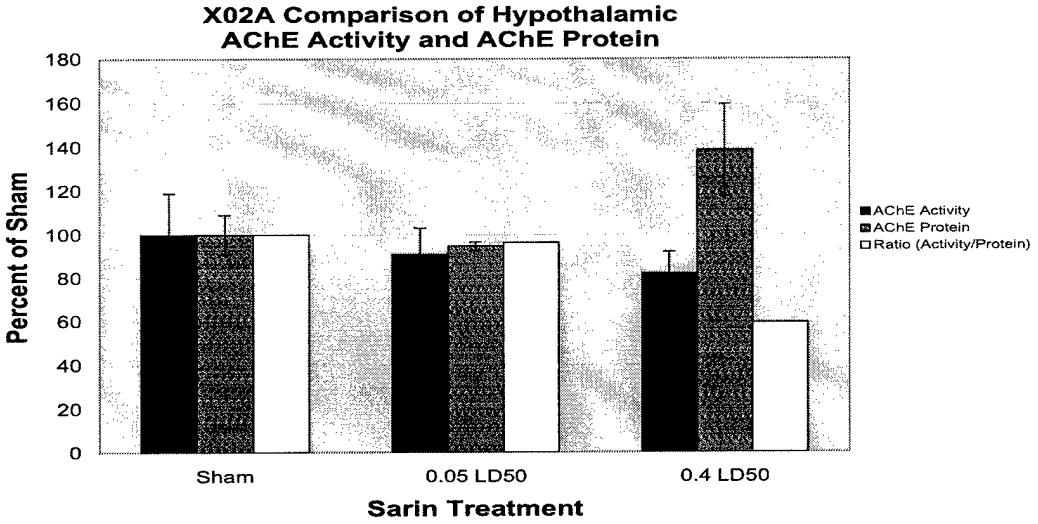


Figure 1-42. Comparison of hypothalamic AChE activity with AChE protein levels. Three injections of sarin at 0.4 and 0.05 LD50 (64 and 8 μg/kg) were given over three successive days.

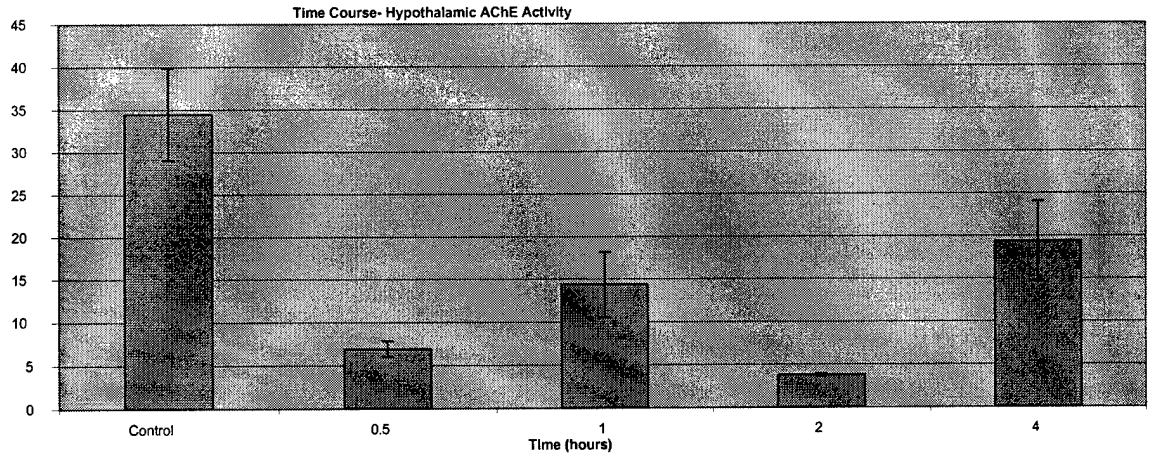


Figure 1-43: Time course for sarin effects on hypothalamic AChE activity. Mice were injected subcutaneously with sarin 2 times (0.6 LD50-9.6μg/kg) and sacrificed at 0.5, 1 2 and 4 hrs post injection.

The long-term effects of sarin were examined following 3 subcutaneous injections of sarin (0.4 LD50). Eight weeks after the final injection, tissue was collected from the mice and assayed for AChE activity. In hypothalamic tissue, but not cortex, the AChE activity was increased compared with shams (Figure 1-44). Likewise, for both tissues, the AChE protein levels as determined by semi-quantitative Western blots were also increased compared to shams.

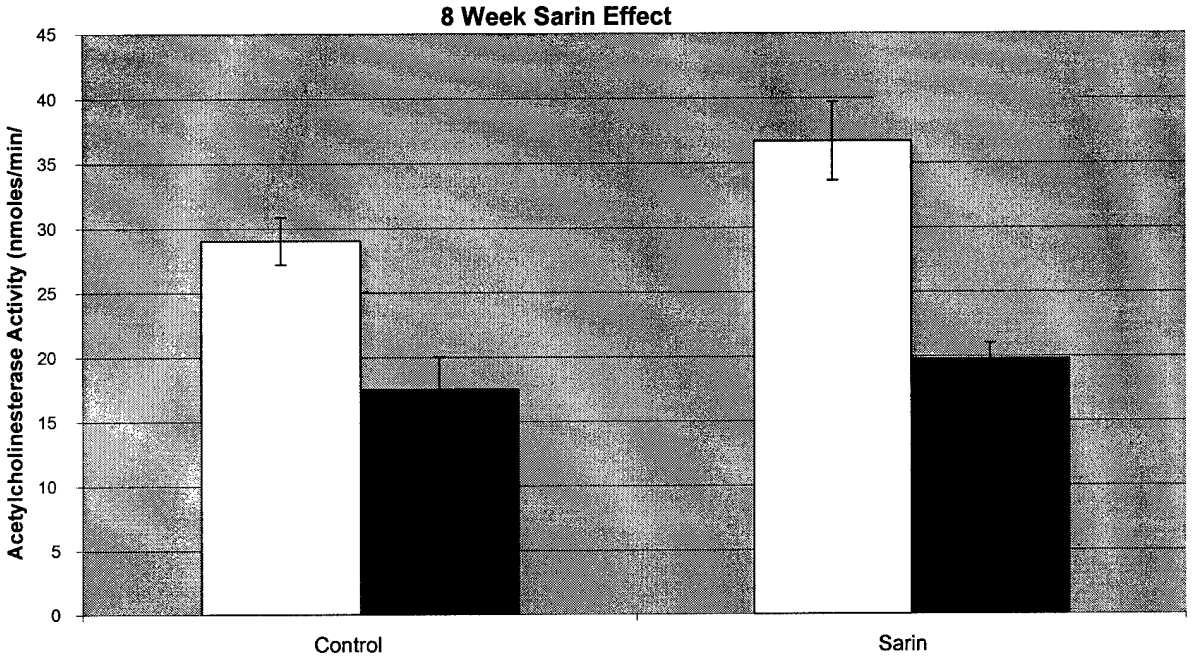


Figure 1-44. Long term effect of sarin on Hypothalamic and Cortex AChE activity. Eight weeks after injection of sarin, the mice were sacrificed and AChE activity was measured in the two tissues.

Single Injection Sarin Effects- To determine if there were acute effects of a single dose of sarin, a follow-up experiment was conducted in which a single injection of sarin (0.4 LD50-6.4μg/kg) was given. At 30 min, 4 hr, 24 hr 48 hr and 72 hr time points, tissue was collected and the AChE activity determined in hypothalamus, cortex and testes. There was no effect on AChE activity in any of the tissues (data not shown).

Summary and Conclusions from Sarin Experiments:

- No effect of multiple injections of sarin (0.05 or 0.4 LD50--.8 or 6.5μg/kg, respectively) on hypothalamic or cortex AChE activity measured 24 hours after last injection.
- Substantial inhibition of hypothalamic and cortex AChE activity after two injections of sarin (0.6 LD50-9.6μg/kg) measured 30 min to 4 hr after last injection.
- Increased AChE protein levels in hypothalamus and cortex of sarin treated mice (3 X 0.4 LD50-6.4 μg/kg).
- Increased AChE activity and protein levels in hypothalamic and cortex after extended period (8 weeks post multiple injection).

- No effect of a single injection of sarin (0.4 LD50-6.4 μg/kg) on AChE activity at 30 min – 72 hrs.
- Slight increase in AChE activity and protein levels 8 weeks after multiple injections of sarin (0.4 LD50-6.4 μg/kg).

We propose that the endogenous peripheral AChE acts as a "sink" for sarin, and prevents immediate effects in central tissues, i.e., hypothalamus and cortex. However, once a threshold has been passed, i.e., multiple injections of sarin, the amount of "free" peripheral AChE is less, thus allowing sarin to have more of an effect on AChE in peripheral and central tissues. A new experiment has been conducted to determine if this is the mechanism of action.

B) Acute and Subacute Effect of Pyridostigmine and Physostigmine on AChE Activity and Protein levels.

Acute Effects- Physostigmine is proposed to freely cross the blood brain barrier (BBB) while pyridostigmine is proposed to not cross the BBB, due to its being a quanternary amine. A direct comparison was made on the effect of pyridostigmine and physostigmine on acetylcholinesterase in these experiments. Mice were injected (subcutaneous) with pyridostigmine, physostigmine or saline for 15 minutes. The mice were then sacrificed and blood, hypothalamic and cortex tissue was removed and analyzed for AChE activity and protein levels. Pyridostigmine and physostigmine caused a significant and similar decrease in blood AChE activity at 15 min (Figure 1-45). Physostigmine but not pyridostigmine caused a decrease in cortex AChE Activity (Figure 1-46). Physostigmine and pyridostigmine both caused a similar decrease in hypothalamic AChE activity (Figure 1-46). AChE protein levels were not affected in hypothalamic or cortex tissue by either physostigmine or pyridostigmine.

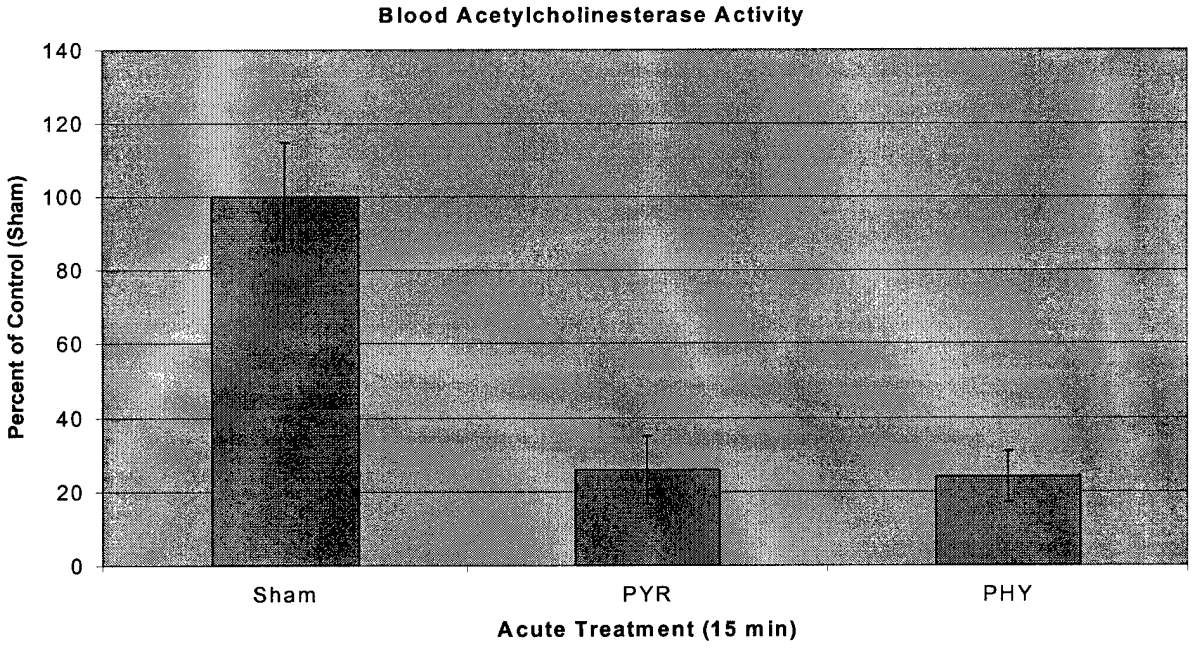


Figure 1-45. Effect of pyridostigmine or physostigmine on blood acetylcholinesterase activity. After acute treatment of blood with the drugs, blood acetylcholinesterase activity was measured.

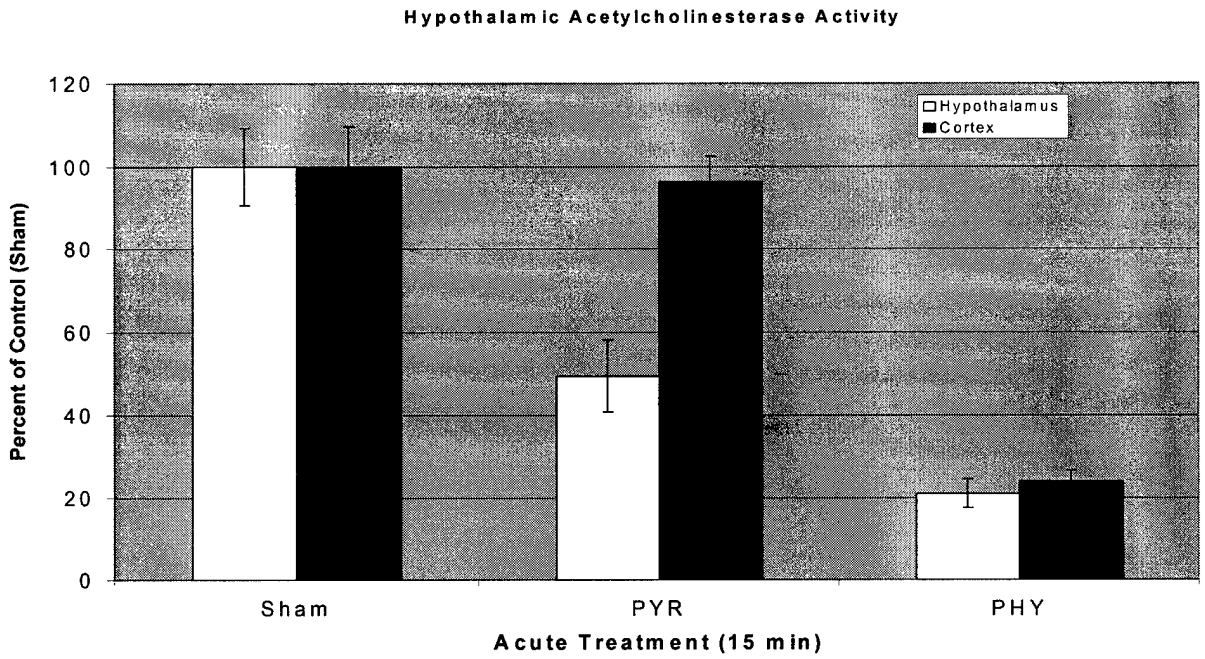


Figure 1-46. Effect of pyridostigmine or physostigmine on AChE activity in hypothalamic and cortex tissue. Due to differences in the AChE activity, values are compared with sham and reported as Percent of control (sham).

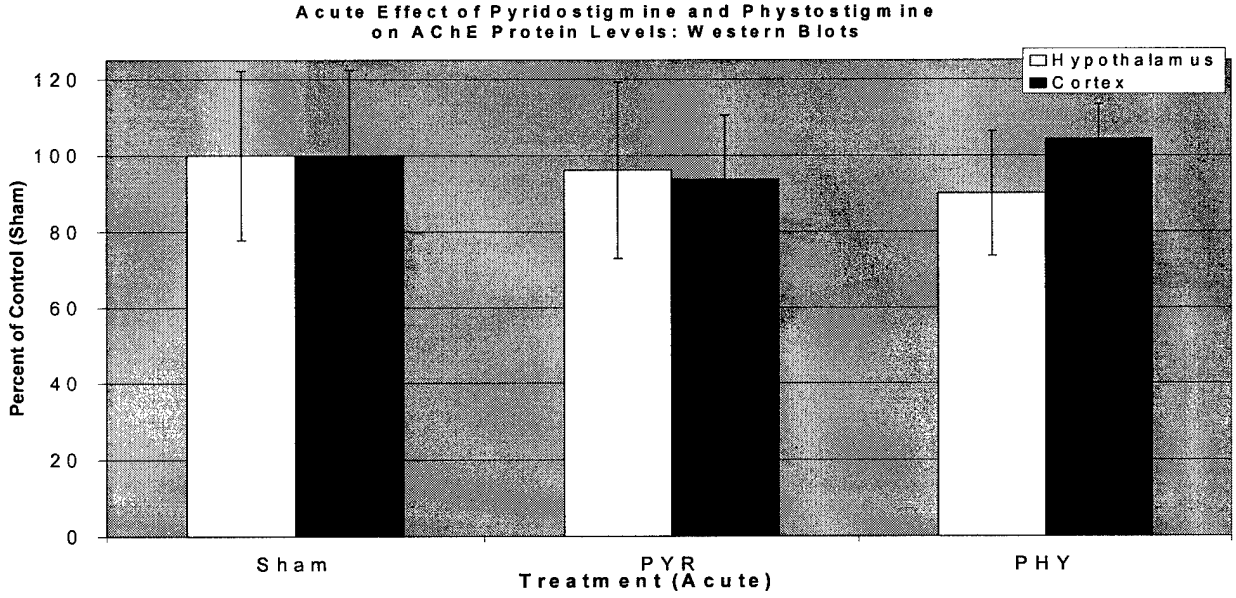


Figure 1-47. Effect of pyridostigmine and physostigmine on AChE protein levels. All AChE protein levels were determined by semiquantitative Western blots using a monoclonal Ab for the AChE and a polyclonal antibody for actin to normalize gel loading.

Subacute Effects- Mice were infused by Alzet mini-pumps for 7 days with saline, pyridostigmine (10 mg/kg/day) or physostigmine (2.88 mg/kg/day). At 7 days, the mice were sacrificed and AChE activity and protein was analyzed in blood, hypothalamus and cortex. There was a clear inhibition of AChE activity in the blood (Figure 1-48). In contrast, pyridostigmine caused an increase in AChE activity in hypothalamic tissue while physostigmine caused a decrease (Figure 1-49). However, in the cortex, physostigmine caused a decrease in AChE activity while pyridostigmine had no effect (Figure 1-49). AChE protein levels increased in the cortex from physostigmine-treated mice whereas pyridostigmine had no effect. Both pyridostigmine and physostigmine caused an increase in AChE protein levels in the hypothalamus (Figure 1-50).

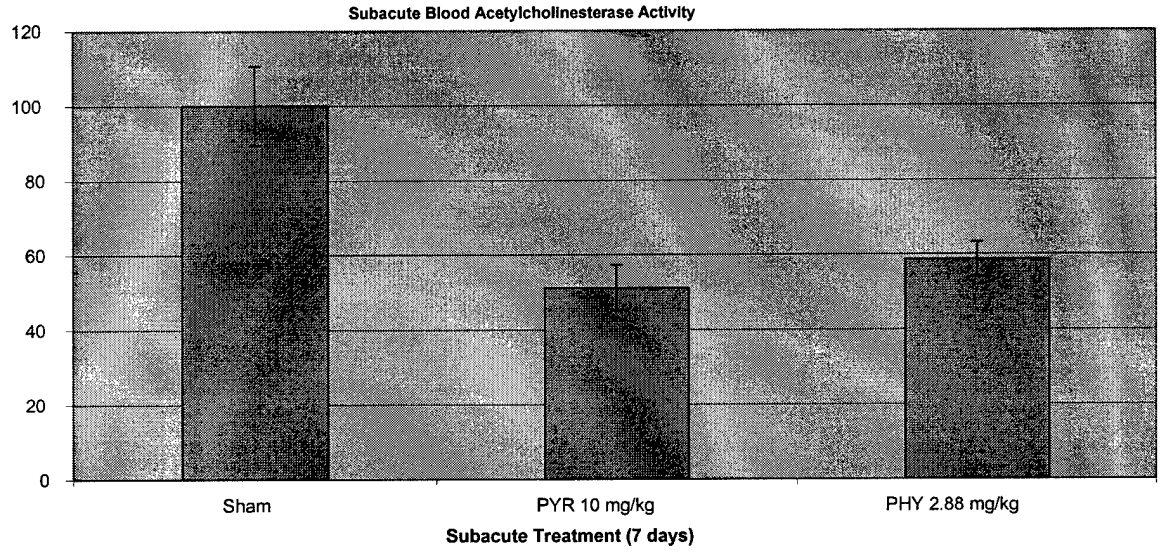


Figure 1-48: Subacute effect of pyridostigmine or physostigmine on blood AChE activity.

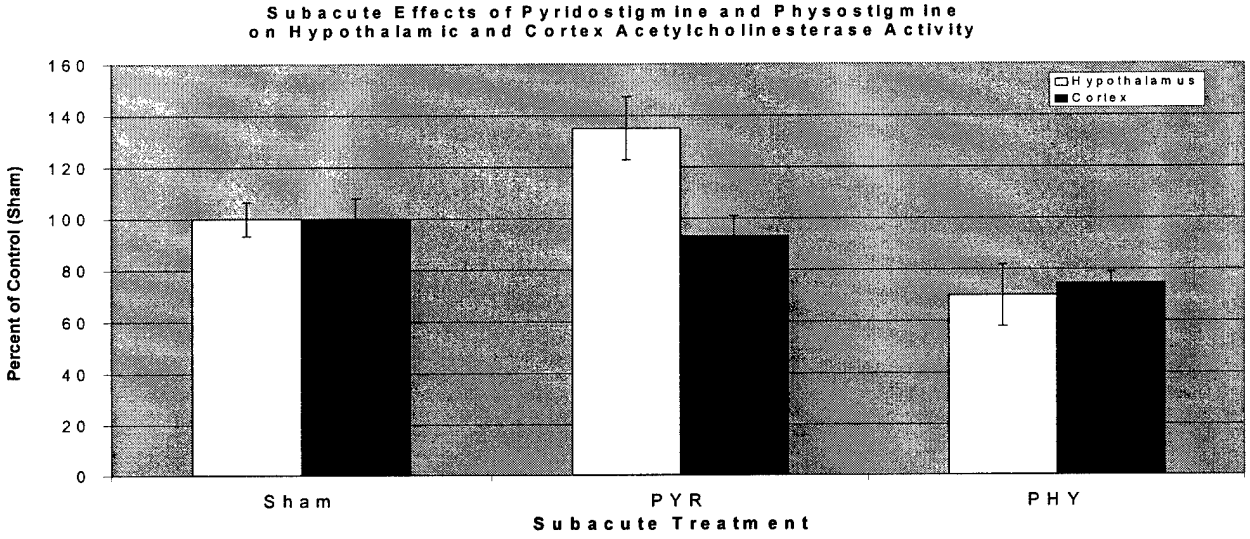


Figure 1-49. Effect of subacute effect of pyridostigmine and physostigmine on hypothalamic or cortex AChE activity.

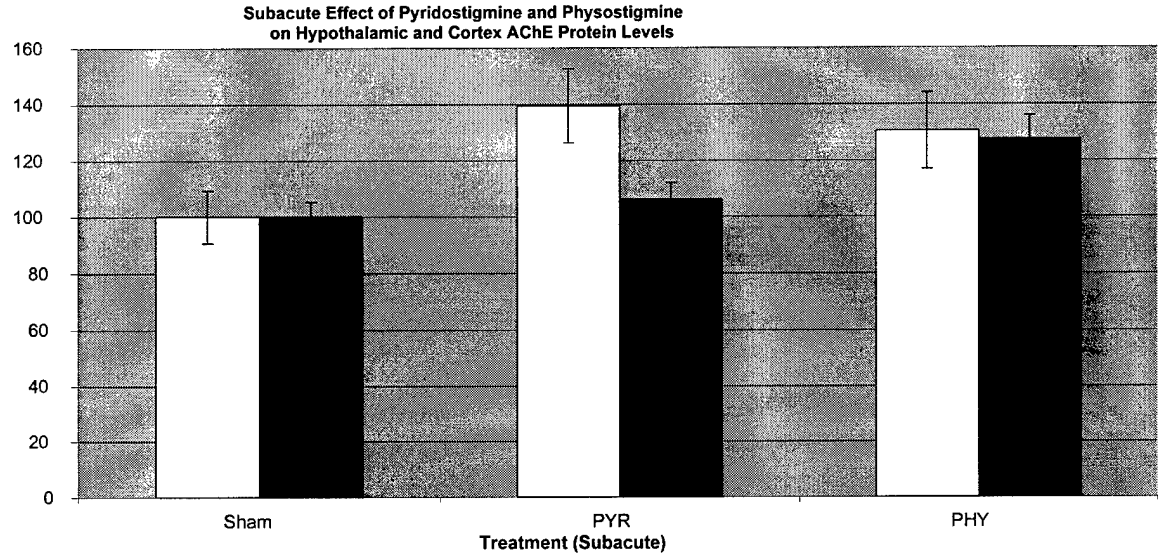


Figure 1-50. Effect of pyridostigmine and physostigmine on hypothalamic and cortex AChE protein levels. Tissues were collected after 7 days of treatment, separated by SDS-PAGE and probed with antibodies to AChE and actin. The relative amount of AChE was normalized to actin.

Summary and Conclusions from Pyridostigmine and Physostigmine Experiments-

- Acute treatment of the mice with pyridostigmine or physostigmine caused a decrease in AChE activity in blood and hypothalamus.
- Acute treatment with physostigmine but not pyridostigmine caused a decrease in cortex AChE activity.
- There was no acute effect of either drug on AChE protein levels.
- Subacute treatment with pyridostigmine caused a decrease in AChE activity in blood and an increase in hypothalamic AChE activity.
- Subacute treatment with physostigmine caused a decrease in AChE activity in the blood and cortex.
- Physostigmine but not pyridostigmine caused a decrease in cortex AChE activity.
- Both physostigmine and pyridostigmine caused an increase in AChE protein in the hypothalamus and cortex.

The inhibition of hypothalamic but not cortex AChE by pyridostigmine in the acute-treatment experiments strongly suggests that pyridostigmine is entering the hypothalamus but not the cortex. This is consistent with the hypothalamic region being more permeable to pyridostigmine than other areas of the brain. Subacute experiments showing an increase in AChE activity and protein also suggest that AChE is entering the hypothalamus to eventually cause upregulation of AChE.

B) Proteomic Analysis: The experiments outlined in the preceding section suggest that pyridostigmine is entering the hypothalamus. Since this region regulates the function and response of the pituitary, we have been interested in identifying changes in peptide hormones in these tissues. As part of the general proteomic analysis, the same 50 peaks in each of the sample have been analyzed by mass spectrometry, the integrated area of each peak annotated and stored in a database. This is currently being analyzed using bioinformatics programs that can search for pattern changes and can identify changes in expression or linkage of different proteins. Also of particular interest in this project is the analysis of the C-termini of peptide hormones, since most peptide hormones are generated by cleavage of the prohormone at specific paired-basic residues followed by removal of the basic residues and modification of the peptides. Thus, an accurate analysis of the C-terminus would provide more insight into the identity of the peptide than the N-terminus. We have developed a method for sequencing peptides in both simple and complex mixtures using the unique proteinchip properties of the Ciphergen proteinchip Array.

For sequencing peptides, 1 μg of peptide hormone, i.e., $ACTH_{1-39}$, was diluted in deionized H_2O to 100 ng/μl. From this, 1 μl was applied to a spot on an H4 (hydrophobic) proteinchip (Ciphergen, Palo Alto, CA) and air-dried. The proteinchip was placed in a "moist chamber", i.e., pipet tip box (with lid) containing a wet paper towel. For the sequencing reaction, CPY (Pierce) (30 units/μl) was diluted to 2.4×10^{-3}, 1.2×10^{-3} and 8×10^{-4} units/μl in 50 mm MES, ph 6.5. One microliter of each dilution was added to each spot on the proteinchip and the proteinchip placed in a moist chamber at 37°C for 5 min. After incubation, 1 μl of a supersaturated solution of matrix, alpha-cyano-4-hydroxy cinnamic acid (CHCA) in 50% acetonitrile containing 0.1% trifluoroacetic acid, was added to each spot to stop the reaction. The proteinchip was analyzed in a Ciphergen PBSII proteinchip reader.

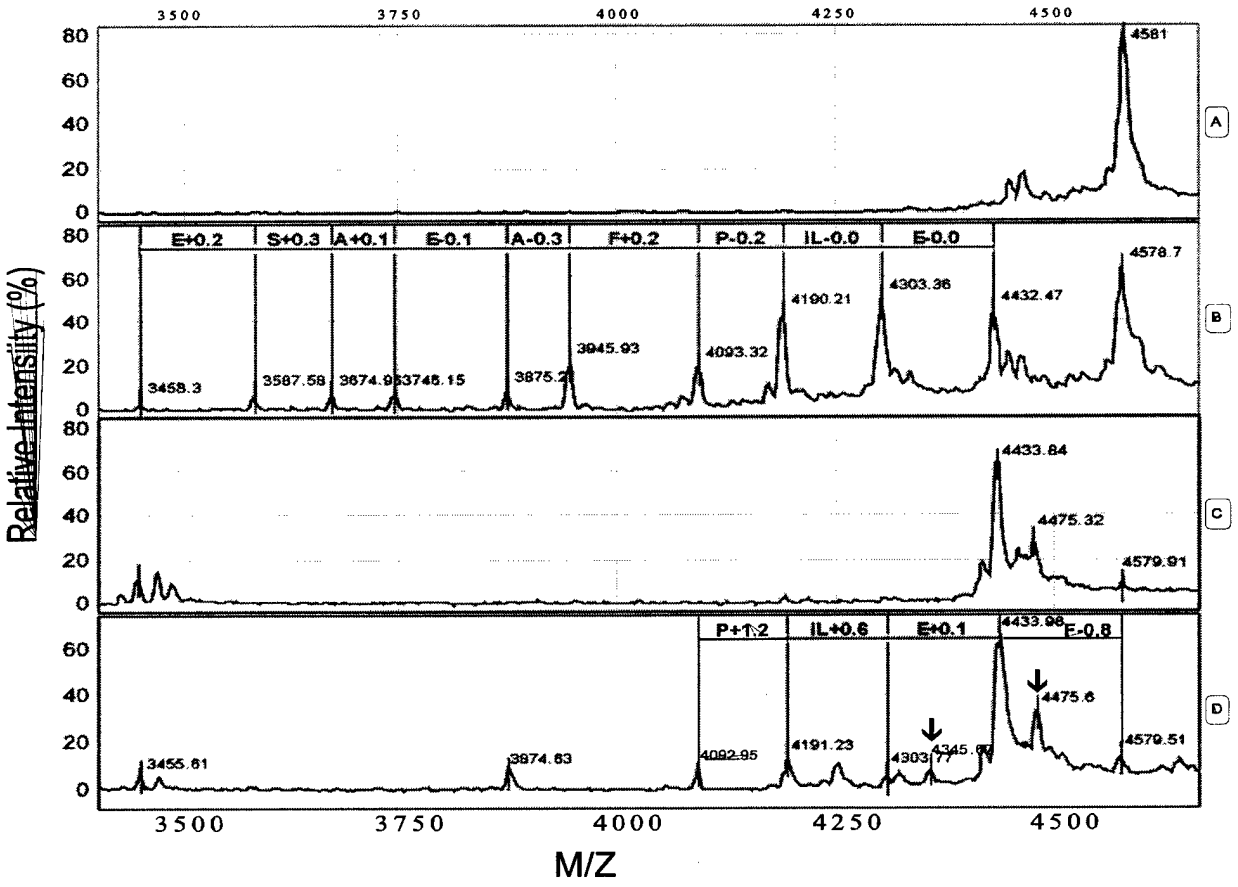
The amino acid sequence for the C-terminus of $ACTH_{1-39}$ (Figure 1-51A) was derived using this protocol (Figure 1-51B). For the 2.4 X 10^{-3} dilution of CPY, the spectra revealed a "ladder" of 11 ion peaks beginning with the original peak for $ACTH_{1-39}$ (4578.7 d). Each successively smaller ion peak represents the removal of an amino acid from the C-terminus of the peptide by CPY. The difference in mass for each peak represents the mass of an amino acid. The single-nomenclature amino acid sequence identified by the Ciphergen 3.1 software was esaeafpi/lef (Figure 1-51B). Since isoleucine and leucine have the same mass (~113.1 d) the Ciphergen software identified the third amino acid from the C-terminus as both isoleucine and leucine. The amino acid sequence generated on the proteinchip for ACTH was identical to that of the known sequence, i.e., ESAEAFPLEF. Further dilution of CPY yielded similar results, the only exception being that only 9 and 5 amino acids could be easily identified at 1:50 and 1:75 dilutions, respectively. Longer incubation times or a higher concentration of CPY resulted in generation of smaller fragments, i.e., 20-26 amino acids long.

