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TITLE: Low Level Chemical Toxicity: Relevance to Chemical Agent Defense

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14. ABSTRACT Wright State University conducted a multidisciplinary project to study the influence of low-level exposure to chemical warfare agents which act via inhibition of acetylcholinesterase (AChE). The problem was covered from the level of the cell to the human subject. Project 1 demonstrated that treatment with AChE inhibitors I (sarin or pyridostigmine, PB) in conjunction with stress produced changes in brain gene and protein expression, autonomic function, muscarinic receptor function and behavior. There was evidence that PB entered the brain to exert its physiological actions. An important finding was that a dose of sarin which produced no effect on blood ChE, caused dramatic changes in autonomic neural function and hypothalamic and cerebral cortical genomic and proteomic expression. Data suggest that it is important not to overlook the importance of low level nerve agent exposure in humans. Project 1 also developed a method for sarin exposure which used pretreatment with a carboxylesterase inhibitor. This method produced an enhancement of sarin's central actions. Project 2 tested the effect of DEET, PB and sarin coupled with stress on brainstem function, brain and muscle metabolism in vivo, and brainstem energy metabolism. DEET/PB/stress caused no significant changes, while the sarin/stress combination antagonized the ability of mitochondria to reoxidize NADH. Project 3 focused on investigation of enzymes involved in chemical metabolism, aldehyde dehydrogenase, chi alcohol dehydrogenase, paraoxonase, and aryl esterase. Results suggest that human chemical sensitivity to formaldehyde and organophosphate correlates well with levels of specific enzyme activities in particular blood fractions. Project 4 focused on the study of genetic expression in neuronal cultures. Results showed that PB or sarin had no effect on the patterns of gene expression.					
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INTRODUCTION

The U.S. Army Research and Material Command (USAMRMC) contracted with Wright State University (WSU) to conduct a multidisciplinary project to study the influence of low-level exposure to chemical warfare agents which act via acetylcholinesterase (AChE) inhibition. The project (Contract No. DAMD17-100-C-0020) began in 2000 and ended in 2005. The research team was composed of faculty investigators from the Departments of Pharmacology and Toxicology and Biochemistry and Molecular Biology with expertise in neuroscience, molecular biology, enzymology, cardiovascular pharmacology, neuropharmacology, behavioral sciences, proteomics and genetics. The focus was on testing the effect of low dose exposure to pyridostigmine bromide (PB), N,N-Diethyl-meta toluamide (DEET), and sarin with investigation of 1) effect of combined chemical and stress exposure on behavioral, cardiovascular, endocrine and cholinergic function as well as possible genetic mediators 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 neuronal cell lines. The overall objective was to study the effect of chemical exposure from the single cell/gene level to the whole animal and ending with the human condition. The report is presented with information on the highlights of each of the subprojects.

BODY

The details of the research accomplishments over the granting period are organized according to the research programs as described in the introduction. Each of these groups is composed of faculty from Pharmacology/Toxicology and Biochemistry and Molecular Biology.

Project 1. Studies of chemical (PB and sarin) and stress exposure on behavioral, cardiovascular, autonomic and central nervous system function as well as low dose sarin effects on genetic and protein expression in brain.

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

Project 3. Studies 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. Studies of gene expression using a DNA microarray system to test the effect of chemicals (PB and sarin) on a neuronal cell line.

Considerable progress was made during the course of the project in terms of research productivity, presentations at national meetings, publications and awards. It has also led to the submission of new grant application and the receipt of an NIH supplement to continue the study of sarin. The program has coalesced into a functional research unit with development of specific program as described above as well as establishment of state-of-art facilities for proteomics and genomic analysis, animal physiology (behavior, cardiovascular and endocrine) and biochemical enzymology. For the conduct of studies with sarin, a low level facility was constructed and approved for use in 2003.

Project 1. Effect of chemical (PB and sarin) and stress exposure on behavioral, cardiovascular, autonomic, endocrine and central nervous system function, including use of Affymetrix gene array system for determination of genomic changes.

The key investigators are Morris, Lucot, Cool, Grubbs, McDougal and Sulentic. 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 Neurotransmitter Systems (Grubbs), Proteomics (Cool) and Toxicological Genomics (McDougal and Sulentic).

The research aims in project 1 focus on stress/chemical interactions with use of the same animal model. The first set of experiments combined 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 (2; 3). The basic design uses C57Bl adult male mice exposed to intermittent shaker stress combined with drug induced inhibition of AChE. The drugs tested were PB, physostigmine (PHY) or sarin. Drugs were administered by osmotic minipump implanted sc under the back of the mouse or via subcutaneous injection. Studies investigated both the acute and chronic effects of the treatment because of suggestions that there were delayed incidence in the pathologies associated with the Gulf War Syndrome. The key research accomplishments are described for each group.

Sub-Project 1: Central Cholinergic Systems (Robert Grubbs, Ph.D.)

Key Accomplishments:

- Characterized the mouse model for simultaneous exposure to stress and AChE inhibitors with respect to changes in blood and brain AChE activity.
- Determined that exposure to PB at a dose sufficient to produce substantial inhibition of blood AChE did not produce apoptosis in mouse brain as previously reported.
- Demonstrated that exposure to PB at a dose sufficient to produce substantial inhibition of blood AChE produces few changes in muscarinic receptor densities in brain. In contrast, exposure to stress or PHY produced changes in muscarinic receptor densities that are subtype specific.
- Characterized the effects of low-level sarin exposure on muscarinic receptor density and expression in mouse brain. Results showed regional and subtype specific effects.
- Characterized the effects of low-level sarin exposure on muscarinic receptor density and expression in mouse heart atria. Results showed acute, but not chronic effects of sarin exposure on the heart.
- Modified the sarin exposure protocol to promote the central effects on AChE activity. We coupled sarin with pretreatment with a specific carboxylesterase inhibitor, 2-(O-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide (CBDP). Results showed an enhancement of central sarin toxicity.

In developing shaker stress model for assessing the combined effects of stress and AChE inhibition on brain and cardiovascular function, an important early milestone was to validate that the dose and exposure protocols were adequate to produce blood AChE inhibition. This information provided a positive control for all subsequent studies that focused on characterizing central effects. We determined that with possible exception of the hypothalamus, exposure to PB at peripherally

effective doses did not significantly inhibit ChE activity in mouse brain. Studies were published and presented at national meetings (1, 2).

PB/Apoptosis studies. To better understand the context of any changes in cholinergic function induced by the exposure to PB, we examined brain tissue from mice exposed to PB for evidence of apoptosis. Mice were treated with 10mg/kg/D PB, 2.88 mg/kg/D PHY, or saline. Drug delivery for all time points other than 8-hours was by subcutaneously implanted Alzet mini-osmotic pumps (Durect Corp., Cupertino, CA). For the 8-hour time point, drug was administered by two subcutaneous injections of 1.667 mg/kg PB, 0.480 mg/kg PHY or saline four hours apart following a mock surgery to mimic treatment for other time points. Anesthesia for the surgeries was a ketamine/xylazine (6:1) mixture injected intramuscularly. Mice were sacrificed after 8, 24, 48 hours or 7 days of treatment with PB or PHY. Each treatment group for the 8, 24 and 48-hour time points contained 4 animals. Treatment groups for the day 7 time points each contained 10 animals. A separate group of mice (n = 4) was injected subcutaneously with 40 mg/kg 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) as a positive control. Initial studies showed unreasonably high levels of TUNEL positive cells in multiple brain areas (Table 1). When the assay was optimized for the high lipid content of brain tissue, no evidence that PB or PHY induced apoptosis was observed.

Table 1, Project 1. Characterization of TUNEL assay. TUNEL positive cells were measured before and after addition of delipidization and blocking steps to TUNEL protocol. Assay was conducted in dentate gyrus, striatum and cortex of PB treated mice (10mg/kg/Day)

	Dentate Gyrus	Striatum	Cortex
Before	48.7%	8.4%	63.3%
After	0.0%	0.0%	0.0%

The assay was validated using mice exposed to MPTP, a brain neurotoxin. Thus, any changes observed following PB exposure cannot be ascribed to be secondary to apoptosis.

Using quantitative autoradiography to map muscarinic receptor levels in multiple brain areas, we determined separately the effect of exposing mice to stress, PB or PHY (positive control for PB). We observed statistically significant changes, usually decreases, in receptor density that were subtype and brain region specific. Most of these changes were observed in M1 and M3 subtypes; the M2 subtype rarely changed. Stress alone stimulated increases in M3 density in the amygdala and the vertical diagonal bands and in M2 density in the amygdala and limbic cortex. PB alone was relatively ineffective in producing changes in muscarinic receptor density as compared to PHY which has easy access to the brain. When stress and drug treatments were combined, stress generally negated the changes produced by PHY alone and showed no interaction with PB.

Sarin effects on brain AChE activity and mAChR expression. A single sc injection of sarin (0.4 x LD₅₀, 64µg/kg) produced no effect on brain AChE activity even though blood AChE activity was inhibited more than 80%. We then determined that a second injection given 24 hr after the first, produced a substantive reduction in brain AChE activity. For this reason we used the two injection protocol for all subsequent studies. We then measured muscarinic receptor density 4 and 24 hr after the second sarin injection and also assessed the message level for three receptor subtypes using real time PCR at the 4 hr time point.

Real-time RT-PCR technique was used to measure mRNA expression of M1, M2, and M3 subtypes in brain regions of interest. The primers and probes were designed using Primer3 software. Each sequence was checked for primer dimerization using Qiagen's Oligo Toolkit. M1, M2, and M3 custom primer and probe sequences were created using PrimerQuestSM (Integrated DNA Technologies, Coralville, IA) and were labeled with the fluorescent reporter, TexasRed. Max gene, labeled with the fluorescent reporter carboxyfluorescein (FAM), was chosen as the housekeeping gene and an Applied Biosystems' Assay-on-DemandTM kit was used (Applied Biosystems Foster City, CA). Each brain section was reverse transcribed using iScript cDNA kit (Bio-Rad Laboratories, Inc., Hercules, CA). A 20µl PCR reaction consisted of 4µl 5X iScript reaction mix, 1µl iScript reverse transcriptase, 10µl nuclease-free water, and 5µl of RNA template (130 ng total RNA). Reaction mix was placed in PCR plate and incubated in iCycler IQ system (Bio-Rad Laboratories, Inc, Hercules, CA) for 5 minutes at 20.0°C, 30 minutes at 50.0°C, 15 minutes at 95.0°C, then held at 4.0°C until sample retrieval. The cDNA product was then amplified using iQ supermix (Bio-Rad Laboratories, Inc., Hercules, CA). The PCR product was detected at the end of extension cycle using Bio-Rad iCycler (Bio-Rad Laboratories, Inc., Hercules, CA). After 50 cycles of amplification the relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (13). Statistical analyses were completed using GraphPad Prism 4 Software. A Grubb's outlier test was run then a one-way ANOVA was done on each section of brain for each receptor subtype. If a significant difference of $P < 0.05$ was observed between doses any groups then the Dunnett's test was used to compare as a post test.

Statistically significant decreases in receptor mRNA were observed in hypothalamus (M1) and piriform cortex (M2 and M3) (Table 2). Trends were observed for other areas, but did not achieve statistical significance due to the limited sample size. These studies show that in contrast to PB, exposure to sarin produces widespread and relatively specific changes in indices of muscarinic transmission in the CNS.

Table 2, Project 1. Effect of sarin on mAChR mRNA expression

mAChR Subtype	M1		M2		M3	
	Sarin 0.05xLD50	Sarin 0.4xLD50	Sarin 0.05xLD50	Sarin 0.4xLD50	Sarin 0.05xLD50	Sarin 0.4xLD50
Brain Region						
Caudate	NC	-	+	+	-	-
Hypothalamus	-	-	NC	NC	-	-
Amygdala	-	-	+	+	NC	-
Hippocampus	-	NC	-	-	-	NC
Piriform Cortex	NC	-	-	-	-	-

Mice were injected sc with two daily injections of sarin and sacrificed 4 hrs after the last injection.

- + = Fold change increased compared to control
- = Fold change decreased compared to control
- NC = No change compared to control

To characterize changes in muscarinic receptor density by sub-type, a differential binding assay was used (10). M₁ receptors were labeled by incubating slices with 3 nM ³H-pirenzepine in 20 mM Tris buffer (pH 7.4) with 1 mM MnCl₂ for 45 minutes at 25°C followed by 3 brief dips (approximately 3-5 seconds) in buffer at 4°C. M₂ receptors were labeled by preincubating slices with 0.3 μM pirenzepine in 50 mM PO₄ buffer (pH 7.4) with 1 mM MgCl₂ for 1 hour at 25°C followed by 3 brief dips in buffer at 4°C. The tissue slices were then incubated in 0.5 nM [³H]-NMS in 50 mM PO₄ buffer (pH 7.4) with 1 mM MgCl₂ for 2 minutes at 25°C followed by 3 brief dips in buffer at 4°C. M₃ receptors were labeled by preincubating tissue slices in 0.5 nM NMS in 50 mM PO₄ buffer (pH 7.4) with 1 mM MgCl₂ for 5 minutes followed by 3 brief dips in buffer at 4°C. The slices were then incubated with 0.5 nM [³H]-NMS in 50 mM PO₄ buffer (pH 7.4) with 1 mM MgCl₂ for 60 minutes at 25°C followed by 3 brief dips in buffer at 4°C. Dissociation was accomplished by incubating slices with 1 μM atropine in 50 mM PO₄ buffer (pH 7.4) with 1 mM MgCl₂ for 60 minutes at 25°C followed by 3 brief dips in buffer at 4°C. Nonspecific binding for each subtype was determined by the addition of 1 μM atropine to the incubation buffer. Receptor density was analyzed using the GLM procedure of SAS. Data were analyzed as a 3 x 2 factorial design with the main effects of stress, drug treatment and stress x drug treatment interactions. Means were considered to be statistically different if the p value was < 0.05.

Table 3, Project 1. Effect of sarin on mAChR cell surface expression

mAChR Subtype	M1		M2		M3	
	Sarin 0.05xLD50	Sarin 0.4xLD50	Sarin 0.05xLD50	Sarin 0.4xLD50	Sarin 0.05xLD50	Sarin 0.4xLD50
Brain Region						
Caudate	↑	↓	↓	↓	NC	NC
Hypothalamus	↑	↓	↑	↓	↓	↓
Amygdala	NC	NC	NC	↓	↓	NC
Hippocampus	↑	NC	NC	NC	NC	NC
Nucleus Basalis	↑	↑	↓	↓	↑	↑
Vert. Diag. Band	↑	↓	↑	↑	↑	↑

NC = No change compared to control.

The large arrows represent statistically significant changes from control; small arrows represent changes where significance was 0.05 < p < 0.1, judgment suspended.

Statistically significant changes in receptor densities were observed in multiple brain areas at both the 4 and 24 hr time points; in several areas we observed increases in receptor density that were subtype specific (Table 3). These areas include the vertical and horizontal diagonal bands, nucleus basalis/substantia inominata, hippocampus, amygdala, and hypothalamus. In some areas these changes were observed at one time point and not the other.

To better understand the nature and site of the sarin induced changes in cardiovascular function (cardiovascular/endocrine section), we characterized the effect of sarin exposure (two injections of either 0.05 or 0.4 x LD₅₀ 24 hr apart) on M2 muscarinic receptor density and mRNA expression levels at 4, 24 hrs and 3 months post exposure. We observed no effect of the lower dose on receptor

density, but found that the higher dose increased muscarinic cell surface receptors at both early time points. There were no long lasting effects seen at the three month time point.

Development of single sarin exposure model that produces CNS effects. An important shortcoming of the mouse model for sarin exposure has been the necessity to use two exposures to produce CNS effects. We reasoned that the first exposure was effectively neutralized by the high levels of peripheral esterases, notably butyrylcholinesterase and carboxylesterase. For this reason, we decided to see if pretreatment with the selective carboxylesterase inhibitor, CBDP, would sensitize the mice to sarin, enabling a single exposure to produce CNS effects. A study by Maxwell et al. (14) had shown that treatment with CBDP effectively inhibited plasma carboxylesterase in mice and altered the ED50 for soman. We found that pretreating mice with 1.5 mg/kg CBDP produced no inhibition of blood AChE activity, but enhanced CNS effect of sarin significantly. Mice were sensitized to the effects of sarin to the point that a single exposure at 0.2 x LD₅₀ was lethal to 66% of animals. Single exposure to 0.1 x LD₅₀ (16 ug/kg) produced statistically significant inhibition of brain AChE activity in all brain areas analyzed (Fig. 1). Thus, pretreating mice with CBDP “humanized” them in the sense that they exhibit sensitivity to a single sarin exposure that is much closer to that seen in humans. This improved model could be developed and used to test for potential neuroprotective agents.

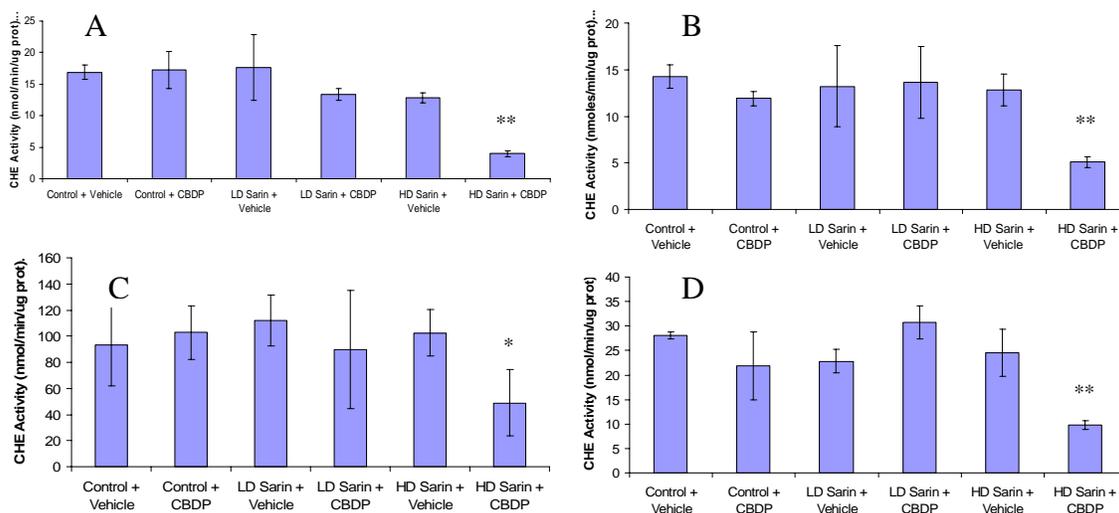


Figure 1, Project 1. Brain ChE activity 24 hr after exposure to sarin +/- pretreatment with CBDP. A - prefrontal cortex; B - caudate; C - amygdala; D- brainstem. * $p < 0.05$ vs control; ** $p < 0.001$ - vs control and all other groups. LD = low dose (0.05 x LD₅₀); HD = high dose (0.2 x LD₅₀ sarin).

On the basis of our data, it does not appear that PB produces much of an effect on the CNS by itself. We could find no evidence that the addition of stress altered this fact. However, this does not mean that PB is not involved in producing the symptoms of the Gulf War Syndrome. The potential irony here is that while PB can save the life of someone exposed to a lethal dose of soman, it would seem that it might sensitize an individual to the effects of a non-lethal exposure to any organophosphate. By inhibiting peripheral ChE activity, more sarin remains available longer in the blood for absorption into the brain where it can exert toxic effects. This was essentially what we observed when we used CBDP to block peripheral esterase activity. In this situation there was enhanced sensitivity and toxicity to sarin.

Sub-Project 2: Neurobehavioral Systems (James Lucot, Ph.D.)

Key Findings

- Three different AChE inhibitors decreased locomotor activity, PB, PHY and sarin
- Stress decreased the locomotor activity effect of PB, but not that of sarin
- The three AChE inhibitors had different effects on the response to acoustic startle, suggesting this as an appropriate measure for non-AChEI actions.
- The combination of stress and sarin produced a delayed onset adrenal hyperplasia and reduced catecholamine content. This may be important in the delayed onset of some Gulf War symptoms
- A minority of mice exhibited a delayed onset self mutilation following the combination of sarin and stress. The genetic variability underlying this may predict those sensitive to combinatorial toxicities
- Two doses of low level sarin reduced the use of dopamine in the amygdala and its predicted consequence, the inability to learn fear potentiation of the startle response. This measure is unusually sensitive to low level exposures.

DESCRIPTION OF MAJOR ACCOMPLISHMENTS.

The framework for the neurobehavioral investigations was to test stress, PB and sarin singly and in combinations to evaluate both the effects of each factor singly and in interaction. Most studies incorporated evaluation of prolonged/delayed effects for at least four weeks after the experimental manipulation. Additional studies were conducted with physostigmine as a positive control.

Statistical analyses were performed using a mixed ANOVA (unless there were no measurement across days) for the appropriate number of factors using the program STATISTICA, '99 Edition (StatSoft, Inc., Tulsa, OK). Significant main effects and interactions were followed by Fisher's LSD post hoc test with all references to significant effect reflecting a $p < 0.05$. The results are presented as a means \pm S.E.M. ($p < 0.05$).

1. Effects of shaker stress.

Animals: All experiments were conducted using male C57Bl/6J mice (HARLAN, Chicago, IL n=83, body weight 24 - 26 g, age 2 month), singly housed in plastic cages with wooden shavings bedding in a temperature controlled room ($T=22-23^{\circ}\text{C}$) with 12:12-h light:dark cycle (lights off from 1700). Standard pellet diet and tap water were provided *ad libitum*. After 10 days of acclimatization the mice were subjected to surgery and behavioral testing.

Stress: The Shaker Power Unit No.5901 (Eberbach Ann, Arbor, MI) was used to induce chronic shaker stress. The Power Unit was set for a 1.13 inches (2.86 cm) stroke and a

speed of 150 cycles per minute. No handling of animals was required. Mice 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 by computer into 45, two min shaking periods separated by randomized still periods with a mean duration of 30 min (13-44 min).

Results: Acute stress lasting fifteen minutes had no effect on locomotor activity, thigmotaxis or rearing when evaluated either during the light phase or during the dark phase. Similarly, chronic intermittent stress lasting one week did not change any motor activity parameter during either the light phase or the dark phase (data not shown). Chronic intermittent stress did not change the acoustic startle response to 100 or 120 dB on the first, third or sixth day of stress nor thereafter. The prepulse inhibition of startle was also unchanged (data not show).

2. Effect of Stress combined with PB exposure

C57/BL mice were treated with PB (Sigma Chemical Co., St. Louis, MO, USA) at the dose of 10 mg/kg/day by means of Alzet minipumps (model 1007D, volume 0.5 μ l/hr over 7 days; DURECT Corporation, Cupertino, CA). Osmotic minipumps were implanted subcutaneously on the back of the mice under anesthesia using a ketamine - xylazine mixture (6 : 1 mg/kg intramuscularly). In the sham-operated group, minipumps were filled with isotonic saline. Mice allocated to chronic stress exposure were housed on the shaker seven hours after implantation of the minipumps, and then they were intermittently shaken for seven days. Control sham-operated and PB mice spent the whole experiment in their home cages, except for the time assigned to behavioral testing.

Results: PB produced an indeterminant ($0.05 < P < 0.1$) decrease in locomotor activity on all parameters. This is consistent with the effects of AChE inhibitors, even though we obtained no evidence that it penetrated into the CNS. The effect PB was eliminated by co-exposure to stress (Figure 2).