The analysis of complex samples, e.g., pituitary homogenates, is made more difficult due to the presence of other peptides and proteins. However, in areas without other ion peaks close-by, some limited sequence data can be obtained. Mouse neuro-intermediate pituitary tissue was homogenized in 0.1 M HCl, one microgram of protein was added to a hydrophobic H4 proteinchip and 1 µl of CPY (0.0024 units/µl) added in the moist chamber. The protein chip was scanned in the Ciphergen proteinchip reader by Surface Enhanced Laser Desorption/Ionization-Time-of-Flight mass spectrometry (SELDI-TOF MS). A separate spot was analyzed without CPY digestion to determine the original ion peaks for comparison.

After digestion with CPY (Figure 1-51D), several new ion peaks were formed that were be identified as ACTH1-39, 1-38, 1-37, 1-36 and 1-35 (Figure 1-51D), based on comparison with the ion peaks generated from synthetic $ACTH_{1-39}$ treated with CPY. A sixth and seventh ion peak were identified as having masses identical to $ACTH_{1-33}$ and $ACTH_{1-31}$ (3874.83 and 3455.61 daltons, respectively). The C-terminal amino acid sequence determined after treatment with CPY was similar to the sequence derived with the standard in Figure 1B, i.e., Pi/LEF. In addition, two interesting ion peaks were observed after CPY digestion, at 4345 and 4475 daltons. These represent a 42-dalton increase in mass compared to ion peaks identified as ACTH-derived. This 42-dalton difference corresponds to the mass of an acetyl group and suggests that these peptides are acetylated forms of ACTH.

Conclusions: Advanced proteomic analysis of simple and complex mixtures of peptide hormones is critical to understanding the physiological role that prohormone processing enzymes and peptide hormones play in regulating the endocrine system in the body. By using "on-chip" C-terminal amino acid sequencing with CPY enzyme digestion followed by SELDI-TOF MS analysis, we provide evidence that limited peptide sequence data can be derived from complex mixtures of peptides. Changes in proteins identified by the bioinformatics analysis will be compared with peptides sequenced using this technique. In addition, we are in the process of developing a protein purification protocol using HPLC to fractionate proteins directly to the proteinchips. In this manner, we will be able to sequence specific proteins "in mass" from a particular tissue.

Figure 1-51. C-terminal sequencing of peptides using CPY enzyme digestion. ACTH1-39 (A) was sequenced in panel B using CPY enzyme. Panel C shows pituitary extracts with the sequencing of a possible ACTH1-39 peak. Arrows indicate acetylated ACTH.



CARDIOVASCULAR/ENDOCRINE

Investigators: Mariana Morris, Ph.D., Iveta Bernatova, Ph.D., Vera Farah, Ph.D. and Luis F Joaquim, M.S.

The objectives of this phase of the investigation were the following: 1) complete the cardiovascular studies on pyridostigmine and stress interactions 2) setup and characterize the radiotelemetry method for long term measurements of blood pressure and heart rate in mice 3) develop the methodology for the spectral analysis of cardiovascular parameters, including characterization of the response to stress and stress/pyridostigmine exposure 4) conduct studies on the influence of stress in an animal model of altered stress responsiveness 5) begin studies on the effect of sarin exposure on cardiovascular parameters and 6) present and publish the data. We have had success on most of these objectives as described below.

KEY ACCOMPLISHMENTS:

- Completed studies to determine the effect of combined exposure to stress and pyridostigmine in mice. The results show that there are no global changes in blood pressure or heart rate after treatment. However, spectral analysis showed dramatic alterations in heart rate variability and baroreflex index in the combined stress/pyridostigmine group. This has important clinical implications since reduced variability is associated with cardiovascular diseases. A paper is in preparation that will be submitted to Nature.

- Completed studies to determine the day/night time course of stress responsiveness (corticosterone, oxytocin and brain c-fos expression). Results showed that there were pattern in stress responsiveness that were similar to that seen for the cardiovascular system.

- Determined the pattern of blood pressure and heart rate variability produced in response to acute and chronic stress. Results show that acute stress produces an increase in pulse interval (PI) and arterial pressure variability, while chronic stress produced a decrease in variability. This is important since it suggests that chronic stress modulates the cardiovascular system in a manner that may be detrimental to health.

- Determined the autonomic mechanisms that come into play in the response to stress. We evaluated the influence of the autonomic nervous system in the cardiovascular responses to stress. The cholinergic blockade increase the heart rate but reduced stress-induced tachycardia and blocked the increase in PI variance. Atropine also markedly reduced the LF and HF components of the PI variability. The α-sympathetic blockade by prazosin abolished the stress-induced hypertension and blocked the increase in PI and SAP variabilities. Prazosin also reduced the LF component of the PI and SAP variabilities.

- Implemented the method for telemetric measurement of blood pressure and heart rate in conscious mice.

- Determined the effect of deletion of the oxytocin gene on the response to stress. Results showed that the stress induced cardiovascular changes were greater in the oxytocin deletion model. This work has been submitted for publication.

- Presented eight abstracts, published two papers, submitted two papers (one in press) and have two papers in preparation.

1. Time related changes in stress reactivity

1a. Cardiovascular response to acute stress

Radiotelemetry: A specialized laboratory was established for testing of cardiovascular responses using radiotelemetric methods for monitoring blood pressure (BP) and heart rate (HR). With the telemetric method, a catheter is inserted into the carotid artery and used for measurement of BP and HR in freely moving mice. The advantage is that the catheter is viable for long periods. The system can be used for measurement of cardiovascular parameters under control and stress conditions. The sampling rate can also be adjusted for high frequency sampling as required for the spectral analysis.

Studies were conducted to determine the effect of acute stress on cardiovascular parameters. C57B1 mice were prepared with arterial telemetric catheters and studied under baseline conditions (30 min) and after cessation of an acute bout of shaker stress (5 min, 150 cycles/min). The animals were allowed to recover from surgery for at least 7 days. Arterial blood pressure (AP) was recorded at 5Kz. Results showed that acute stress increases the AP (27%) and heart rate (26%) (Figure 1-52).

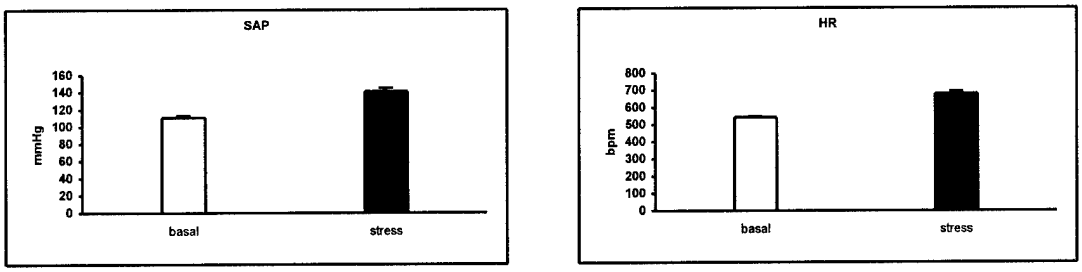


Figure 1-52: Systolic arterial pressure (SAP) and heart rate (HR) in mice before and after acute stress. Stress increased significantly the SAP and the HR (ANOVA repeated measures). Values are mean ± SEM

For a more detailed analysis of cardiovascular parameters, spectral analysis offers a useful tool for the measurement of blood pressure and heart rate variability. It is well known that reduced HR variability is associated with cardiovascular pathologies. We have chosen to use the autoregressive method, which applies a statistical function to processed arterial pressure data. Results showed that acute stress promotes an increase on the pulse interval (PI) variability with changes in both spectral components: low (LF, 0.1-1.0 Hz) and high (HF, 1-5 Hz) frequency ranges (Figs. 1-53 and 1-54). The acute stress also promotes an increase in the SAP variability with changes on LF component (Fig 1-54).

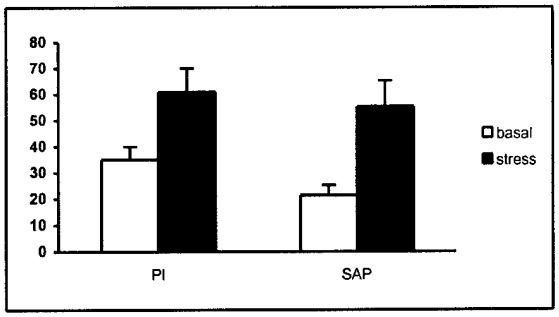


Figure 1-53. Pulse interval (PI, ms^2) and systolic arterial pressure (SAP, $mmHg^2$) variabilities in mice before and after stress. Stress increased significantly the PI and SAP variabilities (ANOVA repeated measures). Values are mean ± SEM. N = 6.

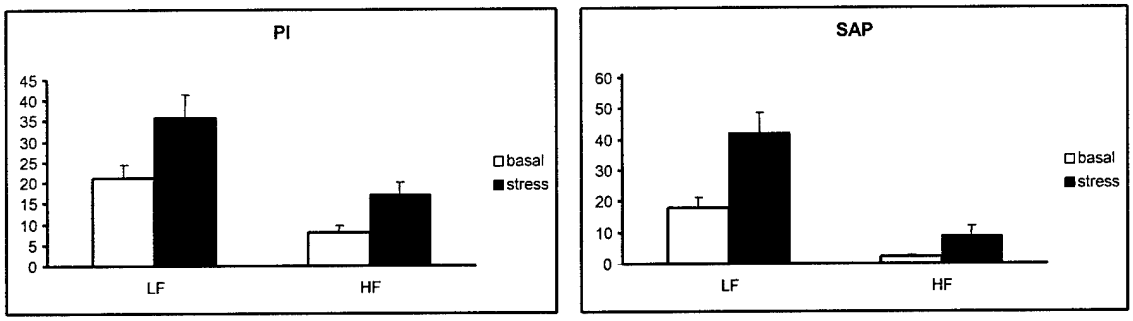


Figure 1-54. Low-frequency (LF) and high-frequency (HF) components of the pulse interval (PI) and systolic arterial pressure (SAP) variabilities in mice before and after stress. Stress increased significantly the LF and HF components of the PI variability but only LF component of the SAP variability (ANOVA repeated measures). Values are mean ± SEM

1b. Cardiovascular responses to chronic shaker stress

A similar study was conducted to test the effect of chronic stress. For this study C57Bl mice that were prepared with chronic carotid arterial catheters according to the method of Li et al. (Li P et al., Am J Physiol 1999; 276:R500-R504). Arterial pressure were recorded with a sampling rate of 4Kz.The specially designed caging system was attached to a shaking device. The shaking device was programming to provide shaker stress over the period of 24h for 3 days.

Stress responses of arterial pressure and heart rate to individual shaking sessions were evaluated on day 1 (before stress) and on day 3 (10 min after the last stress session). Results showed no significant differences in arterial blood pressure:117±5 vs 112±1 mmHg and heart rate 529±13 vs 589±18 bpm (Fig 1-55).

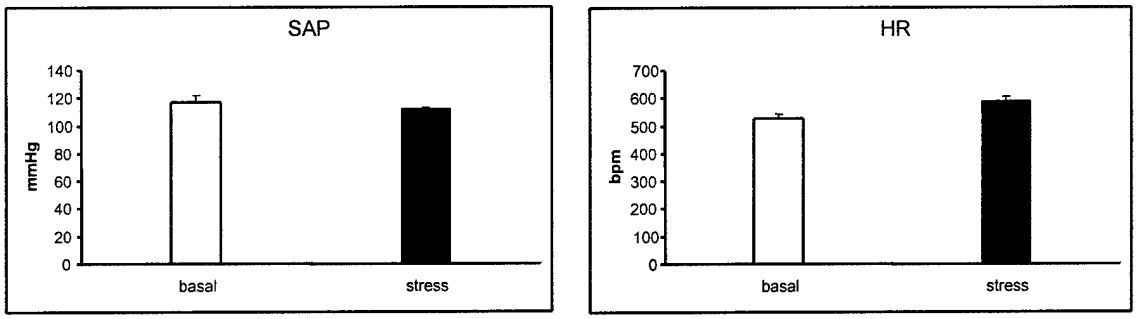


Figure 1-55. Systolic arterial pressure (SAP) and heart rate (HR) in mice before and after chronic stress (3days). Stress does not changes significantly the SAP but the HR was not increased after stress (ANOVA repeated measures). Values are mean ± SEM

Using spectral approaches we evaluated the SAP and PI variabilities before and after chronic stress. Results showed no difference in PI variability, however the SAP variability and its LF oscillations were decreased after chronic stress (Fig 1-56 and 1-57).

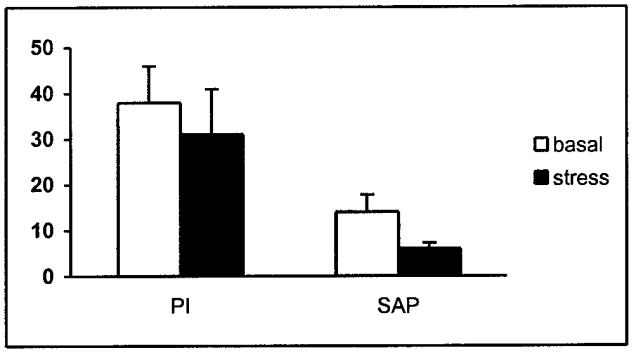


Figure 1-56. Pulse interval (PI, ms^2) and systolic arterial pressure (SAP, $mmHg^2$) variabilities in mice before and after chronic stress. Stress decreases significantly the SAP variability (ANOVA repeated measures). Values are mean ± SEM

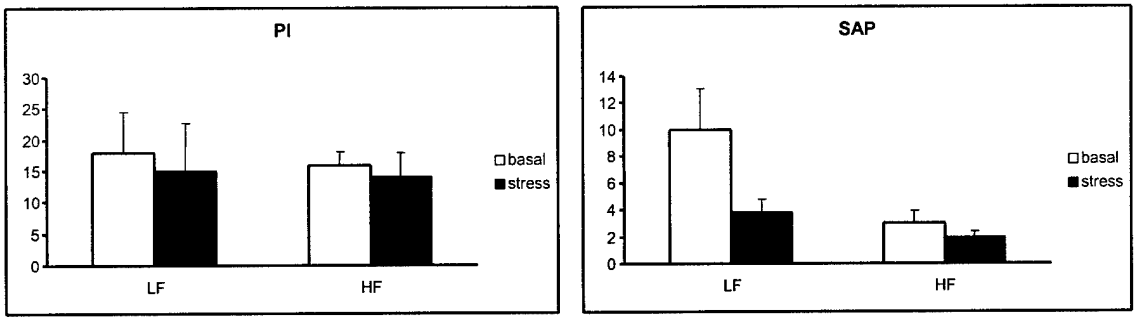


Figure 1-57. Low-frequency (LF) and high-frequency (HF) components of the pulse interval (PI) and systolic arterial pressure (SAP) variabilities in mice before and after chronic stress. Stress decreases the HF component of the SAP variability (ANOVA repeated measures). Values are mean ± SEM

2. Autonomic Control of the Cardiovascular Response to Stress

It is well known that loss of normal autonomic neural control of heart rate and rhythm is an important risk factor of cardiovascular events, including sudden death. Heart rate variability and baroreflex sensitivity are used to assess the activity of the autonomic nervous system. Using pharmacological interventions it is possible to block the autonomic neural control of circulation.

Radiotelemetric recording with autoregressive spectral analysis was used to study the cardiovascular responses under baseline conditions and after stress (one single session of shaker stress, 5min, 150 rpm), autonomic blockade and autonomic blockade/stress. Arterial pressure was recorded in C57/Bl mice at 5 kHz. Systolic arterial pressure and pulse interval were submitted to spectral analysis with variability measured in the low (LF, 0.1-1.0 Hz) and high (HF, 1-5 Hz) frequency ranges. Differences were evaluated by ANOVA 2-way (treatment x stress).

2a. Cardiovascular responses before and after cholinergic blockade:

The cholinergic blockade was performed by administration of Atropine (4mg/Kg, ip).

Stress increased the systolic arterial pressure from 111±2 to 141±4 mmHg and the heart rate from 541±8 to 680±15 bpm (Fig 1-58). The cholinergic receptors blockade by atropine caused a small increase in the heart rate (59 bpm) and no change in arterial pressure. Cholinergic antagonism reduced stress-induced hypertension and tachycardia. (~58%).

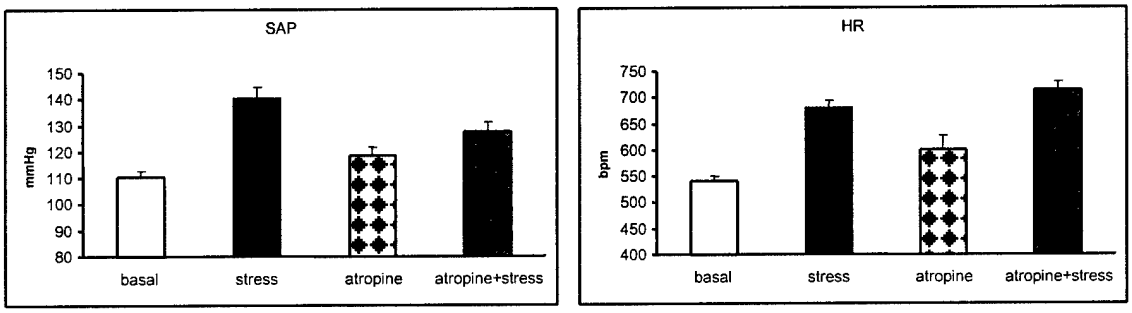


Figure 1-58. Effect of cholinergic blockade on systolic arterial pressure (SAP) and heart rate (HR). Atropine increases the HR with no change in the SAP. The Cholinergic antagonism reduced the stress-induced tachycardia and hypertension (ANOVA 2-way). Values are mean ± SEM

The systolic arterial pressure variance and pulse interval variance also increased after stress: 34 $mmHg^2$ and 26 ms^2, respectively (Fig 1-59). Atropine markedly reduced the PI variance (83%) with reducing the LF (~95%) and HF (~63%) components but no change in systolic arterial pressure variance (Fig 1-60 and 1-61). The cholinergic antagonism also blocked the increase in PI variance after stress.

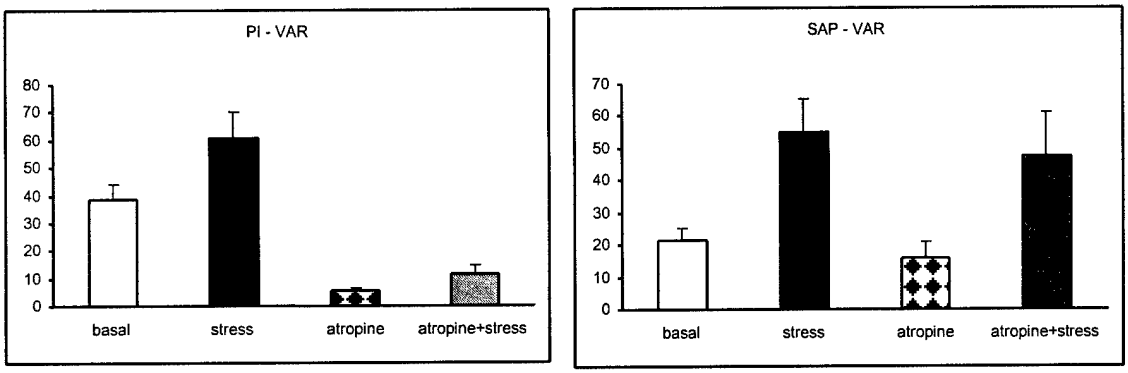


Figure 1-59. Effect of cholinergic blockade on pulse interval (PI, ms^2) and systolic arterial pressure (SAP, $mmHg^2$) variabilities. The cholinergic antagonism reduced the PI variance and blocked the stress-induced increase in PI variance. (ANOVA 2-way). Values are mean ± SEM

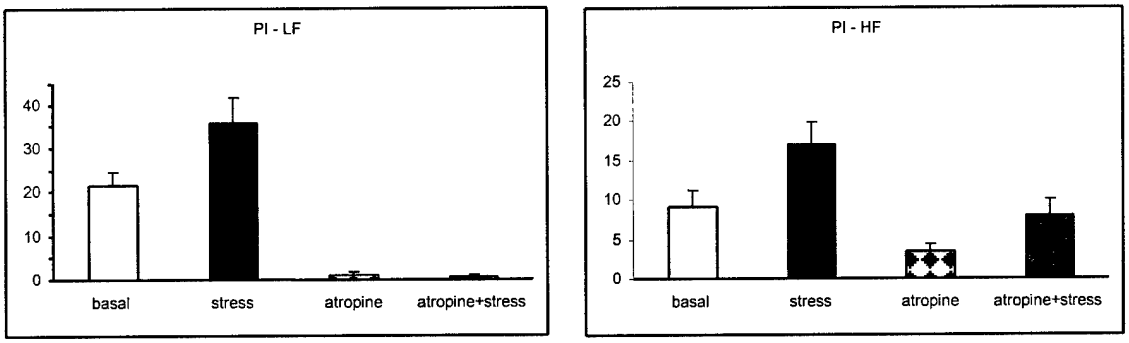


Figure 1-60. Effect of cholinergic blockade in the low (LF) and high (HF) frequency ranges of the pulse interval (PI) variability. The cholinergic antagonism reduced the LF and HF components of the PI variance and blocked the stress-induced increase in LF. (ANOVA 2-way). Values are mean ± SEM

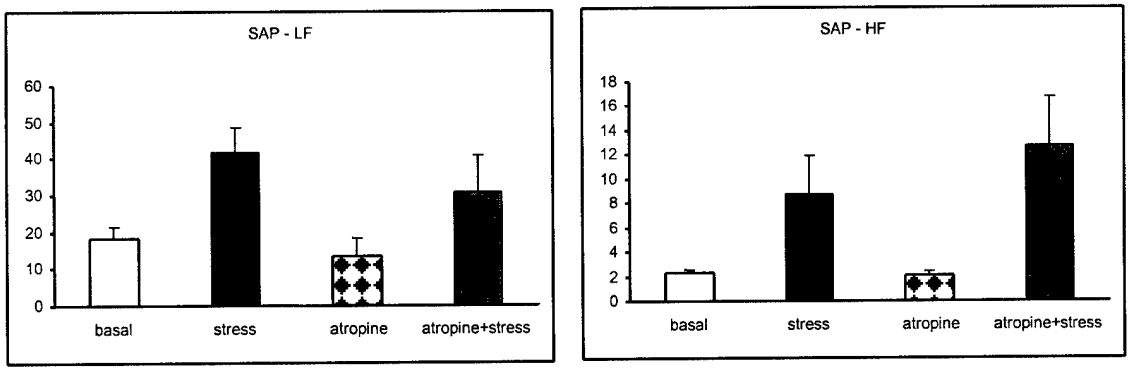


Figure 1-61. Effect of cholinergic blockade in the low (LF) and high (HF) frequency ranges of the systolic arterial pressure (SAP) variability. The cholinergic antagonism no changed the LF and HF components of the SAP variance. (ANOVA 2-way). Values are mean ± SEM

2b. Cardiovascular responses before and after sympathetic blockade:

The α-sympathetic blockade was performed by Prazosin (1mg/Kg, ip).

The α-sympathetic receptors blockade by prazosin caused a small decrease in the systolic arterial pressure (10 mmHg) and increase in heart rate (Fig 1-62). Sympathetic antagonism blocked stress-induced hypertension.

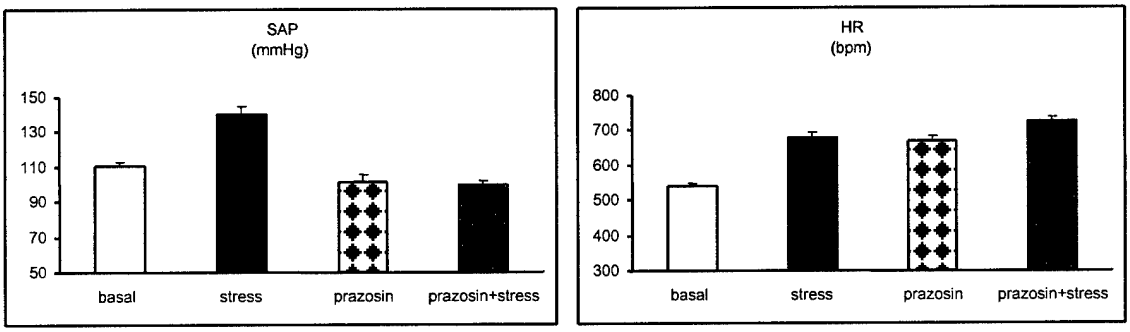


Figure 1-62. Effect of α-sympathetic blockade on systolic arterial pressure (SAP) and heart rate (HR). Prazosin reduced the SAP and increases the HR. The sympathetic antagonism blocked the stress-induced hypertension (ANOVA 2-way). Values are mean ± SEM

Prazosin markedly reduced the PI variance (77%) with reducing the LF (~95%) component (Figs 1-63-1-65). The sympathetic blockade also diminishes the SAP variance (57%) with reducing the LF (~78%) component. The α-sympathetic antagonism also blocked the increase in PI and SAP variance after stress.

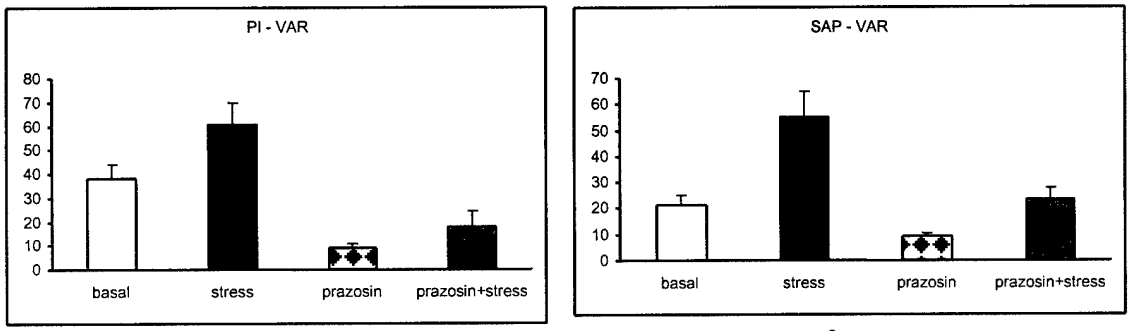


Figure 1-63. Effect of α-sympathetic blockade on pulse interval (PI, ms^2) and systolic arterial pressure (SAP, $mmHg^2$) variabilities. The sympathetic antagonism reduced the PI and SAP variabilities and abolished the stress-induced increase in PI and SAP variabilities. (ANOVA 2-way). Values are mean ± SEM.

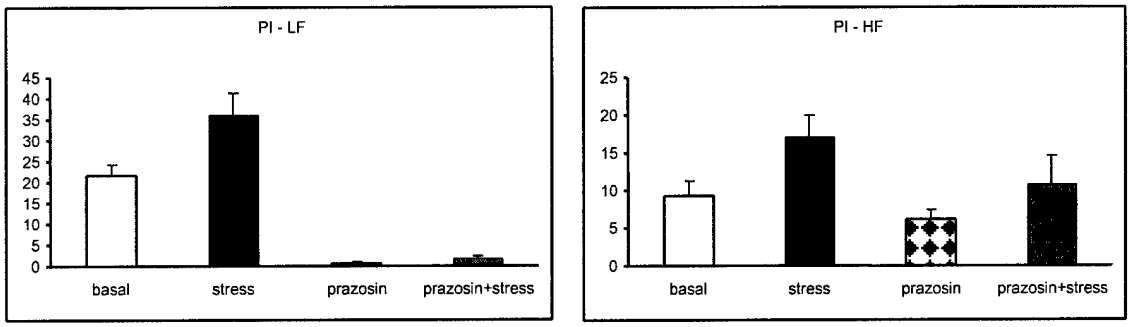


Figure 1-64. Effect of α-sympathetic blockade in the low (LF) and high (HF) frequency ranges of the pulse interval (PI) variability. The sympathetic antagonism reduced the LF component of the PI variance and blocked the stress-induced increase in LF. (ANOVA 2-way). Values are mean ± SEM

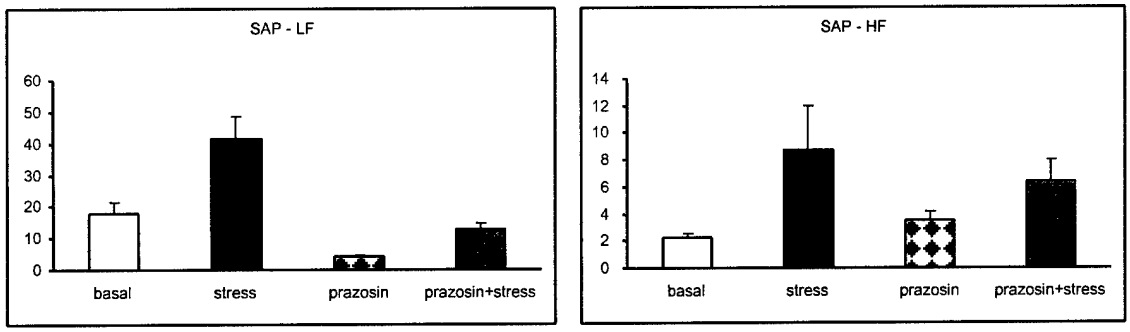


Figure 1-65. Effect of α-sympathetic blockade in the low (LF) and high (HF) frequency ranges of the systolic arterial pressure (SAP) variability. The sympathetic antagonism reduced the LF component of the SAP variance and blocked the stress-induced increase in LF. (ANOVA 2-way). Values are mean ± SEM

3. Stress/PB interactions

Experiments were performed in CB57Bl mice with chronic arterial catheters. The mice were treated for 3 days with PB (10mg/Kg/day, osmotic minipumps), stress (intermittent shaker stress, SS) or stress plus PB. Arterial pressure was recorded (5KHz) under basal condition and after treatment. Beat-to-beat time series of arterial pressure and pulse interval were generated, with evaluation of variability using autoregressive spectral analysis for low and high frequency domains (LF:0.1-1.0 Hz; HF: 1.0-5.0 Hz). There were no differences in cardiovascular parameters among groups before treatments (Fig 1-66). The results showed no differences in arterial pressure. Treatment caused a significant tachycardia only in SS group.

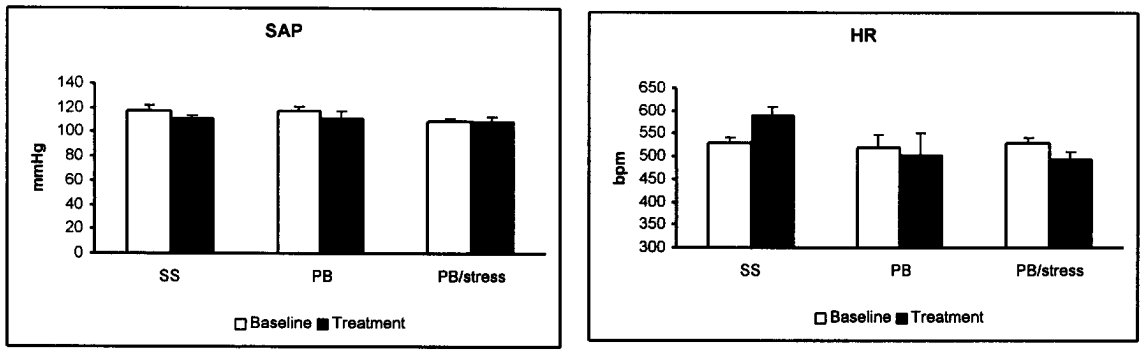


Figure 1-66. Systolic arterial pressure (SAP) and heart rate (HR) in mice treated for 3 days with PB (PB), stress (SS) or stress plus PB (PB/stress) under basal conditions and after treatment. Treatment caused a significant tachycardia only in SS group (ANOVA repeated measures). Values are mean ± SEM.

Using spectral approaches we evaluated the SAP and PI variabilities before and after treatment. Results showed that PI variability and its LF oscillations were markedly increased only in the PB/stress group (Fig 1-67-1-69). There are no differences in SAP variability and their components after treatment.

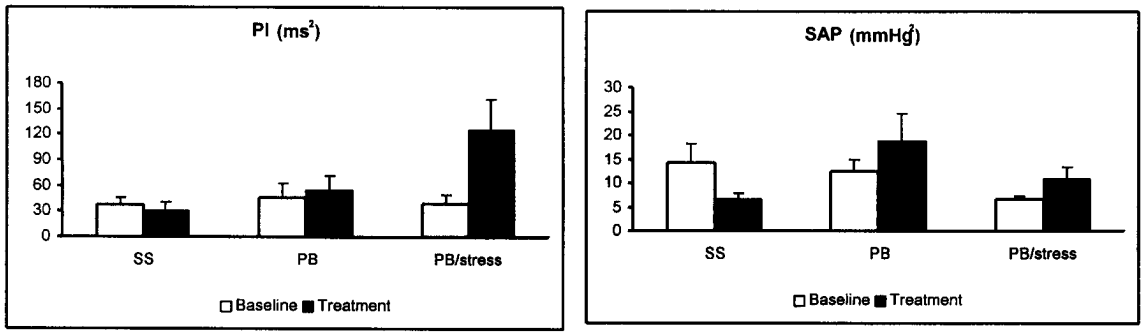


Figure 1-67. Pulse interval (PI) and systolic arterial pressure (SAP) variabilities in mice treated for 3 days with PB (PB), stress (SS) or stress plus PB (PB/stress) under basal conditions and after treatment. PI variability was increased in the PB/stress group (ANOVA repeated measures). Values are mean ± SEM.

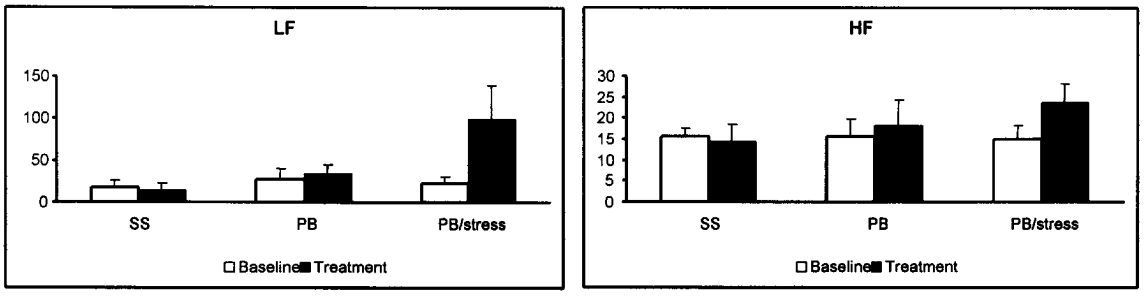


Figure 1-68. Low (LF) and high (HF) frequency ranges of the pulse interval variability in mice treated for 3 days with PB (PB), stress (SS) or stress plus PB (PB/stress) under basal conditions and after treatment. The LF oscillations were increased in the PB/stress group (ANOVA repeated measures). Values are mean ± SEM.

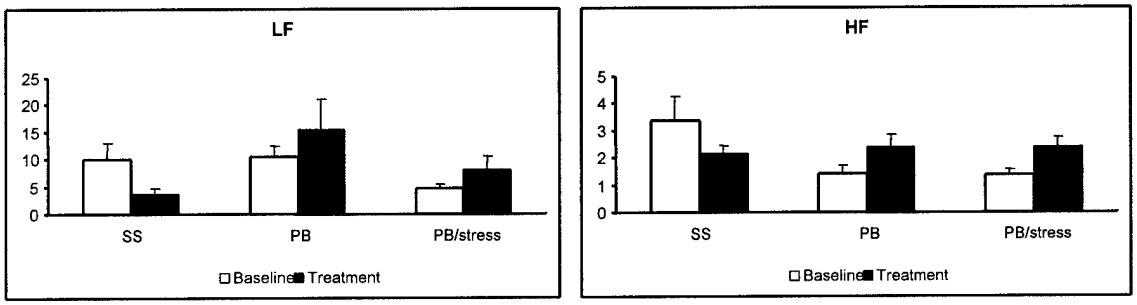
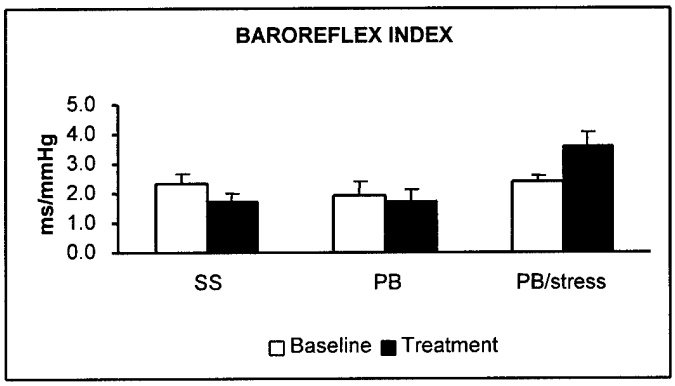


Figure 1-69. Low (LF) and high (HF) frequency ranges of the systolic arterial pressure variability in mice treated for 3 days with PB (PB), stress (SS) or stress plus PB (PB/stress) under basal conditions and after treatment. There were no differences after treatment (ANOVA repeated measures). Values are mean ± SEM.



Cross-spectral analysis was applied between arterial pressure and pulse interval spectra for calculation of baroreflex index. Baroreflex index was augmented only in PB/stress group: 2.4±0.3 vs 3.6±0.5, ms/mmHg.

Figure 1-70. Baroreflex index in mice treated for 3 days with PB (PB), stress (SS) or stress plus PB (PB/stress) under basal conditions and after treatment. There was a difference only in PB/stress group after treatment (ANOVA repeated measures). Values are mean ± SEM.

4. Diurnal Rhythm in Stress Responsiveness

Experiments were conducted to determine the nature of the day/night responses in C57/Bl mice. The rationale was based on published studies that showed a day/night rhythm in cardiovascular responses (Bernatova et al., Hypertension 40:768, 2002). Intermittent shaker stress delivered 3 days (2-min sessions, 45 sessions/day, 150cpm) and the stress responses evaluated at 0900 or 1900h. Plasma oxytocin and corticosterone (cort) levels determined by radioimmunoassay. C-fos determined by immunocytochemistry using Ni-DAB. Stressed and control mice were decapitated with collection of blood for cort and oxytocin measurements and brains (fixed in

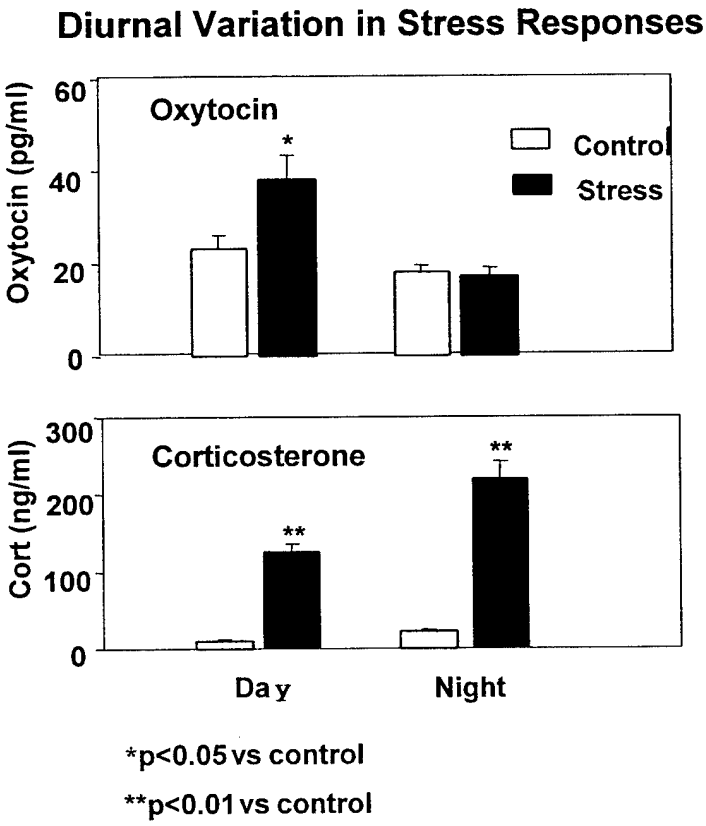


Figure 1-71. Day/Night rhythm in the endocrine responses to chronic stress. ** = $p < 0.01$ from control.

paraformaldehyde) for c-fos staining. Results showed that there was an enhanced oxytocin response during the day while the cort response (% change) was similar between the day and night (Fig 1-71). C-fos expression is observed consistently in the suprachiasmatic nucleus (SCN), with greater expression in the light phase. There was no difference in the SCN responses between stressed and control mice. There is little activation of the paraventricular nucleus (PVN) neurons in response to chronic stress in contrast to the response in acute stress.

Sarin effects on central neural function – a genomic approach

Investigator: James McDougal, Ph.D.

Experiments are underway for the investigation of the effects of sarin on central nervous system function. Mice have been dosed with sarin (2 sc injections, 0.4 LD_{50}) and sacrificed with the collection of blood and brain tissues (hypothalamus and cortex). The analysis will focus on mRNA changes using the DNA Microarray system (Gene Expression Laboratory). AChE activity will be monitored in blood and tissues to determine the efficacy of the sarin treatment. The tissues are stored at -80C, awaiting extraction and analysis. These experiments should provide a good complement to the proteomic studies in progress in Dr. Cool's laboratory. They will also complement the in vitro studies on neural cell lines conducted in Dr. Berberich's laboratory.

Project 2: The effect of stress and chemical exposure on auditory brain responses (ABR), energy metabolism and tissue chemical constituents in an animal model.

PIs: Ina Bicknell, Nicholas V. Reo, and Lawrence J. Prochaska; Department of Biochemistry & Molecular Biology, Wright State University

Project 2 was formerly denoted as Modules 2, 3, and 4 in the contract. Funding for this Project began on 01-Oct-2000. Because the research activities in these three modules are closely interrelated, the research efforts of Project 2 are summarized in one report. This report concerns the activities for the period 01-July-2002 to 30-June-2003.

The PIs, technicians and students involved in Project 2 meet on a biweekly basis to plan and coordinate experiments, discuss data, and review the current literature in the field. A summary of the overall accomplishments is given below. This is followed by a detailed Progress Report that is organized into three sections: Section A concerns the ABR studies (PI: I. Bicknell), Section B concerns the NMR studies (PI: N.V. Reo), and Section C concerns the studies of mitochondrial energy metabolism (PI: L.J. Prochaska).

Third Year Goals/Accomplishments

1. Completed data analysis of chronic low-dose study involving DEET treatment ± noise stress.
2. Completed data analysis of chronic low-dose study involving pyridostigmine bromide (PB) + noise stress.
3. Completed replication study of 24-hour post-injection effects of PB ± noise stress.
4. Completed chronic low-dose study of synergistic effects of DEET + PB + noise stress.
5. Completed dose-response study for sarin; compared subcutaneous (sc) and intravenous (iv) methods of toxin administration (determined LD_{50} for iv injection).
6. Completed first 4-week chronic low-dose study of sarin + noise stress.
7. Prepared and examined rat cochleae by scanning electron microscopy for evidence of hair cell loss/damage following toxin and noise exposure.
8. Completed assessment of the effects of DEET, PB and stress on mitochondrial energy coupling (respiratory control) activities.
9. Completed assessment of the effects of DEET, PB and stress on mitochondrial electron transfer activities.
10. Performed initial isolation of mitochondria from animals treated with low dose of sarin and stress and measured energy coupling and electron transfer activities of the preparation.
11. Determined absolute specific activities for cytochrome c oxidase in brain stem mitochondrial preparations from treated and control animals using intact membrane assays, soluble enzyme assays, and immunoblotting.

12. Determined cytochrome c content (as a marker for apoptosis) of mitochondria isolated from rats treated with DEET, PB and/or stress.

13. Conducted NMR in vitro analyses of brainstem extracts for PB + noise stress protocol and DEET + PB + noise stress protocol.

14. Determined pre- and post-noise exposure levels of blood serum corticosterone levels.

15. Hired and trained new laboratory personnel.

16. Abstract/Presentation: N.V. Reo, I. Bicknell, L. Prochaska, A. Neuforth, S. Shah, L. Shroyer, and D. Moyer. "Effects of Low-Dose Chronic Exposure to Persian Gulf War Chemicals and Stress on Rat Brainstem Function and Energy Metabolism." Proc. Intl. Soc. Magn. Reson. Med. (In press). International Society for Magnetic Resonance in Medicine, Eleventh Annual Meeting, Toronto, Ontario, Canada, July 2003.

Project 2, Section A: Auditory brainstem response (ABR) studies (PI: Ina Rea Bicknell)

1. Introduction/Materials and Method

Male Sprague Dawley rats, obtained from Harlan (Indianapolis, IN) at 40 days of age, were housed in the Cox Institute animal facility. At the time of the pre-exposure ABR and NMR measurements, the rats were eighty to eighty-three days of age. Animals were fed Harlan rodent maintenance diet 2014 (Harlan, Madison, WI); food and water was available *ad libitum*. Animals were housed two per cage in a cyclic light environment (12h light, 12h dark) under quiet rearing conditions [overall noise level: 76dbspl (re: 20μPa); most acoustic energy was below 2khz].

Repeated measures studies

Details of this repeated-measures protocol for the study of the effects of chronic low doses of DEET, pyridostigmine bromide (PB), and sarin, alone and in combination, and the effects of noise stress have been discussed in previous reports. Briefly, eighty-day-old male Sprague Dawley rats were treated once weekly for 4 consecutive weeks with toxin. DEET (225 mg/kg in arachis oil) was given by intraperitoneal (ip) injection. PB (5mg/kg in saline) was given by intragastric gavage. Sarin (190 μg/ml in saline) was given at a concentration of 60μg/kg. The toxin was administered subcutaneously (sc) at the nape of the neck in a volume of 107-125 μl. The animal was placed in a plastic restrainer during the injection. Vehicle was administered to control animals in a volume equal to an average of the volumes used with test animals.

For the study of the synergistic effects of toxins, DEET was administered 10 min before PB. Controls were administered vehicle in a volume equal to an average of volumes used with toxin-injected animals. Immediately after the weekly administration of toxin or vehicle, the animals were exposed to 8h of 85dbspl (re: 20μPa) white noise. Animals were housed individually in cages during noise exposure; cages were rotated every two hours to prevent positional effects. After exposure, animals were returned to their normal quiet rearing environment.

ABR measurements were made 24 hours prior to the administration of the first dose of toxin or vehicle and noise exposure. Repeated measurements were made at: 24 hours after the first toxin or vehicle administration/noise exposure and 6 days after each subsequent weekly toxin or vehicle administration/noise exposure. The duration of the experiment was 4 weeks.

The ABR waveform consists of a series of peaks and valleys; Peak II is a putative indicator of neural activity occurring primarily in the brainstem cochlear nucleus, which receives input from the auditory nerve (1). Peak II is the strongest peak in the rat ABR, and, because it is the last to disappear with decreasing intensity of the auditory signal, it is used for threshold evaluation. Thresholds were obtained using a modified method of limits. The acoustic signals were presented in an intensity series, beginning at 90 dbspl and decreasing in 10-db steps until near threshold, at which point the decrements were 3-db steps. Threshold was defined as the lowest stimulus intensity that elicits a reliably identifiable ABR response. Threshold evaluations were made independently by two judges.

Four acoustic signals were used to collect data: 3-ms tone bursts with frequencies of either 32-, 4-, or 8-khz and alternating condensation/rarefaction clicks having a duration of 100-μs. Clicks were presented at a rate of 23/s. Auditory stimuli were presented binaurally via pediatric ear inserts.

Statistical analyses are done in consultation with the Statistical Consulting Center at Wright State University. The data are evaluated by a univariate analysis of variance (ANOVA) for repeated measures models. Analyses were done using the Statistical Package for the Social Sciences (SPSS) (SPSS, Inc., Chicago, IL). Mauchly's test of sphericity was used to test the assumption of equal variance between pairs of treatment conditions in the repeated measures model. In instances of violation of the assumption of sphericity, the Greenhouse-Geisser procedure was used to correct for positive bias.

The repeated measure factor in this study was time. There were six timepoints: one pre-toxin/vehicle exposure, and five post-toxin/vehicle exposures: 24 h, 1 week, 2 week, 3 week, and 4 week. Group and stress were between-subject factors in the DEET study and group in the PB and DEET + PB studies.

Three ABR parameters were analyzed: hearing threshold, peak latency and peak amplitude. Thresholds were evaluated for each of the four acoustic stimuli used in this study: clicks and 4-, 8-, and 32-khz tones. The characteristics of these stimuli have been described in previous reports. Latencies and amplitudes of Peak II were analyzed statistically only for the 32-khz stimulus.

<u>24-hour post injection PB study</u>

In a previous study of the effects of PB and noise stress, an decrease in latency and increase in amplitude of Peak II of the ABR waveform was noted 24 h after administration of the acetylcholinesterase inhibitor PB, followed by 8h of noise stress, in three of seven test animals. The morphology of ABR waveforms obtained from PB-treated/+ stress animals was abnormal compared to ABR waveforms of control animals. The study of the 24-h post-

PB effects was repeated for three reasons: 1: the effect, while statistically significant, was small, 2: effects were observed in only some of the test animals, 3: orally administered PB is rapidly eliminated from the rat body (2). Because 24-hour NMR and biochemical measurements were not made routinely in the studies, replication of the experiment would provide an opportunity for making those measurements. To distinguish between PB and stress effects, two additional experimental conditions were tested: PB without noise stress and saline (vehicle) with noise stress.

Each test group was composed of four male 80-day-old Sprague-Dawley rats. As the study was a repeated measures design, pre-treatment ABR and NMR in vivo measurements were made. PB dosage was the same as used in the previous study, 5 mg/kg (by gavage); saline (vehicle) was administered to control animals (by gavage). Noise exposure was immediately after administration of PB or vehicle. ABRs were run 24 hours after administration of PB or vehicle. NMR measurements were done approximately 30 min after the ABR. To maintain a schedule of testing 24 hours after dosage with PB or saline and to accommodate the time needed for NMR measurements, animals were exposed to only 4h of white noise at 85 dbspl, compared to 8h in the previous PB-plus-stress study.

In addition to the acoustic stimuli used in the previous PB and stress experiment, (4-, 8-, 32-khz tone bursts of 3-ms duration and 100 μs alternating condensation/rarefaction clicks at the rate of 23 per sec), a click rate of 60 per sec was used in this replication study. The faster click rate acts as a stressor to the auditory system; thus, deficiencies may be more apparent.

Sarin dose/response study

Numerous problems were encountered that related to the logistics of transport of sarin from the main Wright State campus to our laboratory at Cox Institute. We now have a satisfactory transport mechanism in place. We were unable to solve the problem of bubble formation in sarin-filled 25-μl Hamilton syringes stored overnight at –80°c. As standard procedure now, syringes are filled and transported on the morning of injection, and animals are injected immediately after the syringes are delivered to the laboratory.

For the dose/response studies, 50 to 130 μg/kg sarin (1.9mg/ml in saline) was administered in a volume $\leq$ 25μl, via tail vein injection (n=5-11 per dose tested). Rats were placed in a plastic restrainer immediately prior to the injection. Animals were returned to their housing cages and were closely observed for 8h post-injection.

Pre- and post-noise exposure levels of blood serum corticosterone

In the 2002 annual report, we indicted that we were unsuccessful in showing an elevation in corticosterone levels after exposure to noise stress. We proposed that the problem was in the handling of the animals and in the time at which measurements were made. To resolve this issue, stress and non-stress corticosterone measurements were made 1.5 to 4 hours after the onset of the light cycle, a time when levels of the hormone would be expected to be low. In addition, we altered the way in which animals were handled and blood collected.

Individual animals from each group were placed in a customary housing unit for 30 min in the noise-exposure room, but no noise was presented. A second group of animals was

treated in the same way, but these animals were exposed to 30 min of 85-db white noise. At the end of the 30-min periods, the animals were exposed to CO for a few minutes, removed to another room and decapitated. Blood was collected at the time of decapitation. Blood serum was prepared from the blood samples and analyzed, in conjunction with Dr. Mariana Morris's laboratory, for corticosterone levels.

2. Results

Toxins without and with noise stress

DEET +/- noise stress

The following paragraphs are a summary of data discussed in Quarterly Report July 1, 2002 to September 30, 2002.

Significant ABR Peak II threshold differences were observed, both across time and between groups. Thresholds were significantly higher for the + noise stress group compared to the no-noise group ($p \leq 0.05$); however, the differences were of the order of 3 db or less; hence, they were not behaviorally significant. Differences in threshold were not significant for DEET-injected compared to vehicle-injected controls. Both the control and DEET-injected animals had elevated thresholds 24 hr after the first injection and subsequent 8 hours of noise exposure. This increase was most likely a noise-induced temporary threshold shift. This temporary shift generally disappeared during the first week after noise exposure. There were no significant differences across time or between groups in ABR waveform Peak II amplitude or latencies.

PB + noise stress

The following paragraphs are a summary of data discussed in Quarterly Report July 1, 2002 to September 30, 2002.

As was observed in the DEET study, the only significant differences in Peak II thresholds across time were at the 24-h timepoint (4- and 8-khz signals only). Again, this elevation in threshold was considered to be a noise-induced temporary threshold shift. There were no significant differences in Peak II thresholds between PB-exposed animals and vehicle-administered controls for any of the acoustic signals, indicated that no hearing loss occurred as a result of administration of PB and exposure to 8h of 85-db white noise.

Latencies of Peak II for the 32-khz signal, at intensities of 90, 80, and 60 db, were significantly lower at 24h than at the pre-exposure timepoint. Although between group effects were not significantly different, the mean Peak II latencies were shorter and the mean amplitudes of the + PB animals were higher than those of the control animals at every intensity level. Re-examination of the raw data indicated that 3 of the 8 PB-injected animals had abnormalities in the ABR waveform for the 32-khz signal in the region between Peak I and Peak II (Figure 2-1). Analysis of the ABR waveform of the 8-khz signal showed no evidence for this PB-related effect. It appears, therefore, that the effect was frequency specific.

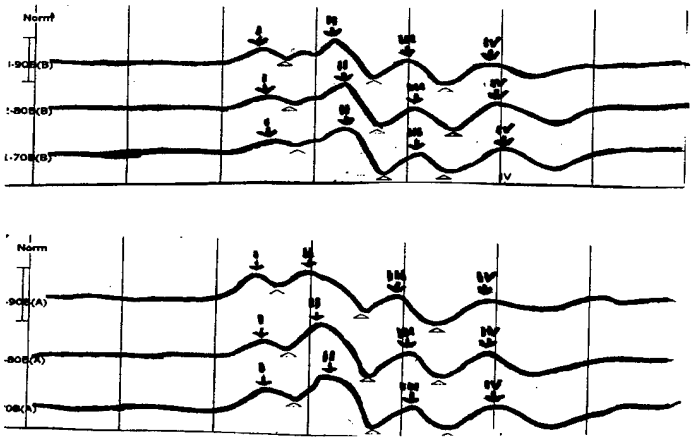


Figure 2-1. Waveform (top panel) of control animal 24h after Receiving vehicle and 8h of noise stress compared to waveform From a test animal 24 h after administration of PB (5mg/kg) and 8h of noise stress (lower panel). In each panel, the ABR waveforms are shown for a 32-khz signal presented in a descending intensity series of 90, 80, and 70 dbspl (y axis). Latency (x axis) is in ms. Major peaks are labeled sequentially as I, II, III, and IV.

Replication of 24-h timepoint in PB + stress study

The following paragraphs are a summary of data discussed in Quarterly Report October 1, 2002 to December 31, 2002.

This study confirmed that oral administration of 5mg/kg of PB does produce shorter 32-khz Peak II latencies and greater amplitudes. Abnormal ABR waveform morphologies were also observed in the replication study. Differences between average pre- and post-exposure Peak II-I latencies were greater in animals administered PB followed by exposure to 85-db white noise (0.1 ms) than they were in animals that were treated with PB without noise (0.06ms). This result indicates that noise enhanced the PB effect. Longer noise exposures (4h vs. 8h) produced greater distortion of the Peak I to Peak II portion of the ABR waveform.

Increasing the rate of presentation of the click to 60/sec had no effect on differences between pre and post-exposure Peak II-I latency intervals in PB-treated animals.

The effect of PB was seen primarily at higher signal intensities of 70 to 90 db, indicating that, while no hearing loss is associated with PB administration, a greater susceptibility to auditory damage from high-intensity noise may be a consequence. As was seen in the earlier study, not all of the test animals exhibited treatment effects. One of four test rats showed no differences in pre- and post-exposure latencies or waveform morphology. Statistical analysis of the replication study data is being done at this time.

DEET + PB + noise stress

There were no significant effects for time, group, or time x group interaction for the 32-, 8-khz or the click stimulus. There was a significant difference between the PB + DEET + noise stress group and the vehicle-administered controls for the 4-khz thresholds . However, the differences in thresholds were <4-5 db and, therefore, as noted earlier, were behaviorally insignificant.

The statistical analysis of Peak II latency and amplitude has been completed. The data, however, must be examined more closely because there were instances, in both parameters, of no significant effects for time, but significant within-subject effects, indicting that latency and/or amplitude varied over time in only some animals in the group. Considering that effects of PB alone were observed in only some animals in the test group, this result is not surprising. There were significant differences between toxin-administered and vehicle-administered groups in Peak II latency at 80, 70, and 60 db, but not at 90 db. A preliminary re-analysis of the data suggests that this difference may reflect the response of only a few animals within the test group.

SARIN

Dose/response relationship and determination of LD_{50} (iv)

Our initial goal in the sarin study was to determine an LD_{50} for sarin administered via tail vein injection and to establish a dose-response relationship using specified percentages of the LD_{50}. With sarin administered via tail vein, in doses ranging from 50 to 130 μg/kg, the symptoms of toxicity for a given dose ranged from none, to temporary fine body tremors or exaggerated mouth movements, to moderate body tremors and lethargy, to severe body tremors, to death (Table 2.A.1, Jan 1, 2003-March 31, 2003 quarterly report). The LD_{50} for sarin administered iv was determined to be 130μg/kg (n=8).

To ascertain whether the variability in symptoms observed with specific doses of sarin was a consequence of the mode of injection, we changed the method of delivery to subcutaneous (sc) injection. According to the literature, this is a common method of sarin administration. An amendment proposing a change in delivery of sarin from iv to sc was submitted to the Wright State University Laboratory Animal Care and Use Committee; approval was given.

Seven rats were injected sc, at the nape of the neck, with 120 μg/kg of sarin (0.19mg/ml in saline). Symptoms, which appeared within 1 to 5 minutes after injection, ranged from transitory salivation to severe body tremors. All of these animals were euthanized by microwave fixation and the brain tissue used for *in vitro* NMR analyses. Because a sc dose of 120 μg/kg sarin produced observable effects, without death, we elected to define "low dose" as 50% of this value. This low dose, 60 μg/kg, was used for the 4-week study of the effects of sarin + noise stress.

Sarin + noise stress: 4-week study

The first 4-week study of 4 sarin-injected and 3 vehicle-injected rats ended mid June 2003. As noted in an earlier report, because of the time involved in NMR measurements, no more than 7 animals can be tested during a 4-week period. Each study is repeated, therefore, for a total of 8 test animals and 6 controls. Only a preliminary analyses of the data is discussed here.

All sarin-injected animals were closely monitored during the 8 hours following injection of the toxin. No overt signs of toxicity were observed after the first three weekly injections. However, within minutes after the 4th weekly injection, 3 of the 4 test animals showed signs of lethargy and/or became immobilized in their hind quarters. The fourth animal had no overt signs of toxicity. Five days after the 4th injection, one of the three affected animals died; the other two animals recovered.

In addition to the seven animals used in the in vivo study, two animals received 4 weekly injections plus noise exposure; these animals were for in vitro NMR studies. Of these two animals, one exhibited convulsions and exophthalmia after the 4th injection,. Because the animal did not recover during the 24 hour post-injection period, it was euthanized. The second in vitro rat showed no overt signs of toxicity after the 4th weekly injection.

ABR measurements

Peak II Threshold

Preliminary analysis indicates no significant differences in threshold for any of the acoustic stimuli, either across time or between groups after 4 weekly injections of sarin (data not shown). One animal did have elevated thresholds (6-13 db) for all stimuli at the 24-h timepoint. Examination of the ABR waveforms did not reveal any notable differences in morphology, again, either across time or between groups. This is in contrast to the observation of abnormal morphologies in the Peak I to Peak II region, shown in Figure 2-1, at 24h after administration of the acetylcholinesterase inhibitor (PB)

Latency

Latency/intensity functions for Peak II of the 32k-Hz signal showed a small increase in latency at intensities below 50 dbspl (Figure 2-2A) in sarin-injected animals. No post-injection time-related differences were observed in the control animals (Figure 2-2B).