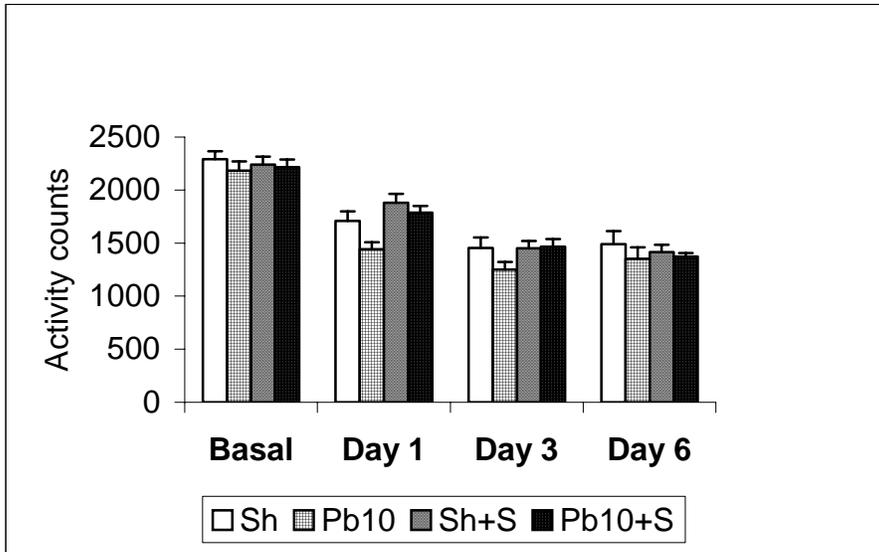


Figure 2, Project 1. Effect of PB and stress, both alone and combination, on open field activity.

PB increased the acoustic startle response on the second and seventh day of infusion but had no lasting effects. We interpret this to result from an action to potentiate ACh action at the neuromuscular junction. There were no changes in prepulse inhibition, which requires forebrain modulation of the basic brainstem reflex (not shown). Co-exposure to stress eliminated the PB effect on startle responses (Fig 3). It was clear that the stress reduced the effects of PB on both open field activity and ASR, but the underlying mechanism for this antagonism was not elucidated.

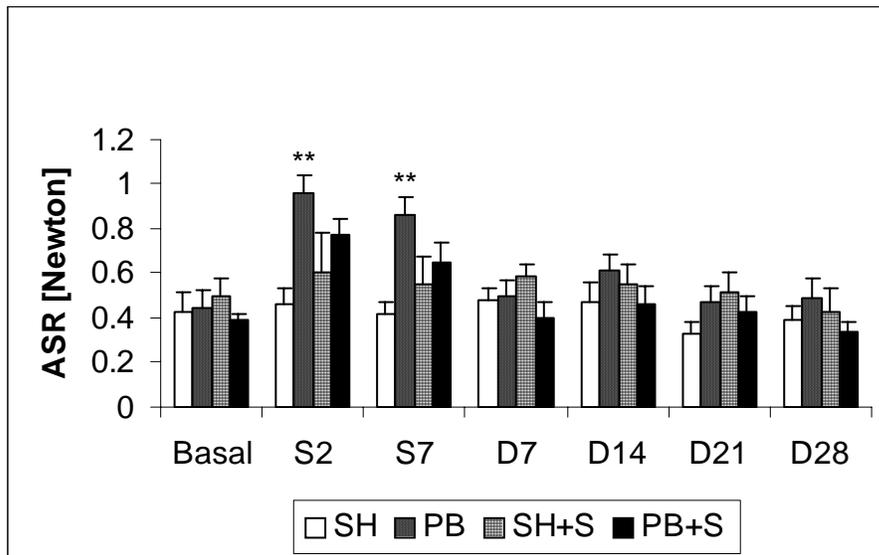


Figure 3, Project 1. Effect of PB and stress, both alone and in combination, on open field activity. S2 and S7 are the second and seventh day of PB infusion and/or shaker stress. Others labeled “D” are days after termination of PB and/or stress.

3. Effect of Physostigmine.

PHY (eserine sulfate, control no. 6864, Nutritional Biochemicals Corporation (Cleveland, OH)) was dissolved in physiological saline and administrated intraperitoneally (i.p.) 30 min before testing (1 ml/100g).

Results: As was the case with PB, PHY decreased locomotor activity (Fig. 4). It also greatly increased thigmotaxis as measured by time in the zone adjacent to the walls (data not shown).

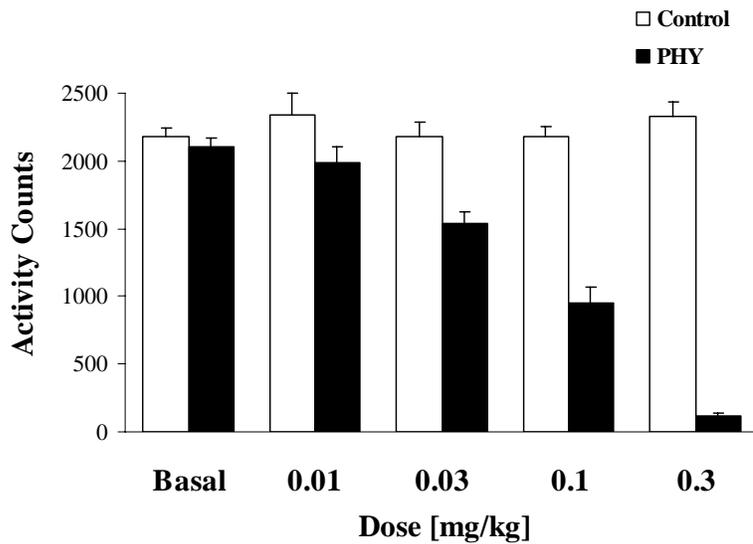


Figure 4, Project 1. Effect of different doses of PHY on open field activity. $P < .001$

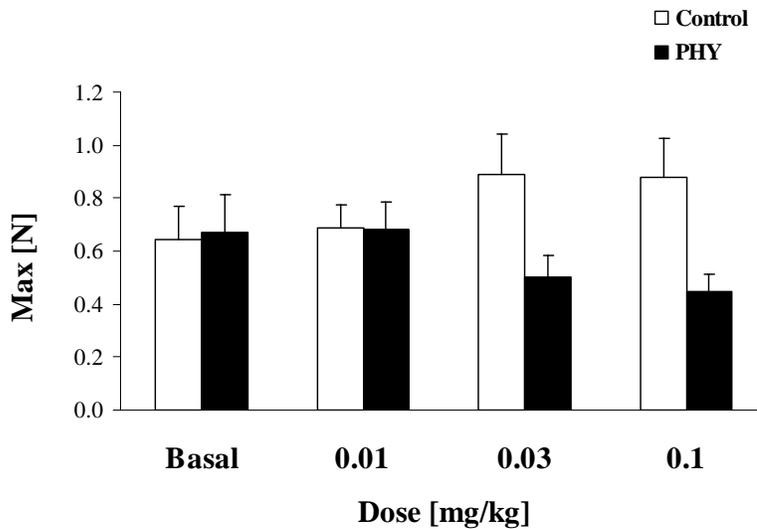


Figure 5, Project 1. Effect of different doses of PHY on the acoustic startle response.

PHY, at a dose producing 40% inhibition of red blood cell AChE activity (Subproject 1) which was comparable to that of PB, greatly reduced the acoustic startle response (Fig. 5) without altering prepulse inhibition (data not shown). Thus, the CNS action of PHY was able to overcome the increased neuromuscular reactivity produced by its peripheral component while not altering the forebrain mechanisms modulating the basic acoustic startle response.

4. Effects of Sarin

The behavioral work was conducted using three daily SC doses of sarin (0.4XLD50 or 64.5 ug/kg), which produced ~ 80% inhibition of red blood cell AChE activity (Fig 6). The schedule of treatments and interactions for Figure 7 are: 1D is after first dose of sarin and 1DA is first day after third dose of sarin. If stress was co-presented, it began four days before the first dose of sarin and ended the afternoon after the third dose of sarin. Like PHY, sarin decreased locomotor activity, though only as a main effect. Stress had no interaction with sarin. Seven days later, the motor response returned to normal despite a continued inhibition of AChE activity, suggesting adaptation (Fig 7).

There was some indication of self mutilation in the mice exposed to the daily doses of sarin plus stress. This was seen after a three to four week delay in approximately 30% of the mice. The mutilation was primarily directed at the fur on the abdomen and forelimbs.

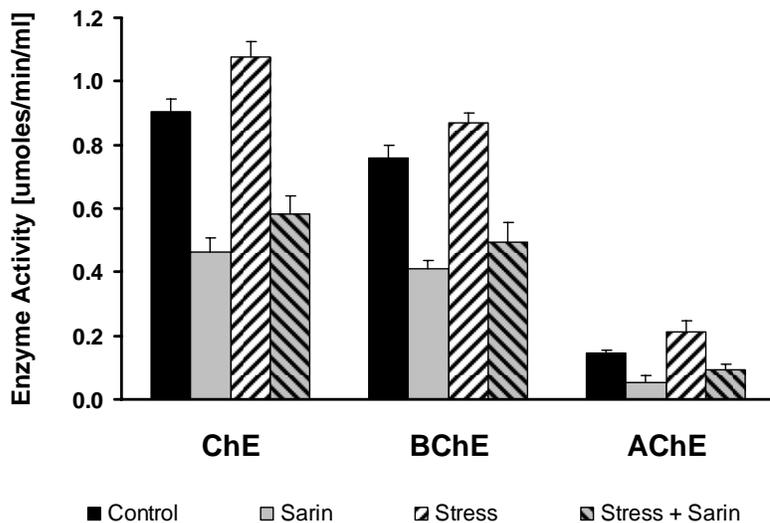


Figure 6, Project 1. ChE, BChE and AChE activity levels 24 hr after last sarin injection in sarin, stress and sarin plus stress mice.

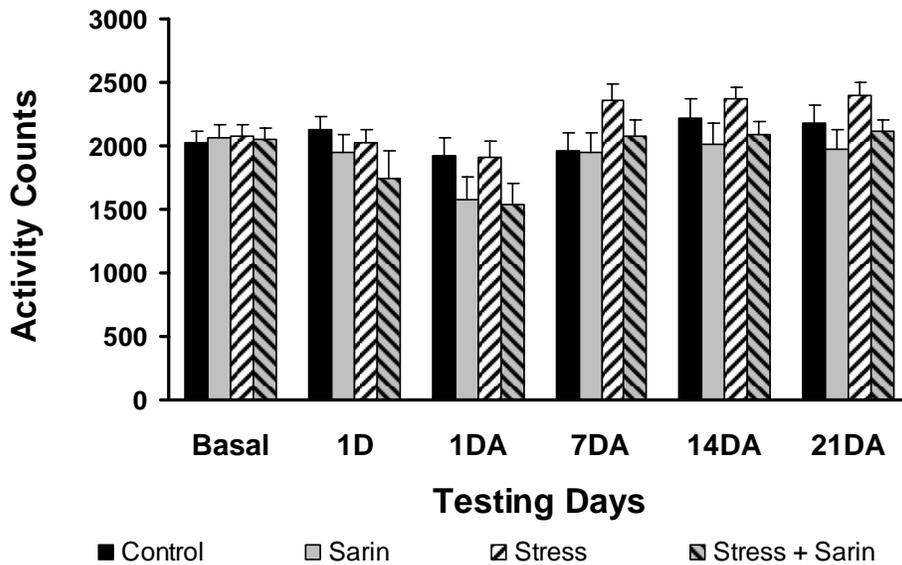


Figure 7, Project 1. Effect of sarin and stress, both alone and in combination, on open field activity.

Unlike either PB or PHY, sarin produced no measurable effects on acoustic startle responses at any time point. Stress did not have an interaction with sarin (Fig 8). Prepulse inhibition was again unaffected (not shown).

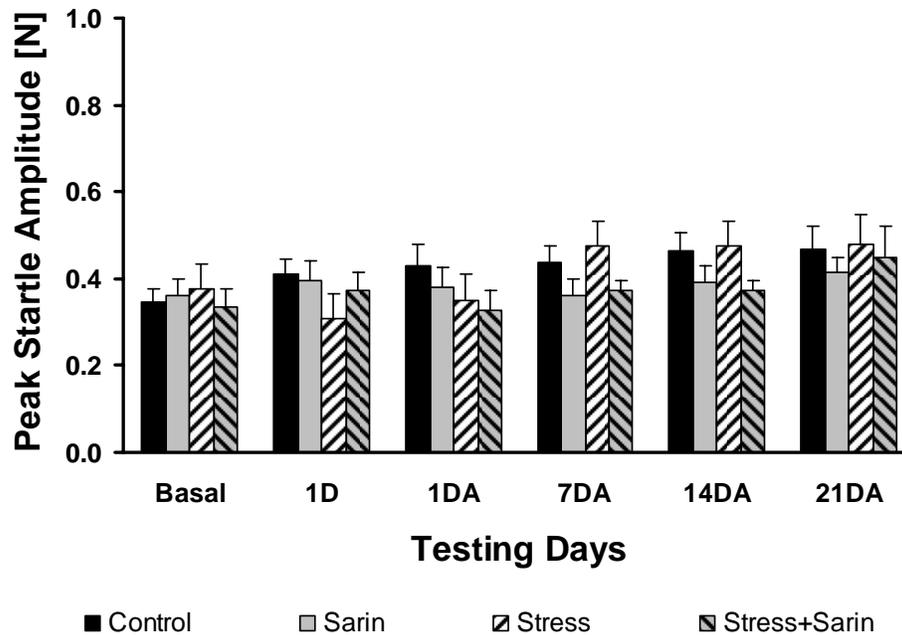


Figure 8, Project 1. Effects of sarin alone and stress, both alone and in combination, on acoustic startle response.

Fear potentiation of acoustic startle responding (FPS)

Two doses of sarin were administered before three days of training in which light was paired with shock. On the fourth day, the fear potentiation of startle was tested and found to be eliminated in the group exposed to sarin (Fig 9).

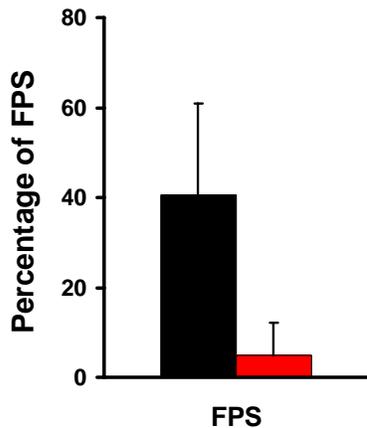


Figure 9, Project 1. Percentage of fear potentiation of startle in control (left bar) and sarin-treated mice (right bar).

As FPS learning depends on dopamine function in the amygdala, we examined dopamine levels in this structure four days after the second dose of sarin. It was found that the ratio of dopamine to DOPAC was marginally increased. As both dopamine and the intracellular metabolite, DOPAC were elevated, an effect consistent with a reduction of dopamine transmission. The large increase in the ratio of dopamine to the extracellular metabolite,

HVA, confirms that dopamine transmission was reduced (Fig JL 9). Thus there was a neural substrate with a behavioral consequence resulting from only 2 sub clinical doses of sarin.

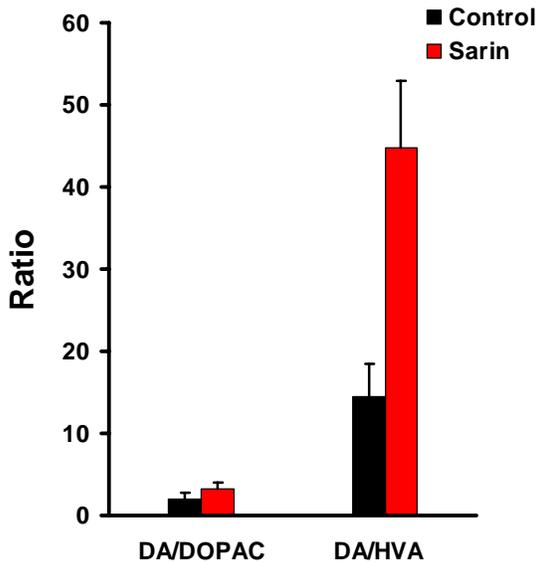


Figure 10, Project 1. Dopamine to metabolite ratio in the amygdala of mice treated with sarin in the FPS experiment.

5. Effect of sarin on the adrenal gland.

Experiments were conducted to evaluate adrenal catecholamines in view of the evidence for delayed mutilation. Sarin did not produce any measurable effects on adrenal content within one week of administration (data not shown). There was a nonsignificant trend to increased levels of norepinephrine, epinephrine and dopamine at seven weeks after sarin exposure. Co-exposure of sarin and stress produced a significant decrease in the concentration all three catecholamines at seven weeks (Fig. 11). However, the weights were more than doubled, so the total content of each amine was unchanged. This delayed effect was presumed to result from hyperplasia of the glands after the sarin treatment coupled with a compensatory downregulation of catecholamine formation. Further work is necessary to determine if the increased weight was in protein or simply hydration level.

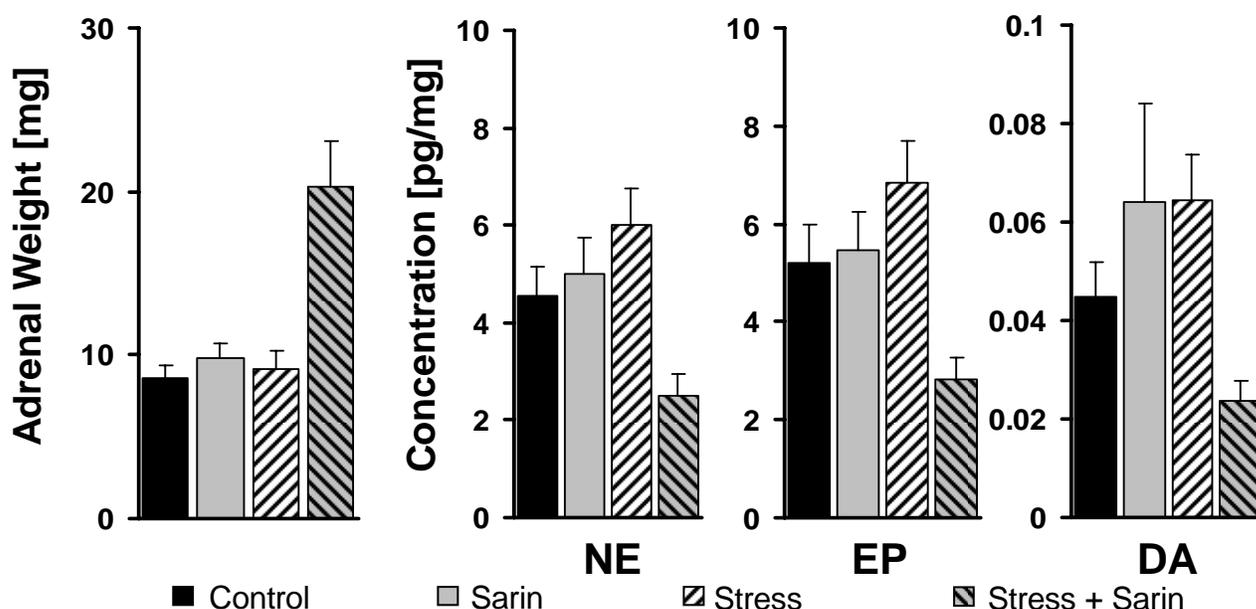


Figure 11, Project 1. Effects of sarin, stress and the combination on adrenal weight and catecholamine concentrations. Measurements were made seven weeks after the last dose of sarin.

Sub-Project 3: Proteomics (David Cool, Ph.D.)

The objective of this experimental section was to study the central cholinergic and endocrine changes associated with chemical (sarin and PB) and stress exposure in mice. The work involved the establishment of a core facility for proteomics including a SELDI-TOF protein chip array system (5; 6; 16; 17).

Key Findings:

- PB is capable of crossing the blood brain barrier to inhibit AChE activity in specific brain regions, particularly the hypothalamus. Proteomic analysis revealed that PB had effects on peptide expression in hypothalamus.

- In mice, peripheral carboxylesterase enzymes act as a sink to prevent sarin inhibition of brain and neuroendocrine function, i.e., hypothalamic AChE activity.
- CBDP, a carboxylesterase inhibitor acts to protect peripheral sarin metabolism, allowing sarin greater access to brain. This results in inhibition of hypothalamic and pituitary AChE activity over extended periods of time

The first set of experiments determined the effects of PB and PHY (delivered via minipumps or injection) on hypothalamic and pituitary AChE and peptide hormone systems. Acute PB and PHY (40 mg/kg and 11.5 mg/kg, respectively) or saline were delivered through sc injections on the back of the mouse. The concentrations of PB and PHY used for all subacute experiments were determined experimentally by those doses that produced approximately 40% inhibition of blood AChE, i.e., 10 mg/kg/day PB, and 2.88 mg/kg/day PHY. Experiments determined the effect of acute (15 min) and subacute (7 day) PB and PHY treatment on regional brain AChE activity. Hypothalami, pituitaries and frontal cortex were collected and analyzed for AChE activity, AChE protein, AChE mRNA and gross protein changes. The presence of PB in the blood was also determined using mass spectrometry.

Plasma PB Analysis

To determine the presence of PB in the blood, plasma from sham and PB-treated animals was analyzed using electrospray ionization mass spectrometry and compared against a standard curve of varying concentrations of PB determine concentration. The concentration of PB observed in the blood was 50 ± 6 ng/ml (Fig 12).

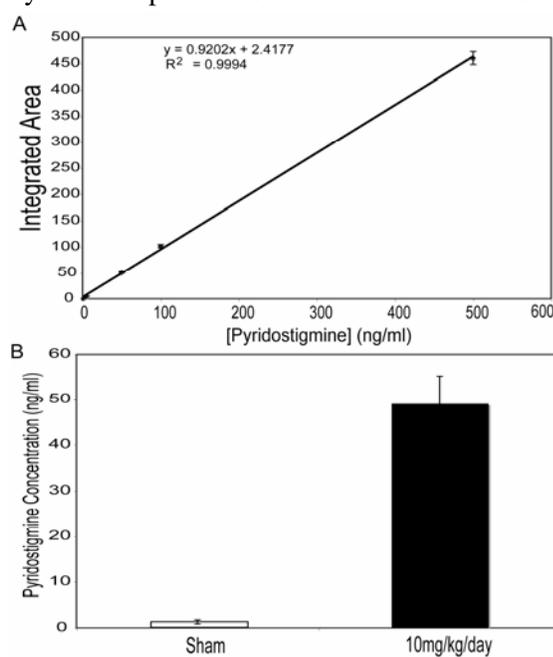


Figure 12, Project 1. Detection of PB in blood. The presence of PB in blood was determined using mass spectrometry. A standard curve was established using increasing concentrations of PB in serum that were then compared to serum from mice implanted with Alzet pumps with a PB dose of 10 mgs/kg/day. The results are the mean \pm SEM from three separate mice.

ACHE Activity Assays

The presence of specific AChE inhibitors in any tissue, i.e., PB or PHY, is expected to inhibit AChE activity. Our first goal was to test the effect of PB or PHY on AChE activity. Mice were treated acutely (15 min) and subacutely (7 days) with vehicle, i.e., saline (Sham), PB or PHY. Following treatment, blood and brain tissues (pre-frontal cortex and hypothalamus) were removed and analyzed for AChE activity.

When blood AChE activity was assayed in acute-treated animals, similar levels of inhibition were found for both PB and PHY treatment, i.e., 75 and 76% inhibition, respectively (Fig 13). Likewise,

in subacute-treated animals, PB and PHY caused 49% and 42% inhibition of blood AChE, respectively (Fig 13). The results clearly show that PB and PHY affect blood AChE activity.