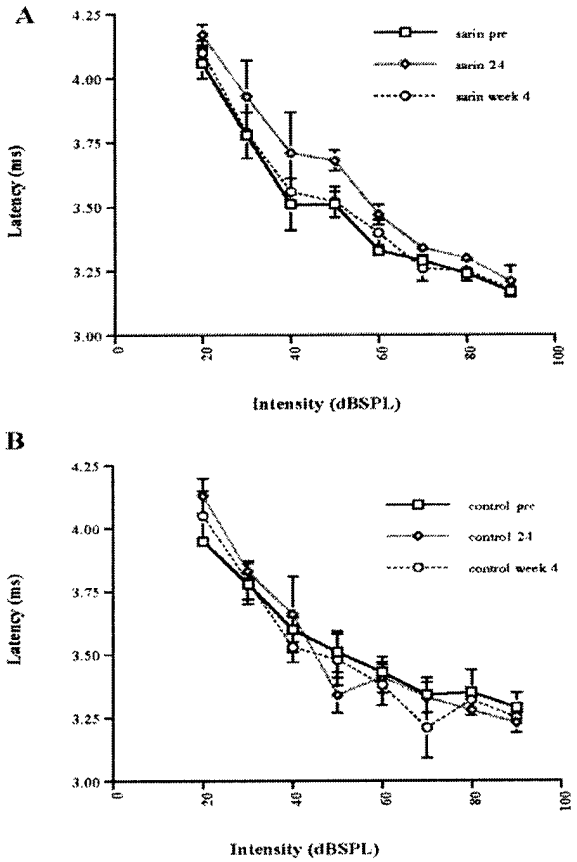


Figure 2-2. 32-khz Peak II latency/intensity functions before Exposure (pre) , 24h after first injection (24) , and 6 days After 4th weekly injection (week 4) of 60 μg/kg sarin (sc) or saline Vehicle (control). Each of four weekly injections was followed By exposure to 8h of 85-db white noise. Values are expressed As the mean from either 4 (sarin, Panel A) or 3 (controls, Panel B) Independent observations (N). SEM are shown as ± horizontal error bars.

Amplitude

Amplitude is not a robust ABR measurement; therefore, until the entire set of 8 animals is tested, a statement cannot be made about the effect of sarin on this parameter.

Temperature and weight

Temperatures (Figure 2-3) and weights (Figure 2-4) were monitored during the 4-week sarin study. No major differences were noted between controls and sarin-injected animals.

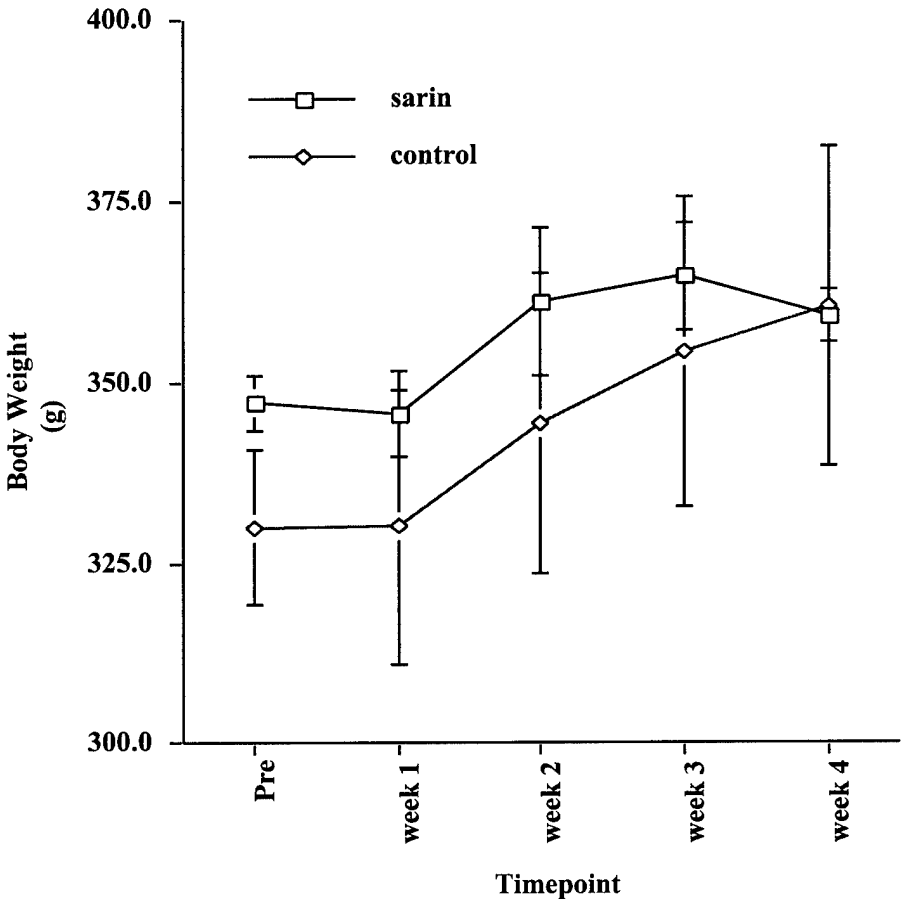


Figure 2-3. Pre- and post-exposure body weights in sarin- and vehicle-Injected rats. Measurements made immediately prior to weekly injection. Values are means of weights of all animals in group (sarin n=4; week 4 n=3; controls n=3). SEM are shown as ± horizontal error bars.

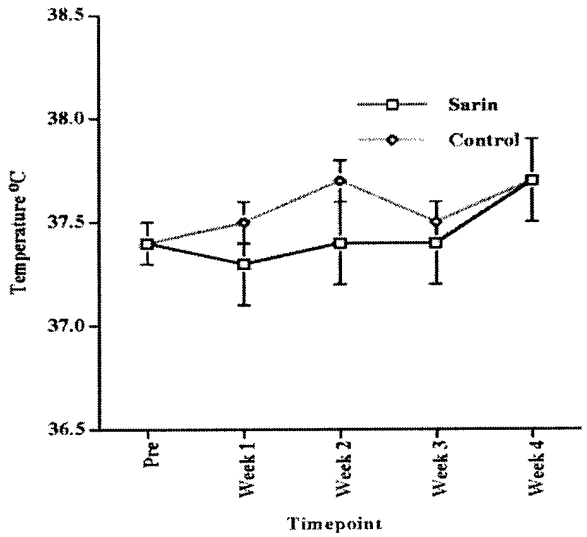


Figure 2-4. Pre and post-exposure core body temperatures in Sarin- and vehicle-injected rats: Measurements were made immediately Prior to collection of ABR data. Values are means of all animals in group (Sarin n=4; control n=3). SEM are shown as ± horizontal error bars.

A summary of the effects observed in the DEET, PB, DEET + PB and sarin studies is shown in Table 2-1.

Table 2-1: Summary of effects of DEET, PB, DEET + PB, and Sarin: Agent administered once per week for four weeks. Stress was 8h of white noise at 85dbspl (re:20μPa). Animals were exposed to noise immediately after administration of agent.

Agent	Peak II Threshold	Peak II Latency	Peak II Amplitude	Waveform Morphology
DEET (225mg/kg) ip +/- stress	Temporary threshold shift at 24h under conditions of + noise	No effect	No effect	Normal
PB (5mg/kg) Gavage + stress	Temporary Threshold shift at 24h	24h shorter than pre; 32-khz signal Only**; Noise enhanced Reduction; Test group not sig. Different from Control group¶	24h higher than Pre; 32-khz signal only**; Test group not sig. Different from control group¶	Abnormal
PB (5mg/kg) Gavage No stress		24h shorter than Pre; reduction less Than under conditions of + noise		Normal
DEET + PB (225/5) mg/kg + stress	4-khz signal only*; not behaviorally sig.	Significant differences*	Significant differences at 90db#	Not yet analyzed
Sarin(60μg/kg) Iv + stress	No significant differences across time or for group (one animal with Elevated thresholds compared to control)	Small increases at intensities <50 db	Not analyzed	Normal

** Statistically significant differences across time ($p \leq 0.05$).
¶ Effect observed in only some animals in the test group.
* Statistically significant differences compared to control group.
#Not significant across time or group($p<0.05$); significant for within group (observed only in some animals in group).

Elevation of blood serum levels of corticosterone after exposure to 30 of noise stress

The following paragraph is a summary of data discussed in Quarterly Report July 1,2002 to September 30, 2002.

Exposure to 30 minutes of 85-db white noise produced a nearly twelve-fold increase in blood serum corticosterone levels (38.64 ± 8.8 ng/ml vs. 458.3 ± 46.7 ng/ml), indicating that the animals were stressed by the noise.

Scanning electron microscope (SEM) studies

No evidence has been seen in SEM studies of the basilar membrane for damage resulting from administration of DEET or PB alone or for DEET+ PB. No evidence of damage from the level of noise used in these studies has been seen. Cochleae from sarin-treated animals have not been examined.

Conclusions

Threshold data indicates that none of the agents tested caused a loss of hearing. Because animals are administered "low doses" of agent, other auditory deficiencies ensuing from the agent may be small and, therefore, difficult to detect. Our data indicates that, at least for PB and sarin, there was differential sensitivity to the chemicals among the rats.

It is apparent from the data, that PB caused a decrease in 32-khz Peak II latency. Acetylcholine acts as an inhibitory neurotransmitter in the central nervous system (CNS) auditory pathway (Brugge, 1992). If PB does cross the blood-brain barrier into the CNS and does bind to acetylcholinesterase, one might expect a disruption in the normal inhibitory responses to an acoustic stimulus. Disruption of the inhibition process might result in shorter latencies and higher amplitudes of the neural response.

In addition, acetylcholine is the major neurotransmitter mediating efferent input to the hair cells of the cochlea (Sewell, 1996). A number of studies have demonstrated that acetylcholine activates a potassium conductance in hair cells, which requires external calcium. Acetylcholine may activate a calcium receptor. An accumulation of acetylcholine, due to inhibition of the acetylcholinesterase, may facilitate calcium entry into the hair cell, thus, shortening the time constant required for hyperpolarization of the hair cell. Sziklai and Dallos (1993) also suggest that acetylcholine has an effect on the electromotility of outer hair cells. The outer hair cell response to an acoustic stimulus may, therefore, be modified.

It is contradictory that sarin, also an acetylcholinesterase inhibitor, was observed to cause small increases in 32-khz Peak II latency. These increases may prove to be insignificant when the data from the entire set of 8 sarin-injected animals is analyzed. Also in contrast to PB effects, no abnormalities in ABR waveforms were observed after administration of sarin. If these results hold true for the second set of animals, it suggests sarin and PB may have different sites of action in the auditory system. There are two muscles in the middle ear, the stapedius and the tensor tympani. If sarin is acting at neuromuscular junctions of one or both of these muscles, the efficacy of transmission of acoustic energy from the outer to the inner ear may be compromised.

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New Personnel

Ms. Kathleen Rainey has replaced David Moyer as Dr. Bicknell's research assistant.

PROJECT 2, SECTION B – NMR Studies of Metabolism (Dr. Nicholas V. Reo)

2B.1 Low-Dose Chronic Protocol: DEET, Pyridostigmine Bromide (PB), and Noise Stress.

The following is a culmination of our data for the three studies involving a 4 wk exposure regimen with chemical agents and stress: (1) DEET + stress, (2) PB + Stress, and (3) DEET + PB + Stress. DEET was administered by ip injection at 225 mg/Kg. PB was given by intragastric gavage at 5 mg/Kg. For the protocol involving the combined treatment, PB (5 mg/Kg) was given first followed by DEET (225 mg/Kg) ten minutes later. Details of the methods are described above in Dr. Bicknell's section of the report.

NMR In Vivo. NMR experiments in vivo were conducted prior to treatments, and at 2 and 4 weeks post-treatment. These studies involve ^{1}H and ^{31}P NMR of rat head (brain), and ^{31}P and ^{13}C spectra of leg muscle. The ^{1}H spectra provide a measure of choline-containing metabolites (Cho), creatine (Cr), and N-acetylaspartate (NAA). The Cr signal represents total creatine (creatine + phosphocreatine), which is assumed to remain constant and provide a good intracellular metabolite reference. Thus, metabolites measurements are expressed as a ratio to total creatine. The ^{31}P NMR spectrum provides a measure of phosphocreatine (pcr) and nucleotide triphosphates (NTP). The β-phosphate signal from ntps is predominantly due to adenosine triphosphate (ATP) and our data is expressed as a ratio pcr/ATP. The results of these studies are provided below.

The pretreatment measurements were conducted prior to treatments or noise stress exposure. Thus, animals in all groups are not distinct at this measurement timepoint. A one-way ANOVA was conducted with the pretreatment data as the dependent variable to assess the variance among all groups. The independent factor was the group identifier: DEET; Control-(DEET); PB; Control-(PB); DEET+PB; Control-(DEET+PB). This statistical analysis showed no significant differences among the groups prior to treatment or stress ($p \leq 0.05$). Table 2-2 shows the metabolite ratios (mean ± SE) measured in rat brain and muscle in vivo at the pretreatment timepoint for treated and control groups. In some NMR spectra the signal-to-noise ratio (S/N) was poor and prohibited an accurate measure of signal intensities. These data were eliminated from the analyses. This accounts for the differences in n-values for some of the experimental groups. Since there were no statistical differences between groups at this timepoint ($p \leq 0.05$), the data were combined to give an overall mean value for each metabolite ratio (Table 2-2). Figures 2-5, 2-6, 2-7, and 2-8 show the metabolite ratios (Mean ± SE) measured via NMR during the experimental timecourse. Specifically, this involved measurements at pretreatment, 2 wk, and 4 wk. Each figure shows the data for a given metabolite measurement for all groups. A repeated measures ANOVA was conducted to determine whether treatment produced any significant changes in the data over times post-dose. All data showed no effects of treatment during the experimental timecourse ($p \leq 0.05$).

These in vivo NMR studies provide an assessment of cellular viability. The pcr and ATP are high-energy phosphate metabolites that provide a sensitive measure of the cellular energy status while N-acetylaspartate (NAA) and creatine (Cr) are useful indicators of neuronal viability. Neuronal cell death leads to a precipitous loss in NAA. Our in vivo NMR results indicate that treatment with either DEET+stress, PB+stress, or DEET+PB+stress does not produce any deleterious effects on neuronal metabolism. These results are corroborated by measurements of brainstem mitochondria (Dr. Prochaska) that also indicate a normal energy status.

Table 2-2. NMR-measured metabolite ratios (Mean ± SE) in rat brain and muscle prior to chemical or noise exposure (pretreatement data). Key: Cho, choline; Cr, creatine; NAA, N-acetylaspartate; pcr, phosphocreatine; ATP, adenosine triphosphate.

Group	Brain Cho/Cr	Brain NAA/Cr	Brain Pcr/ATP	Muscle pcr/ATP
DEET	1.59 ± 0.15 (n=7)	1.94 ± 0.32 (n=7)	1.71± 0.09 (n=10)	2.4 ± 0.05 (n=12)
Control (DEET)	1.58 ± 0.08 (n=5)	1.92 ± 0.26 (n=5)	1.59 ± 0.08 (n=9)	2.5 ± 0.09 (n=6)
PB	1.55 ± 0.08 (n=4)	1.40 ± 0.01 (n=4)	1.69 ± 0.07 (n=7)	2.4 ± 0.07 (n=8)
Control (PB)	1.38 ± 0.11 (n=4)	1.12 ± 0.08 (n=4)	1.62 ± 0.07 (n=5)	2.4 ± 0.08 (n=6)
DEET+PB	1.43 ± 0.13 (n=4)	1.53 ± 0.25 (n=4)	1.49 ± 0.08 (n=11)	2.3 ± 0.04 (n=12)
Control (DEET+PB)	1.16 ± 0.16 (n=6)	1.40 ± 0.36 (n=6)	1.56 ± 0.08 (n=9)	2.2 ± 0.05 (n=9)
All groups combined	1.45 ±0.06 (n=30)	1.59 ± 0.12 (n=30)	1.60 ± 0.03 (n=51)	2.3 ± 0.03 (n=53)

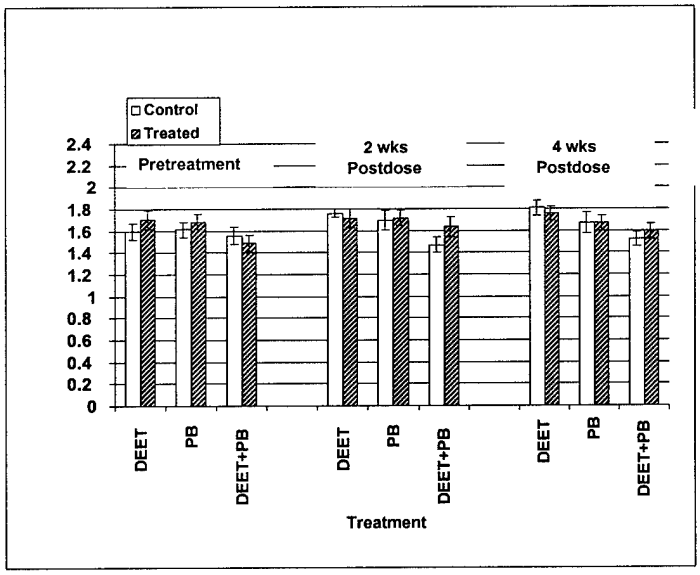


Figure 2-5. Rat brain pcr/NTP ratios (Mean ± SE) measured by ^{31}P NMR *in vivo* during the chronic treatment protocol with chemical agents + noise stress. All control groups received vehicle + noise stress.

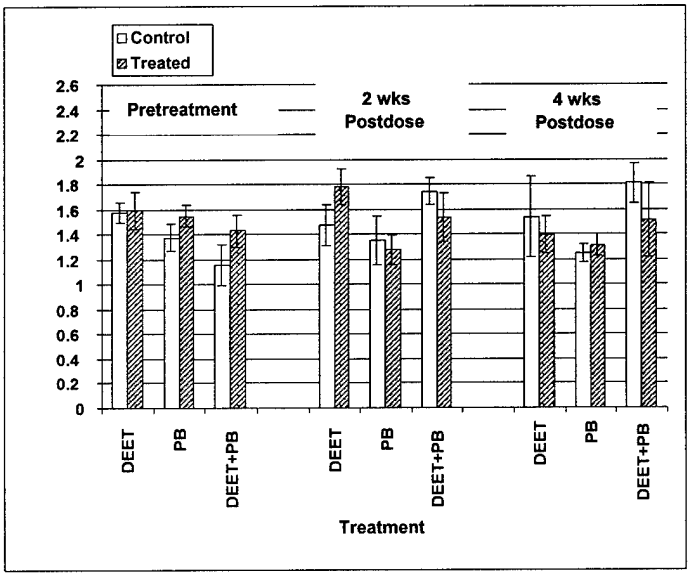


Figure 2-6. Rat brain Cho/Cr ratios (Mean ± SE) measured by ^{1}H NMR *in vivo* during the chronic treatment protocol with chemical agents + noise stress. All control received vehicle + noise stress.

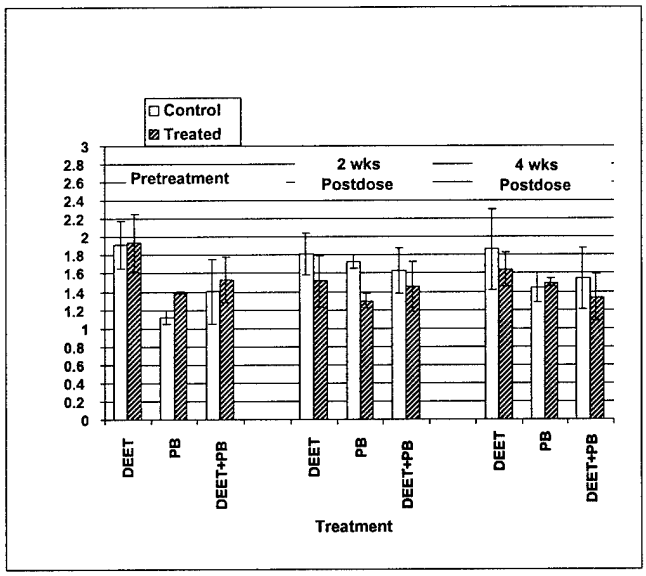


Figure 2-7. Rat brain NAA/Cr ratios (Mean ± SE) measured by ^{1}H NMR *in vivo* during the chronic treatment protocol with chemical agents + noise stress. All control received vehicle + noise stress.

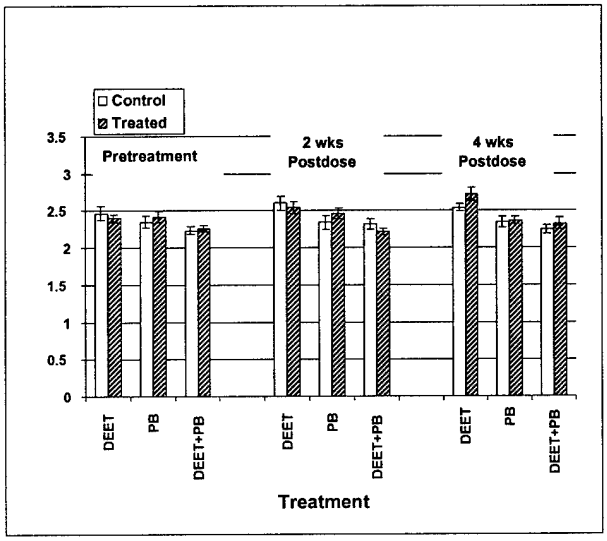


Figure 2-8. Rat muscle pcr/ATP ratios (Mean ± SE) measured by ^{31}P NMR *in vivo* during the chronic treatment protocol with chemical agents + noise stress. All control received vehicle + noise stress.

NMR In Vitro. At the completion of the in vivo experiments the rats were sacrificed by high-power microwave fixation and brainstems were removed for chemical extraction. The brainstem extracts were then analyzed by high-resolution NMR. Data were not obtained for the DEET study due to technical problems at the early stages of the project (these have been discussed in previous reports). We are still experiencing problems due to the large size of these animals (>350 g) and the efficiency of the microwave fixation technique. Brainstem tissues are not always thoroughly 'cooked' and the high-energy phosphate metabolites are very labile and sensitive to hydrolysis. Thus, the ATP and pcr metabolite data are suspect. The metabolite data obtained by proton NMR, however, should be reliable. This is clearly seen in Figure 2-9, which shows a much greater variability in the pcr, ATP, and inorganic phosphate (Pi) data than what is seen for the NAA/Cr ratio. Other metabolites as measured by proton NMR are also being examined but these analyses are not yet complete. The data in Figure 2-9 show no significant differences in Treated vs. Control groups for any of the treatment protocols ($p<0.05$).

Overall, all treatment protocols with chemical agents and stress have failed to produce any significant effects on brain or muscle metabolism as observed by NMR spectroscopy.

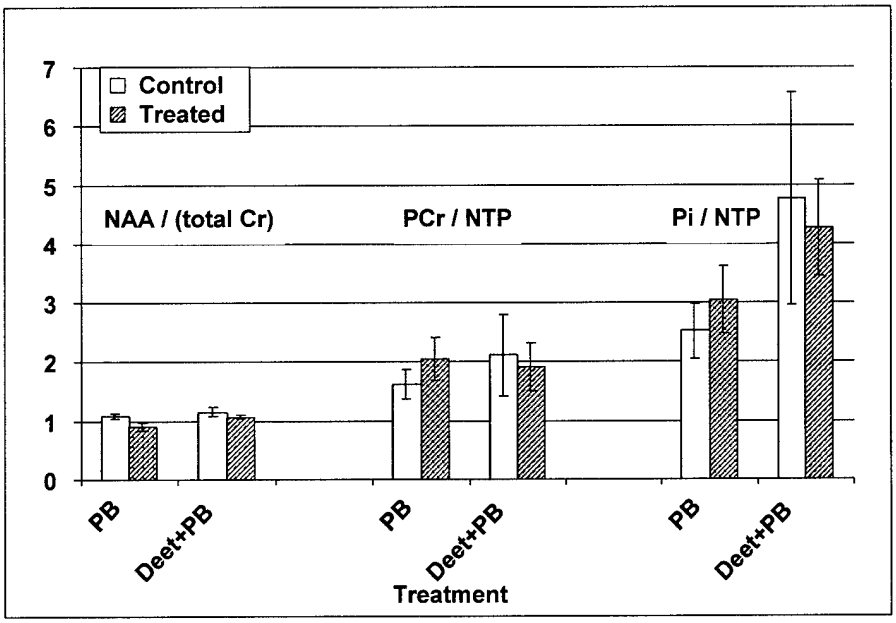


Figure 2-9. NMR-measured metabolite molar ratios (mean ± SE; n=3-7) in rat brainstem extracts prepared at the completion of the 4-wk treatment with chemical agents + stress. Concentration values were computed on a per gram tissue basis. Nucleotide triphosphates (NTP) are predominately ATP; Pi is inorganic phosphate.

2B.2 Effects of Pyridostigmine Bromide (PB) at 24-hour Post-dose.

During this report period a study was conducted to investigate the effects of PB at 24 h post-dose. This study was initiated after Dr. Bicknell had observed some effects of PB on the ABR measurements. These findings were revealed in the chronic low-dose PB + stress study at the 24-h timepoint. Therefore, we repeated these studies to conduct NMR analyses at this same timepoint (normally the NMR analyses are not done at 24 h).

Experimental Protocol. The experimental protocol is as follows. In vivo ABR and NMR measurements were made prior to treatment (Day 0). On Day 1 rats were administered either 5 mg/Kg PB (ig) (treated) or vehicle (control), and then immediately subjected to 4 h of noise stress. At 24 h post-dose, the rats were examined by ABR and NMR in vivo. Following the NMR measurement the animals were sacrificed either by microwave fixation (for our NMR studies in vitro) or by CO_2 inhalation for preparation of brainstem mitochondria and subsequent in vitro biochemical assays (Dr. Prochaska).

In Vivo NMR Results. The pretreatment measurements were conducted prior to treatments or noise stress exposure. Thus animals in all groups are not distinct at this measurement timepoint. A one-way ANOVA was conducted with the pretreatment data as the dependent variable to assess the variance in the two groups. The independent factor was the group identifier, PB (+)stress or Control (+)stress. This statistical analysis showed no significant differences among the groups prior to treatment or stress ($p \leq 0.05$). Table 2-3 shows the metabolite ratios (mean ± SE) measured in rat brain and muscle in vivo at the pretreatment timepoint for treated and control groups. The Cho/Cr and NAA/Cr ratios were measured in the ^{1}H NMR spectra, while

the pcr/ATP ratios were measured by ^{31}P NMR. In some spectra the signal-to-noise ratio (S/N) was poor and prohibited an accurate measure of signal intensities. These data were eliminated from the analyses. This accounts for the differences in n-values for some of the experimental groups. Since there were no statistical differences between groups at this timepoint ($p \leq 0.05$), the data were combined to give an overall mean value for each metabolite ratio (Table 2-3).

TABLE 2-3. Metabolite ratios (Mean ± SE) in rat brain and muscle determined by NMR in vivo. Data were obtained prior to treatment with PB or vehicle or noise stress. Key: Cho, choline; Cr, creatine; PCr, phosphocreatine; NAA, N-acetyaspartate.

Group	Brain Cho/Cr	Brain NAA/Cr	Brain Pcr/ATP	Muscle Pcr/ATP
Control	1.44 ± 0.12 (n=4)	2.16 ± 0.37 (n=4)	1.35 ± 0.10 (n=4)	2.35 ± 0.04 (n=4)
Treated	1.36 ± 0.10 (n=6)	1.84 ± 0.27 (n=6)	1.51 ± 0.05 (n=7)	2.33 ± 0.07 (n=8)
All groups Combined	1.40 ± 0.08 (n=10)	1.97 ± 0.21 (n=10)	1.45 ± 0.05 (n=11)	2.34 ± 0.05 (n=12)

Figure 2-10 shows the metabolite ratios (Mean ± SE) measured via NMR before treatment (Pretreatment) and at 24-h post PB + Noise Stress for treated and control groups. Statistical analyses of the control group posed a problem since 2 of the 4 animals yielded poor spectra at the 24 h timepoint. These data were eliminated and, thus, the sample size (n=2) is too small to conduct statistical analyses. The treated group, however, was analyzed by a paired t-test to compare pretreatment versus 24 h post-treatment data. These data showed no effects of treatment for all metabolites ($p \geq 0.05$).

At the completion of the NMR in vivo experiments, the rats were sacrificed by high-power microwave fixation. Brainstems were excised and stored under liquid N2 for subsequent chemical extraction. Brainstem extracts were then examined by high-resolution ^{1}H and ^{31}P NMR. Figure 2-11 shows the results for the various metabolite ratios for treated and corresponding control groups. A Student's t-test confirms that there are no differences between the treated versus control groups ($p \geq 0.05$). Thus PB + noise stress did not impact brain or muscle energy metabolism at 24 h post-dose as determined by NMR in vivo and in vitro.

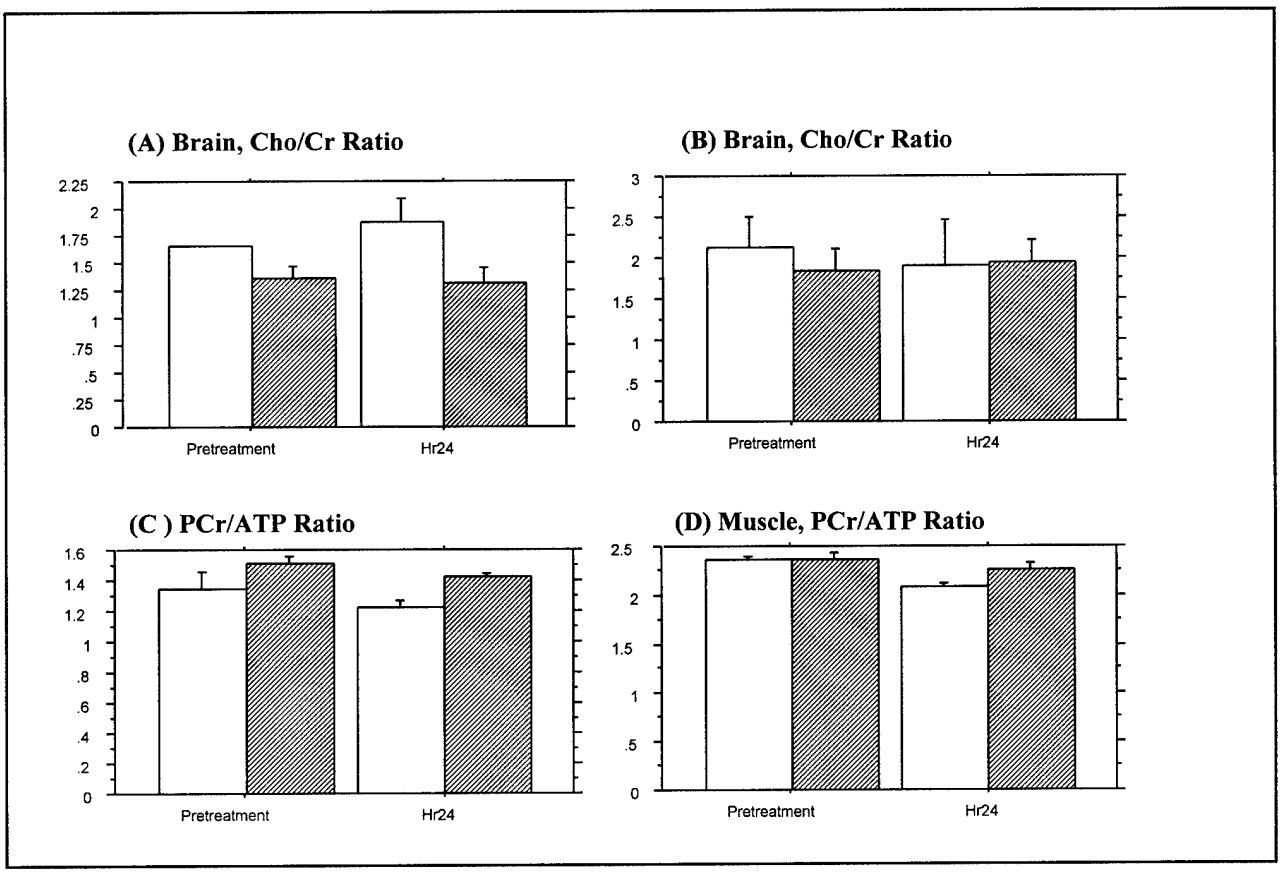


Figure 2-10. Metabolite ratios (mean ± SE) determined from NMR studies of rat head (brain) and leg muscle in vivo. Data were obtained before and 24 h after treatment with either PB + noise stress (Treated; hatched bars), or vehicle + noise stress (Control; open bars). Some animals were eliminated from this analysis due to poor spectral quality, thus n-values may differ from those shown in Table 2-3. For the ^{1}H-derived data in brain (NAA/Cr and Cho/Cr), the n-values are 2 and 6 for Control and Treated groups, respectively. For the ^{31}P-derived data in brain and muscle (pcr/ATP), the n-values are 4 and 7 for Control and Treated groups, respectively. Error bars not shown are below the scale for the plot.

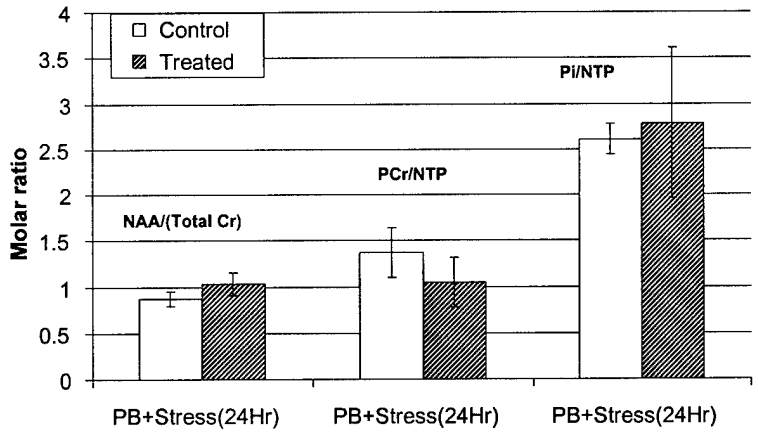


Figure 2-11. NMR-measured metabolite molar ratios (mean ± SE; n=5-7) in rat brainstem extracts prepared at 24 h post-dose with PB + noise stress. Concentration values were computed on a per gram tissue basis. Nucleotide triphosphates (NTP) are predominately ATP; Pi is inorganic phosphate.

2B-3. Low-Dose Chronic Protocol: Sarin + Noise Stress.

Recently, we have initiated studies involving sarin. First we determined an appropriate dose of sarin, then the first phase of experiments involving a 4-wk chronic protocol was initiated. Details of this experimental protocol are discussed above in Dr. Bicknell's section of this report. Similar to our previous studies, the NMR measurements in vivo were conducted prior to treatment with chemical agent or noise stress, and then again at 2 wks and 4 wks post treatment. The data analyses from these initial experiments are not yet complete. Additionally, these studies are conducted in several phases (usually two or three) in order to obtain an appropriate sample size (N-value). Since this is only the first phase of the experiment, we do not have enough animals to conduct statistics.

2B-4. Installation of new NMR equipment and optimization of NMR experimental parameters. In January 2003 we installed a new Varian Inova 600 NMR spectrometer. Some of our high-resolution NMR spectra of brainstem chemical extracts have been acquired using this instrument. Also, in March 2003 we installed a new Tecmag Discovery NMR console on our 8.5 T magnet (our 360 mhz system). This is the system used for all in vivo NMR studies of rat brain and muscle. Due to this installation, it was necessary for us to re-write the NMR pulse sequences and to optimize the acquisition parameters for our surface-coil NMR experiments. Thus, during March and April 2003 we were busy installing and testing this new equipment for our particular in vivo experiments. The first phase of the sarin studies were conducted using this new NMR system. The NMR spectra obtained are of excellent quality, similar to our previous experiments.

Annual Report Project 2: Part C, Energy Metabolism

Lawrence J. Prochaska, PhD., P. I.

Activities (July 1, 2002-June 30, 2003)

Results

The Effects of DEET, Pyridostigmine Bromide and Stress on Mitochondrial Energy Coupling (Respiratory Control) Activities.

Rats were treated with: 1) 37.5% of the LD_{50} dose of DEET; 2) 37.5% of the LD_{50} dose of DEET in the presence of noise stress; 3) 5 mg pyridostigmine bromide (PB)/ Kg body weight in the presence of noise stress; 4) 37.5% of the LD_{50} dose of DEET and 5 mg pyridostigmine bromide (PB)/ Kg body weight in the presence of noise stress using a protocol presented in the Auditory Biology/Magnetic Resonance portion of this report (Sections 2A&B). Thirty days after the initial treatment, animals were sacrificed and mitochondria were isolated from brain stems. Energy coupling ratios were measured using two different substrates (Table 2-4).

Table 2-4 summarizes the mitochondrial respiratory control ratios that we have measured after the experimental treatments. In general, the data show that there was little or no effect of long-term exposure to the chemical agents on the respiratory control ratios of isolated mitochondria when using succinate (FAD-linked) or pyruvate and malate (NADH-linked) as substrates.

However, upon close inspection of the data, there are some statistically significant decreases in the specific activity of the ADP stimulated rate of electron transfer when using succinate as a substrate (for example, control vs. Noise stress control and DEET and PB-treated animals with noise stress). Accompanying changes in specific activity also occur in the basal rate of electron transfer supported by succinate oxidation. Due to variability of the assay, there is no observed statistically relevant difference in the respiratory control ratio in these preparations. This variability in the specific activities will be discussed in the third section.

The results in Table 2-4 also suggest that the treatments have no effect on the ability of mitochondria to make ATP and that the inner membrane permeability of mitochondria was unaffected by the treatments. The total yield of mitochondria from the brain stem tissue of all preparations was similar, emphasizing that there was no decrease in integrity of the mitochondria in the tissue upon all treatments.

Table 2-4. The Effects of a 37.5% LD_{50} Dose of DEET, 5 mg/Kg Body Weight Dose of PB, and Stress on Rat Brain Stem Mitochondrial Energy Coupling

Treatment	Substrate	Activity ($\frac{\text{nmoles } O_2}{\text{(mg protein-min)}}$)[a]	Respiratory Control Ratio
I. Control			
	Succinate	22 ± 1	
	+ADP	61 ± 7	2.8 ± 0.3
	Pyruvate/malate	12 ± 6	
	+ADP	37 ± 6	3.5 ± 1.0
II. Control with Noise Stress			
	Succinate	13 ± 2	
	+ADP	47 ± 4	3.7 ± 1.0
	Pyruvate/malate	10 ± 3	
	+ADP	38 ± 6	4.0 ± 0.8
III. DEET-Treated (30 days after injection)			
	Succinate	18 ± 4	
	+ADP	68 ± 14	3.8 ± 1.1
	Pyruvate/malate	11 ± 5	
	+ADP	29 ± 6	3.6 ± 0.7
IV. DEET-Treated with Noise Stress (30 days after injection)			
	Succinate	14 ± 5	
	+ADP	55 ± 6	4.2 ± 1.8
	Pyruvate/malate	11 ± 8	
	+ADP	32 ± 8	3.6 ± 1.7
V. PB-Treated + Noise Stress (30 days after injection)			
	Succinate	17 ± 3	
	+ADP	61 ± 12	3.8 ± 0.9

Pyruvate/malate	8 ± 4	
+ADP	32 ± 7	5.2 ± 2.9

VI. PB and DEET-Treated + Noise Stress (30 days after injection)

Succinate	12 ± 3	
+ADP	41 ± 10	3.5 ± 0.7
Pyruvate/malate	9 ± 2	
+ADP	34 ± 5	4.3 ± 1.2

[4]all assays were performed in a buffer consisting of 10 mm KH_2PO_4, 250 mm sucrose, 50 mm kcl, 5 mm $mgcl_2$, 1mm EGTA, ph 7.4. Substrate concentrations used were 3 mm succinate, 3 mm pyruvate and 1.5 mm malate, and 0.2 mm ADP. Error measurements are standard deviations. The number of preparations was 10 for control, 4 for control with noise stress, and 3 for DEET-treated with noise stress, 2 for DEET-treated without noise stress, 2 for PB-treated with noise stress, and 3 for PB and DEET-treated with noise stress. Each assay was performed at least six times for each preparation.

In summary, Table 2-4 shows the final form of the data for the effects of the Gulf War chemicals, DEET, PB, and noise stress on the ability of brain stem mitochondria isolated from treated rats to synthesize ATP. Our results suggest that using a low dose of the chemicals over a long time period has no effect on rat brain mitochondrial energy transduction.

The Effects of Sarin and Stress on Mitochondrial Energy Coupling (Respiratory Control) Activities.

Our most recent experiment was a low dose treatment of rats with sarin and noise using a protocol similar to our previous work and as described in Section 2A of this report. Thirty days after the initial treatment, animals were sacrificed and mitochondria were isolated from brain stems. Energy coupling ratios were measured using two different substrates (Table 2-5).

Table 2-5 shows that succinate-supported respiration and the energy coupling ratio induced by ADP is unaffected by the low dose sarin treatment when compared to the appropriate control. The energy coupling ratio of mitochondria from sarin-treated rats is unaffected using NADH linked substrates, but the NADH oxidase activity exhibits a 35% decrease in specific activity, suggesting that there is a specific site of inhibition of mitochondrial electron transfer between NADH and ubiquinone. This area of the respiratory chain is known to be the most labile to oxidative damage caused by reactive oxygen species (See later discussion).

Table 2-5. The Effects of Sarin and Stress on Rat Brain Stem Mitochondrial Energy Coupling

Treatment	Substrate	Activity ($\frac{\text{nmoles } O_2}{\text{(mg protein-min)}}$)[a]	Respiratory Control Ratio
I.	**Control with Noise Stress**		
	Succinate	13 ± 4	
	+ADP	45 ± 14	3.6 ± 0.6
	Pyruvate/malate	9 ± 3	
	+ADP	36 ± 6	4.3 ± 1.6
II.	**Sarin-Treated with Noise Stress (30 days after injection)**		
	Succinate	12 ± 4	
	+ADP	39 ± 11	3.3 ± 0.3
	Pyruvate/malate	4	
	+ADP	23	6.7

[A]all assays were performed in a buffer consisting of 10 mm KH_2PO_4, 250 mm sucrose, 50 mm kcl, 5 mm $mgcl_2$, 1mm EGTA, ph 7.4. Substrate concentrations used were 3 mm succinate, 3 mm pyruvate and 1.5 mm malate, and 0.2 mm ADP. Error measurements are standard deviations. This is a result of our first sarin experiment. The number of assays performed was 6 for each substrate in the mitochondria isolated from control + stress treated animals and three for succinate and two for pyruvate/malate for the mitochondria isolated from sarin treated animals.

The Effects of DEET, Pyridostigmine Bromide and Stress on Mitochondrial Electron Transfer Activities.

Rats were treated with: 1) 37.5% of the LD_{50} dose of DEET; 2) 37.5% of the LD_{50} dose of DEET in the presence of noise stress; 3) 5 mg pyridostigmine bromide (PB)/ Kg body weight in the presence of noise stress; 4) 37.5% of the LD_{50} dose of DEET and 5 mg pyridostigmine bromide (PB)/ Kg body weight in the presence of noise stress using a protocol presented in the Auditory Biology/Magnetic Resonance portion of this report (Sections 2A&B). Thirty days after the initial treatment, animals were sacrificed and mitochondria were isolated from brain stems.

Table 2-6 shows the effects of long-term exposure of the Gulf War chemicals with noise stress on partial electron transfer reactions of the rat brain stem mitochondrial respiratory chain when assayed in the absence of a membrane potential or a pH gradient. Our original approach was to use three different assays to dissect the effects of the chemicals on different portions of the mitochondrial electron transfer chain. They were cytochrome oxidase activity (cytochrome *c* to oxygen), succinate dehydrogenase (succinate to PMS/DCIP in the cytochrome bc_1 complex) and NADH (from NADH dehydrogenase through the cytochrome bc_1 complex) to ferricyanide. We found that both the succinate dehydrogenase and NADH dehydrogenase activities were not reproducible from preparation to preparation. Thus, we decided to use NADH as a substrate to

transfer electrons through the bc_1 complex to cytochrome *c* in the presence of cyanide (to block cytochrome oxidase) to assess most of the respiratory chain's activity. Table 2-6 shows that this assay gave more reproducible results. The rates of electron transfer from NADH to cytochrome *c* are very similar for most treatments except that there is a noted decrease in activity in the stress treated controls and also animals treated with PB, DEET, and noise stress. We observed 15% and 32% inhibition of activity in these treatments, respectively. These decreases in electron transfer activity were further corroborated by measuring NADH to ubiquinone (Complex I) activity in the presence of antimycin and cyanide. Inhibition was observed in the same samples (26% for control + stress and 29% for DEET, PB, + stress), suggesting a possible site of inhibition in Complex I of the respiratory chain. Both the NADH to cytochrome *c* and the NADH to ubiquinone assays are dependent on electron flow through Complex I of the respiratory chain, the most labile site of oxidative damage in the respiratory chain. Thus, our results suggest that in part, stress affects mitochondrial functioning by inhibiting electron transfer through Complex I and that the Gulf War chemicals synergistically increase oxidative damage in Complex I in brain stem mitochondria. Since the doses used in our studies are at such low levels, the effects of the chemicals are small. Yet if exposure to the chemicals was long enough or alternatively at higher doses, the effects of oxidative stress on brain stem mitochondria could account for many of the symptoms described.

Supporting the concept that the low doses used in our studies cause slight but observable effects on mitochondrial electron transfer, Table 2-6 shows that there is variability in Complex I activity within groups of treated animals (DEET, PB, + stress). Two sets of treated animals showed the inhibition of electron transfer in Complex I as assessed by the NADH to cytochrome c and NADH to ubiquinone assays. However, one set of animals showed no effects. Our interpretation of these results is that individual rats (as humans) may have different susceptibility to the chemicals when under stress, suggesting that at low doses of the chemicals effects may be very difficult to distinguish in groups of four animals.

Table 2-6. The Effects of a 37.5% LD_{50} Dose of DEET, 5 mg/Kg Body Weight Dose of PB, and Stress on Rat Brain Stem Mitochondrial Electron Transfer Activity

Treatment	Specific Activities (nmol/min/mg-protein)[a]	
	Cyt. $C \Rightarrow O_2$	**NADH $\Rightarrow$ Cyt. C**	**NADH $\Rightarrow$ UQ**
I. Control		
	248 ± 20	187 ± 13	57 ± 9
II. Control with Noise Stress		
	205 ± 31	159 ± 15	43 ± 6
III. DEET-Treated		
	243 ± 18	206 ± 10	59 ± 10
IV. DEET-Treated with Noise Stress		
	219 ± 12	187 ± 30	56 ± 11
V. PB-Treated + Noise Stress		
	235 ± 7	176 ± 5	63 ± 4
VI. PB and DEET-Treated + Noise Stress		
	212 ± 13	127 ± 5 200 ± 12	41 ± 2, 62 ± 7

[A]activity measurements are presented in nmoles oxygen consumed, nmoles cytochrome c reduced, or nmoles NADH oxidized/mg mitochondrial protein –min. Error measurements are standard deviations. The number of preparations was 4 for control, 4 for control with noise stress, and 3 for DEET-treated with noise stress, 2 for DEET-treated without noise stress, 2 for PB-treated with noise stress, and 3 for PB and DEET-treated with noise stress. Each assay was performed at least three times for each preparation.

The Effects of Sarin and Stress on Mitochondrial Electron Transfer Activities.

Table 2-7 shows the effects of long-term exposure of sarin and noise stress on partial electron transfer reactions of the rat brain stem mitochondrial respiratory chain when assayed in the absence of a membrane potential or a pH gradient. As shown above, stress induces an inhibition of electron transfer in the Complex I region of the respiratory chain, which is exhibited by decreases in rate of electron transfer in NADH to ubiquinone and NADH to cytochrome *c* specific activities. Table 2-7 shows that sarin induces an additional inhibition of activity in these two assays. NADH to ubiquinone and NADH to cytochrome *c* specific activities are inhibited an additional 20%, suggesting that low doses of sarin induce additional damage to the respiratory chain that coincides with the most labile site for oxidative damage in the respiratory chain. However, this result is from only one experiment and we currently are starting the next set of animals on the same protocol.

Table 2-7. The Effects of Sarin and Stress on Rat Brain Stem Mitochondrial Electron Transfer Activity

Treatment	Specific Activities (nmol/min/mg-protein)[a]		
	Cyt. *C* ⇒ O_2	**NADH ⇒ Cyt. *C***	**NADH ⇒ UQ**
I. Control with Noise Stress			
	183 ± 23	154 ± 2	50 ± 1
II. Sarin-Treated with Noise Stress			
	156 ± 15	124 ± 8	43 ± 2

[A]activity measurements are presented in nmoles oxygen consumed, nmoles cytochrome c reduced, or nmoles NADH oxidized/mg mitochondrial protein –min. Error measurements are standard deviations. These data are the result of one experiment (see text). Each assay was performed at least three times for each preparation.

Our results suggest that noise stress alone induces an inhibition of electron transfer in Complex I (NADH dehydrogenase) in mitochondria isolated from treated animals. In addition, sarin and noise stress and also PB and DEET with noise stress both induce significant decreases in electron transfer rates compared to noise stress alone, suggesting that the Gulf War chemicals and stress may induce an increase in reactive oxygen species within brain stem cells. This increase in reactive oxygen species could lead to damage to the mitochondrial respiratory chain at its most labile site of oxidative damage in Complex I.

Assessment of Specific Activities for Mitochondrial Electron Transfer Assays

We have found that specific activities of our preparations vary and are very difficult to quantitate reproducibly. In an attempt to assess if this variability was due to changes in mitochondrial functioning or whether it was due to differences in purity of our preparations, we measured cytochrome *c* oxidase content in our isolated mitochondria using three independent methods: oxygen reduction activity of intact mitochondrial membranes; oxygen reduction activity using detergent solubilized mitochondria; and immunological detection of cytochrome *c* oxidase by immunoblotting. We measured cytochrome *c* oxidase content on two preparations of mitochondria that exhibited the largest difference of specific activities for intact mitochondrial membrane (Table 2-8.).

Table 2-8 shows examples of preparations of rat brain stem mitochondria where the specific activities of membrane bound cytochrome *c* oxidase are at the highest and lowest levels that we have observed since this project began. One preparation (#1) has 50% lower specific activity than the other when assaying intact, uncoupled mitochondria.

Table 2-8. Cytochrome *c* Oxidase Content of Isolated Rat Brain Stem Mitochondria Determined by Three Different Methods

Preparation	Specific Activities (nmol O_2/min/mg-protein)[a]		**Area on Immunoblot /µg Protein Loaded[b]**
	Membrane Bound Cyt. C ⇒ O_2	**Detergent-solubilized Cyt. C ⇒ O_2**	**Subunit IV of Oxidase**
I.	171	2313	959
II.	**337**	**3946**	**1242**

[A] Membrane bound cytochrome *c* oxidase assays were performed in a buffer consisting of 10 mm KH_2PO_4, 250 mm sucrose, 50 mm kcl, 5 mm $mgcl_2$, 1mm EGTA, ph 7.4. Substrate concentrations used were 7.5 mm ascorbic acid, 25 µM cytochrome *c*, and 0.6 mm TMPD with 5 µgm/mg protein of antimycin a added to inhibit electron transfer at the cytochrome bc_1 site. Detergent solubilized cytochrome *c* oxidase activities were measured as above except 0.5 mm dodecyl maltoside was included in the assay buffer and mitochondria were preincubated in 1% dodecyl maltoside for 15 minutes at 0 °C prior to performing the assay.

[B]immunoblotting was performed as described in Ogunjimi et. Al (2000) using polyclonal anti-cytochrome *c* oxidase sera prepared in our laboratory. This sera contains antibodies to all subunits of the enzyme except subunit III. Subunit IV staining intensity (from alkaline phosphatase linked to a secondary antibody) was measured using a scanning densitometer and areas integrated using Sigma Gel and Image Quant software. The linear range of the staining intensity was determined experimentally using beef heart cytochrome *c* oxidase to calibrate the color development.

A 50% difference between the two preparations was observed with the membrane bound enzyme assay, while a 42% difference was observed with the detergent-solubilized assay. The antibody staining intensity exhibited a 30% difference in cytochrome *c* oxidase expression level.

We then tested cytochrome *c* oxidase activity in detergent-solubilized mitochondria in order to test the idea that perhaps the assay of the membrane bound activity was providing us with unreliable results. The detergent solubilized activity is not likely to be controlled by any localized membrane potential or limited by substrate binding (cytochrome c) to the intact mitochondrial inner membrane. The results show that preparation #1 exhibited a 42% decrease in activity, closely matching the membrane bound activity measurement. Finally, we solubilized the same amount of mitochondrial protein from each preparation with SDS, ran each on SDS-PAGE, transferred the protein to PVDF, and developed the immunoblot with our anti-cytochrome *c* oxidase antibody. Upon scanning with densitometry and quantitating the staining intensity of each by running standards, we found that the cytochrome *c* oxidase content in preparation #1 was 30% less than preparation #2, which is in close approximation to the other activity based measurements. Our conclusions from these data (Table 2-8) are that our cytochrome *c* oxidase measurements presented in Table 2-6 are accurate and that most preparations of rat brain stem mitochondria exhibit similar levels of purity based upon specific activity measurements.

Measurement of Mitochondrial Cytochrome c Content as a Marker for Apoptosis

Figure 2-12 shows the cytochrome *c* content of our isolated mitochondria as detected by immunoblotting using our polyclonal antibodies to cytochrome *c*. There are no statistically significant differences in the cytochrome *c* content of mitochondria isolated from control animals treated with noise stress, DEET and PB-treated animals which received noise stress, and PB-treated animals which also received noise stress. This result taken together with the data where similar yields of isolated mitochondria were obtained from brain stems in control and treated animals suggests that PB and DEET with noise stress does not induce significant apoptosis in rat brain stems during the time period studied. Our immunological analysis of our preparations using the different treatments with the Gulf War agents is still incomplete.

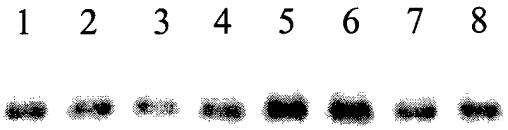


Figure 2-12. The Effects on PB with Noise Stress, PB+DEET with Noise Stress, and Noise Stress on the Cytochrome *c* Content of Isolated Rat Brain Stem Mitochondria.

Electrophoresis, protein transfer to PVDF, and blot blocking were performed as described in Ogunjimi et *al.* (2000) and the blot was developed using polyclonal antibodies against cytochrome *c* (1:1000 dilution) (Kirken et *al.*, 1995). Color development was facilitated using alkaline phosphatase linked to goat antirabbit igg. Lanes 1-4 contain 2.5 μgm mitochondria from animals treated with noise stress; Lanes 5 and 6, 2.5 μgm mitochondria from animals treated with PB and stress; Lanes 7 and 8, 2.5 μgm mitochondria from animals treated with PB + DEET and stress. Scanning densitometry showed no significant differences between lanes 1-8.

Future Directions (July through October, 2003).

We will repeat the low dose exposure of animals to sarin in the presence of noise stress, isolate mitochondria from the treated animals, and perform energy coupling and electron transfer assays. We will also continue to assess the cytochrome c to cytochrome oxidase ratio (to quantitate loss of cytochrome c from the isolated mitochondria) by using immunological methods.

Project 3: The study of enzymes involved in chemical metabolism, activity of dehydrogenases and esterase in human tissues with the goal of establishing whether there are alterations in populations characterized as chemically sensitive.

PI: Gerald M. Alter, Ph.D.

Key Accomplishments:

1. Completed alternate analyses of pilot study data and identified "subgroups" within our experimental and control groups. This suggests novel factors which will increase the reliability of correlations between enzymatic analyses and abnormal or disease states.
2. Substantial progress has been made on the collection and analysis of blood samples from normal, GWS, and chemically sensitive individuals for the main study of this project. These substantially extent results form the present study, validate methodology development and/or results of the pilot study.
3. Developed a sarinase assay that is suitable for all blood components used in this study and identified suitable conditions for completing study assays.

Esterase and Dehydrogenase activities in Blood Fractions from Two Studies:

Here we report results from two studies. One is a pilot study whose results have been discussed in previous quarterly reports, but the data has been analyzed with regard to personal identifiers. The second is a new study using fresh blood fractions from novel individuals in accordance with a double blind protocol. In our new study total enzyme activities for five groups have been collected and are reported in this paper. Esterase enzymes, Aryl esterase and Paraoxonase, and dehydrogenase enzymes, chi Aldehyde dehydrogenase and Alcohol dehydrogenase, have been measured in serum, red blood cells, and white blood cells. The five new groups involve 59 individuals. They include (1) fifteen individuals from the Dayton area used as a control, (2) a known chemically sensitive group of seven individuals from Dayton, (3) eight chemically sensitive patients from Buffalo, N.Y., and two groups, (4) one group of fifteen individuals, and (5) one group of fourteen individuals, from Texas. In the graphs reported here these groups are referred to as groups 1, 2, 3, 4, and 5 respectively. The new Texas groups are comprised of normal, GWS, and chemically sensitive patients, collected and analyzed according to a double blind protocol. Texas samples are placed into two groups because they were received four months from one another. Since receiving information about the identity of individuals from the data from the previously reported pilot study we have been able to classify individuals from the Texas group of that study. The participants in the pilot study were classified according to their sensitivity or put in a control or GWS group. The data were analyzed and repeated and are reported according to this new classification. One significant general result that arises from comparing the pilot and recent studies is that much cleaner assays were obtained. This is probably owing to improved handling, separation, and more timely analysis of samples. As anticipated the improved methods reduce the amount of noise in the kinetic profile and allow more accurate and repeatable results.

Esterase: Paraoxonase and Aryl esterase activities of Blood Components: Total activity of individuals from Two Studies

In the new study esterase activities are measured in the same way described in previous reports, using paraoxon and phenyl acetate as substrates for esterase enzymes in the reaction mixtures of assays. However one significant way the new blood fractions differ from those used in the pilot study is with regard to the anticoagulant used in blood collection. In the pilot study EDTA was used as the anticoagulant in all blood fractions, and in the new study Heparin is the anticoagulant of choice. Serum is the only blood fraction in which no anticoagulant was used. Paraoxonase (PON) and Aryl esterase (AE) total activities are compared between the new and pilot study reported in March. These activities are repeatable and show the same relationship between esterase enzymes with Aryl esterase activities doubling or tripling the activities of Paraoxonase. The previously measured levels of esterases are reported in plasma in Figure 1 within their new subgroups. Figures 3-1A through 3-1D show AE and PON with and without the addition of EDTA in plasma from the pilot study. The groups represented include a group with formaldehyde sensitivity, a group with GWS, a general chemical sensitivity group, and two groups of controls. The first control group, DB control, is comprised of individuals we classified as controls after personal identifiers were revealed. The second control was a known group of Texas controls. The apparent amplitude of activity is higher in the samples with EDTA. As mentioned in the last quarterly report, we believe that the activity is associated with albumin rather than Aryl esterase, which is inhibited by EDTA. Additional EDTA apparently stimulates the albumin hydrolytic activity. In figures 3-2A and 3-2B esterase enzymes are reported from the five new groups in serum. There appear to be similar levels of individual variation within the groups with regard to total activity, but predictably the serum has higher levels of esterase activity than plasma (Figure 3-2A). One striking feature of the comparison appears to be the variation in magnitude of activity. This is because activities in the newer samples represent activities in the assay cuvette while activities in the pilot study are per 2 ml of whole blood. The total activities presented in Figure 3-1 reporting esterase activity in plasma show similar scatter to the esterase activities measured in serum in Figure 3-2. Figure 3-2 reports esterase activity in serum from the new study. However, the scatter seems to be biological rather than methodological because there is very little standard deviation between measurements. The variation of total activity in plasma is similar to other activities measured in the new study. In all measurements the control has higher activity than the known chemically sensitive groups. However whether this variation in total activity is statistically significant is not yet known. In figure 3-2 the greatest amount of variability is present in the last two groups, both groups of double blind Texas participants. Some activities in the Texas groups are within range of the controls, while others are within the range of chemically sensitive individuals. This can be attributed to these groups containing normal, GWS, and chemically sensitive individuals.

Esterase was also measured in red blood cells, rbcs. Figure 3-3A through 3-3D report the total activity of esterase enzymes in RBCs from the pilot study. According to the results there seem to be no significant differences between groups with regard to total activity. The two chemically sensitive groups, first and third series, have more tightly associated activities with less individual activity scatter. The GWS, second series of activities, and the control, fifth series, have the largest amount of methodological standard deviation. In both AE and PON measurements more total activity is measured in the presence of EDTA, Figure 3-3C and 3-3D. When EDTA is added to the reaction mixtures certain individual activities are distinct from the

remainder of the individuals in the group. Whether these differences imply a separate sub group or phenotype is not yet known.

In Figure 3-4 PON activity is reported in RBCs from the new study. The results show about half of the control samples to be within range of the double blind Texas samples and the chemically sensitive individuals. The slightly larger standard deviation in these measurements (figure 3-4B) is the result of the nature of lyzed RBCs and their tendency to create noise in the kinetic profile. One interesting result of the study was that any esterase activity was measured in RBCs. Although it is a small amount of activity, and we cannot be sure the differences are significant, a rate was measurable in all five groups.

In the pilot study esterase levels were measured in WBC (Figure 3-5). The pattern of scatter is similar to that observed with other esterase enzymes. Although esterase activity was assayed in WBC in the new study it was not significant with regard to the blank upon analysis (data not shown).

Formaldehyde Scavenging Dehydrogenase: Aldehyde dehydrogenase and chi Alcohol dehydrogenase (xADH) in blood components

Dehydrogenase activities in plasma, RBCs, and WBCs are displayed in figures 3-6, 3-7, 3-8, and 3-9. In figure 3-6 dehydrogenase activity is reported in plasma from the pilot study. Five groups are represented: (1) Formaldehyde sensitive, (2) GWS, (3) general sensitivity, (4) double blind Texas control, and (5) control, from left to right. This group order is repeated for all data reported from the pilot study. Aldehyde dehydrogenase (ALDH) and chi Alcohol dehydrogenase (xadh) assays were completed in the same manner reported previously using formaldehyde as substrate in the reaction mixtures, and glutathione (GSH) as a co-substrate in xadh assays. In these results the assay variation (error bars) is small relative to variation from subject to subject.

Figure 3-6A and 3-6B report activities of ALDH and xadh in plasma from the pilot study. Figure 3-6A shows little variation in the activities of the first four groups. However, the control group has lower activity than the other four groups, almost indistinguishable. Total activity of ALDH is lower than x ADH by a factor of ten in the RBC's components. No dehydrogenase activity was observed in serum during our recent study. This is in contrast to the activities observed in plasma collected during our pilot study. This likely reflects improved separation of blood components in the recent study that eliminated contamination of the extra cellular blood fraction with the contents of broken cells.

Figure 3-7 reports dehydrogenase activities in rbcs for the same five groups in the pilot study. In figure 3-7A ALDH is reported in RBCs. ALDH activity is lower than xADH by the same factor of ten observed in plasma. Because it is a mitochondrial enzyme we would not expect to observe any activity in rbcs, but it is nonetheless present in small quantities. These activities probably reflect lysis of red cells, which contaminated plasma. In this graph the Dayton control is on average within range, slightly higher, than the other four groups, but there is a greater degree of scatter than in the other groups. Overall the activities in all groups are approximately equivalent. In figure 3-7B xADH activities are reported. The Dayton control activities are also slightly higher than the Texas control and the chemically sensitive groups, with one exception in the formaldehyde sensitive group. If we consider this rate and outlier the Dayton control activity averages higher than the other four groups. Dehydrogenase activities were not observed in WBC

Figure 3-8A and 3-8B report the ALDH and xADH activities in RBC of all five groups from the new study. These results indicate a repeatable pattern. The Dayton control has

consistently higher activity than the chemically sensitive or Texas groups, which is a mixture of normal, sensitive, and GWS individuals. Finding ALDH activity in rbcs is surprising because rbcs contain no mitochondria and ALDH is normally distinguished as a mitochondrial enzyme. This could suggest there are significant cytosolic ALDH isoenzymes. In general activity is higher in these samples than in the pilot study. This is a result of both improved handling and more timely collection of activity after blood is drawn.