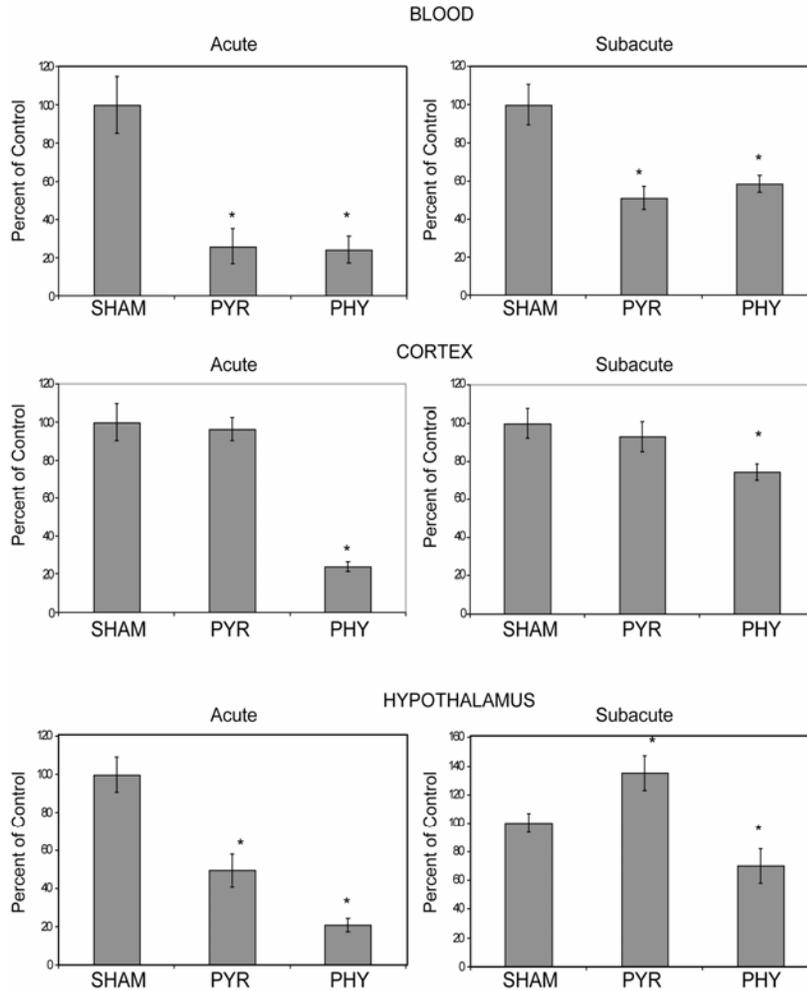


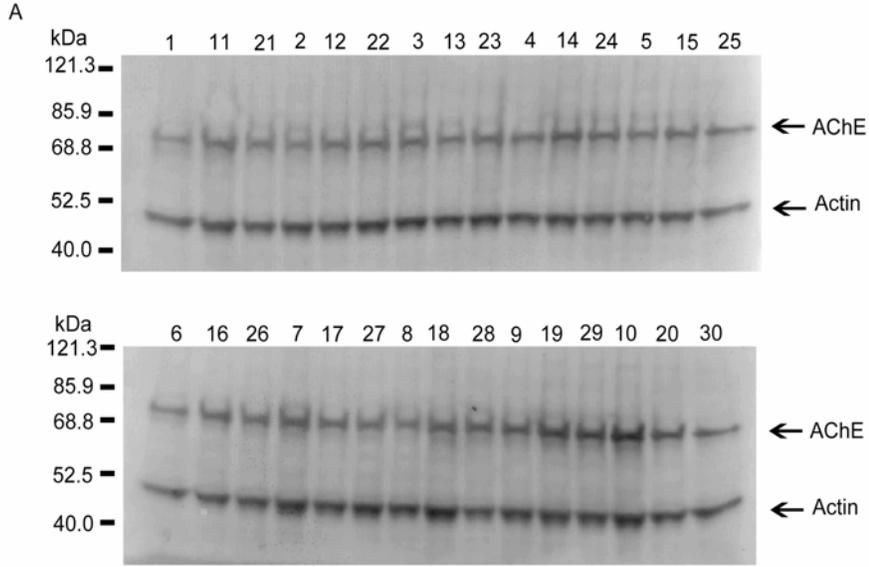
Figure 13, Project 1. Effect of PB and PHY on AChE activity in blood, cerebral cortex and hypothalamus. The data are presented as the average \pm SEM from 10 animals per group assayed in triplicate, i.e., N=10 group.

Having determined that AChE activity in blood was inhibited by both PB and PHY, we next studied their effects on hypothalamic and frontal cortex AChE activity. Cortical AChE was inhibited 76% and 26% by acute and subacute PHY treatment, respectively, (Fig 13). Conversely, PB had no effect on cortical AChE activity (Fig 13).

In comparison to blood and cortex, the hypothalamic response to PB and PHY treatment was more complex. As shown in Figure 13, both treatments, i.e., acute and subacute, resulted in significant changes in hypothalamic AChE activity. Acute treatment with PB and PHY resulted in 51% and 79% inhibition of AChE activity, respectively. In contrast, following subacute treatment with PB,

AChE activity increased 35%, while PHY caused a 31% inhibition of activity. Since PB has an effect at both acute and subacute treatments in hypothalamus but not cortex, the results suggest that PB is capable of entering the hypothalamus but not the cortex.

Semi-Quantitative Western Blot Analysis



AChE protein should not be affected by acute treatment with PB or PHY. To test this hypothesis, Western blot analysis was performed on the same cortex and hypothalamic tissues that were analyzed for AChE activity. Figure 14A shows a representative Western blots from the subacute cortex. To prevent membrane to membrane variance, sham, PB and PHY samples from each tissue were analyzed in an alternative manner (Fig 14 legend for details). Figure 14 B shows the Western blot analysis of the hypothalamus following the same treatment with PB and PHY. No significant changes were observed following acute PB or PHY treatment. However, following subacute treatment, 39% and 30% increases in AChE protein were observed with PB and PHY, respectively. Cortical AChE protein expression was not affected by either acute or subacute treatment with PB (Fig 14B). Acute PHY treatment also had no effect. In contrast, subacute treatment with PHY resulted in a 27% increase in cortical AChE protein (Fig 14 B).

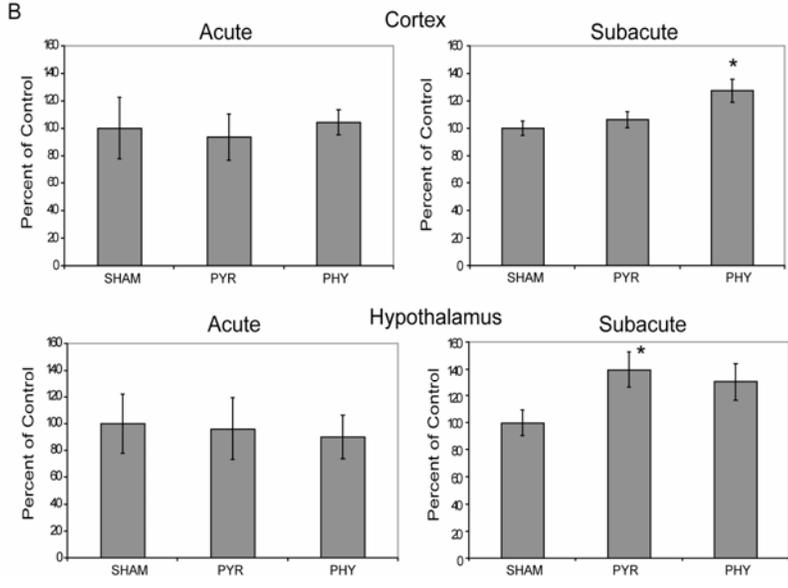


Figure 14, Project 1. Effect of PB and PHY on AChE protein level expression in cortex and hypothalamus. AChE protein was analyzed by western blotting using an antibody to AChE and an antibody to actin (normalize the protein loading). Numbers 1-10 represent Sham; 11-20 represent PB-treatment; and 21-30 represent PHY- treatment. Samples were grouped to prevent inter and intra-blot differences. FUJI software was used to analyze the density associated with each band.

Real-Time PCR

Since there was increased expression of AChE protein in hypothalamic tissue in response to subacute treatment with PB and PHY, we wanted to determine if this could be correlated with an increase in AChE mRNA expression. To study AChE expression, RealTime PCR analysis of AChE mRNA was performed. RealTime PCR analysis of mRNA from acute PB and PHY-treated cortical tissues showed no significant changes in AChE mRNA (Fig 15). In contrast, subacute treatment with PB resulted in a 28% decrease in AChE mRNA. No changes were observed with PHY.

In the hypothalamus, acute treatment with PB caused no increase in mRNA levels, while acute treatment with PHY caused an 80% increase ($p < 0.05$) in AChE mRNA (Fig 15). In contrast, subacute PB -treatment caused a 40% increase, whereas PHY caused no change in AChE mRNA levels.

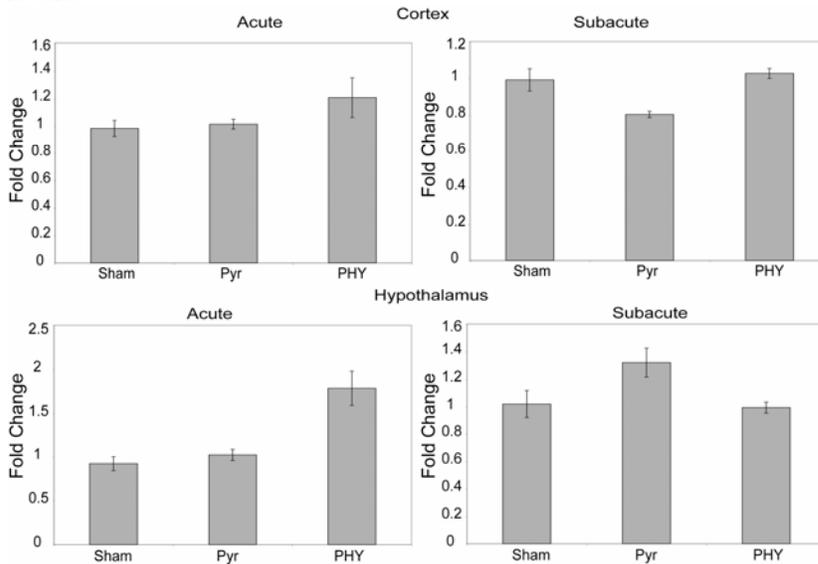


Figure 15, Project 1. Effect of PB and PHY on AChE mRNA levels in cortex and hypothalamus. mRNA was extracted and analyzed by semi-quantitative RealTime RT-PCR using a probe for AChE and the housekeeping gene MAX in each well. The data resulting from this was analyzed using the $2^{-\Delta\Delta CT}$ method (13). The data represent the average \pm SEM from 10 independent samples assayed in duplicate.

Proteomic Analysis

Acetylcholine has the potential to regulate the neuroendocrine system at the level of cholinesterase and also in a non-cholinergic manner, via muscarinic receptors. The hypothalamus and pituitary axis, which represents the neuroendocrine system in this experiment, are ultimately responsible for secretion of peptide hormones that regulate endocrine and metabolic homeostasis throughout the body. Therefore, we wanted to determine if peptides or proteins other than AChE in these tissues were affected by either PB or PHY treatment. To test this hypothesis, we used a relatively new technique, i.e., SELDI-TOF mass spectrometry, to generate a “proteomic” profile of the hypothalamic and cortex proteins and peptides after treatment with PB or PHY. Using the Ciphergen ProteinChip[®] reader, proteomic profiles for the four tissues, i.e., cerebral cortex, hypothalamus, anterior pituitary and neurointermediate lobe pituitary were generated for sham, PB and PHY treated mice from acute and subacute experiments. For each ion peak in the spectral profile, the area under the ion peak was integrated and an average obtained for each tissue from that group. These were then compared using a MANOVA analysis followed by LSD post-hoc test.

MANOVA analysis indicated that only the subacute-treated hypothalamus showed significant changes ($p < 0.047$) (Table 4). Acute-treated hypothalamus showed a strong trend, i.e., $p = 0.076$, while cortex showed no significant change for either treatment group, $p > 0.5$.

Upon examination of the individual ion peaks in the hypothalamus profiles, numerous changes were observed following subacute treatment with PB or PHY, e.g., 33 peaks were affected. Of these, twelve peaks showed similar changes following subacute treatment with PB and PHY. Levels of the smallest ion peak, i.e., 1835 daltons, were decreased with both PB and PHY treatment, while levels of all the other peaks increased. The identity of these peaks is not currently known. However, the results clearly establish that PB and PHY affected the proteomic profile in the hypothalamus but not in the cortex.

Table 4, Project 1. MANOVA Analysis of SELDI-TOF MS Proteomic Data.

Time	TISSUE	p- VALUE
Acute	Anterior Pituitary	0.627
	Posterior Pituitary	0.271
	Hypothalamus	0.076
	Cortex	0.545
SubAcute	Anterior Pituitary	0.545
	Posterior Pituitary	0.530
	Hypothalamus	0.047
	Cortex	0.544

A MANOVA analysis was used to examine proteomic data from the SELDI-MS analysis of each tissue. The resulting p-values indicate the probability of the treatment groups being different than the sham treated. $P < 0.05$ is considered significant. Time indicates the length of time for the treatment with PB or PHY, i.e., acute= 15 minutes, subacute= 7 days.

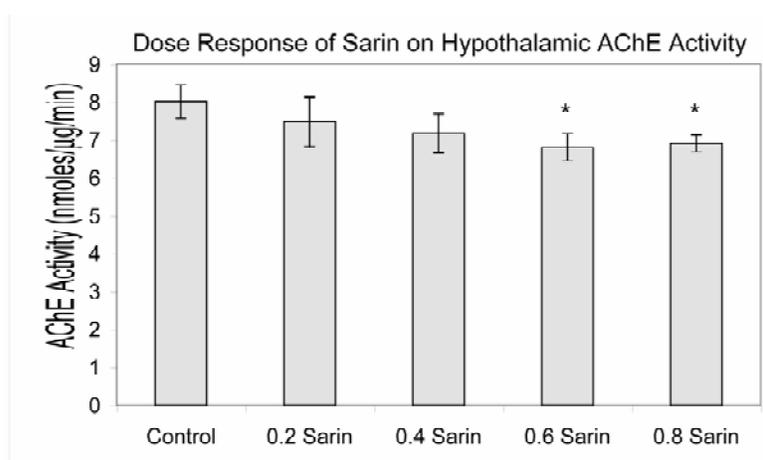
Table 5, Project 1. Subacute (7 day) effects of PB and PHY on hypothalamus proteomic profiles as determined by SELDI-TOF MS analysis of samples on a Ciphergen ProteinChip® Array.

Subacute Hypothalamus							Significance (p < 0.05)		
Peak (M/Z)	Average Integrated Area						Sham vs PB	Sham vs PHY	PB vs PHY
	Sham	S.E.	PB	S.E.	PHY	S.E.			
1835.2	32.3	5.7	12.3	3.2	16.3	5.2	Yes	Yes	-
4471.5	25.6	8.0	58.8	12.6	64.8	11.4	Yes	Yes	-
5444.3	70.1	11.5	173.2	35.2	356.9	27.2	Yes	Yes	Yes
5706.6	56.9	21.8	211.8	54.3	410.5	36.5	Yes	Yes	Yes
6224.8	78.9	27.1	192.4	45.4	313.5	20.5	Yes	Yes	Yes
6573.5	29.1	4.8	54.6	11.6	81.4	7.1	Yes	Yes	-
6901.6	103.0	41.8	419.5	100.9	457.9	53.2	Yes	Yes	-
7013.8	165.5	71.0	737.8	180.7	1014.6	109.3	Yes	Yes	-
8398.4	329.5	63.8	693.6	115.4	1155.3	86.4	Yes	Yes	Yes
9915.1	64.3	22.8	234.8	50.4	353.0	29.3	Yes	Yes	Yes
11325.9	118.9	48.2	466.9	104.6	517.2	66.4	Yes	Yes	-
13810.2	95.1	38.1	324.6	73.3	405.0	43.1	Yes	Yes	-
14035.9	251.5	100.6	869.9	180.8	1146.4	148.0	Yes	Yes	-
14988.4	274.3	95.4	1017.0	235.4	1575.0	253.5	Yes	Yes	-
16791.7	358.1	88.5	1132.5	173.3	1528.9	168.0	Yes	Yes	-

Average integrated areas for 45 ion peak M/Z values for sham, PB and PHY were compared using an ANOVA array in order to determine significance (Yes = significant difference, p < 0.05). S.E. indicates SEM.

Effect of sarin and CBDP: The second major project determined the combinatorial effects of sarin and CBDP on the hypothalamic and pituitary systems. In preliminary experiments, single or multiple doses of sarin injected sc into mice were found to have little effect on brain AChE activity (Figure 16).

Figure 16, Project 1. Dose response of sarin on hypothalamic AChE activity. Four increasing concentrations were injected into C57 mice; 0.2 LD50, 0.4 LD50, 0.6 LD50 and 0.8 LD50. Tissues was collected after 24 hours and processed for AChE activity. The data represent the mean ± SEM from 10 mice. * p < 0.05 vs. control



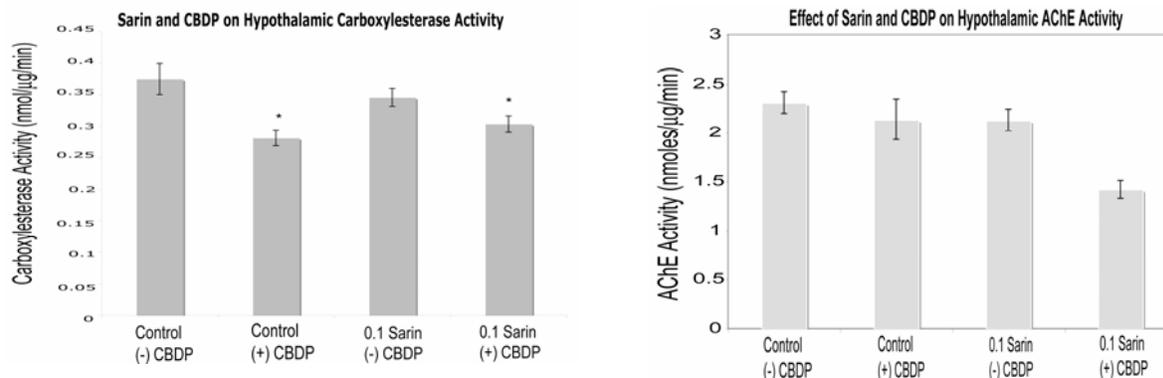


Figure 17, Project 1 (left). Effect of CBDP on carboxylesterase activity in the presence or absence of sarin. CBDP activity was measured in hypothalamus 24 hr after injection. Results show the mean \pm SEM from n of 10. * $p < .05$ vs. without CBDP

Figure 18, Project 1 (right). Effect of combination of CBDP and sarin on AChE activity in the presence or absence of sarin. CBDP activity was measured in hypothalamic tissue 24 hours after injection. The results represent the mean \pm SEM from 10 animals. $p < 0.05$. with CBDP sarin without CBDP sarin

The hypothesis to explain this phenomenon was that peripheral ChE and/or carboxylesterases were binding or “soaking up” the sarin. To overcome this peripheral effect, the carboxylesterase inhibitor, CBDP was used to inhibit carboxylesterases. CBDP (1.5 mg/kg) was injected 1 hour prior to sc injection of sarin (16 μ g/kg). After 24 hours, hypothalamic carboxylesterase activity was significantly inhibited in the CBDP-treated mice, but not in mice treated only with sarin (Fig 17). AChE activity was analyzed in the same hypothalami and results showed that the only combination that caused AChE inhibition was sarin in the presence of CBDP (Fig 18).

Further analysis of mRNA levels by Semi-Quantitative RealTimeRTPCR showed a decrease in the AChE mRNA after 24 hr of exposure to either CBDP, sarin or both (Fig 19). The changes in mRNA expression suggest that CBDP and sarin share similar pathways for down regulating AChE mRNA expression at 24 hours. The results strongly suggest that sarin is “absorbed or bound up” by peripheral ChE/carboxylesterase and is not present in high enough concentrations to inhibit brain activity, i.e., hypothalamus and pituitary. There were no significant differences in proteins/peptides in hypothalami as observed by SELDI-TOF MS. This may be a reflection on the shorter time for changes to occur, i.e., <24 hours compared with the PB study described above where changes were observed at 7 days.

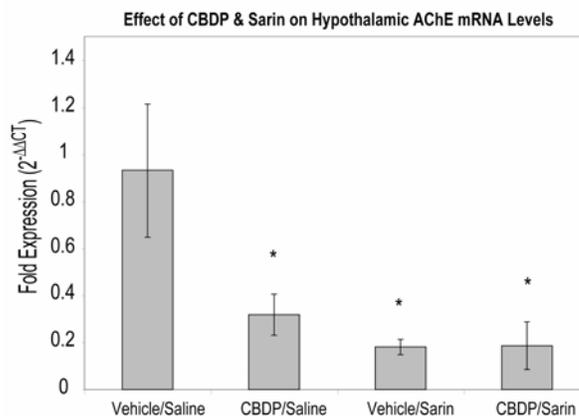


Figure 19, Project 1: Effect of CBDP and sarin on hypothalamic AChE mRNA levels. A portion of the hypothalami from the CBDP and AChE assays was used for mRNA extraction and semiquantitative RealTime RTPCR analysis of AChE mRNA. Saline is the vehicle used for dilution of sarin in the Vehicle and CBDP alone. The results represent the mean \pm SEM from 5 hypothalami for each treatment group. * $p < 0.05$.

Sub-Project 3: Cardiovascular/Autonomic (Mariana Morris, Ph.D.)

The objective of this sub-project was to examine the short and long term effects of chemical exposure (PB and sarin) and stress on cardiovascular parameters in mice. There is much evidence linking cardiovascular pathologies and stress and there is data showing that persons diagnosed with Gulf War syndrome have autonomic imbalances (7; 11). For these studies we established methods for measuring blood pressure and heart rate in the conscious mice and coupled this with a chronic stress exposure (2-4; 9) We also applied sophisticated statistical analytical methods for the measurement of blood pressure and heart rate variability (9; 12). This data is useful for the evaluation of autonomic balance in humans and animals.

Key Accomplishments:

- Developed model for chronic cardiovascular monitoring in mice using chronic direct and telemetric catheters.
- Determined the effect of long term shaking stress on cardiovascular and endocrine parameters in mice. Results showed that there were consistent increases in blood pressure and heart rate associated with shaker stress. There was also evidence of a day/night rhythm in responsiveness. There was also an accommodation of the adrenal corticosterone response with time.
- Determined the effect of chronic treatment with PB on cardiovascular parameters. There were no long term changes noted even though there was significant inhibition of AChE.
- Determined the effect of acute and chronic stress on cardiovascular parameters including testing of autonomic balance.
- Determined the short and long term cardiovascular effects of sarin exposure, 1 day to 90 days. Results showed that there were no changes in blood pressure or heart rate although there were dramatic alterations in heart rate variability.
- Determined the short and long term effects of sarin on central catecholaminergic systems.
- Determined the cardiovascular effects of stress in an animal model of altered responsiveness, mice lacking the ability to produce the peptide stress hormone, oxytocin.

Studies began with examination of the effect of chronic AChEI with PB (1, 3 or 10 mg/kg/day, 0.5 ul/hr for 5-7 days) on cardiovascular and behavioral parameters in mice (2). The data showed that chronic PB exposure produced few changes in blood pressure, heart rate, body weight, drinking or behavior in the fact of significant inhibition of blood AChE (Fig 20). However, subsequent studies of the combination of stress and PB revealed that under these conditions there were dramatic alterations in heart rate variability (12). There were no changes in the absolute BP or HR or the stress response (Fig 21). However, using spectral analytical methods, we found that there was an increase in heart rate variance and it low spectral component, almost a 2 fold change (Fig 22). The

data indicates that the combination of stimuli acted via central neural mechanisms to alter cardiac function. Further studies were conducted to evaluate the effect of PB/stress combination on cardiac and vascular structure (1). Using histological markers for cell death and measurement of vascular wall thickness, we found that there were changes induced by PB and PB/stress. There was an

increase in apoptotic index in heart with a reduction in the aortic endothelium. There was also a reduction in thickness/diameter ratio in aorta in PB treated mice. Together the data provide support for the idea that PB even at low doses may cause clinical side effects related to autonomic balance and cardiac/vascular structure

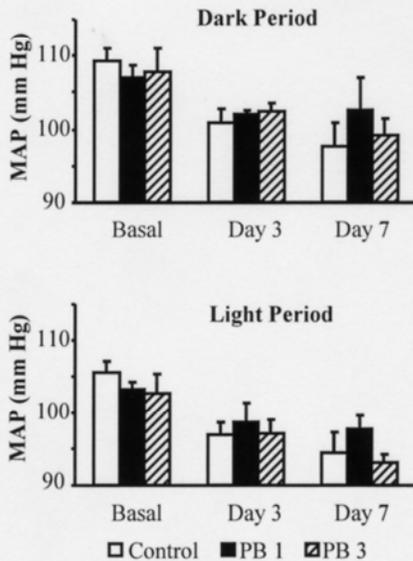


Figure 20, Project 1. Effect of PB (1 & 3 mg/kg.day, 0.5ul/hr for 7 days) on MAP during the dark and light periods. N = 6-7/group. There was no significant effect of PB on blood pressure

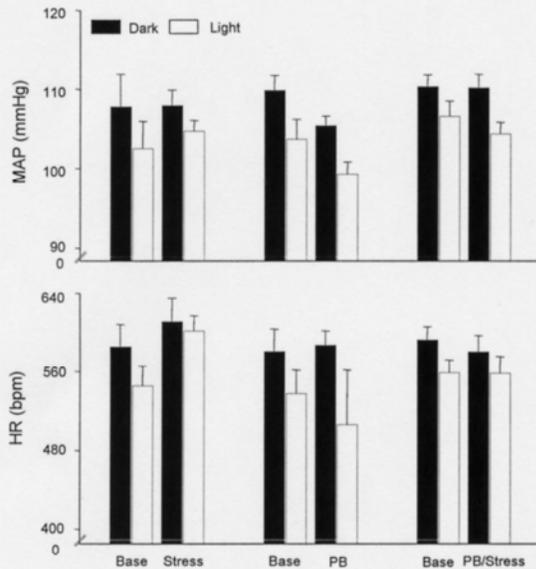


Figure 21, Project 1. Day/night average values of mean arterial pressure (MAP, upper panel) and heart rate (HR, bottom panel) recorded over 24 hours during basal (Base) and after three days of stress, PB (10 mg/kg/day, 5 days) treatment, or PB treatment coupled with stress. There were no significant effects of treatment.

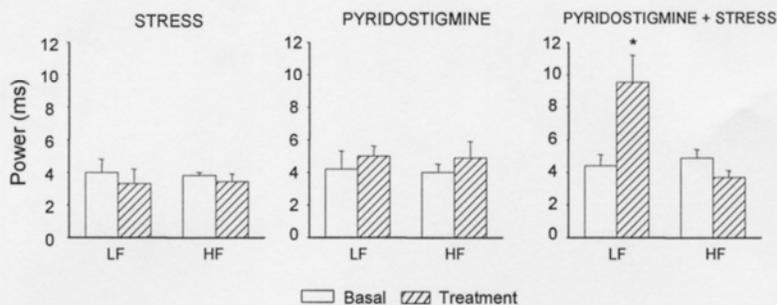


Figure 22, Project 1. Power spectral density (ms) of low (LF; 0.1-0.5 Hz) and high (HF; 1-5 Hz) frequency components of PI (pulse interval) variability calculated by spectral analysis for each experimental group (Stress, PB, and PB + Stress) during basal and after treatment. * $p < 0.05$ compared to basal. Data from figure 21.

Studies of chronic stress exposure revealed that there were no long term changes in blood pressure, heart rate or behavior (3; 9; 12). However, there were alterations in the pattern of reactivity (3). This was seen as increased pressor responses during the light period (the inactive phase in rodents). Chronic stress also produced an attenuation of stress induced corticosterone response suggesting a habituation to the response or an exhaustion of adrenal function.

To further evaluate the cardiovascular response to stress, we used spectral analytical methods which allow for the evaluation of autonomic function under basal, non-stress conditions (9; 12). The results showed that acute stress produced an increase in blood pressure variability and increase in the low frequency spectral component. There were also increases in blood pressure and heart rate after acute shaker stress. The chronic response was a reduction in blood pressure variability, suggesting that there are alterations which occur with time. A second aspect of the stress study, was to determine the effect of autonomic blockade on the cardiovascular responses to stress (8). The data showed that there were specific effects with cholinergic and adrenergic blockade which allowed for a further discrimination of the sympathetic and parasympathetic components. For example, cholinergic blockade reduced the stress tachycardia and blocked the increase in pulse variance and its low frequency component. Finally, stress studies were conducted in a model of altered endocrine status, the oxytocin gene deletion mice. The rationale for this is that these mice show an accentuated response to stress. In this protocol, we tested the effect of chronic stress on blood pressure, heart rate, drinking and plasma corticosterone levels (4). Results showed that blood pressure was lower in the oxytocin deficient and there was an enhanced pressor response. We did not follow up these studies with tests of chemical exposure as proposed in the original application because the changes were not robust.

The final set of experiments used sarin and produced interesting findings related to the cardiovascular effects of low dose exposure. This work was presented at the Toxicology Meeting and the Experimental Biology Meetings in 2005. A manuscript has been submitted to Experimental Neurology (15). The data show that exposure to a low dose of sarin (sc, 0.05 LD50, 8 µg/kg, two injections) which produced no change in blood AChE activity produced profound alterations in cardiac function. These were seen as

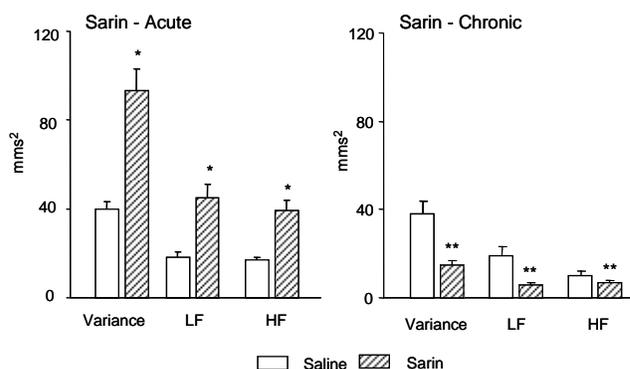


Figure 23, Project 1. PI (cardiac pulse interval) variability in time (variance) and low and high frequency domains; saline vs. sarin, acutely and chronically (1 and 10 weeks, respectively)(n = 6-9, group). * = p < 0.001 vs. sarin acute and ** = p < 0.003 vs. sarin chronic.

time-related changes in heart rate variability. Within 3 days there was a marked increased in variance and low frequency component (associated with sympathetic activity) which was followed by a delayed decrease at 3 months after treatment. There were no changes in blood pressure or blood pressure variability.

There is clinical evidence which associates low heart rate variance with cardiovascular pathologies such as heart attack and failure. There is also data that persons with “Gulf War Syndrome” and survivors from the Tokyo subway attack show alterations in autonomic balance similar to that seen in our studies (11; 18). The data raises concern even with exposure to long, virtually non-detectable doses. The long term time point also showed increased brain tyrosine hydroxylase mRNA (Fig 24). Studies in Sub-Project 2 (Neurobehavioral) also found changes as seen by the delayed adrenal hyperplasia present in mice treated with sarin and stress. The overall conclusion is that a low dose of sarin produced acute and delayed effect on cardiac function and autonomic balance. The long term action results in sympathetic activation specific for the heart, changes in brain stem catecholaminergic activation and changes in adrenal medullary function.

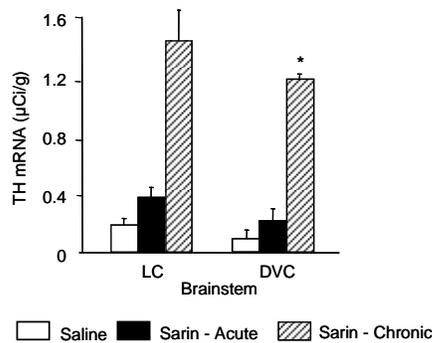


Figure 24, Project 1. Effect of saline or sarin treatment on TH mRNA expression in locus coeruleus (LC) and dorsal vagal complex (DVC) (n = 5-7/group). Measurements were made acutely and chronically (1 and 10 weeks, respectively) after sarin. There were effects of treatment in LC, [F(2,15)=26.47, P<0.001] and DVC, [F(2,10)=102.31, P<0.001]. * = p <0.001 vs. saline.

Sub-Project 4: Brain Genomic Expression (James McDougal, Ph.D. and Courtney Sulentic, Ph.D.)

The objective of this series of studies was to determine if low level exposures to sarin would cause changes in gene expression in brain, specifically in the hypothalamus and prefrontal cortex. These are brain areas which are critical in control of behavior, cognitive and autonomic function. Complementary studies had shown that low doses of sarin produced dramatic, long term alterations in cardiac function. We hypothesized that sarin would produce changes in genetic expression, up and down regulation, and that this information would provide clues as to the mechanism of low dose sarin toxicity. Genetic expression was measured using the Affymetrix gene array system with the assistance of Dr. Steven Berberich (Director of the Genomic Research Center and PI of Project 4).

Key Finding:

- The key finding was that treatment with a low dose of sarin (8 µg/kg, 0.05 LD₅₀) produced changes in brain gene expression in the absence of any effects on AChE activity. This is important since it may explain some of the reasons for the delayed sarin toxicity seen in humans.

Our approach was to inject male C57Bl mice (Harlan; 25-30 g) sc on two consecutive days with a lower dose sarin (8 µg/kg or 0.05 LD₅₀), a higher dose sarin (64 µg/kg or 0.4 LD₅₀) or isotonic

saline. Animals were sacrificed 4 hr after the second injection and total RNA was isolated from prefrontal cortex and hypothalamus (Fig 25).

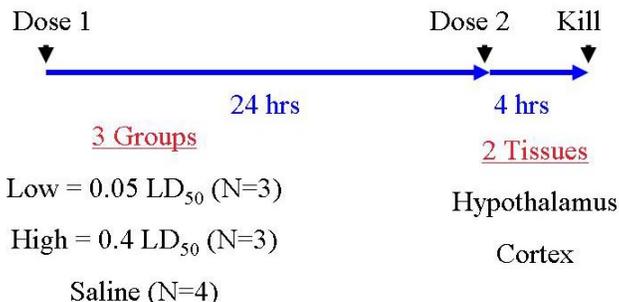


Figure 25, Project 1. Experimental Protocol.

Biotin labeled cRNA targets were prepared according to Affymetrix standard protocols and hybridized to Affymetrix MGU74Av2 mouse arrays. In the prefrontal cortex and the hypothalamus, raw signal and presence or absence calls for each of the 12,400 genes on each of the ten arrays for each tissue (4 control, 3 low dose treated and 3 high dose treated) were analyzed using GeneSpring™ software (Silicon Genetics, Redwood City CA) and PathwayAssist© (Stratagene, La Jolla CA). The raw value for each gene in a treated array from the cortex or hypothalamus was normalized by dividing by the median value of that gene in the control arrays from the same tissue. The process of sorting to find the most interesting changes in transcription was: 1) Genes that were not present (according to the Affymetrix algorithm) in at least 2 of the 10 arrays were eliminated (leaving 6,388 in cortex and 6,070 in hypothalamus). 2) Genes that showed less than a two-fold increase or decrease in expression in both the low and high doses were eliminated (leaving 105 genes in the cortex and 103 in the hypothalamus). These genes were analyzed in more detail using PathwayAssist which uses neural language processing of the abstracts in PubMed to identify relationships (such as binding, metabolism, positive and negative changes in expression) between genes or proteins that were changed.

Gene expression changes occurred in the cortex and hypothalamus at a dose that caused no change in blood or tissue AChE (Fig 26).

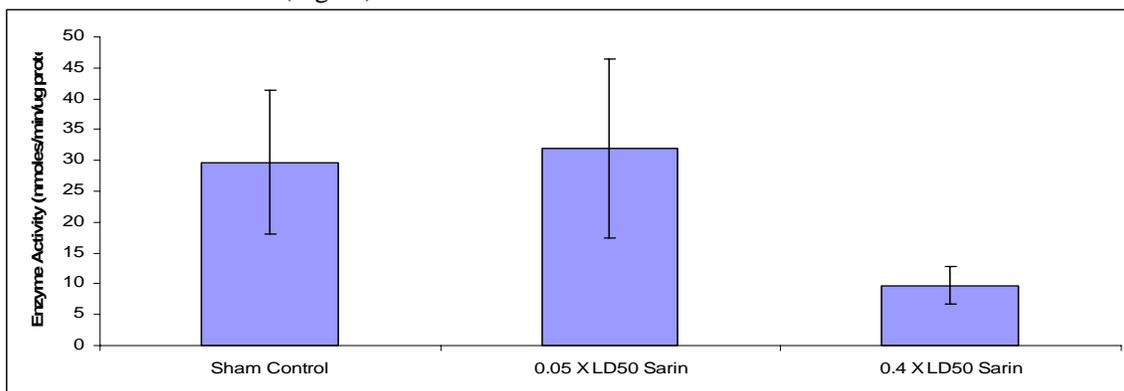


Figure 26, Project 1. Total cholinesterase activity (\pm S.D.) in the prefrontal cortex in mice treated with two doses of sarin.

Gene expression was altered in the prefrontal cortex (Fig 27) and in the hypothalamus (data not shown) by both sarin doses. The lower dose response is particularly interesting because gene transcripts were changed at a dose that showed no change in total cholinesterase activity.

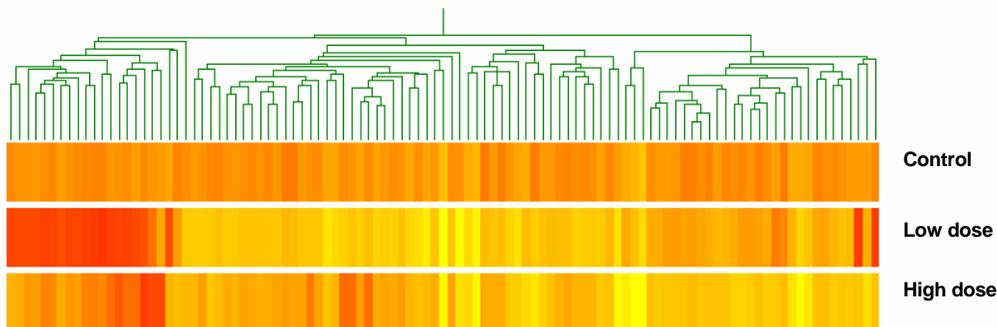


Figure 27, Project 1. Average expression levels of the genes detected as present in the cortex compared with dose. Orange represents the normal expression of these genes. Red indicated genes down regulated. Yellow indicates genes up regulated. The green tree structure shown at the top shows the relationship between genes that responded similarly.

Of the 6,388 gene transcripts detected as present in the frontal cortex, 105 were changed two-fold (Table 6).

Table 6, Project 1. Number of genes increased with sarin treatment in the prefrontal cortex

	Up Regulated	Down Regulated
Lower dose	51	19
Higher dose	47	3

Of the 6,070 gene transcripts detected as present in the hypothalamus, 103 were changed two-fold (Table 7).

Table 7, Project 1. Number of genes increased with sarin treatment in the hypothalamus

	Up Regulated	Down Regulated
Lower dose	31	20
Higher dose	13	51

In the cortex more genes were up regulated than down regulated and in the hypothalamus more gene down regulated than up regulated, emphasizing the difference in response. In the cortex, more genes were affected with the lower dose than with the higher dose. The transcripts that were detected as present in the hypothalamus and the frontal cortex had an 86% overlap, but only 8 transcripts were changed two fold in both the frontal cortex and hypothalamus at either dose (Fig 28). This suggests that although many of the genes are expressed in both tissues, the response of the tissues to sarin is very different. Although the genes were changed in both tissues the response of some of these genes was in opposite directions (Table 8).

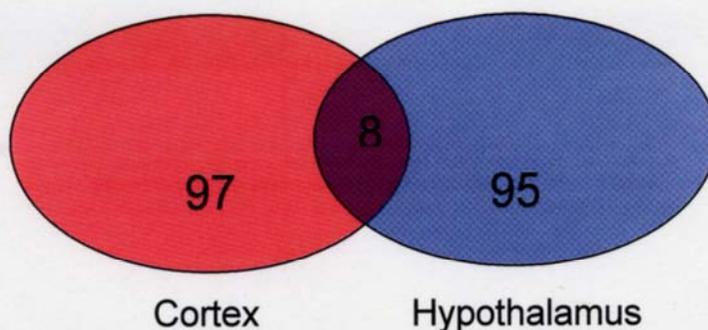


Figure 28, Project 1. Comparison of the number of gene changes (two-fold) common to both the cortex and hypothalamus. The 8 transcripts are those changed (either up or down) in both tissues.

Table 8, Project 1. Identity of genes changed universally and their direction in brain tissues.

Gene	Cortex	Hypo
Osteopontin (SPP1)	up	down
Tyrosine phosphatase (PTPRJ)	down	down
Chromogranin B (CHGB)	up	down
Prostaglandin D2 synthase (PTGDS)(2 genes)	up	down
Hephaestin, cu-oxidase (HEPH)	up	up
Doublecortin (DCX)	up	up
Zinc finger protein 26 (ZFP26)	down	down

Genes changed were primarily from the simplified Gene Ontology (GO) molecular function categories of enzymes, structural proteins, nucleic acid binding and transport. Interestingly, the GO categories of genes increased were the same categories that were decreased. This suggests that compensatory changes occur within gene categories such as enzymes. Figure Q illustrates the molecular function categories and number of genes that were up and down regulated at the lower dose in the prefrontal cortex. Some enzyme genes were increased and other enzyme genes were decreased suggesting that an increase in one aspect of a function must be compensated by a decrease in another aspect. Gene array studies provide an important tool to understanding the mechanisms of toxic and subtoxic effects of agents such as sarin.

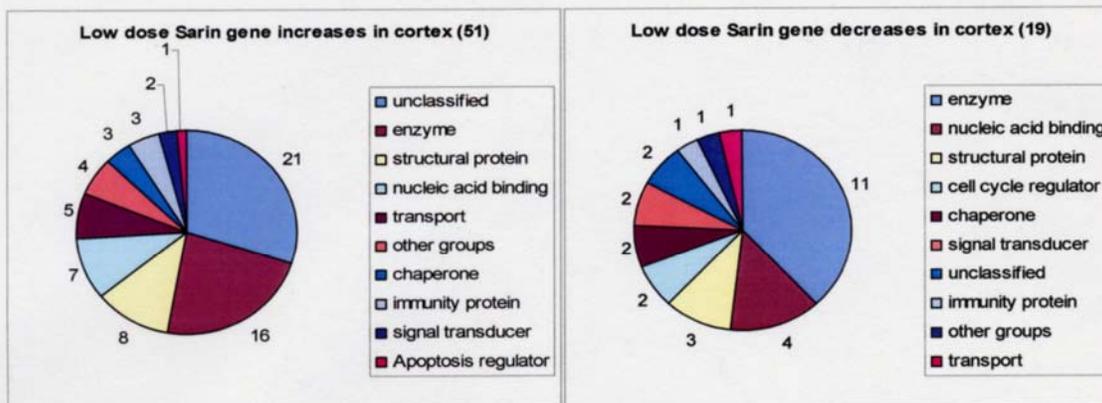


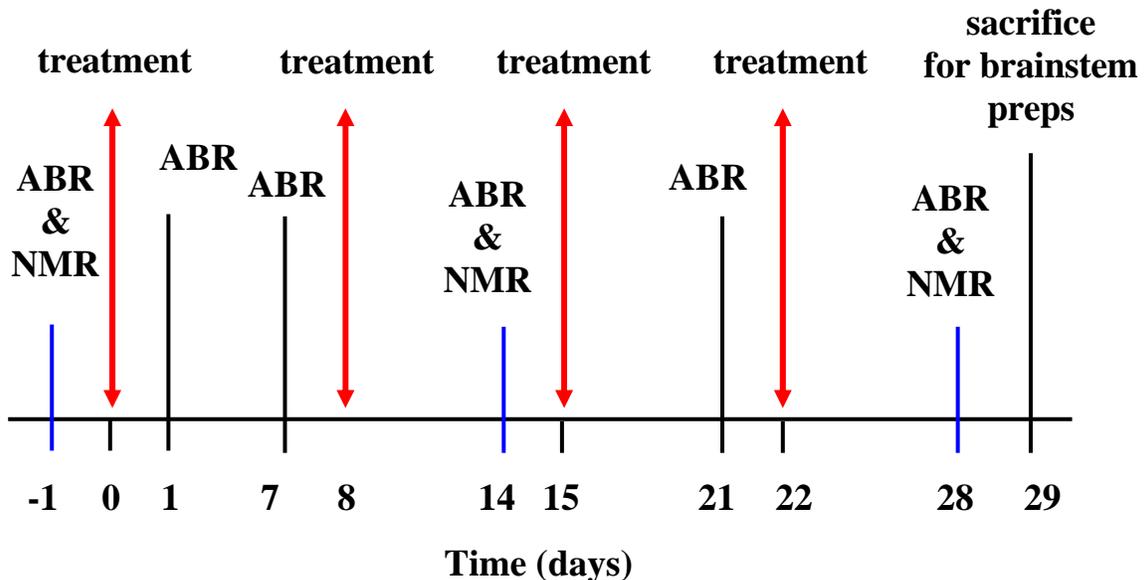
Figure 29, Project 1. Comparison of the number and categories of gene transcripts increased (two-fold) at the lower dose of sarin

Project 2. Studies of stress and chemical exposure on auditory brainstem responses, energy metabolism and tissue chemical constituents in an animal model (Ina Rea Bicknell, Ph.D., Nicholas V. Reo, Ph.D., Lawrence J. Prochaska, Ph.D.)

Overview and Experimental Protocol

In the original contract, Project 2, denoted as Modules 2, 3, and 4, represented a team investigation involving three independent laboratories, all sharing the same animal model system. Because the three investigators shared the same groups of animals, the final report for Project 2 will summarize the results from the three different approaches. The goal of Project 2 was to assess the effects of low chronic doses of three chemicals: PB, DEET, and sarin on brainstem function, brain and muscle metabolism *in vivo*, and brainstem energy metabolism. The project used ~ 80 day-old male Sprague-Dawley rats. Chemicals were administered alone, or in combination, in the presence or absence of stress (noise, presented at an intensity of 85 dB SPL, re: 20 μ Pa, for 8 h). The treatment protocol is illustrated in Figure 1. Controls were administered vehicle under identical experimental conditions. Long-term exposure to low doses of toxin, rather than a single acute high-dose exposure, was employed in an effort to mimic battlefield conditions. Because stress may alter a response to a toxin, it was included as a factor in these experiments. Noise of the intensity employed in these experiments induced a twelve-fold elevation in stress hormones in the blood serum. Electron microscopy indicated no damage to cochlear tissues of the ear (not shown). At the termination of each toxin study, blood was obtained for AChE determination.

Figure 1, Project 2. Experimental Paradigm. Experiments were conducted before treatment (ABR + NMR) and at 1 day (ABR), 7 days (ABR), 14 days (ABR + NMR), 21 days (ABR), and 28 days (ABR + NMR) post-treatment. On day 29, brainstems were removed for NMR and biochemical assays.



The auditory brainstem response (ABR), a measure of electrical activity generated in the brainstem auditory pathway in response to sound, was used to monitor changes in the central nervous system. Nuclear magnetic resonance (NMR) was employed to measure both *in vivo* and *in vitro* increases and decreases in high-energy metabolites and metabolic intermediates in brain/muscle/brainstem in treated animals. Additional biochemical activities were measured to assess *in vitro* alterations in energy metabolism in mitochondria, the subcellular organelle responsible for energy metabolism in living cells. As indicated in Fig. 1, ABR and *in vivo* NMR measurements were made pre- and post-toxin dose. Mitochondrial and *in vitro* NMR measurements were made by sacrificing animals and removing brainstems after the fourth week of exposure to toxin.

Key Accomplishments

- Blood chemistry evaluations validated the stress model. Pre- and post-noise stressor levels of serum corticosterone (Cort) were measured and found to be elevated after noise exposure.
- Completed studies of chronic low-dose exposure to DEET plus noise stress. ABR, NMR, mitochondrial energy coupling (respiratory control) and electron transfer activities of isolated brain stem mitochondria and cytochrome *c* (as a marker for apoptosis) and cytochrome *c* oxidase content of isolated brainstem mitochondria (using immunoblotting) were measured. These endpoints were used in all subsequent studies.
- Completed studies of chronic low-dose exposure to PB plus noise stress.
- Completed studies of acute (24 hour post-injection) effects of PB plus noise stress.
- Completed studies of synergistic effects of chronic low-dose exposure to DEET + PB + noise stress.
- Completed dose response study for sarin comparing subcutaneous (sc) and intravenous (iv) methods of chemical administration and determined LD₅₀ for iv injection in rats.
- Completed studies of chronic low-dose exposure to sarin plus noise stress.