Dehydrogenase activities were not observed in wbcs collected for the pilot study. This may be the result of handling or dilution of samples in the pilot study making it difficult to measure a significant rate. We believe the ability to detect activity in WBC is the result of improved handling of blood fractions.

Figure 3-9A and 3-9 B report ALDH and xADH activities in WBC. The activity pattern observed in xadh is similar to that observed for ALDH, with the highest activity observed in the control and Texas groups and the chemically sensitive groups consistently lower than the control. These results have less methodological standard deviation associated with them. We are currently analyzing these results with the personal identifiers provided from Texas to see if any sub groups might exist within these groups.

Phenotype: Grouping of Normal and Test individuals

Though, as whole experimental measurements are quite repeatable in our pilot and recent studies, there is large variation among individuals within the same group. This might reflect multiple groups or individuals within our groupings. This could be as significant as any of our original groupings. To probe for this possibility activities of individuals were compared in two ways. We followed the genotyping method of Robert Haley, which characterizes individuals on the basis of the ratio of esterase and paraoxonase activities (Robert W. Haley, Toxicology and Applied pharmacology, 157, (227-223) 1999. Figure 3-10 shows the results from the pilot study plotted in such a way with PON data on the X-axis and AE results on the Y-axis. The groupings that emerge show a consistent pattern where the controls are in the upper right portion of the graph indicating the R type or heterozygous phenotype, and all the sensitive individuals are tightly grouped in the lower left of the graph indicating Q type or homozygous phenotype. These results indicate that two phenotypes exist in the pilot study. The data from the current study is being further analyzed for phenotype differences and will appear in the next quarterly report.

A second method of examining groups of individuals was to compare the profile of all enzyme activities measured for each person. Representative results are shown in figure 3-11A and 3-11B. The different line shapes seen within figure 3-11 imply that different "sub groups" may be present. We are currently correlating individual histories with the profile differences to determine the basis for these "sub" groupings. Preliminary analysis suggests similar patterns have been observed. These data were reconfigured in order to see if any patterns with regard to enzyme profile would emerge. The pattern of activity is the same with some notable exceptions in the two Texas groups. Two individuals in the Dayton chemically sensitive group have different patterns with regard to dehydrogenase profile. We cannot be certain that this data indicates entirely different groupings, but it would appear that some of the individuals in each group have different patterns with regard to dehydrogenase activity in particular. In the Texas groups there appear to be different groups with regard to amplitude in all enzymes, but the pattern is consistent overall.

Sarinase Assays

Figure 3-12 is a velocity versus substrate plot for sarinase saturation. As the graph shows complete saturation has not yet been achieved. We cannot however use saturating levels of sarin due to its availability, and the large volume required for repeatable results. Currently, sarinase assays are being completed for all samples in serum, and will be reported in the coming months.

Figure . 3-1

Esterase total activity in plasma

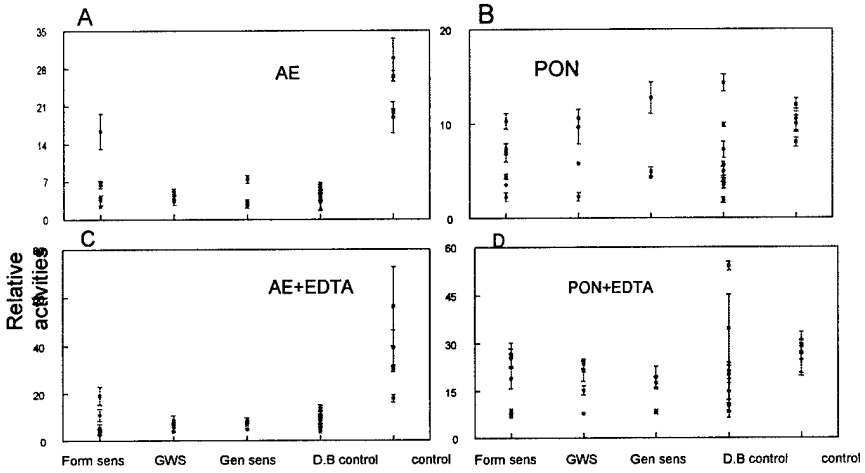


Figure. 3-2A

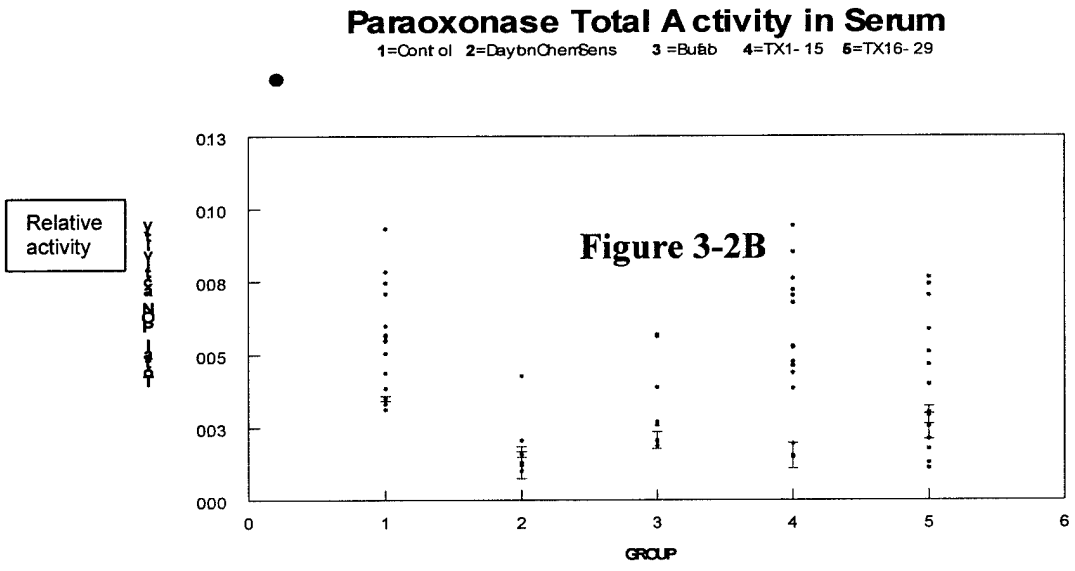


Figure 3-2B

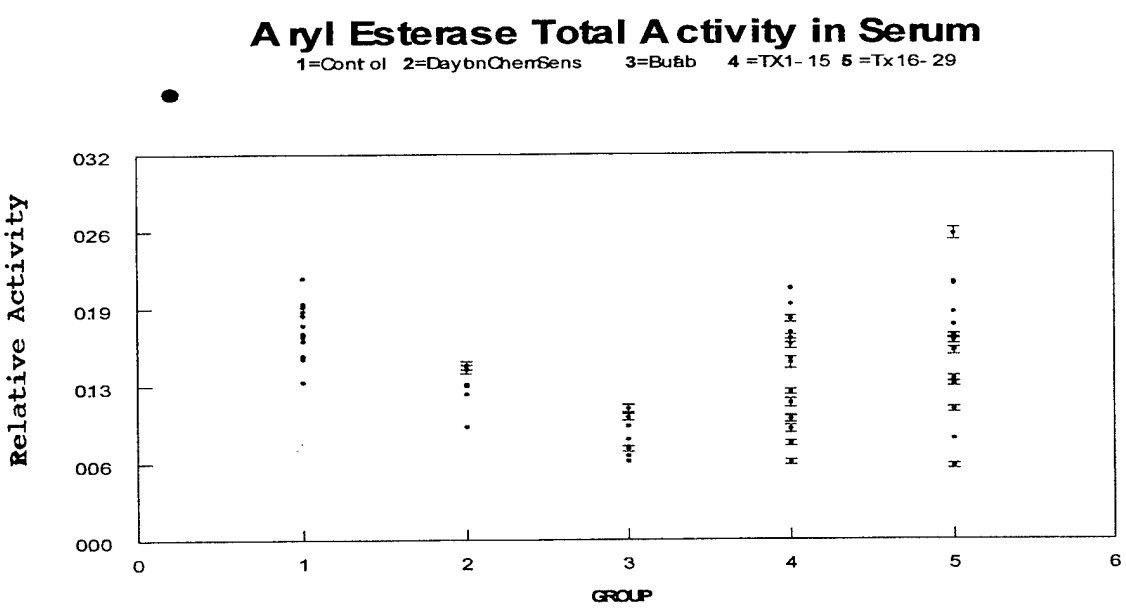


Figure 3-3

Esterase total adivityin RBC

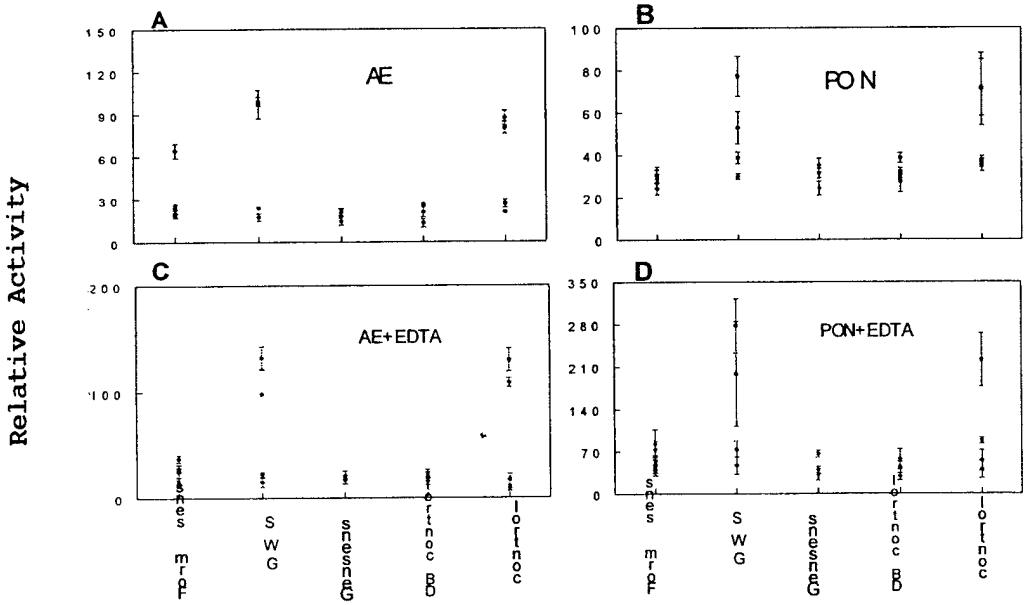


Figure. 3-4A

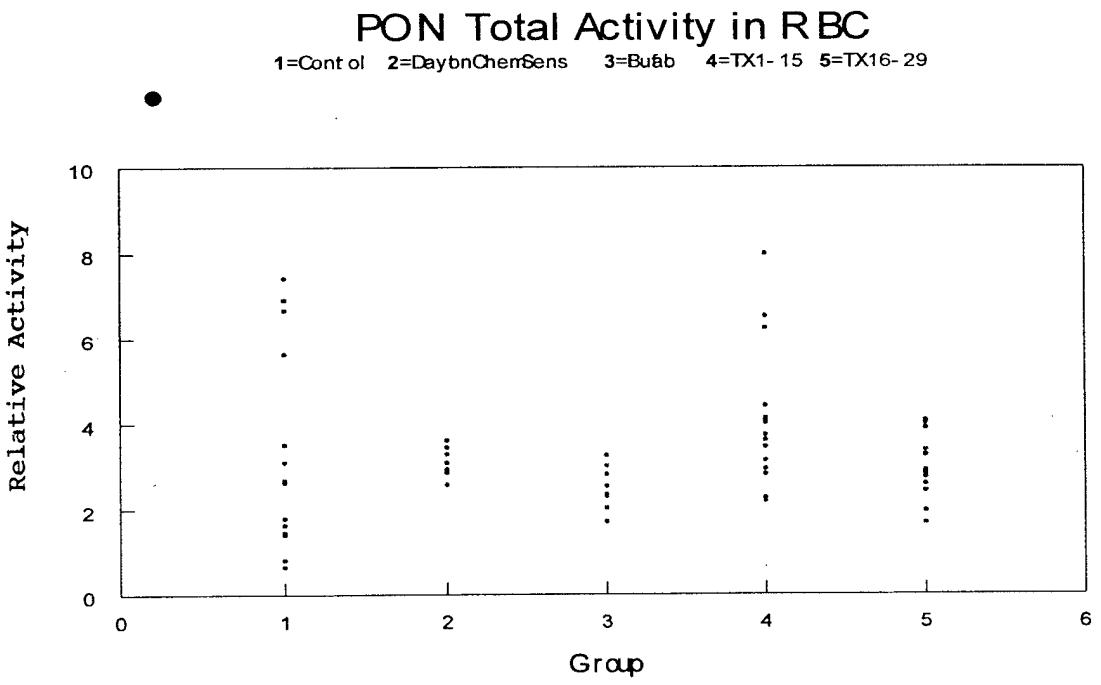


Figure. 3-4B

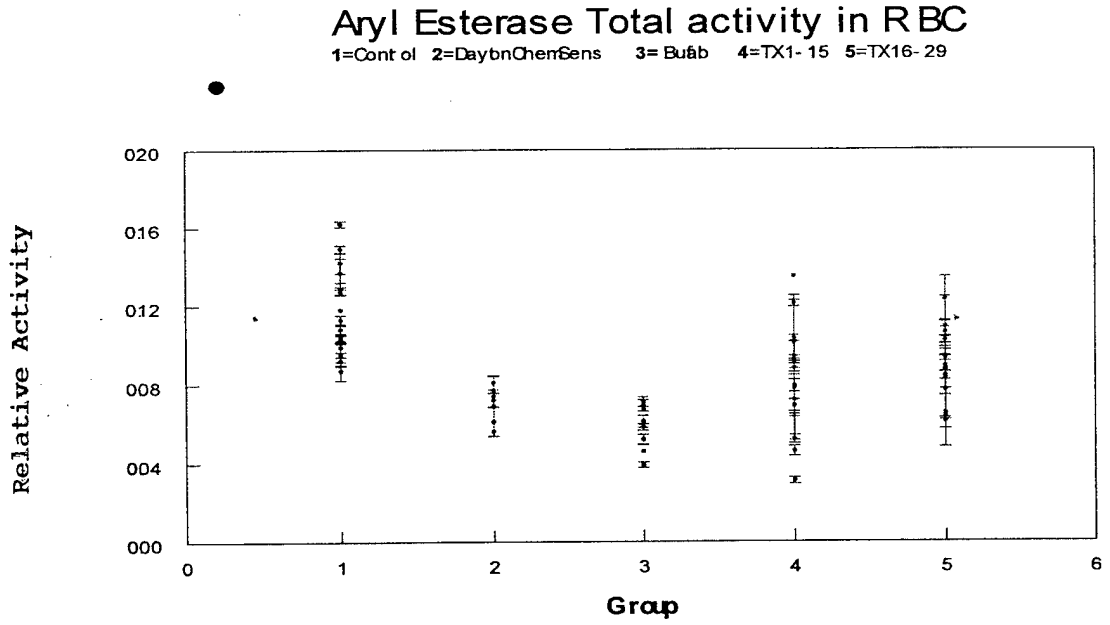


Figure 3 - 5

Esterase total activity in WBC

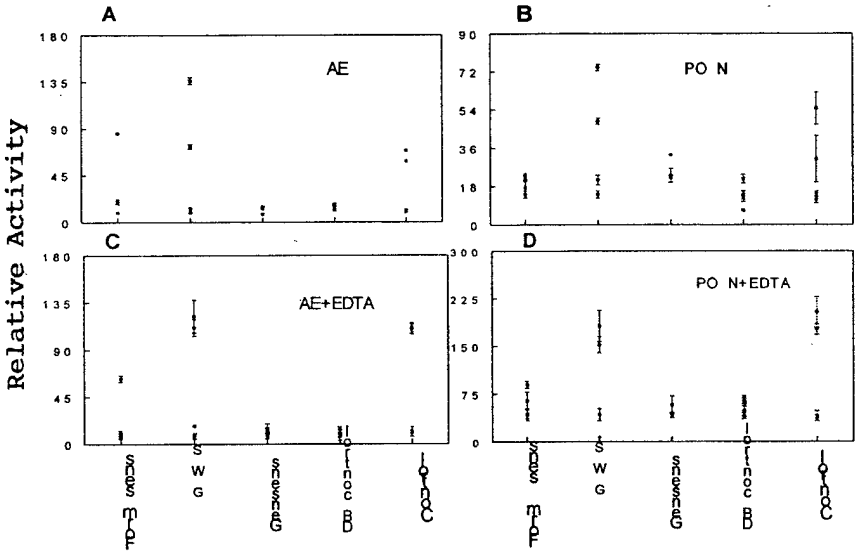


Figure 3 - 6

Dehydrogenase total activity in plasma

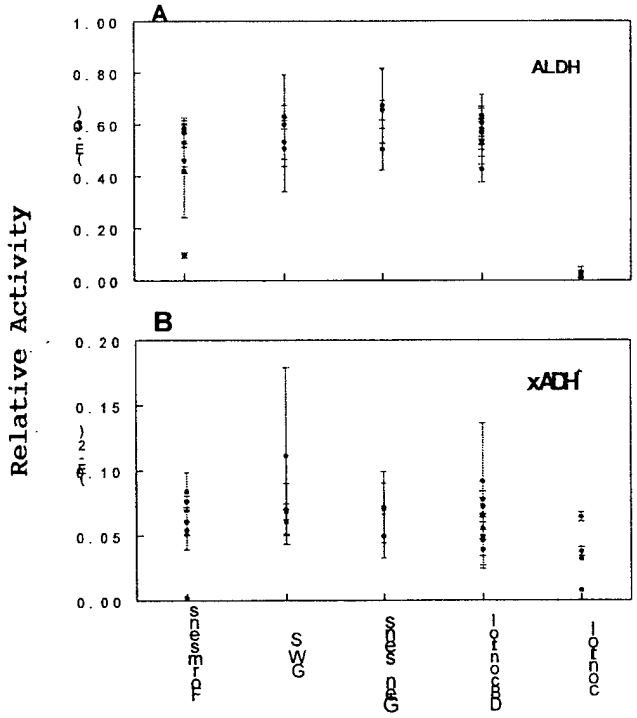


Figure 3 - 7

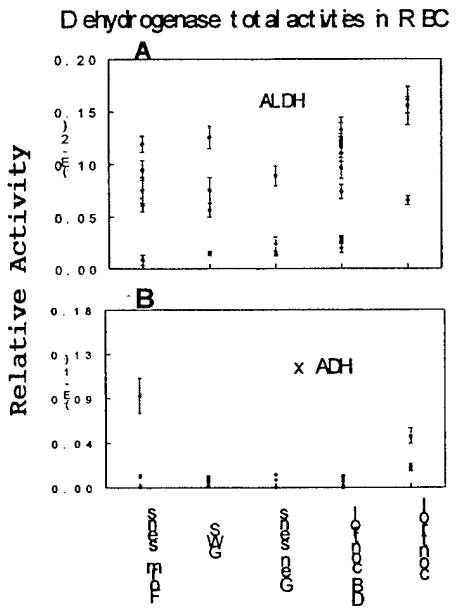


Figure. 3-8A

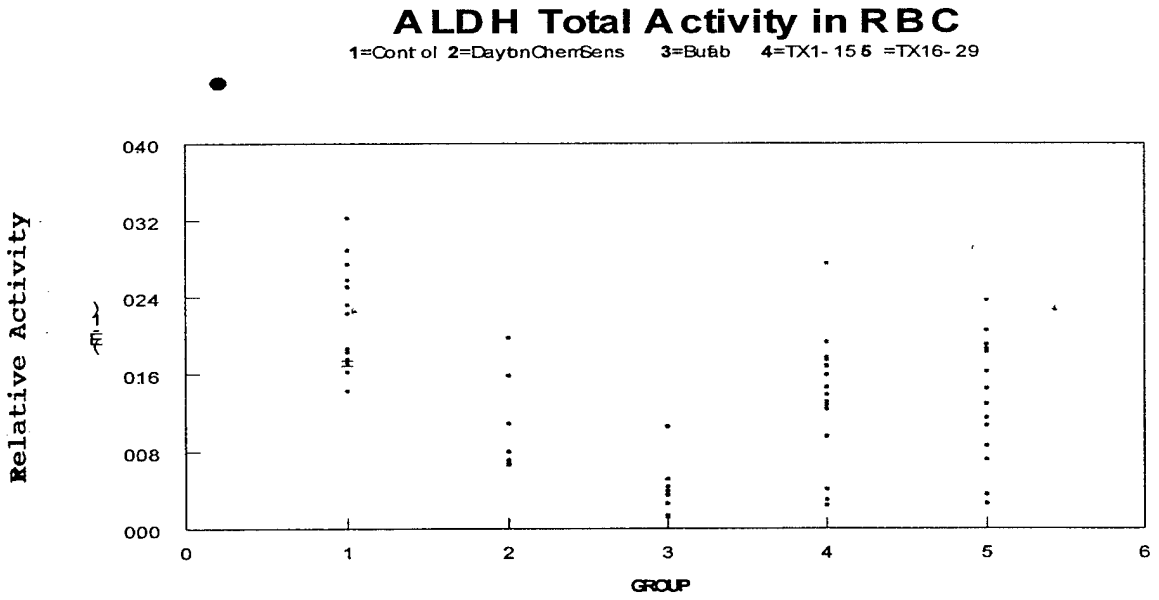


Figure 3-8B.