Key Findings:

Chronic low-dose studies with DEET. Chronic low doses of DEET (225 mg/kg, intraperitoneal injection, [ip]) caused no functional or biochemical changes in rat brainstem, and no interactions with noise stress were observed.

Chronic low-dose studies with PB. No statistically significant differences were observed between ABRs of control animals and animals treated with chronic low doses of PB (5mg/kg by gavage), under conditions of noise stress. Little or no biochemical changes were observed in rat brainstem. Noise stress, however, caused a decrease in NADH-supported mitochondrial electron activity.

Chronic low-dose studies with DEET + PB. There was no change in blood AChE induced by DEET + PB or the noise stress. Few changes were seen in rat brainstem after combined administration of DEET (225 mg/kg, ip) + PB (5 mg/kg, gavage) + noise stress. However, different animals exhibited different sensitivities to the toxins suggesting the possibility that endogenous chemical sensitivity may be an animal-dependent phenomenon

Chronic low-dose studies with sarin. There was no change in AChE activity in blood induced by sarin or the noise stress. A clear negative effect on the ability of mitochondria to reoxidize NADH was observed under conditions of sarin and stress. This effect was dependent on the dose of sarin administered. Little or no effect of sarin plus stress was seen in the ABR response. NMR spectroscopy showed little to no effect in endogenous high energy compound metabolic stores in rat

brain and muscle (*in vivo*) or in rat brainstem (*in vitro*). Changes in the phosphorylation state of unknown mitochondrial proteins were observed. Interpretation of the results was colored by animal-to-animal variability in sensitivity to sarin.

Summarized Results and Data for Key Findings:

Chronic low-dose studies with DEET. Chronic low doses of DEET (225 mg/kg, intraperitoneal injection, [ip]) caused no functional or biochemical changes in rat brainstem, and no interactions with noise stress were observed.

As a preliminary experiment to the chronic low dose studies using DEET, the noise treatment was evaluated as mechanism stress by measuring serum corticosterone (Cort) as a marker for stress hormone release (Table 1, Project 2). A twelve-fold increase in serum Cort was measured after application of noise, validating noise as a stressor.

Table 1, Project 2. Corticosterone levels under conditions of 30 min of quiet or 30 min of 85 dB SPL white noise as determined by radioimmunoassay (ICN ImmChem radioimmunoassay kit).

Noise condition	Corticosterone (ng/ml) Mean ±SEM*
Quiet	Ave: 38.64±8.8 n=5
+ 30 min noise	Ave: 458.3±46.7 n=5
*SEM: standard error of the mean	

Brainstem Functional Studies. No behaviorally significant differences in ABR peak II thresholds, latencies, or amplitudes were observed between DEET- and vehicle-injected groups or between noise stress vs. no-noise stress groups. Temporary increases in peak thresholds were observed at 24 hours but were considered to be noise-induced as they occurred in both treated and control groups.

NMR Metabolite Studies. NMR experiments were limited to *in vivo* measurements in brain tissue and leg muscle, and were performed at three timepoints during the experimental protocol: pretreatment, and at 2 and 4 weeks posttreatment. The animals were divided into four groups: Control (-) stress (n=8), DEET (-) stress (n=8), Control (+) stress (n=10), and DEET (+) stress (n=12). Relative signal intensities were measured in rat brain for the following metabolites:

¹H spectra: choline (Cho), creatine (Cr), and N-acetylaspartate (NAA).

³¹P spectra: adenosine triphosphate (ATP) and phosphocreatine (PCr).

The ¹H peaks were normalized to the Cr signal intensity and expressed as a ratio relative to Cr. Cr is uniformly distributed in brain and is usually unaffected by pathological processes. The ³¹P peaks were normalized to total phosphorus by integrating the entire ³¹P brain spectrum. The ratio of NAA:Cr is thought to represent functional neuronal mass, while the ratio of PCr:ATP is a measure of the cellular energy status. Cho reflects amounts of phosphocholine and glycerophosphocholine that are associated with phospholipid metabolism.

The metabolite ratios do not necessarily represent the tissue concentration ratios since the NMR data were not acquired under ‘quantitative conditions’ (i.e., full T₁ relaxation). These data are, however, useful for comparative purposes to delineate differences between groups.

There were no discernable differences in brain or muscle ATP or PCr levels between DEET-treated animals in the presence or absence of noise stress and appropriate controls (Table 2).

Table 2, Project 2. The effect of noise stress, DEET, and DEET with noise stress upon high energy compounds in rat brain as measured by NMR.

Group	Cho/Cr	NAA/Cr	PCr/ATP
Control (-)stress	1.86 ± 0.15 (n=4)	1.66 ± 0.14 (n=4)	1.47 ± 0.09 (n=6)
DEET (-)stress	1.56 ± 0.08 (n= 5)	2.40 ± 0.34 (n=5)	1.57 ± 0.04 (n=8)
Control (+)stress	1.50 ± 0.10 (n=8)	1.77 ± 0.17 (n=8)	1.59 ± 0.08 (n=9)
DEET (+)stress	1.52 ± 0.16 (n=10)	1.72 ± 0.24 (n=10)	1.71 ± 0.09 (n=10)
All groups combined	1.57 ± 0.07 (n=27)	1.85 ± 0.13 (n=27)	1.60 ± 0.04 (n=33)

The pretreatment measurements were conducted prior to DEET or noise stress exposure. Thus animals in all groups are not distinct at this measurement time point. A two-way ANOVA was conducted with the pretreatment data as the dependent variable to assess the variance in the four groups. The two independent factors were the group identifiers, treatment and stress. This statistical analysis showed no significant differences among the groups prior to treatment or stress ($p < 0.05$). Table 2 shows the metabolite ratios (mean ± SE) measured in rat brains *in vivo* at the pretreatment time point for each experimental group. The Cho/Cr and NAA/Cr ratios were measured from the ^1H NMR spectra, while the PCr/ATP ratio was 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.

A repeated measures ANOVA was conducted to test whether treatment and/or stress produced any significant changes in the data over times post-treatment. Here the data obtained at pretreatment, 2 wk, and 4 wk were repeated measurements (dependent variables) with treatment and stress as independent factors. All measurements (Cho/Cr, NAA/Cr, and PCr/ATP) showed no effects of either DEET exposure alone or the combination of DEET + stress ($p < 0.05$). Noise stress alone, however, produced a significant effect on the Cho/Cr data as determined by this repeated measures ANOVA ($p=0.05$). The Cho/Cr ratios averaged over all time points (mean ± SE) for animals exposed to stress (1.50 ± 0.06 , $n=51$) were slightly lower than the value for animals not exposed to stress (2.02 ± 0.16 , $n=24$). If this does reflect some changes in membrane components, then the effect was not large enough to impact energy metabolism since PCr:ATP ratios were not affected.

These NMR data corroborate the ABR measurements and the measurements of mitochondrial function: DEET exposure in rats, with or without noise stress, does not compromise brain function or cellular energy metabolism.

Brainstem Energy Metabolism Studies. Assays for energy coupling (an indicator of *in vivo* mitochondrial function) and respiration in isolated brainstem mitochondria were unaffected by treatment of the animals with DEET. Immunoblots showed no change in cytochrome oxidase content of mitochondria isolated from animals treated with DEET (+/- noise stress) compared to mitochondria isolated from control (+/- noise stress). In addition, cytochrome *c* content (as a

marker for apoptosis) of mitochondria did not change in any treatment as assessed by immunoblotting. There was no change in AChE activity in blood induced by DEET or noise stress using a standard non enzymatic assay based upon the addition of dithionitrobenzoate (Elman's reagent).

Chronic low-dose studies with PB. No statistically significant differences were observed between ABRs of control animals and animals treated with chronic low doses of PB (5mg/kg by gavage), under conditions of noise stress. Little or no biochemical changes were observed in rat brainstem. Noise stress, however, caused a decrease in NADH-supported mitochondrial electron activity.

Brainstem Functional Studies. Morphologically abnormal waveforms for the 32-KHz signal (Fig. 2, bottom panel) were observed in 3 of the total 8 PB-treated animals, indicating a differential sensitivity to PB. Figure 2 top panel is the control for this experiment. The rat ABR waveform consists of four dominant peaks (I, II, III, and IV). Peak II is generally the strongest peak and is regarded as a putative indicator of neural activity occurring primarily in the cochlear nucleus of the brainstem auditory pathway. Peak II is usually the last major peak to disappear with decreasing intensity of the auditory signal and, therefore, was used for evaluation.

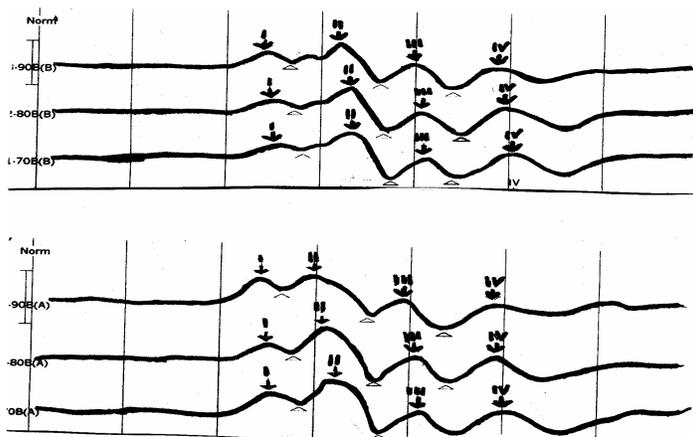


Figure 2, Project 2. Rat ABR Waveform. The rat ABR waveform consists of four dominant peaks (I, II, III, and IV). Peak II is generally the strongest peak and is regarded as a putative indicator of neural activity occurring primarily in the cochlear nucleus of the brainstem auditory pathway. Peak II is usually the last major peak to disappear with decreasing intensity of the auditory signal and, therefore, was used for evaluation. Top panel: control animal 24h after receiving vehicle and 8h of noise stress. Bottom panel: waveform from a test animal 24 h after administration of PB (5mg/kg) and 8h of noise stress. In each panel, the ABR waveforms are shown for a 32-kHz signal presented in a descending intensity series of 90, 80, and 70 dB SPL (amplitude, y axis). Latency (x axis) is in ms.

NMR Metabolite Studies. *In vivo* NMR measurements showed no differences in brain or muscle ATP, PCr, or NAA between rats treated with PB and noise stress compared to rats exposed to noise alone. Two different experiments were conducted: (1) repeated doses once weekly over 4-wks (chronic study), and (2) one single dose with measurements made at 24 h post-dose (acute study). Fig. 3 shows the results for the 4-wk study, which suggested that PB under conditions of noise stress does not produce any deleterious effects on neuronal metabolism.

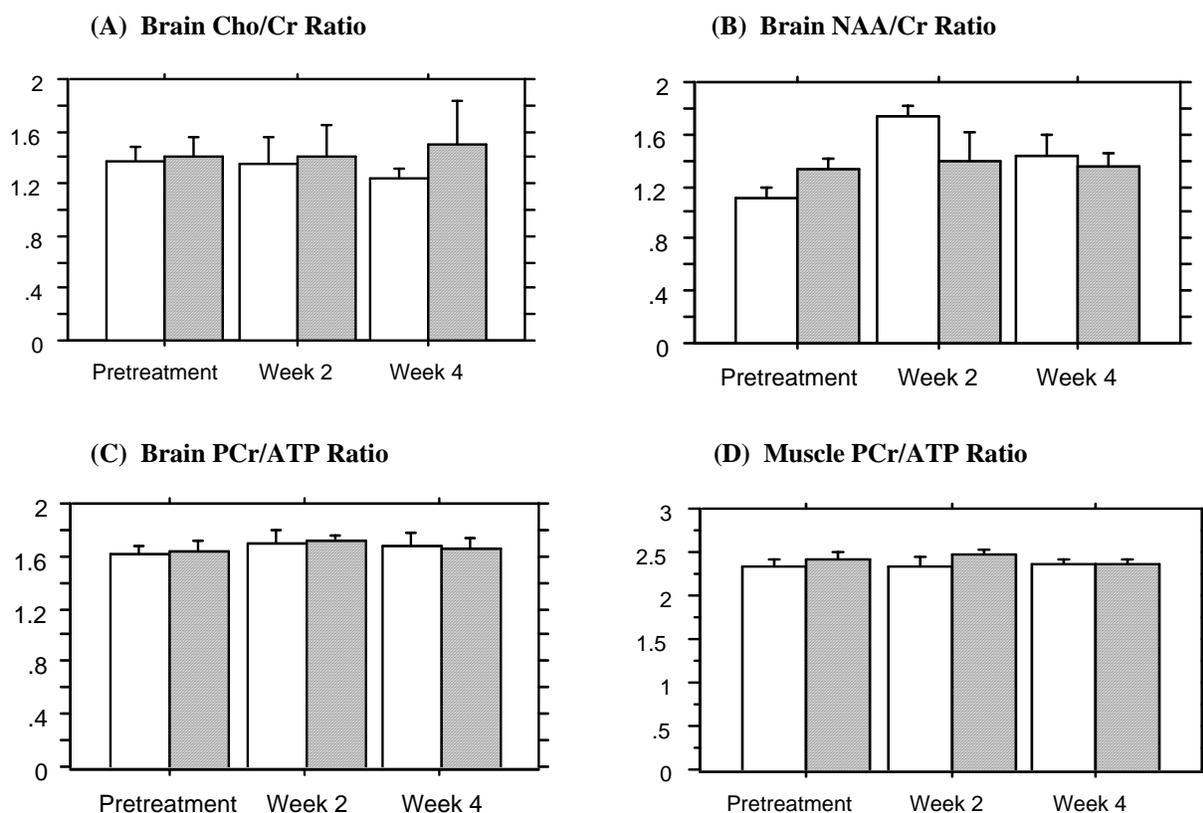


Figure 3, Project 2. NMR measured metabolite ratios (Mean \pm SE) in rat brain and leg muscle in vivo for PB-treated (hatched bars) and control (open bars) groups. Measurements are before (pretreatment) and at 2 and 4 weeks during the chronic exposure protocol involving PB or saline plus noise stress. (A), (B), and (C) are the Cho/Cr, NAA/Cr, and PCr/ATP ratios, respectively, measured in rat brain, while (D) shows the muscle PCr/ATP ratios measured in the same

Brainstem Energy Metabolism Studies. Little or no effect on energy coupling was observed in mitochondria isolated from rats chronically exposed to PB + stress when compared to noise-stressed controls. The ability of mitochondria to make ATP and the inner membrane permeability of mitochondria was unaffected by the treatments. Some inhibition of electron activity was observed in mitochondria isolated from PB + stress and stress-treated animals when using NADH as the substrate, suggesting a possible site of inhibition in Complex I of the respiratory chain (Table 3, Project 2). Stress appeared to affect mitochondrial functioning by inhibiting electron transfer through Complex I with a small additional decrease in activity induced by PB. There was little or no change in cytochrome oxidase or cytochrome *c* content of mitochondria from the PB + stress animals compared to mitochondria from stress-treated animals. There was no change in AChE activity in blood induced by PB or noise stress.

Table 3, Project 2. The Effects of a of PB (5 mg/Kg Body Weight) and Stress on Rat Brain Stem Mitochondrial Electron Transfer Activity

Treatment	Specific Activities (nmol/min/mg-protein) ^a		
	Cyt. <i>C</i> \Rightarrow O ₂	NADH \Rightarrow Cyt. <i>C</i>	NADH \Rightarrow UQ

I. Control with Noise Stress	213 ± 44	148 ± 18	77 ± 15
II. PB-Treated + Noise Stress (24 hours after injection)	235 ± 13	200 ± 6	61 ± 1
III. PB-Treated + Noise Stress (30 days after injection)	224 ± 25	177 ± 8	76 ± 18

^aActivity measurements are presented in nmoles oxygen consumed, nmoles cytochrome c reduced, or nmoles NADH oxidized/mg mitochondrial protein–min. Error measurements are standard deviations and an unpaired Student's t-test was used to examine differences between treated and control groups. All data were determined to be significant at the 95% confidence level ($p \leq 0.05$). Each assay was performed at least three times for each preparation.

Acute Effects of PB (24 hr post injection).

NMR Metabolite Study. Fig. 4 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 time point. 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 ($p \leq 0.05$). Thus PB + noise stress did not impact brain or muscle energy metabolism at 24 h post-dose as determined by NMR *in vivo*.

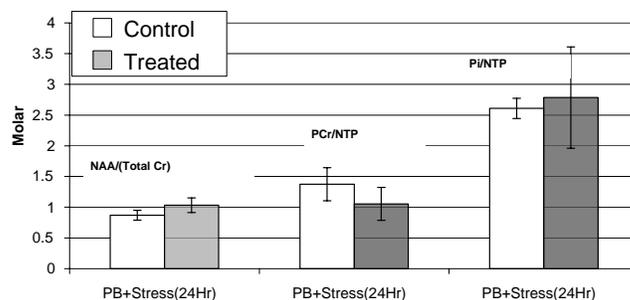


Figure 4, Project 2. NMR-measured metabolite molar ratios (mean ± SE; n=5-7) in rat brainstem extracts prepared at 24 h postdose with PB + noise stress. Concentration values were computed on a per gram tissue basis.

Brainstem Functional and Energy Metabolism Studies. Acute PB effects were similar to those seen in the chronic studies with abnormal ABR waveform morphologies occurring 24 hours after administration of PB (Figure 2, Project 2, bottom panel). In addition, noise stress and PB caused a decrease in NADH-supported mitochondrial electron activity (Table 3, Project 2). Neither *in vivo* nor *in vitro* NMR measurements showed major changes in high-energy metabolites in brain, brainstem, or muscle.

Chronic low-dose studies with DEET + PB. There was no change in blood AChE induced by DEET + PB or the noise stress. Few changes were seen in rat brainstem after combined administration of DEET (225 mg/kg, ip) + PB (5 mg/kg, gavage) + noise stress. However, different animals exhibited different sensitivities to the toxins suggesting the possibility that endogenous chemical sensitivity may be an animal-dependent phenomenon.

Brainstem Functional Studies. The ABR showed that 4-kHz thresholds were significantly different in DEET + PB + noise-stressed rats. However, because these changes were small, they were not considered to be behaviorally significant. Significant differences between Peak II latencies were seen in treated animals compared to controls; significant within-group Peak II amplitude differences were observed in treated animals.

NMR Metabolite Studies. *In vivo* NMR experiments showed no change in brain or muscle ATP, phosphocreatine, or NAA when comparing DEET + PB + noise stress to a vehicle-treated controls with noise stress (data not shown). The *in vitro* NMR experiments also showed no change in NAA, PCr, or ATP levels in brainstems of animals treated with DEET + PB + noise stress (Tables 4 and 5, Project 2). The results suggested that there was no change in metabolism in the brainstem/brain/muscle after chronic toxin exposure.

Table 4, Project 2. Brainstem metabolite concentrations (umol/g tissue; Mean ± SE) determined by ³¹P NMR in rats treated with PB + DEET + Stress (Treated) and corresponding controls (vehicle + Stress).

GROUP	PCr	ATP	Pi
Treated (n=7)	0.75 ± 0.06	0.61 ± 0.13	2.03 ± 0.27
Control (n=3)	0.62 ± 0.30	0.58 ± 0.34	1.63 ± 0.29

Table 5, Project 2. Brainstem metabolite concentrations (umol/g tissue; Mean \pm SE) determined by ^1H NMR in rats treated with PB + DEET + Stress (Treated) and corresponding controls (vehicle + Stress).

GROUP	NAA	Total Cr	Myo-Ins	Lactate
Treated (n=7)	1.49 \pm 0.13	1.41 \pm 0.14	1.03 \pm 0.19	0.40 \pm 0.04
Control (n=5)	1.22 \pm 0.22	1.12 \pm 0.16	0.81 \pm 0.14	0.29 \pm 0.03

Brainstem Energy Metabolism. There was little or no effect on energy coupling in mitochondria isolated from rats chronically exposed to DEET + PB + stress compared to control animals treated with noise stress. An increase in inhibition in NADH-linked electron transfer was observed in some DEET + PB + stress-treated animals compared to stress-treated control animals. This was not observed in all experiments. Noise stress appeared to affect mitochondrial functioning by inhibiting electron transfer through Complex I with a small additional decrease in activity induced by DEET + PB + stress. There was little or no change in cytochrome oxidase or cytochrome *c* content of mitochondria from the DEET + PB + stress animals when compared to mitochondria isolated from stress-treated controls.

Dose-response study for sarin: (not presented in key findings)

Because of the inconsistency in literature values, a dose response curve for the sarin LD₅₀ in male Sprague-Dawley rats was determined. The LD₅₀ for sarin injected intravenously (iv) was determined to be 130 ug/kg of body weight. Due to concerns about consistency of delivery of the toxin, the injection method was changed from iv to subcutaneous (sc). Injection (sc) of 120 ug/kg produced observable symptoms ranging from mild salivation to severe body tremors, but no death. Our first experiment for chronic low doses of sarin used one-half of this amount (60 ug/kg). Due to differential sensitivity of the animals to sarin, a dose of 30 ug/kg was used in subsequent experiments.

Chronic low-dose studies with sarin There was no change in AChE activity in blood induced by sarin or the noise stress. A clear negative effect on the ability of mitochondria to reoxidize NADH was observed under conditions of sarin and stress. This effect was dependent on the dose of sarin administered. Little or no effect of sarin plus stress was seen in the ABR response. NMR spectroscopy showed little to no effect in endogenous high energy compound metabolic stores in rat brain and muscle (*in vivo*) or in rat brainstem (*in vitro*). Interpretation of the results was colored by animal-to-animal variability in sensitivity to sarin.

Brainstem Functional Study. Chronic low doses of sarin had no statistically significant effect on the ABR of the rats. No changes were observed in threshold, latency, or peak amplitude for animals treated with sarin and noise stress when compared to noise-treated control animals.

NMR Metabolite Studies. *In vivo* NMR experiments showed no change in brain or muscle ATP, PCr, or NAA when comparing sarin + noise stress to vehicle-injected animals treated with noise stress.

Figure 5 shows the metabolite ratios (Mean \pm SE) measured via NMR during the experimental time course. Specifically, this involved measurements at pretreatment, 2 wk, and 4 wk. 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 time course ($p \leq 0.05$). Our *in vivo* NMR results indicate that combined treatment with sarin + noise stress does not produce any deleterious effects on neuronal metabolism. At the completion of the *in vivo* experiments the rats were sacrificed by high-power microwave fixation

and brainstems were removed for chemical extraction and NMR analyses. The *in vitro* NMR experiments showed no change in NAA, PCr, or NTP levels in brainstems of animals treated with sarin and noise stress. Together, the *in vivo* and *in vitro* results suggested that there was no change in metabolism in the brainstem/brain/muscle after chronic toxin exposure (Please see next Page).

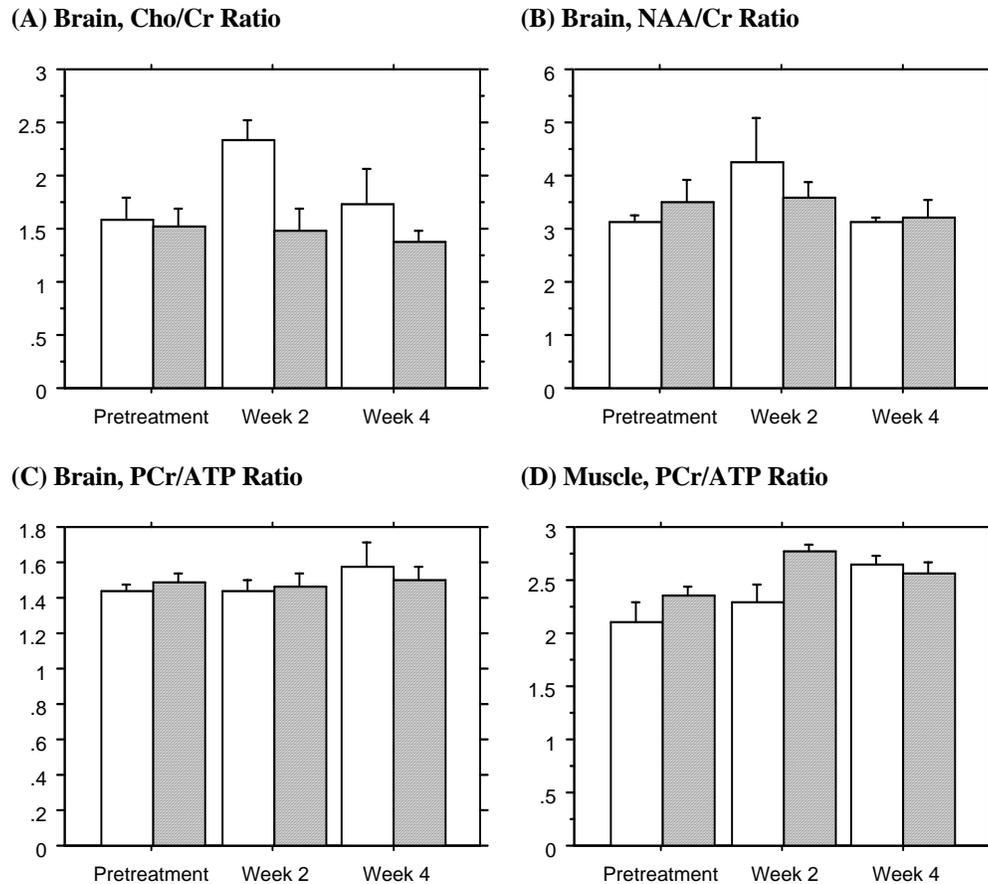


Figure 5, Project 2. NMR measured metabolite ratios (Mean \pm SE) in rat brain and leg muscle *in vivo* for treated (hatched bars) and control (open bars) groups. Rats were treated with sarin + noise stress while controls were given vehicle+noise stress. Treatments were repeated once weekly and measurements were made prior to treatment and at 2 and 4 wks.

Brainstem Energy Metabolism Study. A clear negative effect on the ability of mitochondria to reoxidize NADH was observed under conditions of sarin and stress (Table 6, Project 2). This effect was dependent on the dose of sarin administered. The higher dose of sarin (60 ug/kg) plus stress inhibited succinate-supported mitochondrial respiration, but had no effect on the energy-coupling ratio induced by ADP (Table 7, Project 2). The energy-coupling ratio using NADH-linked substrates was also unaffected, but the NADH oxidase activity exhibited a decrease in specific activity, suggesting a specific site of inhibition of mitochondrial electron transfer between NADH and ubiquinone. This area of the respiratory chain is labile to oxidative damage caused by reactive oxygen species. In addition, the high dose of sarin induced an inhibition of electron transfer activity in cytochrome *c* oxidase (Table 6, Project 2). In mitochondria isolated from animals treated with the lower dose of sarin 30 ug/kg) effects on NADH oxidase activity

were less pronounced. Thus, in the brainstem, high doses of sarin inhibit mitochondria electron transfer activity at multiple sites but do not affect energy metabolism.

Table 6, Project 2. The Effects of High and Low Doses of Sarin 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 with Noise Stress	212 \pm 42	158 \pm 12	84 \pm 10
II. High Dose Sarin-Treated with Noise Stress	156 \pm 15	124 \pm 8	60 \pm 3
III. Low Dose Sarin-Treated with Noise Stress	184 \pm 20	144 \pm 10	72 \pm 6

^aActivity measurements are presented in nmoles oxygen consumed, nmoles cytochrome c reduced, or nmoles NADH oxidized/mg mitochondrial protein \cdot min. Error measurements are standard deviations and an unpaired Student's t-test was used to examine differences between treated and control groups. All data were determined to be significant at the 95% confidence level ($p \leq 0.05$). Each assay was performed at least three times for each preparation.

Table 7, Project 2. The Effects of High (60 μ gm/kg body weight) and Low Doses (30 μ gm/kg body weight) of Sarin and Stress on Rat Brain Stem Mitochondrial Energy Coupling

Treatment	Substrate	Activity (<u>nmoles O₂</u> / (mg protein \cdot min) ^a	Respiratory Control Ratio
I. Control with Noise Stress	Succinate	14 \pm 4	
	+ADP	50 \pm 8	3.3 \pm 0.4
	Pyuvate/malate	7 \pm 2	
	+ADP	33 \pm 3	5.0 \pm 1.0
II. High Dose Sarin-Treated with Noise Stress (30 days after injection)	Succinate	12 \pm 4	
	+ADP	39 \pm 11	3.3 \pm 0.3
	Pyuvate/malate	4	
	+ADP	23	6.7

III. Low Dose Sarin-Treated with Noise Stress (30 days after injection)

Succinate	14 ± 6	
+ADP	48 ± 6	3.4 ± 0.3
Pyuvate/malate	5 ± 1	
+ADP	28 ± 3	6.9 ± 1.3

^aError measurements are standard deviations and an unpaired Student's t-test was used to examine differences between treated and control groups. All data were determined to be significant at the 95% confidence level ($p \leq 0.05$). Each assay was performed at least three times for each preparation.

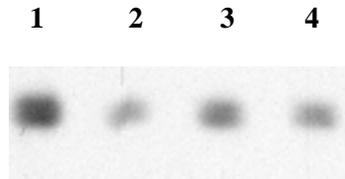
The inhibition of mitochondrial electron transfer and energy coupling data induced by sarin and noise stress could be due to an increase in reactive oxygen species within brain stem cells. This increase in reactive oxygen species could lead to damage to the mitochondrial respiratory chain at its most labile site of oxidative damage in Complex I. Since the doses of chemicals 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 energy metabolism could account for many of the symptoms of the Gulf War Syndrome described.

Apoptotic (programmed cell death) or necrotic (cell death) events could lead to the symptoms exhibited by those with Gulf War Syndrome. Apoptosis can be triggered by many agents, including oxidative stress (hypoxia and ischemia), irradiation by ultraviolet or ionizing radiation, and chemical agents including ethanol, and drugs such as colchicine and cocaine. Although the details of the pathway vary from cell-type to cell-type and from tissue to tissue, there are common properties to apoptotic pathways. The earliest events involve an activation of caspase-8 from its inactive proenzyme form. The caspase 8 then can cleave a BCL₂ like protein, which results in the permeability transition in mitochondria, an event that collapses the mitochondrial membrane potential. The permeability transition causes a rapid depletion of mitochondrial ATP and also causes the mitochondria to lose its ability to maintain vital membrane gradients. The mitochondria then swell changing their ultrastructure, which allows cytochrome *c* to be released from the intermembrane space. Cytochrome *c* then can activate (with caspase-8) caspase 3 which commits the cell to apoptosis. The process is reversible if cellular/mitochondrial ATP is not extensively depleted, but if the ATP content in cells is lowered and the membrane potential is permanently inhibited in the mitochondria by the permeability transition, then apoptosis proceeds.

Since cytochrome *c* is a main player in apoptosis, we measured the cytochrome *c* content of our isolated mitochondria from the brain stems of rats treated with noise stress alone and low doses of sarin and noise stress as detected by immunoblotting using our polyclonal antibodies to cytochrome *c* (Figure 6). Laser densitometric analysis showed that there is less cytochrome *c* content (about a 30% decrease) in mitochondria isolated from animals treated with sarin and noise stress than animals which received noise stress alone. Figure 3 also shows the cytochrome *c* oxidase content of mitochondria isolated from brain stems of animals from the variously treated animals. Cytochrome *c* oxidase content was assessed using a polyclonal antibody to the enzyme and measured by the staining intensity of anti-subunit IV (MW=17,100) in each mitochondrial preparation. There is a decrease (about 50%) in cytochrome *c* oxidase content in mitochondria isolated from animals treated with sarin and noise stress compared to animals treated with noise alone.

Both cytochrome *c* and cytochrome *c* oxidase content of isolated mitochondria from brainstems of rats treated with 30 µg/kg of sarin and noise stress were less than that in mitochondria isolated from animals receiving noise stress alone. These results suggest that cytochrome *c* content of mitochondria from sarin + stress-treated animals was depleted by an unknown mechanism.

A Anti-Cytochrome *c*



B Anti-Subunit IV of Cytochrome *c* Oxidase

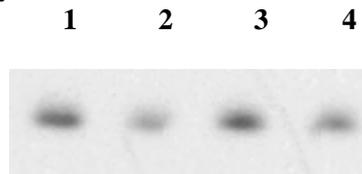


Figure 6, Project 2. The Effects of Low Doses of Sarin with Noise Stress and Noise Stress on the Cytochrome *c* (Panel A) and Cytochrome *c* Oxidase Content (Panel B) of Isolated Rat Brain Stem Mitochondria. Electrophoresis, protein transfer to nitrocellulose, and blot blocking were performed and the blot was developed using polyclonal antibodies against cytochrome *c* (1:1000 dilution) in panel A. Chemiluminescence was used to detect antibody binding development using horseradish peroxidase linked to goat antirabbit IgG. Lanes 1 and 3 contain 2.5 µgm mitochondria from animals treated with noise stress; Lanes 2 and 4 contain 2.5 µgm mitochondria from animals treated with sarin and stress. In Panel B, all conditions were similar to Panel A, except the blot was developed using polyclonal antibodies against beef heart cytochrome *c* oxidase subunit IV (1:2000 dilution). Lanes 1 and 3 contain 2.5 µgm mitochondria from animals treated with noise stress; Lanes 2 and 4 contain 2.5 µgm mitochondria from animals treated with sarin and stress.

To assess the mechanism of the observed decrease in cytochrome *c* and cytochrome *c* oxidase content, the specific activities of enzymes located in the mitochondrial matrix, citrate synthase and malate dehydrogenase, were determined and found to decrease in brainstem mitochondria isolated from animals treated with sarin and stress. The extent of this decrease, however, was not as great as the decrease in cytochrome *c* content of these preparations. Our conclusion is that sarin does not damage all mitochondria in the brainstem or alternatively, different animals have different sensitivities to the toxin.

Oxidative damage to mitochondria was assessed by immunoblotting with antibodies against the peroxyredoxins, a family of enzymes that monitors the oxidative state of the cell. Little or no change of peroxyredoxin 1 (cytosolic location), 2 (mitochondrial location), or 3 (both mitochondrial and cytosolic locations) was found in mitochondria isolated from rats treated with sarin and noise stress compared to those enzymes from controls receiving noise stress alone, suggesting there was little or no change in redox state of the cell after sarin and noise-stress treatment.

To determine if metabolic processes within the cell were modified by sarin treatment, the tyrosine phosphorylation state of mitochondrial proteins was measured by immunoblotting. A greater than 50% decrease in tyrosine phosphorylation of three proteins was observed, one at 170-200 kDa, the

second at 126 kDa, and third at 55 kDa. These proteins have not been identified yet, as work continues on this part of the project.

Statistical analyses

Statistical analyses of the ABR studies were done in consultation with the Statistical Consulting Center at Wright State University. The data were evaluated by repeated measures ANOVA with Peak II thresholds, latencies, and amplitudes as the measures repeated over time. Groups (test and control) and absence or presence of stress were between variables; time was the within variable. 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.

NMR *in vivo* data were analyzed by an appropriate ANOVA (factorial or repeated measures) to identify differences due to treatment or time of measurement post-treatment. An unpaired Student's t-test was used to examine differences between treated and control groups with regard to NMR data from *in vitro* brainstem extracts. All data were determined to be significant at the 95% confidence level ($p \leq 0.05$).

For mitochondrial energy metabolism studies, error measurements were shown as standard deviations and an unpaired Student's t-test was used to examine differences between treated and control groups. All data were determined to be significant at the 95% confidence level ($p \leq 0.05$). All assays were performed at least three times for each preparation.

Project 3. Studies 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. (Gerald M. Alter, Ph.D.)

We hypothesized that Multiple Chemical Sensitivity (MCS) in humans is linked with abnormal activity levels of the enzymes capable of metabolizing those specific chemicals. To test this assertion for formaldehyde (FORM) sensitivity and organophosphate (OP) sensitivity, we examined the activity of four enzymes in the catabolic pathway for these toxicants in both chemically sensitive and normal control populations. The enzyme activities examined which are important for detoxifying and metabolizing formaldehyde and organophosphates include: aldehyde dehydrogenase (ALDH), chi alcohol dehydrogenase (χ ADH), paraoxonase (PON), and aryl esterase (AE). Our anticipation was that the dehydrogenases would be most directly correlated with formaldehyde detoxification, while the esterases would be important in organophosphate hydrolysis.

In a pilot study, we collected blood, hair, and saliva samples from individuals to determine the best, readily accessible tissues in which to monitor the levels of our targeted activities. Preliminary studies indicated that blood was the only suitable tissue for such an analysis. Therefore we have undertaken analysis of these activities blood from normal, formaldehyde sensitive, organophosphate sensitive and multiple-sensitive groups. We resolved blood samples into red cell enriched, white cell enriched, and serum fractions since we anticipated that enzyme activities would be distributed differentially through out these fractions.

Results suggest that sensitivity to formaldehyde and organophosphate correlates well with levels of specific enzyme activities in particular blood fractions. It follows that the activities are good

biomarkers for the two chemical sensitivities examined here. It also follows that these results suggest novel approaches of treating individuals with these maladies.

Materials and Methods.

We collected blood samples from a total of 69 individuals, 20 from a control population and 49 in test populations, using a double blind protocol, which was lifted after biochemical analysis of the blood preparations was complete. Thirty-three individuals in the test population were screened for sensitivity by the Environmental Health Foundation in Dallas Texas, and sixteen individuals were diagnosed with chemical sensitivity at clinics in either Dayton, Ohio, or Buffalo, New York. Most, but not all “normal” individuals were recruited in the area surrounding Dayton, Ohio. No individuals less than 10 or over 72 participated in this study. **It should be noted that no human volunteers were exposed to organophosphate nerve agents as a part of this project.**

A questionnaire completed by each participant and examined after the double blind protocol was lifted, indicated that 9 individuals were sensitive to formaldehyde only, 10 to organophosphate only, and the remainder of these individuals were sensitive to both (as well as other chemical compounds in most instances).

Blood samples withdrawn from each participant were either allowed to clot, and the serum collected, or drawn into heparin tubes and then separated by zonal centrifugation through Ficoll-Paque to collect white and red cells. Preparations were divided into approximately 1.0 ml aliquots, frozen and shipped on dry ice to Wright State University by overnight mail. Blood samples were stored in a -20° freezer until enzyme activities were measured.

Esterase activities (aryl esterase or paraoxonase) were determined by monitoring the release of protons that accompanies the hydrolysis of aryl esterase or paraoxon. Time courses were followed using a pH sensitive indicator dye (phenol red) to monitor small pH changes spectrophotometrically. Owing to the complexity of the reactions mixtures involving cell lysates, fluorescence-based detections systems proved unreliable. Specific assay conditions for proton-based assays were: 2 mM HEPES (pH 8.0), 2 mM CaCl₂, phenol red, 10 uM and about 10 µl of blood preparation in a 1 ml assay. When paraoxonase activity was measured, the assay solution was made 1.2 mM in paraoxon, and 2.63 M in NaCl. When aryl esterase was measured, the substrate, phenyl acetate was made 3.5 mM in the assay cuvette. In both assays absorbance changes were measured at 557 nm in a Cary 30 Spectrophotometer.

Substrate stock solutions were made as follows: Phenylacetate was diluted to 35 mM in ethanol. Typically 1 ml assays were started with the addition of 100 ul of this stock. In the case of paraoxon, 4.6M (pure) paraoxon was diluted with ethanol to a final concentration of 120 mM. Addition of blood component was used to initiate the paraoxonase assays.

Assays for γ ADH, and aldehyde dehydrogenase were routinely performed as follows: For ALDH, the assay conditions were 50 mM TRIS (pH 8.0), 3 mM PBazole, 1 mM K₃Fe(CN)₆, 5 mM NaCN and 10 µl of blood preparation in a 1 ml assay. Substrate concentrations were routinely 1.25 mM NAD and 0.8 mM formaldehyde. For γ ADH, normal assay conditions were 50 mM TRIS, (pH 8.0), 3.0 mM PBazole, and about 20 µl of blood product in a 1ml assay. Substrate concentrations normally were: 1.25 mM NAD, 0.8 mM GSH, and 0.8 mM formaldehyde. Production of NADH during both these assays was measured spectrophotometrically at 350 nm. The PBazole was added to inhibit alcohol dehydrogenase and the ferricyanide was added to convert hemoglobin to its cyanomet form. In the latter state, the spectrum of the HbCN is insensitive to variations in solution conditions.

White and red cell preparations were thawed, lysed hypotonically, and centrifuged to remove cell debris prior to being added to the reaction mixtures. Activity values are total activities in 2 ml of the original blood sample. They are expressed as μ moles of substrate consumed per minute.

Experimental Design

Blood samples from each participant were fractionated into a red-cell enriched fraction, indicated by “r”, a white blood cell fraction, indicated by “w”, and a serum fraction indicated by “s”. The 4 activities mentioned above (aldehyde dehydrogenase or ALD, chi alcohol dehydrogenase or χ ADH, paraoxonase or PON, and aryl esterase or AE) were determined for each fraction of each individual’s blood.

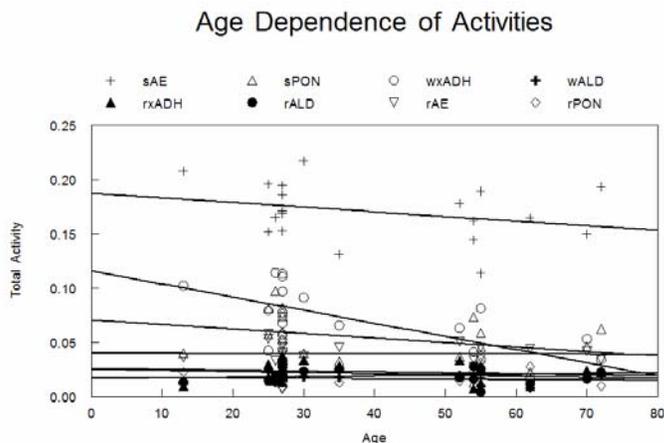
Each activity from each fraction was examined to detect correlations between a particular activity and the clinical evaluation of the patient or the control population. Logistic regression and discriminate analysis were used to search for activities that distinguished control from chemically sensitive individuals.

Results and Discussion

Results of our analyses are summarized in the following figures. Preparations and type of enzyme activity reported are labeled using the abbreviations mentioned in the preceding “experimental design” paragraph.

Figure 1 shows the age dependence of activities. Though activities were generally very reproducible for each patient within about 5%, variability among individuals was large. Each data point represents the appropriate activity of an individual. Lines in Figure 1 are linear regression lines. The largest slope is for χ ADH activity measured in white cell fractions. This suggests that activity may decrease with age. However, the decrease does not appear greater than the scatter in our data. We conclude that there is no statistically significant age-dependence of activities shown in our data.

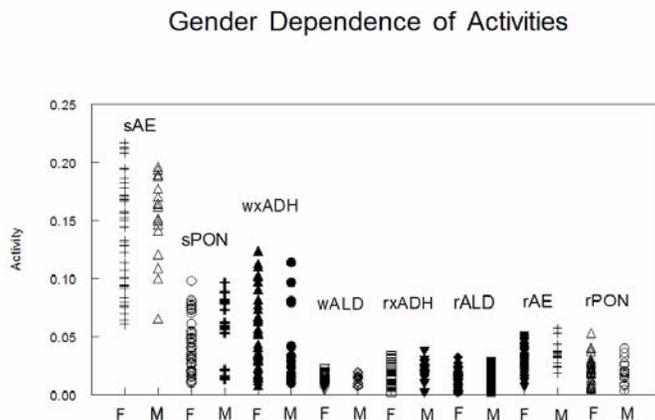
Figure 1, Project 3



Similarly, comparisons of activity values for various activities and blood fractions between female (F) and male (M) participant indicate there is no significant gender-dependence of activities

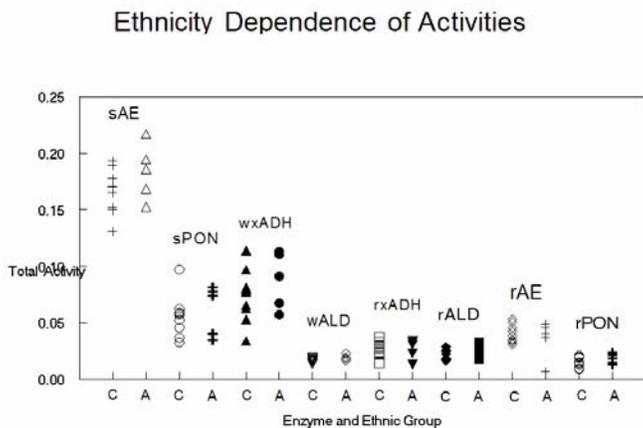
reported here (Figure 2). As in Figure 1, each point represents the activity characteristic of the appropriate blood fraction of a participant.

Figure 2, Project 3



Finally, results in Figure 3 indicate that there is no significant ethnic-dependence of activity values, for the only two ethnic groups involved in this study (C, Caucasian, and A, Asian Indian). Once again, points correspond to the appropriate activities characteristic of the indicated group.

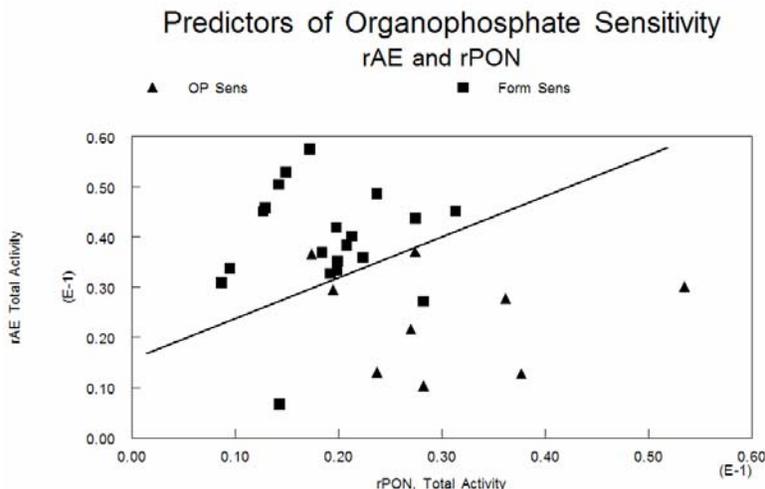
Figure 3, Project 3



Using logistic regression and discriminate analysis we determined the correlation of pairs of activity measurements with chemical sensitivity. Previous reports indicated that PON and AE activities correlate with severity of gulf war syndrome (GWS) symptoms. Using this pair of activities we were able to discriminate OP sensitive from control group individuals (Figure 4). However the statistic significance of the classification on this basis was only marginally statistically significant. In Figure 4 and the following plots, each point represents the values of a pair of enzymatic activities measured in an individual's blood. Those from control group individuals are indicated by squares and those from the OP sensitive (only) group are indicated by triangles. The line represents activities, based on discriminate analysis, that have an equal probability of belonging to normal; and

chemically sensitive individuals. The farther above the line, the greater the probability that the activities come from control group individuals, and the further below the line, the greater the probability that the activities come from organophosphate individuals.

Table 4, Project 3



Statistical parameters describing the statistical significance of the classification are summarized in Table 1. The statistical significance of this classification is marginal, with p values for rPON being greater than 0.05. Further the robustness of this activity pair or distinguishing organophosphate sensitive individuals and control individuals was not very good. When individuals that were multiply sensitive (including OP sensitive), the statistical significance of classification based on these activities decreased substantially.