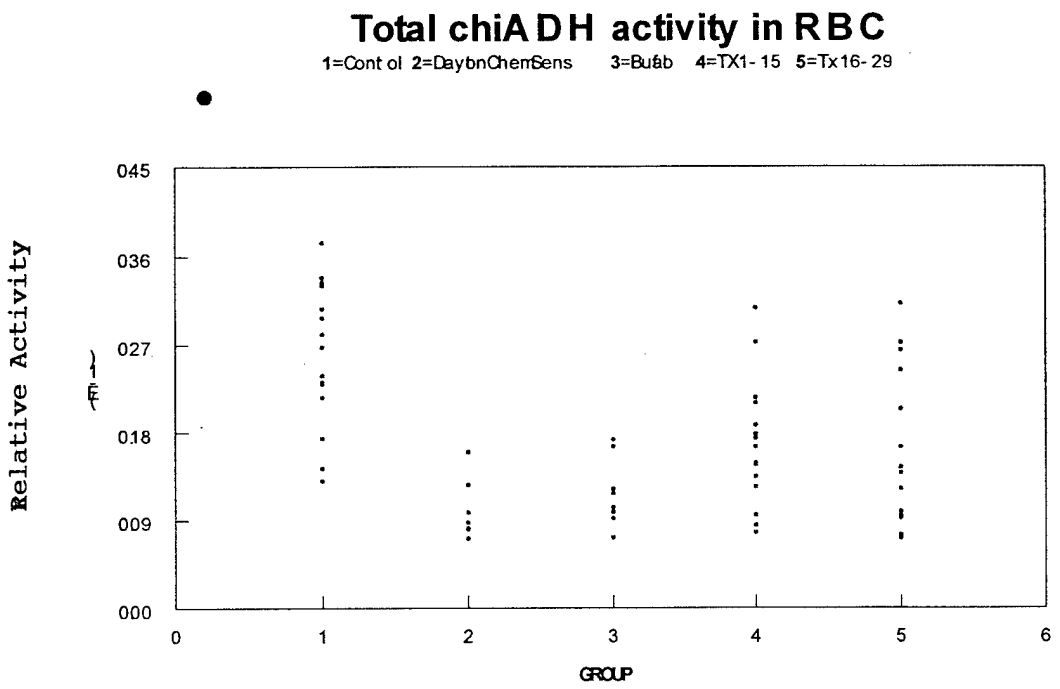


Figure 3-9A

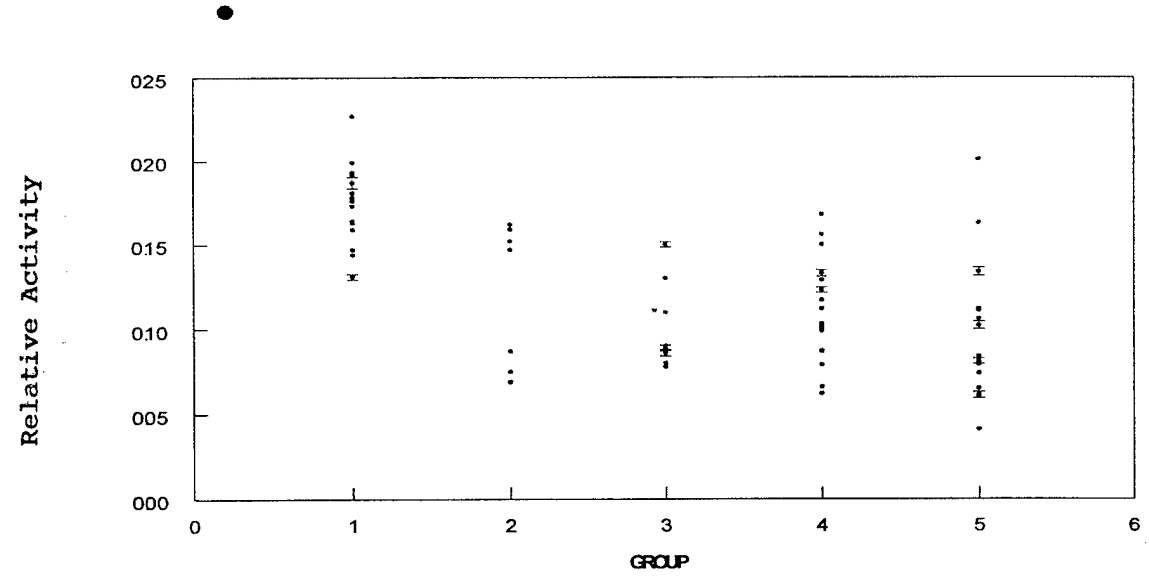


Figure. 3-9B

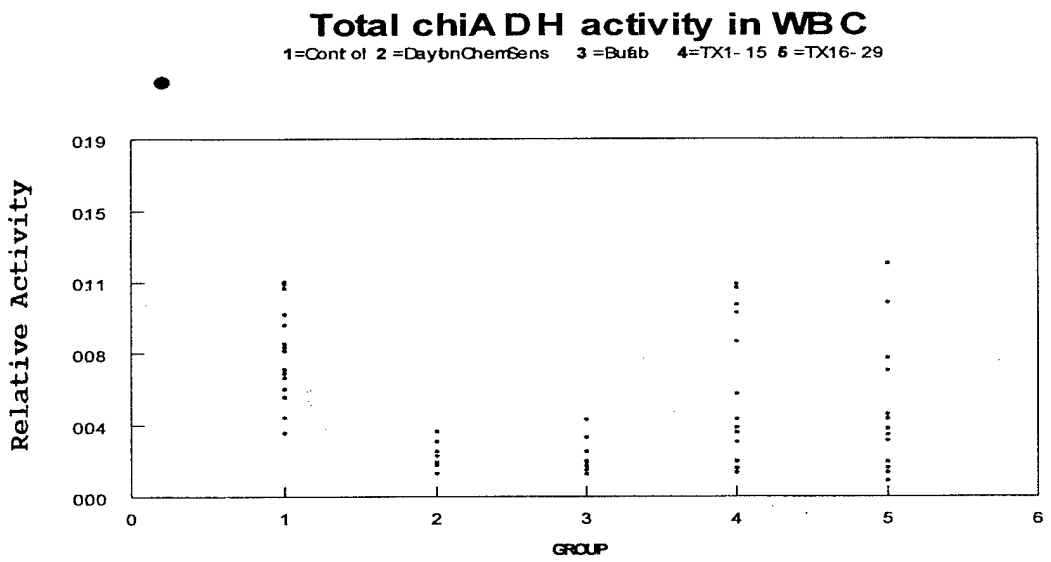


Figure 3- 10

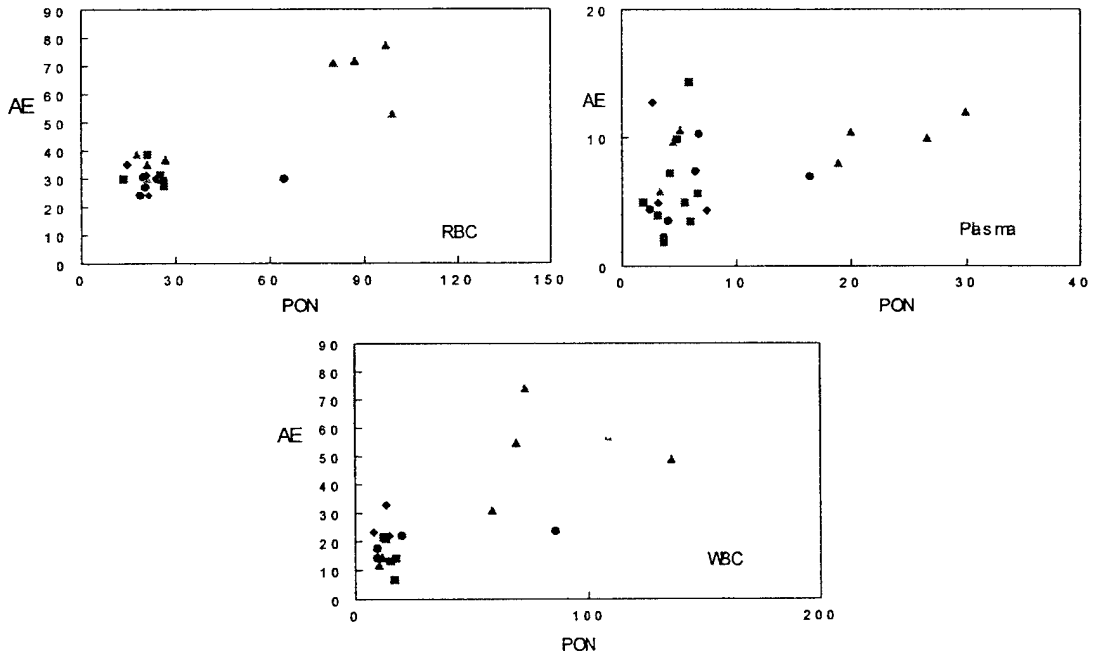


Figure. 3-11A

Total Enzyme Profile for Buffalo Chemically Sensitive Grou

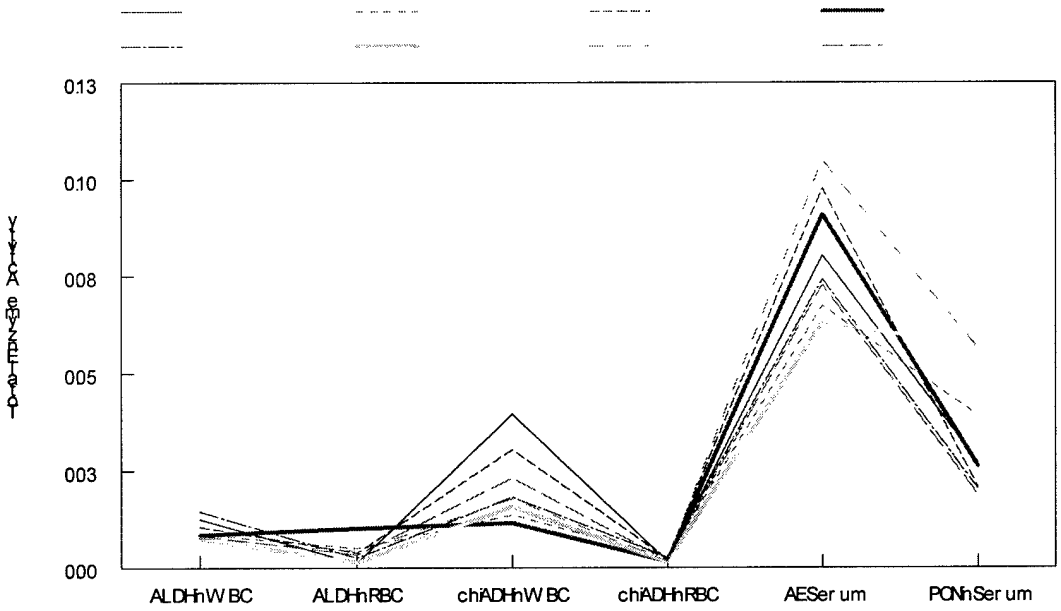


Figure 3-11B

Total Enzyme profile for Dayton Chemically Sensitive Gr

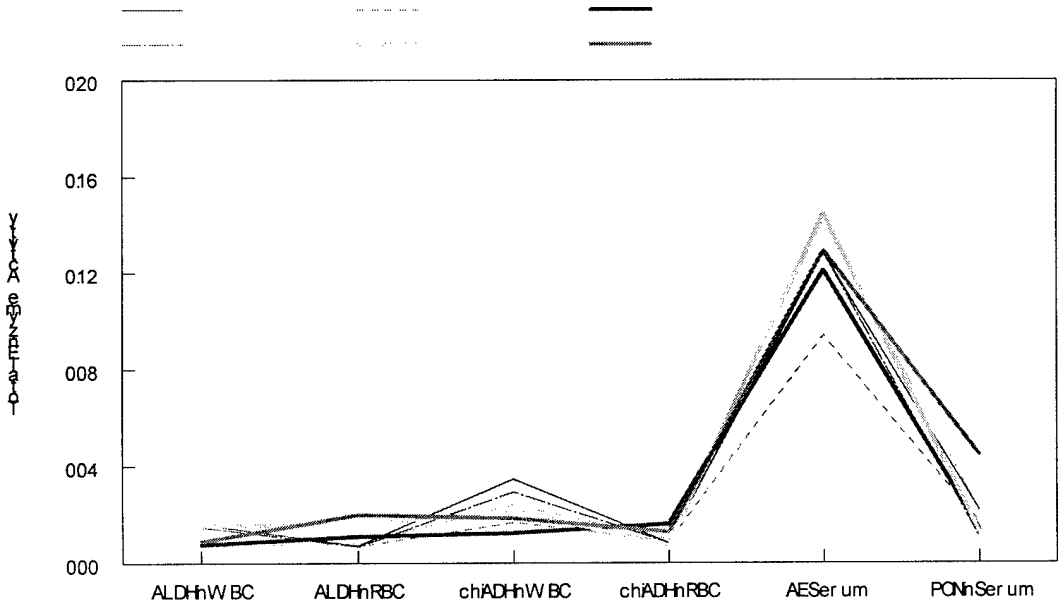
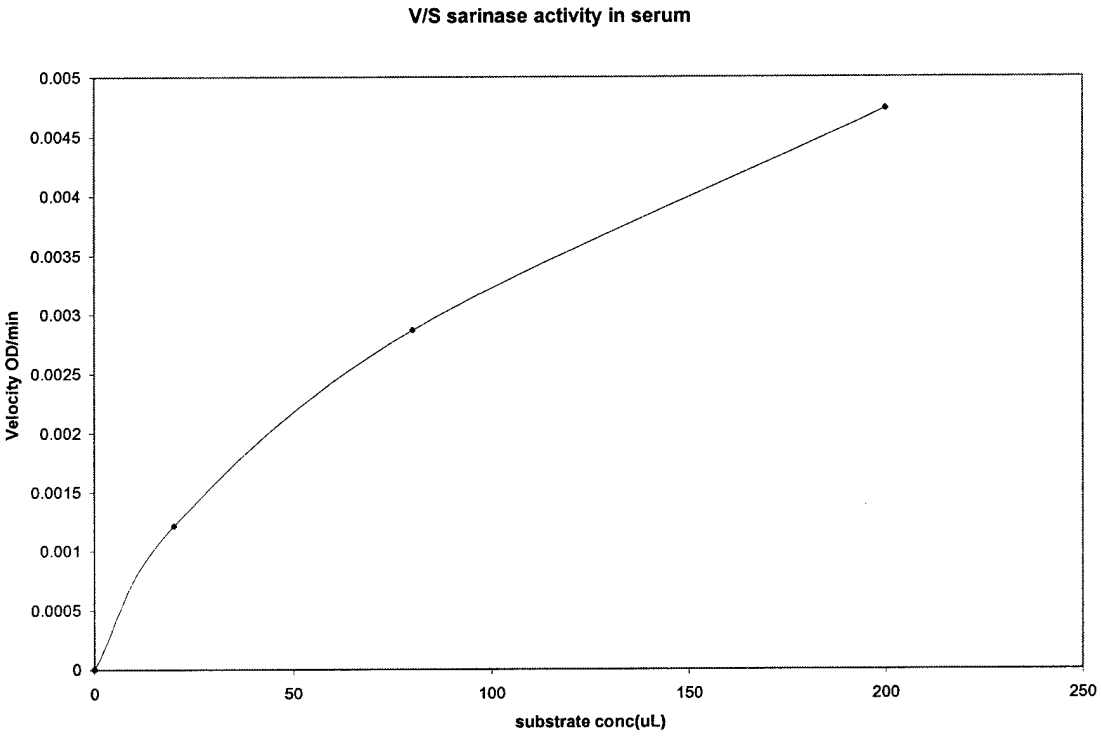


Figure 3-12


V/S sarinase activity in serum
Velocity OD/min
0.005
0.0045
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
50
100
150
200
250
substrate conc(uL)

Project 4: The study of gene expression using a DNA microarray system to test the effect of chemical exposure on a neuronal cell line.

Investigators: Steven J. Berberich, Ph.D., Tina Caserta, M.S., John Paietta, Ph.D. And Madhavi Kadakia, Ph.D.

STATEMENT OF WORK: YEAR 3

1. Completed: Gene expression profiling of sarin exposure of SH-SY5Y neurons.
2. Completed: Gene expression profiling of sarin and pyridostigmine bromide exposure of SH-SY5Y neurons.
3. Completed: Data-mining of gene expression data from pyridostigmine bromide exposed neurons.
4. In progress: Data-mining of gene expression data from sarin and sarin with pyridostigmine bromide exposed neurons.

SUMMARY PROGRESS REPORT

The activities during the third year of this project involved examining the gene expression profiles of differentiated SH-SY5Y cells when exposed for short periods (1-2 hours) with varying doses of sarin (0.19-19 μg/ml). Initially, we undertook experiments to test the viability of cells when placed in a containment device required for sarin exposure. During this past year, we have completed six major sarin exposure experiments, four with sarin alone and two involving sarin with pyridostigmine bromide. A 7^{th} experiment has been completed in late June 2003 but the RNA has not yet been examined using genechips. Unlike our findings with extended exposure (2-3 days) of neurons to pyridostigmine bromide (year two report), we have seen less robust gene changes with short exposure to sarin. Using increased numbers of replicates taken on different days, we have uncovered modest gene expression changes using Mann-Whitney tests after eliminating those genes with expression levels that appear to represent inactive genes (Absent calls). While the genechip experiments proposed are now completed, we anticipate using the final year to systematically analyze the results and where appropriate, undertake validation by RT-PCR from previously isolated total RNA.

PRESENTATIONS

None

PUBLICATIONS

None

GRANTS AWARDED

Last year, the Gene Expression Laboratory (GEL) that maintains the Affymetrix genechip equipment, became a member of the Genome Research Infrastructure Partnership (GRIP). Other members include the Genome Research Institute of the University of Cincinnati, Children's Research Hospital of Cincinnati, Proctor and Gamble and Wright-Patterson Air Force Base, ARFL. GRIP was recently awarded a 2 year, 9 million dollar Biomedical Research and Technology Transfer award from the State of Ohio to improve its genomics infrastructure. GEL will continue to train researchers and work with bioinformatics researchers to develop new tools for genomic analysis.

DETAILED PROGRESS REPORT

Treatment of Differentiated SH-SY5Y cells with HEPES buffered DMEM/F12 media

In order to complete the sarin treatment experiments, we needed to place the cells in a double contained (sealed) chamber during the time in which the cells are exposed to GB (or mock exposed). This necessitated that the cells be cultured in a media that was not dependent of 5% CO_2 in order to maintain a proper pH. After consultation with Invitrogen, we decided to examine how differentiated SH-SY5Y cells would react in MEM/F12 media containing 10% FBS and 15 mM HEPES. The cells were initially grown in our standard media and subjected to differentiation as previously described. On day 7 post-differentiation, the media was removed and replaced with the HEPES containing media. The cells were sealed in the double-chamber, and placed in a 37°c incubator for 0-6 hours. At the time points indicated, the plates were removed from the chamber and cell number and viability determined using trypan blue. As shown in Figure 4-1 (below), the cells begin to lose viability after 4 hours of treatment in this media under the conditions described. Based on these findings we determined that sarin exposure experiments would be performed for less than 2 hours.

Figure 4-1: Mock Sarin Exposure Experiment

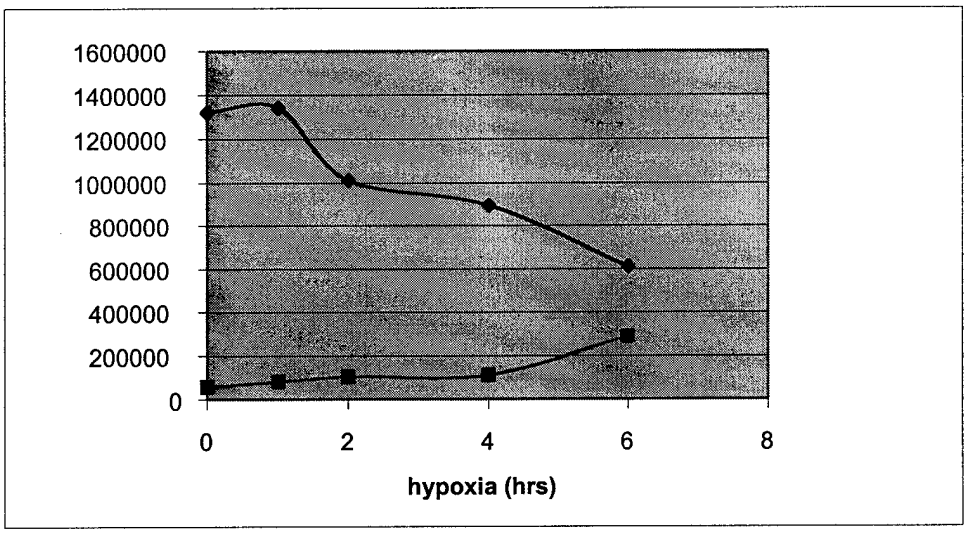


Figure Legend: Day 7, Differentiated SH-SY5Y cells were refed with Differentiation media containing 15 mM HEPES, placed in a sealed container and placed at 37°c. At the indicated time point the plates were removed, cells trypsinized and placed in a trypan blue containing media. Cell counts and viability was performed using a hemacytometer. This experiment was performed in duplicate showing similar trends. Triangles: viable cells, rectangles, non-viable cells.

Gene Expression Profiling of sarin exposed SH-SY5Y neurons.

During the past year we have completed the second of our three-phase project, examining gene expression profiles of SH-SY5Y neurons to varying doses of sarin. The data shown below represents the sarin exposure experiments performed and those genes that showed significant changes in gene expression.

Table 4-1: Exposure to 1.9 or 19 μg/ml sarin for 1 hour.

N=4	DMW	DMD	DMD & DMW	IMW	IMI	IMI & IMW
1.9 v 0 μg sarin	357	5	3	422	1	0
19 v 0 μg sarin	216	15	8	197	0	0
1.9 & 19 v 0 μg sarin	**91**		**2**	**60**		**0**

IMI: Number of genes induced based on a majority of Change calls of either increase (I) or marginally increase (MI) from all possible comparison analyses.
DMD: Number of genes decreased based on a majority of Change calls of either decrease (D) or marginally decrease (MD) from all possible comparison analyses.
IMW: Number of genes induced based on a Mann-Whitney analysis of the signals between the treated and control samples.
DMW: Number of gene suppressed based on a Mann-Whitney analysis of the signals between the treated and control samples.

Conclusions: The majority of genes that either increased or decreased in sarin treated cells compared to controls using the Mann-Whitney test were considerably higher than those identified using Change calls. When comparing the genes from these two searches, there was little overlap. However, based on the Mann-Whitney tests alone we could demonstrate that 91 and 60 (bold) genes were downregulated and upregulated, respectively at both doses of sarin tested in this experiment. In an attempt to establish a time response at the tested sarin doses, we decided to perform our second sarin exposure (experiment 2) with a 2-hour sarin exposure.

Table 4-2: Exposure to 1.9 or 19 μg/ml sarin for 2 hours.

N=4	DMW	DMD	DMD & DMW	IMW	IMI	IMI & IMW
1.9 v 0 μg sarin	329	25	10	229	2	1
19 v 0 μg sarin	690	94	62	511	1	0
1.9 & 19 v 0 μg sarin			**5**			**0**

IMI: Number of genes induced based on a majority of Change calls of either increase (I) or marginally increase (MI) from all possible comparison analyses.
DMD: Number of genes decreased based on a majority of Change calls of either decrease (D) or marginally decrease (MD) from all possible comparison analyses.
IMW: Number of genes induced based on a Mann-Whitney analysis of the signals between the treated and control samples.
DMW: Number of gene suppressed based on a Mann-Whitney analysis of the signals between the treated and control samples.

Conclusions: As in the first experiment, the Mann-Whitney and Change calls algorithms demonstrated a consistent imbalance in the numbers of genes called increased or decreased in the sarin treated verses untreated. Again, there was little overlap in the genes when both datamining approaches were compared. Surprisingly, the genes that met both criteria showed no overlap with the identified significant genes observed in the one-hour treatment of experiment 1 (data not shown). Based on these results we decided to refocus our efforts on 1-hour exposures at three sarin exposure doses (0.19, 1.9 and 19 μg/ml).

Table 4-3: Exposure to 0.19, 1.9 and 19 μg/ml sarin for 1 hour.

N=3	DMW	DMD	DMD & DMW	DMD or DMW
0.19 v 0 μg sarin	610	97	44	663
1.9 v 0 μg sarin	504	47	24	527
19 v 0 μg sarin	563	49	29	583
0.19,1.9 & 19 v 0 μg sarin			**6**	**156**

N=3	IMW	IMI	IMI & IMW	IMI or IMW
0.19 v 0 μg sarin	429	12	6	435
1.9 v 0 μg sarin	412	14	6	420
19 v 0 μg sarin	561	104	30	635
0.19,1.9 & 19 v 0 μg sarin			**0**	**54**

IMI: Number of genes induced based on a majority of Change calls of either increase (I) or marginally increase (MI) from all possible comparison analyses.
DMD: Number of genes decreased based on a majority of Change calls of either decrease (D) or marginally decrease (MD) from all possible comparison analyses.
IMW: Number of genes induced based on a Mann-Whitney analysis of the signals between the treated and control samples.
DMW: Number of gene suppressed based on a Mann-Whitney analysis of the signals between the treated and control samples.

Conclusions: This experiment examined sarin exposures at three doses, each for one hour. Patterns of gene changes similar to those seen in experiment 1 were observed. Using a less conservative selection approach of including genes that met either criteria (DMD or DMW; IMI or IMW), we were able to identify a set of genes that were induced (54 genes) or repressed (156) at all doses of sarin treated plates relative to the untreated plates. To complete the sarin experiments at the lower doses, a final set of one hour exposures were completed (experiment 4.2).

Table 4-4: Exposure to 0.19 and 1.9 μg/ml sarin for 1 hour.

N=4	DMW	DMD	DMD & DMW	IMW	IMI	IMI & IMW
0.19 v 0 μg sarin	303	12	5	41	356	25
1.9 v 0 μg sarin	20	286	9	23	286	9
0.19&1.9 v 0 μg sarin			**1**			**0**

IMI: Number of genes induced based on a majority of Change calls of either increase (I) or marginally increase (MI) from all possible comparison analyses.
DMD: Number of genes decreased based on a majority of Change calls of either decrease (D) or marginally decrease (MD) from all possible comparison analyses.
IMW: Number of genes induced based on a Mann-Whitney analysis of the signals between the treated and control samples.
DMW: Number of gene suppressed based on a Mann-Whitney analysis of the signals between the treated and control samples.

Conclusions: This experiment focused only on the lower doses of sarin exposure (0.19 and 1.9 μg/ml) for one hour. The chart above shows the datamining results within this experiment and are consistent with previous experiments in terms of the numbers of significantly changed genes. This dataset with the previous datasets were compiled and reexamined for significant gene expression fluctuations following sarin exposure.

Table 4-5: Summary of Sarin exposure gene expression changes by Mann-Whitney

Experiments 4-3,4	**N=**	DMW	IMW
0.19_v_0 μg sarin	7	107	69
Experiments 4-1,3,4	**N=**	DMW	IMW
1.9_v_0 μg sarin	11	69	42
Experiments 4-1,3	**N=**	DMW	IMW
19_v_0 μg sarin	7	120	92

IMW: Number of genes induced based on a Mann-Whitney analysis of the signals between the treated and control samples.
DMW: Number of gene suppressed based on a Mann-Whitney analysis of the signals between the treated and control samples.

We observed a number of genes that showed modulation of gene expression based on the Mann-Whitney test when combining the controls and sarin exposures from 2-3 different experiments. Each gene met the p-value of less than 0.05. The table below lists only those genes with p-values of less than 0.01 and demonstrate some gene modulations that are likely significant.

Table 4-6: Genes that modulate with exposure to 1.9 μg/ml sarin for one hour.

1.9 μg/ml sarin

	Mann Whitney		Average signal		Description
N=11	P Value	Change	0 μg sarin	1.9 μg sarin	
36411_s_at	0.002	Down	85.09	61.84	ELAV-like neuronal protein-2 Hel-N2
35858_at	0.005	Down	98.28	80.25	Postmeiotic segregation increased 2-like 9
36391_at	0.008	Down	75.54	57.52	Cyclin T1
31831_at	0.008	Down	128.93	112.1	Smoothelin
37101_at	0.009	Down	221.95	202.55	Breast cancer metastasis suppressor 1
33857_at	0.01	Down	174.11	151.44	P47
1973_s_at	0.01	Down	122.63	108.32	C-myc oncogene
254_at	0.005	Up	1987.06	2271.36	H3.3 histone class C mrna
1675_at	0.008	Up	335.14	369.05	Gtpase-activating protein ras p21
40777_at	0.01	Up	791.16	944.01	Beta-catenin

Gene Expression Profiling of sarin and pyridostigmine bromide exposed SH-SY5Y neurons.

These studies, which represent our final phase of experiments, were just recently completed (May-July, 2003). The RNA has been collected and genechip experiments performed on all but the final experiment. Only preliminary data screening has been done on the genechip experiments completed and initial findings suggest that sarin with pyridostigmine bromide gene changes are not as robust as the phase 1 pyridostigmine bromide alone exposures, similar to what we observed when data-mining the sarin alone exposure profiles (phase 2). By combining the first two experiments (8 genechips per condition) the following table lists the number of genes

showing a change in gene expression based on a Mann-Whitney test, using a p-value of less than 0.05.

Table 4-7: Exposure to pyridostigmine bromide (48 hours, 10-4M) and sarin (1 hour, 0.19 or 1.9 μg/ml).

Exp 1 and 2 (N=8)	DMW	IMW
PB & 1.9 sarin_v_con	95	82
PB & 0.19 sarin_v_con	146	109
PB&1.9 or 0.19 sarin_v_Con	**18**	**16**

IMW: Number of genes induced based on a Mann-Whitney analysis of the signals between the treated and control samples.
DMW: Number of gene suppressed based on a Mann-Whitney analysis of the signals between the treated and control samples.

Conclusions: After analyzing the first two experiments separately and together we have concluded based on the lack of any reproducible gene changes based on the Change Call (>50% of comparison analyzes) that gene changes observed with this experimental protocol will be modest ones, similar to what we have observed with sarin exposure. In the upcoming months we will engage in systematic analysis of each genechip and comparison of different treatments and treatment regimens.

KEY RESEARCH ACCOMPLISHMENTS

Project 1: Investigation of the effect of combined chemical and stress exposure on behavioral, cardiovascular, endocrine and cholinergic function.

- There was an indication that sarin when combined with stress produced a delayed behavioral pathology. A small subset of the mice showed self-mutilation. If this can be replicated, it may shed light on the mechanism of the Gulf War Syndrome.
- There were differences in the behavioral responses (acoustic startle) to PB, physostigmine and sarin. The results do no fit with a simple cause and effect relationship between AChE inhibition and behavior.
- Demonstrated that there were interactions between PB and stress in terms of the effects on corticosterone secretion and brain AChE activity.
- Conducted studies on the effect of PB and stress on apoptosis and muscarinic receptor binding. The result showed that there were specific changes in muscarinic receptor binding in response to PB and stress. There was no evidence of apoptosis.
- Set up the methodology for combined analysis of AChE activity and protein in tissue.
- Established a Proteomics Laboratory (directed by D. Cool) for the study of proteomic changes in response to stress and chemical exposure. Preliminary experiments have identified proteomic changes that are different between PB and physostigmine.
- Developed a method for sequencing peptides "on proteinchip" using carboxypeptidase Y enzyme to remove amino acids from the C-terminus of peptides and proteins.
- Completed studies to determine the effect of combined exposure to stress and pyridostigmine in mice. The results show that there are no global changes in blood pressure or heart rate after treatment. However, spectral analysis showed dramatic alterations in heart rate variability and baroreflex index in the combined stress/pyridostigmine group. This has important clinical implications since reduced variability is associated with cardiovascular diseases. A paper is in preparation that will be submitted to Nature.
- Completed studies to determine the day/night time course of stress responsiveness (corticosterone, oxytocin and brain c-fos expression). Results showed that there were pattern in stress responsiveness that were similar to that seen for the cardiovascular system.
- Determined the pattern of blood pressure and heart rate variability produced in response to acute and chronic stress. Results show that acute stress produces an increase in pulse interval (PI) and arterial pressure variability, while chronic stress produced a decrease in variability. This is important since it suggests that chronic stress modulates the cardiovascular system in a manner that may be detrimental to health.
- Determined the autonomic mechanisms that come into play in the response to stress. We evaluated the influence of the autonomic nervous system in the cardiovascular responses to stress. The cholinergic blockade increase the heart rate but reduced stress-induced tachycardia and blocked the increase in PI variance. Atropine also markedly reduced the LF and HF components of the PI variability. The α-sympathetic blockade by prazosin abolished the stress-induced hypertension and blocked the increase in PI and SAP variabilities. Prazosin also reduced the LF component of the PI and SAP variabilities.

- Implemented the method for telemetric measurement of blood pressure and heart rate in conscious mice.
- Determined the effect of deletion of the oxytocin gene on the response to stress. Results showed that the stress induced cardiovascular changes were greater in the oxytocin deletion model. This work has been submitted for publication.
- Published 14 abstracts and 6 papers. Bernatova won an award for her work in cardiovascular pharmacology at the Experimental Biology Meeting. Atira Hartiman won a graduate student award for her work in Proteomics.
- Conducted a minisymposium on the Stress/PB interactions. Participants were the program investigators, research associates/assistants, and graduate students involved in the DoD project.
- Initiated studies on the genomic effects of sarin administration. The focus is on the central neural alterations.

Project 2: The effect of stress and chemical exposure on auditory brain responses, energy metabolism and tissue chemical constituents in an animal model.

- Completed data analysis of chronic low-dose study involving DEET treatment ± noise stress.
- Completed data analysis of chronic low-dose study involving pyridostigmine bromide (PB) + noise stress.
- Completed replication study of 24-hour post-injection effects of PB ± noise stress.
- Completed chronic low-dose study of synergistic effects of DEET + PB + noise stress.
- Completed dose-response study for sarin; compared subcutaneous (sc) and intravenous (iv) methods of toxin administration (determined LD_{50} for iv injection).
- Completed first 4-week chronic low-dose study of sarin + noise stress.
- Prepared and examined rat cochleae by scanning electron microscopy for evidence of hair cell loss/damage following toxin and noise exposure.
- Completed assessment of the effects of DEET, PB and stress on mitochondrial energy coupling (respiratory control) activities.
- Completed assessment of the effects of DEET, PB and stress on mitochondrial electron transfer activities.
- Performed initial isolation of mitochondria from animals treated with low dose of sarin and stress and measured energy coupling and electron transfer activities of the preparation.
- Determined absolute specific activities for cytochrome c oxidase in brain stem mitochondrial preparations from treated and control animals using intact membrane assays, soluble enzyme assays, and immunoblotting.
- Determined cytochrome c content (as a marker for apoptosis) of mitochondria isolated from rats treated with DEET, PB and/or stress.
- Conducted NMR in vitro analyses of brainstem extracts for PB + noise stress protocol and DEET + PB + noise stress protocol.
- Determined pre- and post-noise exposure levels of blood serum corticosterone levels.
- Hired and trained new laboratory personnel.

Project 3: The study of enzymes involved in chemical metabolism, activity of dehydrogenases and esterase in human tissues with the goal of establishing whether there are alterations in populations characterized as chemically sensitive.

- Completed alternate analyses of pilot study data and identified "subgroups" within our experimental and control groups. This suggests novel factors which will increase the reliability of correlations between enzymatic analyses and abnormal or disease states.
- Substantial progress has been made on the collection and analysis of blood samples from normal, GWS, and chemically sensitive individuals for the main study of this project. These substantially extent results form the present study, validate methodology development and/or results of the pilot study.
- Developed a sarinase assay that is suitable for all blood components used in this study and identified suitable conditions for completing study assays.

Project 4: The study of gene expression using a DNA microarray system to test the effect of chemical exposure on a neuronal cell line.

- Completed: Gene expression profiling of sarin exposure of SH-SY5Y neurons.
- Completed: Gene expression profiling of sarin and pyridostigmine bromide exposure of SH-SY5Y neurons.
- Completed: Data-mining of gene expression data from pyridostigmine bromide exposed neurons.
- In progress: Data-mining of gene expression data from sarin and sarin with pyridostigmine bromide exposed neurons.

REPORTABLE OUTCOMES

Abstracts:

Project 1:

1. Lucot, J.B., M. Dubovicky, J. Wells. Effects of pyridostigmine and chronic shaker stress on acoustic startle response , pre-pulse inhibition and open field behavior of mice. 291.7 2002 Abstracts Viewer/Itinerary Planner, Washington, D.C., Society of Neuroscience, 2002 CD ROM.

2. Mach, M, Morris, M., Lucot, J.B., Behavioral changes after acetylcholinesterase inhibition in C57Bl/6J mice. FASEB. 390.3, 2003.

3. Grubbs, R.D., W.A. Price, B.S. Mauck, M. Mach, J.B. Lucot, S.J. Paton and M. Morris. Effects of exposure to sarin, pyridostigmine bromide and stress on cholinesterase activity in mouse blood and brain. Tox and Risk Assessment Conf. 2003.

4. Ropp, S. A., Paton, S.J., Morris, M. And Cool, D.R. Effects of subacute pyridostigmine and stress on hypothalamic and cerebral cortex acetylcholinesterase and proteomic profiles. FASEB Apr 2003, Abs# 388.13.

5. Joaquim, L. F., Bernatova, I., Farah, V. D., Salgado, H. C., and Morris, M. Spectral analysis of heart period and arterial pressure under chronic stress in mice. Hypertension, in press. 2003

6. Mauck, B. S., Morris, M., and Grubbs, R. D. Effect of pyridostigmine and stress on brain cholinergic receptor density in C57Bl mice. FASEB J, 17: A622, 2003.

7. Bernatova, I., Rigatto, K. V., and Morris, M. Stress-induced cardiovascular responses in oxytocin knockout (OTKO) mice: Is there an antistress effect of oxytocin? FASEB J, 17: A1055, 2003.

8. Key, M., Frietze, T., Bernatova, I., Lucot, J. B., Paton SJ, Dubovicky, M., and Morris, M. Diurnal rhythm in chronic stress responsiveness in mice. FASEB J, 16: A506, 2003.

9. Rigatto, K. V., Bernatova, I., Puryear, R. W., Key, M., and Morris, M. Hypotension and enhanced salt appetite in oxytocin knockout mice. Hypertension, in press. 2003.

10. Farah, V., Joaquim, L.F., Irigoyen, M.C. and Mariana Morris. Cholinergic input is critical in the regulation of heart rate variability and stress reactivity in mice. Hypertension, in press, 2003.

11. Bernatova, I., Dubovicky, M., Price, W.A., Grubbs, R.D., Lucot, J.B., Morris, M., Effect of chronic pyridostigmine bromide treatment on cardiovascular parameters and behavior in mice. In press, 2003.

12. Bernatova, I., Rigatto, K.V., Key, M., and Morris, M. Enhanced Stress Responsiveness in Oxytocin knockout Mice, in press, 2003.

13. Joaquim, L.F., Fazem, R., Bernatova, I., Salgado, H., Morris, M. Hidden cardiovascular effects of pyridostigmine coupled with chronic stress, submitted AHA meeting, 2003.

14. Spectral analysis of heart period and arterial pressure under chronic stress in mice Joaquim, LF[1]; Bernatova, I[1]; Farah, V[1]; Salgado, HC[2]; Morris, M[1]. [1]Department of Pharmacology and Toxicology, Wright State University, Dayton, OH; [2]School of Medicine of Ribeirao Preto, SP, Brazil.

Project 2:

1. N.V. Reo, I. Bicknell, L. Prochaska, A. Neuforth, S. Shah, L. Shroyer, and D. Moyer. "Effects of Low-Dose Chronic Exposure to Persian Gulf War Chemicals and Stress on Rat Brainstem Function and Energy Metabolism." Proc. Intl. Soc. Magn. Reson. Med. (In press). International Society for Magnetic Resonance in Medicine, Eleventh Annual Meeting, Toronto, Ontario, Canada, July 2003.

Manuscripts:

1. Cool, D.R. and Debrosse, D., Extraction of oxytocin and arginine-vasopressin from serum and plasma for radioimmunoassay and SELDI-TOF MS. (2003) *J. Chromatography B*, 792: 375-380.

2. Bernatova I, Key MP, Lucot JB and Morris M. Circadian differences in stress-induced pressor reactivity in mice. *Hypertension* 40: 768-773, 2002.

3. Bernatova I, Dubovicky M, Price WA, Grubbs RD, Lucot JB and Morris M. Effect of chronic pyridostigmine bromide treatment on cardiovascular and behavioral parameters in mice. *Pharmacol Biochem Behav* 74: 901-907, 2003.

4. Rigatto KV, Puryear R, Bernatova I, Morris M. Salt Appetite and the renin angiotensin system: effect of oxytocin deficiency, *Hypertension*, in press, 2003.

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CONCLUSIONS

This is a comprehensive multidisciplinary project that involves nine principal investigators and four major projects. The contract has resulted in the development of a number of sophisticated research methods that will be used to address the issues related to CWA responsiveness and its modulation by stress. The approaches range from gene screening using the Affymetrics DNA microarray to the telemetric monitoring of behavior and cardiovascular parameters in conscious mice. Thus, there is much potential for contribution to the environmental sciences, particularly as related to the military.