Table 1, Project 3

Figure (type of Sensitivity)	Activity Pair	P Value	Group (Size)	% Correctly Assigned	Statistical Sensitivity	Statistical Specificity
4 (OP)	rAE rPON	0.04 0.151	Control (21) OP Sens (9)	80	67	91
5 (OP)	rAE wALD	0.06 0.08	Control (21) OP Sens (9)	83	56	95
5 (OP, Mixed)	rAE wALD	0.005 0.0115	Control (21) OP sens + Mix (29)	82	86	76
6 (FORM)	rPON wALD	0.048 0.0105	Control (21) Form Sens (10)	90	81	90

OP Sens: Organophosphate (only) sensitive group

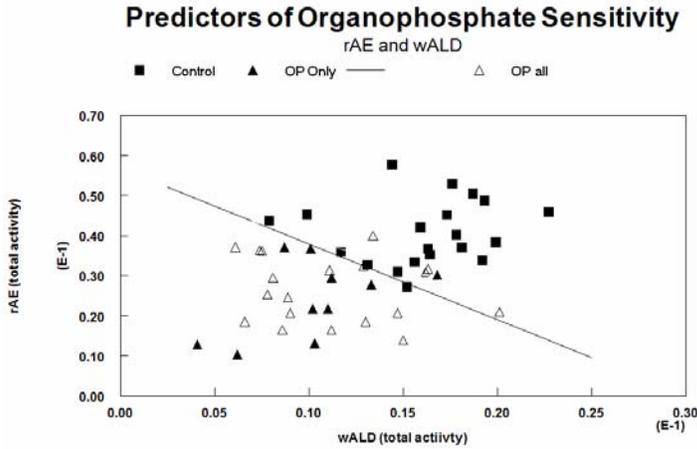
Mix: Multiple chemical sensitive group, including organophosphate sensitive

FORM: Formaldehyde (only) sensitive group

The pair of activities, rAE and wADH were much better for discriminating between OP sensitive and control individuals (Figure 5). Whether OP sensitive only (filled triangles) or multiply sensitive (including OP sensitivity) groups were considered, it was possible to discriminate between sensitive and normal individuals using this activity pair. The statistical significance of the distinction is marginal when the OP (only) sensitive group was used, but became significant when the large,

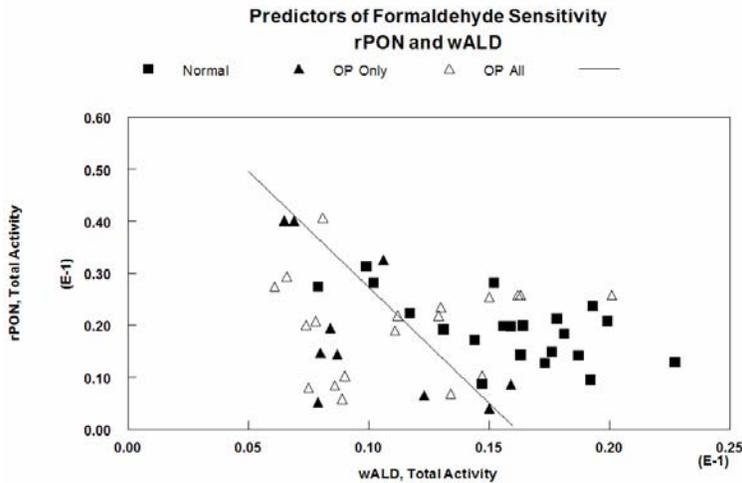
multiply chemically sensitive group was considered (Table 1). It is likely that the marginal statistical significance characterizing the OP (only) is a result of the small size of this test group. Statistical significance is achieved when a larger (multiply chemically sensitive) group is compared with the normal group.

Figure 5, Project 3



Statistically significant segregation of formaldehyde (FORM) sensitive and control groups was achieved using the activity pair, rPON and wALD (Figure 6, Table 1). The greatest statistical significance occurred when comparing FORM sensitive (only) with control groups (Table 1). P values for the comparison rise slightly when mixed sensitive (including FORM sensitive) and control group activities are compared (statistical parameters not shown).

Figure 6, Project 3



It is likely that the statistical significance of comparisons of between control and test groups can be significantly increased by increasing the number of participants. We are currently attempting to do this with funding from an alternative source.

Conclusions:

Based on results of this study, we conclude that the blood fraction activities monitored in this study:

- Have little or no dependence upon the age of the individual.
- Have little or no gender dependence of the individual.
- Have little or no ethnic dependence (between Caucasians and Asian Indians).
- Are objective biochemical markers for organophosphate sensitivity and formaldehyde sensitivity.

These results indicate, for the first time, a novel link between the phenotypic expression of chemical sensitivity and the biochemical markers of enzyme activity in blood that can be quantitatively measured. This information may be clinically useful to help identify or confirm a diagnosis of chemical sensitivity and suggest novel approaches in managing this malady.

Project 4: Studies of gene expression using a DNA microarray system to test the effect of chemicals (PB and sarin) on a neuronal cell line (Steven J. Berberich, Ph.D.)

This project contained 4 tasks designed to determine whether PB and sarin either separately or together altered gene expression profiles of human neuronal cells. As a part of this project, we also established the Genomic Expression Laboratory to use the Affymetrix system for measurement of gene expression patterns. Two cell culture systems were employed to generate differentiated neuronal cells for treatment. Although several gene alterations were observed, none of the patterns of expression suggested that PB or sarin had any significant impact on cultured neurons. A unique gene signature pattern was observed as HCN-2 cells underwent differentiation.

4.1 Experiments were performed to optimize the conditions for neuronal cell growth and differentiation to use as a test system for chemical exposure.

Two human neuronal cell lines, HCN-2 and SHSY5Y, were employed for studies outlined in tasks 4.2-4.4. In this task we optimized the neuronal differentiation of each cell line. The addition of SHSY5Y cells to this study was the result of two outcomes. First, there were only a few reproducible gene expression changes observed with differentiated HCN-2 cortical neurons following 24 hours of PB. Second, because of the delay in obtaining approval for working with sarin, we had additional time to examine PB effects and thus moved to another human neuronal cell line. One noteworthy outcome from this task is a dataset of gene expression alterations seen when HCN-2 cells undergo differentiation. To date, this represents the first gene expression examination of the differentiation signaling pathway in HCN-2 cells.

HCN-1 cell differentiation

Towards determining the optimal differentiation conditions, HCN-2 cells were initially plated at a high (1×10^6 / 60 cm dish) or low (5×10^5 / 60 cm dish) density in DMEM with 10% fetal bovine serum (FBS) for two days prior to differentiation (Day -2). On Day 0, Differentiation plates (DIFF) were refed with DMEM with 10% FBS, 4.5 g/L glucose, 25

ng/mL nerve growth factor, 0.5 mM IBMX and 20 nM TPA while control plates received media minus NGF, IBMX and TPA. Media was replaced following the timeline shown in Figure 4.1. Figure 4.2 represents a time course of HCN-2 differentiation over 6 days. Pictures of the cells were taken at 0, 4 and 6 days post-differentiation. Based on trypan blue exclusion assay (data not shown), the increase in floating cells seen in the DIFF plates at Day 6 does not represent dead cells but rather neuronal cells that have lost adherence to the tissue culture plate.

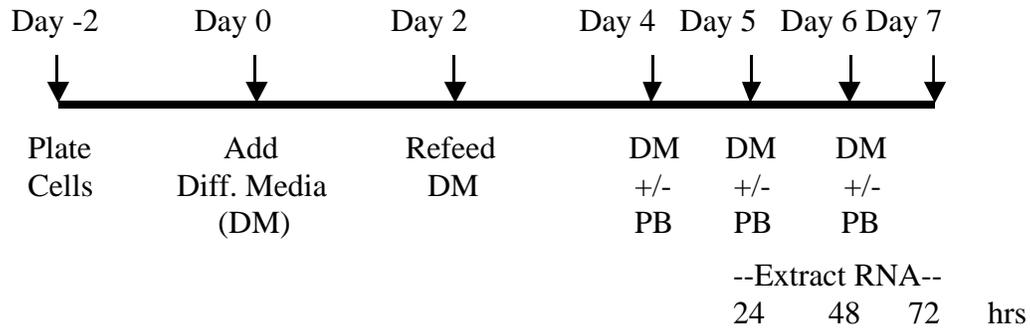


Figure 4.1 Differentiation Timeline for HCN-2 cells.

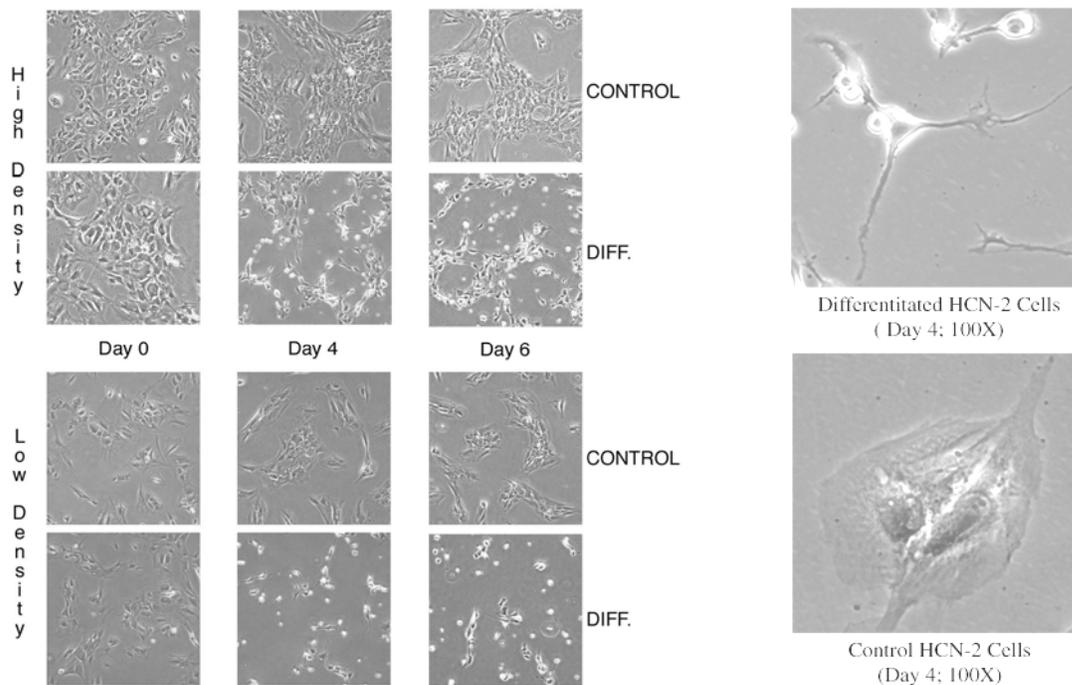


Figure 4.2 Differentiations of HCN-2 cells

To further demonstrate the phenotypic changes associated with HCN-2 differentiation (other than morphology), we generated gene expression profiles of differentiated and undifferentiated HCN-2 cells. Four independent experiments examining the gene expression changes between undifferentiated HCN-2 cells compared to differentiated

HCN-2 cells at day four of the differentiation protocol were performed, with each condition tested in triplicate. Taking the four separate experiments (diff. vs. undiff.) we performed a series of datamining schemes to identify significant alterations in gene expression. The criteria used for this data set were the following:

1. Changes in relative expression of genes ≥ 2 -fold or ≤ -2 -fold (diff. vs. non-diff) AND difference calls of Increased or Decreased.
2. Changes in relative expression of genes (diff. vs. non-diff) that showed a significant P value ≤ 0.05 using the Mann-Whitney test.
3. Clustering relative expressions of genes using Self Organizing Maps (SOM).
4. Compiling the list of common genes found in all methods (1, 2 and 3).

One hundred ninety-six genes from the U95Av2 GeneChips (n=12,656) met the criteria listed above. To assess the cellular networks altered by these gene inductions and repressions the fold changes for the 196 genes were examined using the Ingenuity's Pathway Knowledge Database. The most significant pathway contained 35 genes relating to cell-to-cell signaling/tissue development and cell proliferation. Most evident in this network is the induction of the TGFbeta pathway (Figure 4.3).

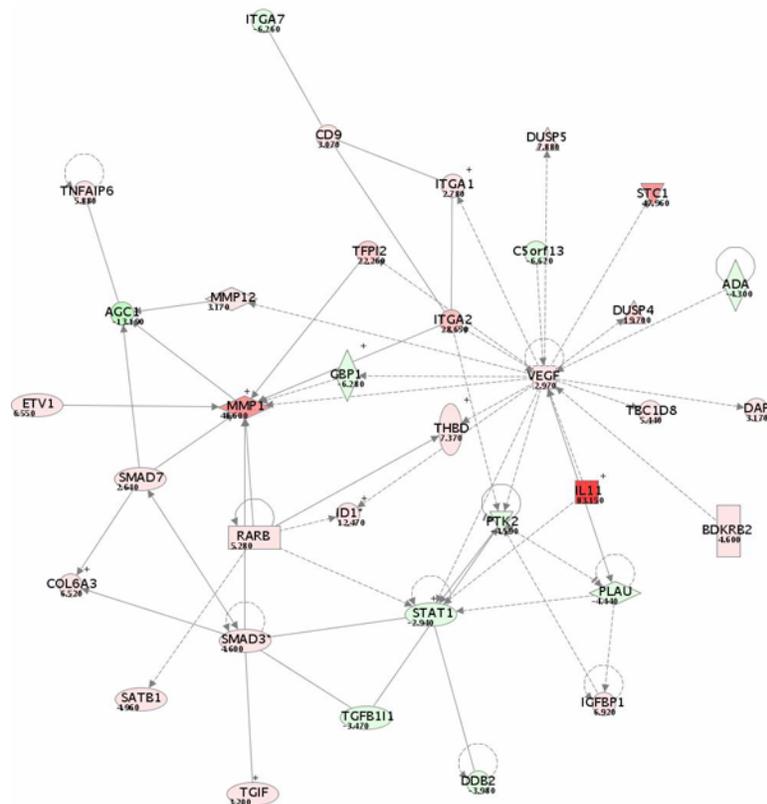


Figure 4.3: HCN-2 differentiation relate to gene network involving cell to cell signaling/tissue development/cell proliferation. Genes in network that were altered during HCN2 differentiation are colored. Shades of red represent increases in HCN2 differentiated cells relative to undifferentiated HCN2 cells. Green shaded genes are repressed in HCN2 differentiated cells. The number associated with these genes are the average fold change (log base 2).

SHSY5Y neuronal differentiation

During year two we tested methods to differentiate SHSY5Y cells, a human neuroblastoma cell line that has the capacity to differentiate into neuronal like cells (Encinas et. al., 2000). Briefly, SHSY5Y neuroblastoma cells were grown in F9/Eagle's media containing 10% fetal bovine serum. Differentiation was initiated by exposing the SHSY5Y cells to media containing to 10 μ M all trans retinoic acid for 5 days. On day 6, the cells were treated with brain derived neurotrophic factor in serum free media. Under these conditions, we were able to obtain a homogenous population of cells with neuronal morphology (Figure 4.4).

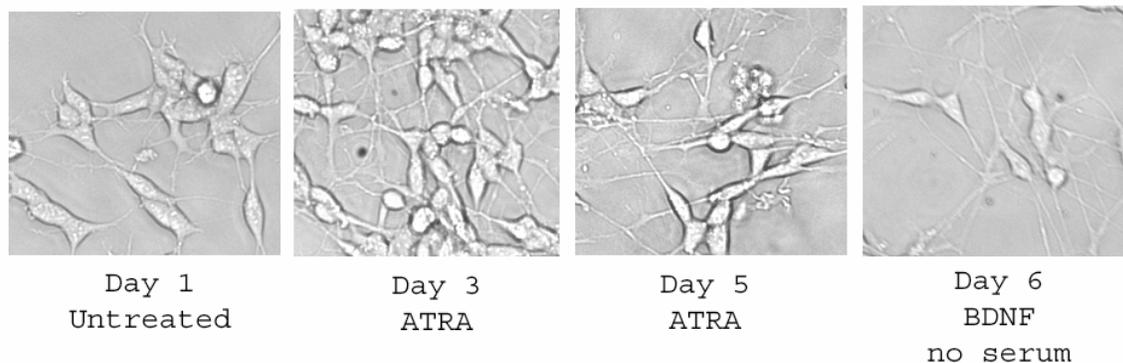


Figure 4.4 Morphological changes following differentiation of SHSY5Y cells treated for 5 days with retinoic acid followed by addition of BDNF in the absence of serum.

4.2 Characterized the changes in neuronal gene expression in response to PB treatment.

For both neuronal cell lines we observed no overt loss of cell viability when the cells were exposed to PB doses ranging from 10^{-8} M to 10^{-3} M for up to 48 hours. However, there were difficulties in maintaining the differentiated cells, independent of PB treatment, for periods of time greater than 72 hours thus the gene expression datasets focused on 24 and 48 hours post treatment. There were no significant alterations in gene expression seen with HCN-2 cells at 24 hours post treatment and only a few reproducible gene alterations reported after 48 hours. We conclude from these results that cultured cortical neurons lacking AChE receptors do not show any significant or detrimental gene expression changes within 2 days post PB treatment. These findings were presented at two toxicological meetings in 2002. Next, we examined the effect of PB exposure on differentiated SHSY5Y neurons. While more gene expression changes were detected at the 10^{-3} M and 10^{-4} M PB dose at 24 and 48 hours, there were only a few genes that were seen to remain altered at both time points (Table 1).

Table 1: Genes with the same pattern of gene expression after 24 and 48 hours of 10^{-3} M PB treatment of SHSY5Y differentiated neurons.

GeneID	24 hr Fold Change	48 hr Fold Change	Description
1369_s_at	-1.42	-1.73	Interleukin 8
32818_at	-1.39	-2.53	hexabrachion (tenascin C, cytotactin)
35879_at	-1.3	-0.94	Pro-galanin
38125_at	-1.36	-2.1	Beta-migrating plasminogen activator inhibitor 1
38772_at	-1.28	-1.89	CYR61
39066_at	1.36	1.67	Microfibril-assoc. glycoprotein 4

Next, to assess if there were signaling pathways impacted by PB, we examined the 274 genes altered following 48 hours of 10^{-3} M PB treatment of differentiated SHSY5Y cells. However only 19 of these genes were altered across all three doses of 48 hour PB exposure (10^{-3} M, 10^{-4} M and 10^{-8} M). Those genes are listed in Tables 2 and 3. Taken together, we believe that PB does not show significant affects on either differentiated cell line.

Table 2: List of Genes that are repressed at all doses of PB (48 hours).

10-3M Fold Change	10-4M Fold Change	10-8M Fold Change	Description
-1.19	-1.21	-1.35	Nucleosome assembly protein 1-like 1
-1.55	-1.36	-1.34	collagen, type XVI, alpha 1
-1.36	-1.55	-1.78	EGF-like-domain, multiple 4
-1.24	-1.12	-1.18	Isocitrate dehydrogenase 3 (NAD+) beta
-1.22	-1.25	-1.24	ELAV (embryonic lethal, abnormal vision, Drosophila)-like (Hu antigen D)

Table 3: List of Genes that are induced at all doses of PB (48 hours).

10-3M Fold Change	10-4M Fold Change	10-8M Fold Change	Description
1.6	1.38	1.74	Pax8
1.45	1.36	1.6	synapsin I (SYN1)
2.31	2.25	3.03	UDP glycosyltransferase 2 family, polypeptide B15
1.83	1.6	1.65	serine protease inhibitor, Kunitz type 1
3.32	2.5	3.23	AL050065:Homo sapiens mRNA; cDNA UNKNOWN
1.75	1.91	2.43	Homo sapiens cDNA, 5' end /clone=IMAGE-588365
1.54	1.37	1.71	Homo sapiens mRNA for KIAA0514 protein
1.77	1.62	1.82	Homo sapiens mRNA for KIAA0841 protein
1.56	1.43	1.6	p126 (ST5)' suppressor of tumorigenicity
1.66	1.46	1.54	phorbolin-like protein MDS019
1.64	1.47	1.65	beta-adrenergic receptor kinase (ADRBK1)
1.71	1.51	1.73	AD024 protein
1.69	1.56	1.77	Homo sapiens cDNA: FLJ23482 fis, clone KAIA03142
1.53	1.32	1.68	myosin-binding protein C, slow-type

4.3 Characterized the changes in neuronal gene expression in response to sarin.

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% CO₂ 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 4-5, 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.

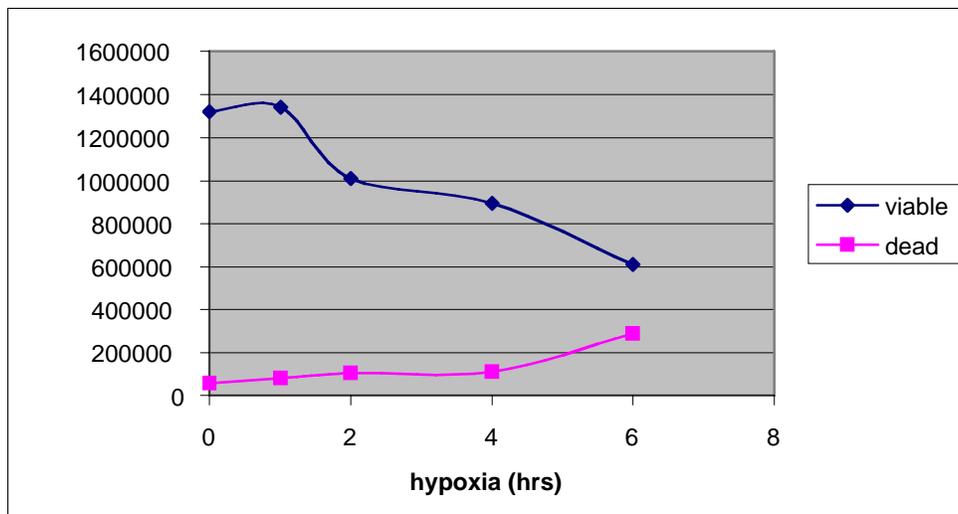


Figure 4.5 Mock Sarin exposure experiment. Day 7, Differentiated SH-SY5Y cells were refed with Differentiation media containing 15 mM HEPES, placed in a sealed container and placed at 37°C. At the indicated time point the plates were removed, cells trypsinized and placed in a trypan blue containing media. 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.

The experiments employing sarin involved treated the differentiated SHSY5Y cells with from 0.19-19 µg/mL of sarin for 1-2 hours using the approach outlined above. Unlike our studies with PB, data analyses using fold change and the Mann-Whitney test resulted in 0-5 genes being altered. Again there was no overlap between doses or time frames of treatment. Based on these findings we conclude that at the doses and times employed in this study sarin has no significant impact on the gene expression of differentiated SHSY5Y cells. A summary of the number of sarin exposure treatments employed from which these conclusions are drawn is below (Table 4).

Table 4: Sarin Treatments

Experiments 3, 4	n/group
0.19_v_0 µg sarin	7
Experiments 1,3,4	n/group
1.9_v_0 µg sarin	11
Experiments 1,3	n/group
19_v_0 µg sarin	7

4.4 Characterized the changes in neuronal gene expression in response to the combined treatment with PB and sarin.

Finally, we employed combination studies of PB and sarin to assess if sarin impacted PB gene alterations. We chose 48 hours at 10⁻⁴M and the low/mid doses of sarin since separately neither of these treatments elicited significant gene alterations. With additional time it would have been interesting to examine the higher dose of PB where gene changes

were detected to assess if sarin impacted these changes. In any event the results demonstrated no significant gene alterations in differentiated SHSY5Y following 48 hours PB treatment (10^{-4} M) followed by 1 hour of exposure to sarin (1.9 μ g/ml). The number of plated treated under each condition are listed below (Table 5).