The key accomplishment section clearly illustrates the progress that was made in the third year of the project. Many of the research projects have come to fruition, resulting in scientific presentations, publication of abstracts, and submission of manuscripts. The net result is an expansion of our knowledge base as related to stress/chemical interactions, genomic and proteomic changes associated with PB and sarin exposure . During the fourth year of the project, the focus will be on completion of projects and presentation at National Meetings and submission of publications.

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APPENDIX

Ropp, S.A., Paton, S.J., Morris, M., and Cool, D.R. Effect of subacute pyridostigmine and stress on hypothalamic and cerebral cortex acetylcholinesterase and proteomic profiles. FASEB J, 17: A622, 2003.

Pyridostigmine (PYR) is an acetylcholinesterase (AChE) inhibitor used prophylactically against possible exposure to organophosphate nerve agents in the Gulf War. PYR is thought to not cross the blood brain barrier (BBB). However, many Gulf War Veterans exhibited impairment of CNS functions suggesting an agent did cross the BBB. To analyze the effects of PYR and stress on discrete brain regions, i.e., hypothalamus and cerebral cortex, AChE activity and expression were assayed along with SELDI-TOF mass spectrometry of the tissue proteins. Mice were administered PYR at doses of 0, 0.5, 1, 2, 3 and 10 mg/kg/day subcutaneously using Alzet minipumps for 7 days with or without shaker stress. Hypothalamic AChE activity was decreased in a dose-dependent manner at 2 mg/kg/day PYR. Higher doses of PYR (10 mg/kg/day) showed an 82% increase in AChE activity. Western blot analysis of the same samples revealed an 80% increase in AChE protein levels in the 10 mg/kg/day PYR treatment group. There was no change in cortex AChE activity or protein levels at any of the PYR doses. Stress had no effect on hypothalamic and cortex AChE activity, either in the sham or 10 mg/kg/day PYR-treated groups. Stress did appear to cause a significant decrease ($p<0.05$) in AChE protein levels in the 10 mg/kg/day treated mice compared to 10 mg/kg/day treatment without stress. There was no difference between the 10 mg/kg/day plus stress versus sham plus stress mice ($p=0.1378$). Proteomic analysis of the same stress and PB-treated hypothalamic and cortical preparations by SELDI-TOF mass spectrometry showed significant and similar changes in the protein profiles compared with AChE activity and protein assays. The results suggest that stress and PYR both affect the peptide protein profiles in the hypothalamus by changes in protein expression, prohormone processing, secretion or enzymatic activity. Supported by DOD contract No. 99214005.

Mach, M, Morris, M., Lucot, J.B., Behavioral changes after acetylcholinesterase inhibition in C57Bl/6J mice. FASEB. 390.3, 2003.

The acoustic startle response (ASR) is a sensitive method for investigation of modulators of sensorimotor activity. The role of the rodent's cholinergic system in modulating ASR is still unclear. In our study we evaluated the effect of the acetylcholinesterase inhibitor physostigmine (PHY) on ASR and pre-pulse inhibition (PPI) as well as motor activity using C57B1/6J mice. The system was set up for 6 types of white-noise burst stimulus trials: no stimulus (background, 60 db), pre-pulse (70 db), pulse (100 db and 120 db), pre-pulse plus pulse (70 db+100db and 70 db+120 db). Locomotor activity was measured using an open field during 15 min sessions. PHY was administered IP at the doses of 0.0, 0.01, 0.03, 0.1 mg/kg. The ASR and locomotor activity were evaluated 30 min after injection. Administration of PHY affected motor activity tested in the open-field in a dose-dependent fashion, with notable impairment in activity at 0.03 and 0.1 mg/kg. The results also showed that animals at 0.1 mg/kg spent more total time in the peripheral zone rather then in the central zone. We also found that ASR was significantly decreased after 0.03 and 0.1 mg/kg. PPI of 120 db startle was significantly decreased by 0.03 mg/kg PHY and there was non-significant trend of a decrease by 0.1 mg/kg PHY. The results showed that PHY administration impaired ASR, which could be due to the general motor effects observed in the open field test, whereas the effects on PPI reflect actions in forebrain structures.

Mauck, B. S., Morris, M., and Grubbs, R. D. Effect of pyridostigmine and stress on brain cholinergic receptor density in C57Bl mice. FASEB J, 17: A622, 2003.

Pyridostigmine bromide (PB), a reversible cholinesterase inhibitor, in conjunction with stress, has been suggested as a possible cause of Gulf War Syndrome. Prolonged exposure to PB and stress may lead to increased stimulation of cholinergic receptors due to slower degradation of acetylcholine, leading to changes in receptor levels. This work explores the hypothesis that PB coupled with stress will alter cholinergic receptor density. Male C57Bl mice were exposed to PB (3 or 10 mg/kg/day) for 7 days via Alzet mini-osmotic pumps implanted subcutaneously. The mice were stressed by shaking at random intervals (avg of 2 min/30 min) for one week, which was sufficient to increase blood cortisol levels. Brain tissue for autoradiographic analysis was collected on day 7 of treatment or 30 days after treatment. While we examined many brain regions, analysis revealed that most significant changes ($P < 0.05$) were seen in the cholinergic nuclei. Stress typically increased muscarinic and nicotinic receptor density, while PB generally decreased muscarinic and increased nicotinic receptor density. Stress effects were apparent on day 7 of treatment while PB effects were seen thirty days after treatment. The delayed effect of PB is significant in that the development of Gulf War symptoms by the soldiers was also a delayed effect. This research is funded by DoD contract DAMD17-00-C-0020.

Grubbs, R.D., W.A. Price, B.S. Mauck, M. Mach, J.B. Lucot, S.J. Paton and M. Morris. Effects of exposure to sarin, pyridostigmine bromide and stress on cholinesterase activity in mouse blood and brain. Tox and Risk Assessment Conf. 2003.

Soldiers who participated in the 1991 Persian Gulf War received pyridostigmine bromide (PB), as a prophylactic against nerve gas attack. PB has been proposed as one possible causative agent of the symptoms of Gulf War Syndrome; exposure to sarin has been proposed as another possible cause. We have developed a model in which mice are exposed to the chemical warfare agent sarin, PB and stress to test this hypothesis. The experiments reported were designed to compare the effects of two doses of PB (3 or 10 mg/kg/day) with or without stress on ChE activity in C57Bl mice. The mice were surgically implanted with osmotic mini-pumps to deliver a steady dose of PB over 7 days and subjected to shaker stress at random intervals with an average intershake interval of 2 minutes every 30 minutes. For the sarin experiments, groups of mice were injected subcutaneously (s.c.) With sarin at 0.05 X LD_{50} or 0.4 X LD_{50} for three consecutive days. Total cholinesterase (ChE) activity was then determined by a modified version of the colorimetric assay of Ellman, *et al.* Total ChE activity was measured in diluted whole blood and in brain homogenate samples. Blood acetylcholinesterase (AChE) activity was determined by inhibiting butyrylcholinesterase (BChE) with iso-OMPA (tetraisopropylpyrophosphoramide). BChE activity was then calculated by subtracting AChE activity from total ChE activity. Following 7 days of exposure to PB alone or PB and stress, blood ChE activity decreases of 18.6% and 39.8% (and 36.5% and 40.0% when stressed) were seen in the 3 and 10 mg/kg/D dosage groups, respectively, when compared to sham control levels. Treatment with 0.4 X LD_{50} sarin reduced blood ChE activity by approximately 50% 30 minutes after the final injection; ChE activity recovered slowly over 72 hours. By day 23 post-injection, the blood ChE activity in sarin treated mice recovered to sham control levels. Mice treated with 0.05 X LD_{50} sarin did not differ in ChE activity from sham control. We found no significant difference in blood ChE activity between stressed and non-stressed sham-implanted control animals, nor between animals given PB 10 mg/kg/day and PB 10 mg/kg/day + stress. Analysis of brain stem and prefrontal cortex showed no significant change in AChE activity after exposure to PB or stress. Similarly, there was no difference in prefrontal cortex AChE activity in mice treated with sarin. Our results indicate that exposure to sarin produced the expected decreases in blood cholinesterase activity which returns to normal over the course of 3 weeks. We also find that PB alone or with concurrent stress does not alter ChE activity in the brain areas analyzed. Together these data provide the necessary validation of our model for assessing the roles of PB and sarin in contributing to symptoms of Gulf War Syndrome. This research is funded by DoD contract DAMD17-00-C-0020.

Spectral analysis of heart period and arterial pressure under chronic stress in mice

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Objective: Chronic stress is associated with human cardiovascular dysfunction such as hypertension, stroke and lower variability of heart rate and blood pressure. We evaluated the variability of cardiovascular parameters in time and frequency domains in CB57I mice submitted to chronic stress. Methods: Carotid arterial catheters were inserted into male CB57l mice (n=04) for recording arterial pressure (AP). AP was measured during a basal period and after 3 days of intermittent shaker stress. The stress paradigm used random exposure to shaking (2 min periods, 150 cycles/min, 45 sessions/24 hr). Systolic arterial pressure (SAP) and pulse interval (PI) were calculated and beat-to-beat time series of SAP and PI were created. The variability in time domain was obtained using the variance of the mean value. In frequency domain, we used autoregressive spectral analysis to estimate the components of oscillations and their absolute values: low frequency (LF: 0.1-1.0 Hz) and high frequency (HF: 1.0-5.0 Hz). Results: Measurement of average cardiovascular parameters showed that there was an increase in HR after the stress period (598+/-21 vs 526+/-16, bpm) with no change in MAP (100+/-4 vs 101+/-6, mmhg). With regard to variability of the signals, there was a trend for a reduction in the stressed condition for both SAP and PI (Table). Spectral analysis showed two components of variation for SAP and PI with no difference between basal and stress. The range for LF and HF were 0.4-0.5 Hz and 3.2-3.7Hz, respectively. Conclusion: The lower variability of SAP and PI suggest that chronic stress produces alterations in the cardiovascular system in a period as short as 3 days. Spectral analysis showed that the changes were focused on the LF component, suggesting an autonomic imbalance, which may be important in pathologies such as myocardial infarction and stroke.

	Systolic arterial pressure			Pulse interval		
	Variance $mmhg^2$	LF $Mmhg^2$	HF $Mmhg^2$	Variance Ms^2	LF Ms^2	HF Ms^2
BASAL	11.4+/-3.1	7.2+/-1.9	3.4+/-1.1	37.6+/-10.2	17.2+/-8.4	15.9+/-2.7
STRESS	5.8+/-1.2	3.0+/-0.8	2.0+/-0.3	22.8+/-7.2	7.4+/-2.5	13.3+/-4.8

Lucot, J.B., M. Dubovicky, J. Wells. Effects of pyridostigmine and chronic shaker stress on acoustic startle response , pre-pulse inhibition and open field behavior of mice. 291.7 2002 Abstracts Viewer/Itinerary Planner, Washington, D.C., Society of Neuroscience, 2002 CD ROM.

Pyridostigmine bromide (PB) was used to protect soldiers from nerve agents. Chronic stress may alter the BBB and allow toxins or drugs to enter the brain. We evaluated the effect of PB dosing during chronic intermittent shaker stress (CISS) on acoustic startle response (ASR), prepulse inhibition (PPI) and open field (OF) behavior of mice. Mice were treated with PB at the doses of 0 or 10 mg/kg/day. One half was shaken for 7 days (2 min shakings separated by an average of 30 min rest). The others were housed in standard conditions. Mice were tested in the startle monitor system for ASR and PPI. The system was set up for 6 types of stimulus trials: no stimulus (background, 60db), prepulse (70 db), pulse (100 db and 120 db), prepulse plus pulse (70 db+100 db and 70db+120db). The mice were tested during the CISS on days 2 and 7 (S2, S7) and 7, 14, 21 and 28 days after ending CISS. Another group was tested in the open field in 15 min sessions on day 1, 3, and 6 during CISS. The ASR for 120 db was significantly increased in the PB group on day S2 and S7 compared to SH controls. The ASR for 100db was significantly higher in the PB group only when tested on day S2. Open field test revealed a significant decrease in locomotion in the PB group compared to SH controls on day 1 of treatment. There was no significant effect of coexposure on the ASR and OF test. The results suggest an acute effect of PB on ASR and locomotion and may indicate that CISS can reduce the PB induced changes in mouse behavior.
Supported by DoD, contract No. 99214005.

Bernatova, I., Rigatto, K. V., and Morris, M. Stress-induced cardiovascular responses in oxytocin knockout (OTKO) mice: Is there an antistress effect of oxytocin? FASEB J, 17: A1055, 2003.

The objective was to study the role of oxytocin (OT) in the regulation of blood pressure (BP) and stress reactivity using an OT gene deletion model. Male OTKO and control OT+/+ mice were prepared with chronic arterial catheters. Mice were exposed to 7-day shaker stress (2-min periods, 45 times/day). Mean arterial pressure (MAP) and heart rate (HR) were recorded continuously (24h) before stress, on stress days 1, 3 and 7, and on recovery. Basal MAP and HR were lower in OTKO than OT+/+ ($p<0.005$) with a significant diurnal rhythm in both parameters ($p<0.001$). Stress-induced responses of MAP and HR were recorded in the dark (1900h) and light (0800h) periods. In the light (nonactive) period, stress-induced responses of MAP were observed on all days in OTKO while in the OT+/+ group only on days 1 and 3. Stress delivered in the dark (active) period induced a significant MAP response only on day 1 in OTKO. There were no genotype-related changes in HR responses. OTKO mice also showed a decreased corticosterone response to stress (298 vs. 411%, $p<0.05$, OTKO vs OT+/+). In conclusion, deletion of the OT gene produced an animal model of low blood pressure and altered endocrine and blood pressure stress responsiveness. The data provides support for an antistress effect of endogenous OT on blood pressure regulation. Supported by the US DoD contract # 99214005.

Key, M., Frietze, T., Bernatova, I., Lucot, J. B., Paton SJ, Dubovicky, M., and Morris, M. Diurnal rhythm in chronic stress responsiveness in mice. FASEB J, 16: A506, 2003.

Cardiovascular pathologies in humans show a diurnal rhythm with a greater incidence seen during the morning hours. We reported that mice exhibit a day/night rhythm in stress-induced blood pressure responses (hypertension 40:768, 2002). The objective of this study was to determine the pattern of central activation (expression of neuronal *c-fos*) and endocrine secretion (corticosterone and oxytocin) in response to chronic stress. C57bl mice were exposed to intermittent shaker stress (2 min periods, 45 times/day) for 3 days. Animals were sacrificed after the last stress, 0900-1000 or 1900-2000h. Brains were collected for *c-fos* immunocytochemistry and plasma for assay of oxytocin (ot) and corticosterone (cort). Results indicate that chronic stress increased *c-fos* expression in the suprachiasmatic, medial preoptic and periventricular regions. Preliminary examination suggests that the pattern of activation was different in the day and night periods. Baseline cort was higher during the night (22±2 vs10.5±1.7, dark vs light) but stress responsiveness was similar during the day/night periods (~1000% increase). In contrast, the ot stress response was greater during the light phase (67 vs 2% increase, light vs dark), as was the blood pressure increase. These findings suggest that there is a diurnal rhythm in the neuroendocrine response to chronic stress, seen as increased oxytocin and neuronal activation.

Rigatto, K. V., Bernatova, I., Puryear, R. W., Key, M., and Morris, M. Hypotension and enhanced salt appetite in oxytocin knockout mice. Hypertension, in press. 2003.

The objective was to evaluate the importance of oxytocin in the regulation of blood pressure (BP) and fluid balance. These experiments examined day/night BP and heart rate (HR) rhythms and the response to volume depletion. BP and HR were measured continuously (24h) in male control (OT+/+) and OTKO mice using a chronic carotid arterial catheter. ANOVA showed diurnal variations in BP and HR ($p<0.02$ and 0.003, respectively), a reduction in BP in OTKO ($p<.02$) and no change in HR.

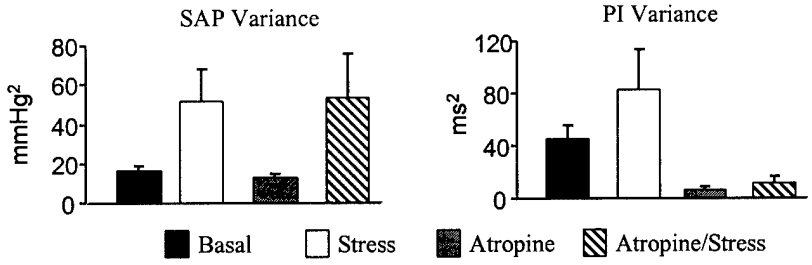
GROUP	**OT+/+**	**OTKO**
MAP (mm Hg)		
Dark	111±2	106±1^{+}
Light	104±2*	100±1*
HR (bpm)		
Dark	595±8	581±7
Light	563±6	548±12

* $p<0.05$ vs dark, +$p<0.05$ vs OT++

Volume depletion was induced by ip treatment with polyethylene glycol (0.5ml, 30%sc) with measurement of salt (2%NaCl) and water intake using a two bottle choice. There was a 3-fold difference in salt intake in OTKO as compared to controls (90±38 vs 276±77 licks/24hr,OT+/+vs OTKO). There were no differences in water intake. Taken together, these data show that removal of OT amplifies the salt-seeking behavior and produces a small, but consistent hypotension. The fact that OTKO mice have decreased BP and voluntarily consume an aversive salt solution, further implies that OT is a powerful regulator of circadian salt appetite, fluid balance and consequently blood pressure.

Farah, V., Joaquim, L.F., Irigoyen, M.C. and Mariana Morris Cholinergic input is critical in the regulation of heart rate variability and stress reactivity in mice. Hypertension, in press, 2003.

Stress is a risk factor in cardiovascular disease, however, the mechanisms through which stress mediates its cardiovascular effects are not defined. Radiotelemetric recording with autoregressive spectral analysis (SA) was used to study the response to shaker stress (5 min, 150 rpm) with and without cholinergic blockade (atropine, 4mg/kg ip). Arterial pressure was recorded in C57/Bl mice (n=4-6) at 5 kHz under baseline conditions and after stress, atropine and atropine/stress. Systolic arterial pressure (SAP) and pulse interval (PI) were submitted to SA with variability measured in the low (LF, 0.1-1.0 Hz) and high (HF, 1-5 Hz) frequency ranges. Stress increased SAP (28 mmHg), heart rate (136 bpm), SAP variance (35 $mmHg^2$) and PI variance (38 ms^2.) Atropine caused a small increase in HR (40 bpm), a large reduction in PI variance (85% decrease) and no change in SAP variance. Cholinergic antagonism reduced stress-induced tachycardia and blocked the increase in PI variance. Atropine also markedly reduced the LF (~89%) and HF (~79%) components of PI variability with no change in SAP variability. Results document the importance of cholinergic input in the modulation of heart rate variability in mice and further suggest a role in stress responsiveness.



Bernatova, I., Dubovicky, M., Price, W.A., Grubbs, R.D., Lucot, J.B., Morris, M., Effect of chronic pyridostigmine bromide treatment on cardiovascular parameters and behavior in mice. In press, 2003.

Experiments were performed to determine the effect of chronic low dose of pyridostigmine bromide (PB) on blood acetylcholinesterase (AChE), cardiovascular (CV) function and behavior in C57BL6 male mice. PB was administered sc using osmotic minipumps at 1 and 3 mg/kg/day for 7 days. Blood pressure and heart rate (HR) were measured continuously for 24h before treatment and on days 3 and 7 after minipump insertion using chronic carotid arterial catheters. Mean arterial pressure (MAP) of the control group was 108±2 and 104±2 mm Hg during the dark and light periods, respectively. HR was 510±18 and 493±19 beats/min during the dark and light periods, respectively. PB treatment had no effect on MAP or HR. Basal blood AChE activity was 0.42±0.1 μmol/min/ml, with no changes observed with PB at 1 mg/kg/day. The higher PB dose (3 mg/kg/day) decreased blood AChE activity by 85% on day 7. Despite the reduction in blood AChE activity, there were no alterations in open field behaviors (locomotor activity, rearing, distance traveled, rest time, number of entries and pokes). In conclusion, chronic low dose PB exposure decreased blood AChE activity but had no effect on CV function or behavior in mice.

Bernatova, I., Rigatto, K.V., Key, M., and Morris, M. Enhanced Stress Responsiveness in Oxytocin knockout Mice, in press, 2003

Studies investigated the role of oxytocin (OT) in blood pressure regulation and stress reactivity in OT knockout (OTKO) mice. Male OTKO (OT-/-) and control (OT+/+) mice with chronic arterial catheters were exposed to 7 days of shaker stress (2-min periods, 45 times/day). The immediate MAP and HR responses were analyzed in the dark and light periods (19.00h and 08.00h). In the light, stress-induced MAP increases were seen on stress days 1, 3, 7 and 1 day post-stress recovery in OTKO and on stress days 1 and 3 in controls. In the dark, stress induced pressor responses were seen only in OTKO (stress days 1 and 3), but no in controls. There were no genotype-related differences in HR responses. Plasma corticosterone was measured before stress exposure and 30 min after the last shaking session on day 7. OTKO mice showed lower responses than controls (increase 298% *vs.* 411%, $p<0.05$). In conclusion, deletion of the OT gene altered endocrine and blood pressor stress responsiveness in mice. The data provides evidence for an antistress effect of endogenous OT in blood pressure regulation.

Joaquim, L.F., Fazem, R., Bernatova, I., Salgado, H., Morris, M. Hidden cardiovascular effects of pyridostigmine coupled with chronic stress, submitted AHA meeting, 2003.

Introduction: Pyridostigmine (PB), a reversible inhibitor of acetylcholinesterase, is used clinically and causes few overt symptoms. However, there are questions as to whether PB may have central interactions under stressful conditions. We evaluated the combined effects of chronic stress and PB exposure on cardiovascular parameters, including spectral analysis (SA) for heart rate and pressure variability in mice. Methods: C57BL6 mice with chronic arterial catheters were treated for 3 days with PB (10mg/kg day, osmotic minipumps, PB), stress (intermittent shaker stress, SS) or stress plus PB (PB-SS). Arterial pressure was recorded (5KHz, 15 min) under basal conditions and after treatment. Beat-to-beat time series of mean arterial pressure (MAP) and pulse interval (PI) were generated, with evaluation of variability using standard deviation and autoregressive SA for low and high frequency domains (LF:0.1-1.0 Hz; HF:1.0-5.0 Hz). Cross SA was applied between MAP and PI spectra for calculation of baroreflex index. Results: Treatment caused a significant tachycardia only in the SS group, but no change in MAP. PI variability and its LF oscillations were markedly increased in the PB-SS group (Table). Baroreflex index was also augmented in PB-SS (SS: 2.3±0.4 *vs* 1.8±0.3; PB: 1.9±0.5 *vs* 1.7±0.4; PB-SS: 2.4±0.3 *vs* 3.6±0.5, ms/mmHg) with coherence >75%. MAP variability showed no differences among groups. Conclusion: Chronic stress unmasked an effect of pyridostigmine on cardiovascular function. There was a marked increase in low frequency PI variation in association with an increase in baroreflex index. Data suggests that PB could present relevant clinical effects under chronic stress conditions.

	Baseline			**Treatment**		
	SS	**PB**	**PB-SS**	**SS**	**PB**	**PB-SS**
PI (ms)						
Variance	5.9±0.6	6.3±1.2	5.9±0.8	5.0±0.9	7.1±1.0	11.0±1.4 *†
LF	4.0±0.8	4.4±1.3	4.4±0.7	3.3±0.9	5.3±0.6	9.5±1.7 *†
HF	3.8±0.2	3.8±0.5	3.7±0.4	3.4±0.5	4.2±0.8	4.9±0.5

* $p<0.05$ *vs* baseline; † $p<0.05$ *vs* SS and PB groups

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Effects of Low-Dose Chronic Exposure to Persian Gulf War Chemicals and Stress on Rat Brainstem Function and Energy Metabolism

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Synopsis

During the Persian Gulf War (GW) US troops were exposed to DEET, pyridostigmine bromide (PB), and low levels of sarin nerve gas. Combined with stress, such exposures may be a contributing cause to GW Illness. Using a 4-wk chronic exposure in rats we measured auditory brainstem response (ABR) evoked potentials, and brain metabolism by ^{1}H and ^{31}P NMR *in vivo*. NMR of brainstem extracts and biochemical assays of brainstem mitochondrial energy metabolism were also conducted. To date, ABR shows an effect on brainstem function at 24 h post PB+stress; NMR indicates that DEET+PB+stress causes a decrease in brainstem taurine.

Introduction

Many GW veterans are afflicted with a neurological and musculoskeletal disease syndrome characterized by chronic fatigue, muscle pain, ataxia, brain abnormalities, and behavioral symptoms. The cause of this illness is unknown, but it has been proposed that chronic exposure to chemicals and stress may have adversely affected deep brain regions. In support of this, MR spectroscopy of GW veterans showed decreased N-acetylaspartate:creatine ratios (NAA:Cr) in the basal ganglia and the brainstem suggesting neurological damage (Haley, RW, *et al.* Radiology 215, 807, 2000). We are investigating the effects of chemical agents and stress in a rat model using a multidisciplinary approach to assess brainstem function and energy metabolism. The protocol involves individual and combined exposures to DEET (an insecticide), PB (a prophylactic drug to combat nerve agents), and sarin (a warfare nerve agent). These studies involve ABR and NMR measurements of brain *in vivo*, NMR analyses of brainstem extracts, and biochemical assays of energy metabolism in brainstem mitochondria. This approach provides a comprehensive assessment of brainstem function and biochemistry.

Methods

Study protocols include: DEET (225 mg/Kg, ip) ± stress; PB (5 mg/Kg, ig) + stress; and DEET (225 mg/Kg) + PB (5 mg/Kg) + stress. Studies involving sarin have not yet been initiated. Rats were exposed to chemical(s) or vehicle (controls) by single injection once weekly for four consecutive weeks. Stress was induced by subjecting rats to white noise (85 dB) for 8 h following each treatment with chemical or vehicle. *In vivo* measurements (under anesthesia) were conducted pretreatment (ABR and NMR) and at the following times postdose: 24 h (ABR), 1 wk (ABR), 2 wk (ABR and NMR), 3 wk (ABR), and 4 wk (ABR and NMR). Rats were then sacrificed and brainstems removed for either NMR analyses of chemical extracts, or biochemical assays of mitochondria preparations. Four different acoustic stimuli were used to evaluate ABR threshold, peak latency, and peak amplitude. Surface coil ^{1}H and ^{31}P NMR (8.5 T) of rat head *in vivo* yielded the choline:creatine (Cho:Cr), NAA:Cr, and PCr:ATP metabolite ratios. High-resolution NMR of brainstem extracts provided metabolite concentrations. Measurements in brainstem mitochondria included the respiratory control ratio using succinate or pyruvate/malate as substrates, and cytochrome oxidase activity.

Results and Discussion

In a separate study, serum corticosterone levels were determined (radioimmunoassay) to be 12-fold higher in rats exposed to the noise stressor for 30 min. relative to unexposed controls (458 ± 47 *vs.* 39 ± 9 ng/ml, mean ± SE, n=5). This clearly indicates that this protocol is an effective stressor.

In general, DEET ± stress did not produce any significant effects in brainstem function or metabolism assessed by all methods. The PB + stress group showed differences in the ABR peak latency. Latencies of Peak II measured at acoustic intensities >50 dB were shorter in some animals at 24 h post-exposure relative to pre-exposure ($p<0.05$). The effect was not observed in subsequent measurements made at 1-wk intervals postdose, suggesting PB + stress causes acute but reversible changes between the cochlea and the cochlear nucleus. Studies are in progress to determine if this effect is manifested in the brainstem metabolic profile. Chronic exposure to PB + stress did not produce any significant effects in brain metabolites assessed by NMR *in vivo* (Fig. 1), NMR of brainstem extracts, or biochemical assays of brainstem mitochondria (Table 1). The DEET + PB + stress experiments are currently in progress, but preliminary NMR data (Fig. 2) show lower levels of brainstem taurine in treated (n=5) *vs.* control (n=2) rats (0.06 ± 0.01 *vs.* 0.50 ± 0.17 umol/g; $p=0.005$). Taurine serves as an osmolyte for cell volume regulation. Vectorial movement of taurine to the extracellular space may help to reduce cell swelling. Thus low taurine in treated animals may be indicative of brainstem edema.

Conclusions

These studies demonstrate a powerful experimental paradigm to investigate brainstem function and biochemistry in an animal model. ABR and NMR data *in vivo* corroborate the biochemical data from NMR analyses of brainstem extracts and assays of oxidative phosphorylation in mitochondria preparations. Although the effects of low-dose chronic exposure to DEET and/or PB may be minimal, we anticipate that combined exposure with sarin may have profound effects.

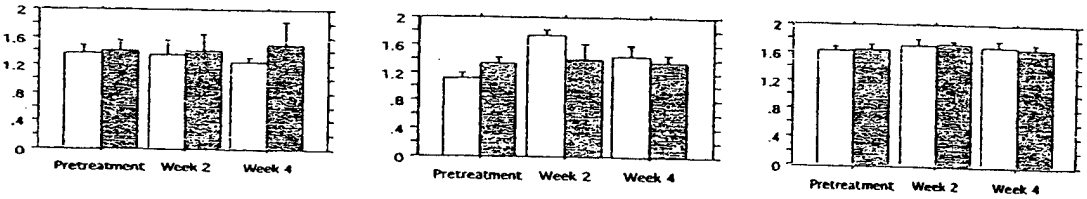

Figure 1. NMR measured metabolite ratios (Mean±SE) in rat brain in vivo for PB+ stress (hatched bars) and control + stress (open bars) groups. Measurements are before (pretreatment) and at 2 and 4 wk during the chronic exposure protocol. (A), (B), and (C) are the Cho:Cr, NAA:Cr, and PCr:ATP ratios, respectively.

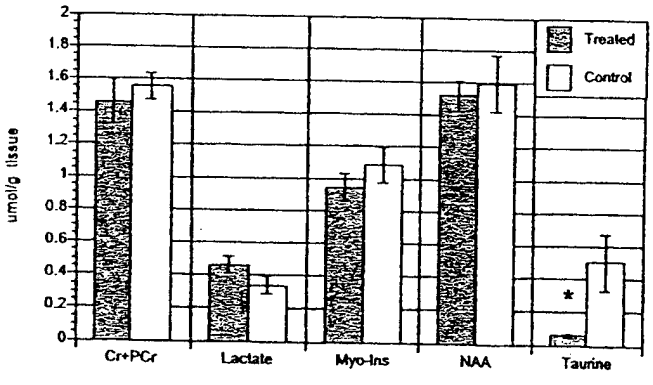

Figure 2. Metabolite concentrations (umol/g; Mean ± SE) from ^{1}H NMR in rat brainstem extracts after a 4 wk exposure to DEET + PB + stress (treated) or the noise stressor only (control). Taurine is significantly lower in treated *vs.* control ($p<0.05$).

TABLE 1. Assessment of rat brainstem mitochondrial energy metabolism in treated and control groups. Values are Mean ± SD. No significant differences were observed for treated v s. control.

GROUPS	Respiratory Control Ratio		Cytochrome c oxidase (nmol O_2/min/ mg protein)
	Succinate + ADP	Pyruvate/malate + ADP	
Control + stress	3.7 ± 1.0	4.0 ± 0.8	205 ± 31
PB + stress	3.8 ± 0.9	5.2 ± 2.9	235 ± 7
DEET + PB + stress	3.2 ± 0.8	3.8 ± 1.0	216 ± 12