Table 5: Dual Treatment Experiments

Treatment	Exp. 1	Exp. 2
PB & 1.9 sarin_v_con	4 plates	4 plates
PB & 0.19 sarin_v_con	4 plates	4 plates

KEY RESEARCH ACCOMPLISHMENTS

Project 1:

- Characterized the mouse model for simultaneous exposure to stress and AChE inhibitors with respect to changes in blood and brain AChE activity, behavior, cardiovascular and autonomic neural function.
- Determined that exposure to PB at a dose sufficient to produce substantial inhibition of blood AChE did not produce apoptosis in mouse brain as previously reported.
- Demonstrated that exposure to PB at a dose sufficient to produce substantial inhibition of blood AChE produces few changes in muscarinic receptor densities in brain. In contrast, exposure to stress or PHY produced changes in muscarinic receptor densities that are subtype specific.
- Characterized the effects of low-level sarin exposure on muscarinic receptor density and expression in mouse brain. Results showed regional and subtype specific effects.
- Characterized the effects of low-level sarin exposure on muscarinic receptor density and expression in mouse heart atria. Results showed acute, but not chronic effects of sarin exposure on the heart.
- Modified the sarin exposure protocol to enhance the central effects on AChE activity. We coupled sarin with pretreatment with a specific carboxylesterase inhibitor, 2-(O-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide (CBDP). Results showed an enhancement of central sarin toxicity.
- Three different AChE inhibitors decreased locomotor activity, PB, PHY and sarin. Stress decreased the locomotor activity effect of PB, but not that of sarin
- The three AChE inhibitors had different effects on the response to acoustic startle, suggesting this as an appropriate measure for non-AChE inhibitor actions.
- The combination of stress and sarin produced a delayed onset adrenal hyperplasia and reduced catecholamine content. A minority of mice exhibited a delayed onset self mutilation following the combination of sarin and stress. This may be important in the delayed onset of some symptoms associate with the Gulf War Syndrome.

- Two doses of low level sarin reduced dopamine use in the amygdala and its predicted consequence, the inability to learn fear potentiation of the startle response. This measure is unusually sensitive to low level exposures.
- PB is capable of crossing the blood brain barrier to inhibit AChE activity in specific brain regions, particularly the hypothalamus. Proteomic analysis revealed that PB had effects on peptide expression in hypothalamus.
- In mice, peripheral carboxylesterase enzymes act as a sink to prevent sarin inhibition of brain and neuroendocrine function, i.e., hypothalamic AChE activity.
- CBDP, a carboxylesterase inhibitor acts to protect peripheral sarin metabolism, allowing sarin greater access to brain. This results in inhibition of hypothalamic and pituitary AChE activity over extended periods of time
- Developed model for chronic cardiovascular monitoring in mice using chronic direct and telemetric catheters.
- Determined the effect of long term shaking stress on cardiovascular and endocrine parameters in mice. Results showed that there were consistent increases in blood pressure and heart rate associated with shaker stress. There was also evidence of a day/night rhythm in responsiveness. There was also an accommodation of the adrenal corticosterone response with time.
- Determined the effect of chronic treatment with PB on cardiovascular parameters. There were no long term changes noted even though there was significant inhibition of AChE.
- Determined the effect of acute and chronic stress on cardiovascular parameters including testing of autonomic balance.
- Determined the short and long term cardiovascular effects of sarin exposure, 1 day to 90 days. Results showed that there were no changes in blood pressure or heart rate although there were dramatic alterations in heart rate variability.
- Determined the short and long term effects of sarin on central catecholaminergic systems.
- Determined the cardiovascular effects of stress in an animal model of altered responsiveness, mice lacking the ability to produce the peptide stress hormone, oxytocin.
- Treatment with a low dose of sarin (8 µg/kg, 0.05 LD50) produced changes in brain gene expression in the absence of any effects on AChE activity. This is important since it may explain some of the reasons for the delayed sarin toxicity seen in humans.

Project 2:

- Blood chemistry evaluations validated the noise stress model. Serum corticosterone (Cort) was elevated twelve fold after noise exposure.
- Chronic low-dose exposure (225 mg/kg, intraperitoneal injection) to DEET plus noise stress had little no effect on the Acoustic Brain Response (indicator of brain function), brain high energy compound metabolism measured by Nuclear Magnetic Resonance Spectroscopy (NMR), and mitochondria brainstem energy coupling (respiratory control) and electron transfer

activities of isolated brain stem mitochondria. No changes in cytochrome c (as a marker for apoptosis) and cytochrome c oxidase content of isolated brainstem mitochondria (using immunoblotting) detected in treated animals.

- Chronic low-dose exposure (5mg/kg by gavage) to PB plus noise stress showed no statistically significant differences between ABRs of control animals and animals treated with noise stress. Little or no biochemical changes were observed in rat brainstem. Noise stress, however, caused a decrease in NADH-supported mitochondrial electron activity.
- Acute (24 hour post-injection) effects of PB plus noise stress had little or no effect on the ABR, NMR of high energy metabolites, or mitochondrial energy metabolism or coupling ability of isolated brainstem mitochondria from treated animals.
- Synergistic effects of chronic low-dose exposure to DEET + PB + noise stress showed that there was no change in blood acetylcholine esterase activity (AChE) induced by DEET + PB or the noise stress. Few changes were observed in rat brainstem after combined administration of DEET (225 mg/kg, ip) + PB (5 mg/kg, gavage) + noise stress. However, different animals exhibited different sensitivities to the toxins suggesting the possibility that endogenous chemical sensitivity may be an animal-dependent phenomenon.
- Completed dose response study for sarin comparing subcutaneous (sc) and intravenous (iv) methods of chemical administration and determined LD50 for iv injection in rats.
- Chronic low-dose exposure to sarin plus noise stress did not cause a change in AChE activity in blood. A clear negative effect on the ability of mitochondria to reoxidize NADH was observed under conditions of sarin and stress. This effect was dependent on the dose of sarin administered. Little or no effect of sarin plus stress was seen in the ABR response. NMR spectroscopy showed little to no effect in endogenous high energy compound metabolic stores in rat brain and muscle (in vivo) or in rat brainstem (in vitro). Changes in the phosphorylation state of unknown mitochondrial proteins were observed. Interpretation of the results was colored by animal-to-animal variability in sensitivity to sarin.

Project 3:

- Set up methods for assay in blood of enzymes important in the metabolism of formaldehyde and organophosphate toxins, aldehyde dehydrogenase, chi alcohol dehydrogenase, paraoxonase, and aryl esterase, and used them to study enzyme activities in blood from individuals with multiple chemical sensitivity.
- There was no relationship between enzyme activities (cited above) and age, gender or ethnic groups (Caucasians and Asian Indians).
- There was a relationship between chemical sensitivity (organophosphate and formaldehyde) and blood enzyme activity (aldehyde dehydrogenase, chi alcohol dehydrogenase, paraoxonase, and aryl esterase). The results indicate that these may be used as objective biochemical markers.

Project 4:

- Established the Genomic Expression Laboratory to use for genetic expression experiments
- Set up and tested two neuronal culture systems for testing the effect of PB and sarin. Results showed that treatment with AChE inhibitors did not alter the pattern of genetic expression.

REPORTABLE OUTCOMES

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CONCLUSIONS:

The outcomes and conclusions are provided in each of the four sections. However, in terms of a global assessment, the research program, "Low level chemical toxicity: relevance to chemical agent defense," provided important new paradigms of study and methods as well as new results on the effects of low level exposure. The problem was addressed from the level of the cultured cell to the human subject. Project 1 demonstrated that treatment in mice with AChE inhibitors (sarin or PB) in conjunction with stress produced changes in central nervous system gene expression, changes in cardiovascular/autonomic function and changes in muscarinic receptor function and changes in behavior. A key finding was that a dose of sarin which produced no change in blood ChE, caused dramatic changes as seen by autonomic reflexes and brain gene and protein expression. These changes were most apparent long term. The data suggests that we must not overlook the importance of low level chemical agent exposure in humans. Experiments using chronic PB exposure showed that there was entry into specific brain regions which elicited effects on enzyme activity, protein expression and autonomic function with PB. Behavioral studies uncovered the most sensitive deficit following sub-clinical sarin to date, loss of learning the fear-potential of startle. This occurred via prevention of dopamine transmission in the amygdala, which could represent the starting point for a long-term cascade of neuronal events that lead to subsequent neurological deficits. There was also a delayed onset hyperplasia of the adrenal glands following the same sarin treatment. Finally, a test of carboxylesterase inhibition showed that pretreatment with this agent enhanced the brain response to sarin. It will be important to repeat our studies and to combine them with tests of countermeasures.

The clinical studies which were a part of Project 3 examined enzyme activities (dehydrogenases and esterases) in a population of chemically sensitive individuals. The enzymes measured are related to the metabolism of formaldehyde and organophosphates. The studies were able to statistically separate the controls from the chemically sensitive. The results are important because they suggest that one might predict using blood tests the nature and the level of chemical sensitivity. Further studies are needed with larger groups and perhaps tests of other chemical alterations.

To determine the direct neural effects of AChEI, project 4 used a tissue culture system with measurement of gene expression using the Affymetrix system. The results of this project were largely negative. Neither PB or sarin at a variety of doses caused reproducible changes in gene expression. Since these experiments used neural cell lines (HCN1 and SHSY5Y), one question is whether these were the most appropriate for testing.

Project 2 used rats to study the effect of stress, DEET, PB, sarin and the combined treatments on neural responses (auditory brainstem response), brain high energy metabolites (NMR spectroscopy) and brain mitochondrial energy metabolism (biochemical assay). The goal was to compare the various outcomes to produce a unifying theory for the chemical/stress effects. The initial studies of DEET, PB and stress showed that there were no changes in brainstem reflexes or metabolism. For sarin, this was also the case for auditory reflexes and energy metabolites. However, there were some intriguing findings as related to electron transfer NADH and ubiquinone, a decrease in activity. This is the area of the respiratory chain which is sensitive to oxidative damage and may have important clinical relevance.

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ABBREVIATIONS

A – Asian Indian
ABR – Acoustic Brain Response
AChE – acetylcholine-esterase
AChEI – acetylcholine-esterase inhibitor
ADH – alcohol dehydrogenase
ADP – Adenosine diphosphate
AE – aryl esterase
ALDH – aldehyde dehydrogenase
ANOVA – analysis of variance
ATP – adenosine triphosphate
C – Caucasian
CBDP – carboxylesterase inhibitor, 2-(O-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide
CHO – c holine
cm – centimeter
Cort – corticosterone
Cr – creatine
Cyt. C – cytochrome c
DEET – N, N' Dimethyl-m-toluamide
DIFF – differentiated plates
DM – differentiation media
DMEM – Dubecco's modified Eagle's media
FBS – fetal bovine serum
FORM – formaldehyde
FPS – fear potentiation of acoustic startle response
g – gram
GWS – Gulf War Syndrome
IBMX – isobutyl-methyl-xanthine
ip – intraperitoneal injection
iv – intravenous injection
L – liter
M – molar
MCS – multiple chemical sensitivity
mL – milliliter
mM – millimolar
MPTP – 1-methyl-4-phenyl-1,2,5,6-tetrahydroPBidine
Myo-Ins – myo-inositol
NAA – N-acetylaspartate
ng – nanogram
NGF – nerve growth factor
nM – nanomolar
NMR – Nuclear Magnetic Resonance
NTP – total nucleotide triphosphates
OP – organophosphate
OTKO – oxytocin knockout
PB – pyridostigmine bromide
PCr – phosphocreatine
PCR – polymerase chain reaction
PHY – physostigmine
Pi – inorganic phosphate

ABBREVIATIONS (continued)

PON – paraoxonase

RNA – ribonucleic acid

S/N – signal to noise ratio

sc – subcutaneous injection

SE – standard error

SOM – self-organizing maps

SYN1 – synapsin I

TGFbeta – transforming growth factor beta

TPA – tissue plasminogen activator

UQ – ubiquinone

USAMRMC – U.S. Army Research and Material Command

Appendix

Effect of chronic pyridostigmine bromide treatment on cardiovascular and behavioral parameters in mice

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Abstract

Experiments were performed to determine the effect of chronic low-dose pyridostigmine bromide (PB) treatment on blood acetylcholinesterase (AChE), cardiovascular (CV) function, and behavior in C57BL/6J male mice. Chronic carotid arterial catheters were used for long-term CV measurements and for collection of blood samples. Separate groups of mice were used for behavioral open field tests. PB was administered subcutaneously using osmotic minipumps at 1 and 3 mg/kg/day for 7 days. Blood pressure and heart rate (HR) were measured continuously for 24 h before treatment and on Days 3 and 7 after minipump insertion. Blood samples were collected on the same days. 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 in either dark or light period. Basal AChE activity was 0.42 ± 0.1 $\mu\text{mol}/\text{min}/\text{ml}$, 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.

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Keywords: Blood pressure; Heart rate; Open field; Acetylcholinesterase; Gulf War Syndrome

1. Introduction

Pyridostigmine bromide (PB) is a quaternary ammonium compound that inhibits the hydrolysis of acetylcholine (ACh) by reversibly binding to acetylcholinesterase (AChE). PB is used clinically in the treatment of myasthenia gravis (Breyer-Pfaff et al., 1985). It has also been used as a prophylactic agent against nerve gas exposure, particularly during the Persian Gulf War (Sapolsky, 1998). The scientific rationale for this treatment is that PB competitively blocks the binding of irreversible organophosphate AChE inhibitors, such as soman (Blick et al., 1994). Additionally, its lipophobicity and charge on the quaternary ammonium

group inhibit its passage across the blood–brain barrier, thus limiting the central nervous system effects. There is little evidence of adverse effects of PB treatment in humans (Israeli et al., 1990; Cook et al., 1992; Wenger et al., 1993).

Despite its relative safety, exposure to PB along with other chemicals and/or chronic stress has been implicated in the development of “Gulf War Syndrome” (Haley and Kurt, 1997; Haley et al., 1997). Chronic fatigue, muscle and joint pain, headache, sensorimotor difficulties, and problems with concentration are just a few of the complaints of veterans of the Persian Gulf War (Institute of Medicine, 1995, 2000; Knoke et al., 2000). Because of the possible involvement of PB in this cadre of symptoms, there has been much interest in studying its effects in various models. Behavioral studies showed that PB had marked behavioral effects in rats. PB at doses less than or equal to 0.10 LD₅₀ interfered with avoidance learning, open-field behavior, and complex coordinated movements (Wolthuis and van Wersch, 1984; Wolthuis et al., 1995). There are also reports of decreased

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locomotor activity, exaggerated acoustic startle response, or impeded response acquisition with immediate or delayed reinforcement after PB treatment (Hoy et al., 2000, 1999; van Haaren et al., 2001; Servatius et al., 2000). Cardiovascular (CV) studies in rats showed that acute PB administration increased blood pressure (Chaney et al., 2002) or caused no change (Bataillard et al., 1990). In humans, there was a decrease in HR with no changes in reflex activity (Castro et al., 2000; Nobrega et al., 2001). These PB effects may be mediated by cholinergic interactions with cardiac function and peripheral cholinergic vasodilatory mechanisms. Central stimulation of muscarinic receptors triggers a BP increase mediated by an increase of sympathetic tone (Buccafusco, 1996; Buccafusco and Brezenoff, 1979) and release of vasopressin (Rascol et al., 1990).

We have chosen to use a model of chronic PB treatment in mice, using osmotic minipumps. The rationale for this model is that the military personnel of the Gulf War were given uncontrolled access to PB tablets. Thus, they were exposed chronically to varying dosage levels of PB. This oral self-treatment may result in underdosing as well as overdosing, both with possible lethal consequences. Thus, in the present study, we evaluated the behavioral and CV effects of chronic subcutaneous PB treatment using osmotic pumps that provide a method for continuous and constant drug delivery in mice.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (Harlan Sprague–Dawley, Indianapolis, IN), 10–12 weeks of age, with body weight of 24–26 g, were used in the present study. The mice were housed at 22 °C with a 12:12-h dark/light cycle (0500–1700 h lights on). Animals were housed individually in plastic cages with wooden shavings. They were maintained on a standard pellet diet (0.5% sodium by weight; Harlan Teklad) with tap water ad libitum. After 10 days of acclimatization, the mice were randomly assigned for individual experiments. The Laboratory Animal Care and Use Committee of the Wright State University approved all experiments.

2.2. PB treatment

PB (Sigma, St. Louis, MO, USA) was infused subcutaneously at 1 mg/kg/day (PB 1) or 3 mg/kg/day (PB 3) by means of Alzet minipumps (model 1007D, volume of 0.5 μ l/h over 7 days; DURECT, Cupertino, CA, USA). This treatment results in blood levels of PB approximately 8 and 12 ng/ml, respectively, in a separate experiment. Osmotic pumps were implanted subcutaneously on the back of the mice under anesthesia using a ketamine–xylazine mixture (6:1 mg/kg im). In the control group, minipumps were filled with isotonic saline.

2.3. Blood cholinesterase (ChE) activity

Total blood ChE, AChE, and butyrylcholinesterase (BChE) activities were determined before treatment (basal values) and on Days 3 and 7 of treatment. Total ChE activity was determined by a modified version of the colorimetric method of Ellman et al. (1961) using a Packard Fusion Microplate analyzer at 25 °C. The ChE measurements were made in whole blood collected from carotid arterial catheters. The blood samples were stored at 4 °C and enzyme activities were determined within 4 h of collection. For assay, the blood was diluted 1:100 with 0.1 M NaPO₄ buffer (pH 7.4). Blood AChE activity was determined by inhibiting BChE activity with 25 μ M tetraisopropylpyrophosphoramidate (iso-OMPA; Sigma) and then by calculating BChE activity by subtracting AChE activity from total ChE activity.

2.4. CV measurements

Mice were prepared with chronic carotid arterial catheters (Li et al., 1999; Bernatova et al., 2002). This method allows for continuous, long-term measurement of BP and HR in conscious animals. After surgery, a heparinized saline solution (100 U/ml) was continuously infused into the catheter at 25 μ l/h using a syringe pump (Model 220; KD Scientific, Boston, MA, USA). The infusion is required in order to maintain catheter patency over the time course of the experiment. The catheter was covered with a metal spring that was attached to a fluid swivel at the top of the cage. The animals were allowed to recover from surgery for at least 4–5 days. Blood pressure (BP) and heart rate (HR) were recorded continuously (24 h) before minipump implantation (basal values) and on Days 3 and 7 of the treatment. Systolic and diastolic BP were recorded directly using a sampling rate of 85 samples per second using the Biopac System MP100 (BIOPAC Systems, Santa Barbara, CA, USA). HR is derived from the BP data. The data were converted from digital to numeric form using acquisition software. Data were processed by calculation of 10-min means of the respective variable. These 10-min means were averaged for the calculation of the dark and light period means.

2.5. Behavioral tests

Separate groups of mice ($n=9–12$) were tested using an automated open field system combined with hole board (infrared photobeam technology, Motor Monitor, Version 3.11, 2000; Hamilton Kinder, Poway, CA, USA). The open field (40 \times 40 cm) contained nine holes (diameter 4 cm, depth 7.5 cm) and was divided into central (20 \times 20 cm), intermediate, and peripheral (both 5 cm wide) zones. Illumination (300 lx) as well as background noise levels were identical in the animal housing room and the testing room. The mouse was placed in the center of the open field and the following variables of motor activity were recorded: loco-

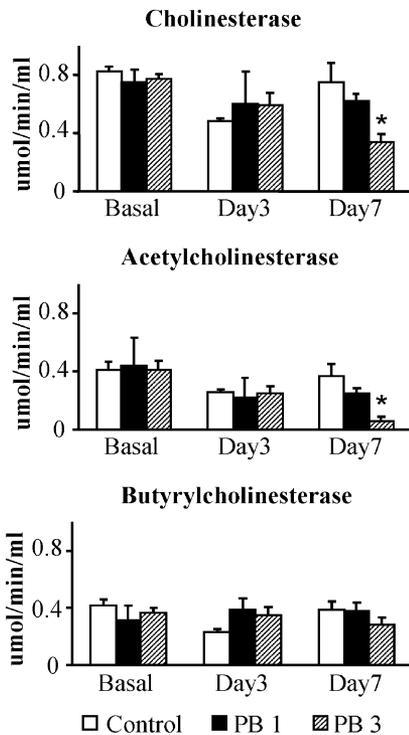


Fig. 1. Effect of PB (1 and 3 mg/kg/day) on ChE, AChE, and BChE activity. There was a significant main effect of PB treatment on ChE [$F(1,41)=12.6$, $P<.001$] and AChE [$F(1,41)=14.8$, $P<.001$] activity. * $P<.03$ vs. basal value. Values are mean \pm S.E.M.

motor activity, fine movements (grooming), rearing, and head dipping. Moreover, distance traveled, total time, rest time, number of entries, head pokes, and head dips into the holes in individual zones were recorded. Mice were assigned to the experimental groups, according to the baseline values of locomotor activity. Animals were tested after 7

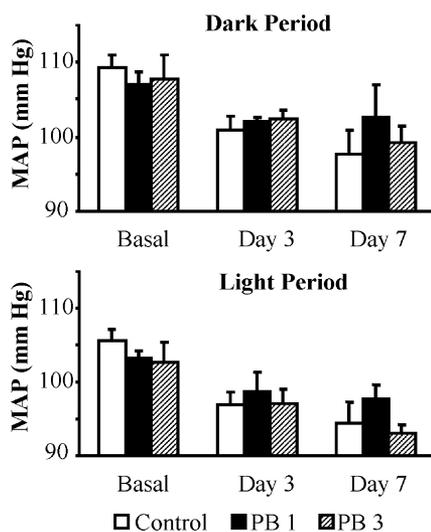


Fig. 2. Effect of PB (1 and 3 mg/kg/day) on MAP during dark and light periods. MAP in the dark period was significantly higher than in the light period [$F(1,82)=13.76$, $P<.0001$ main effect of the circadian factor]. Values are mean \pm S.E.M.

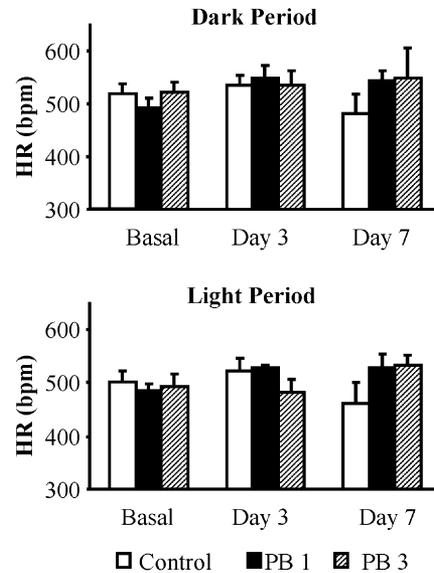


Fig. 3. Effect of PB (1 and 3 mg/kg/day) on HR during dark and light periods. Values are mean \pm S.E.M.

days of continuous PB treatment. Mice were exposed to the open field in 15-min sessions once daily in the morning between 0900 and 1300 h using the same time schedule. After the testing session, the number of fecal boli (defecation rate) was noted for assessment of emotional reactivity. The open field chamber was cleaned with 70% alcohol solution between animals.

2.6. Statistical analysis

Differences in MAP and HR were evaluated by three-way ANOVA (Group \times Day of Treatment \times Day Period)

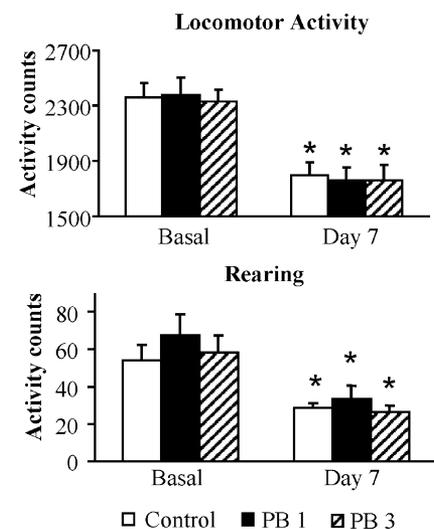


Fig. 4. Effect of PB (1 and 3 mg/kg/day) on locomotor activity and rearing. There was a significant main effect of time in the intensity of locomotor activity [$F(1,59)=42.57$, $P<.0001$] and rearing [$F(1,59)=17.86$, $P<.0001$], which reflects a habituation to the testing. * $P<.01$ vs. respective basal value. Values are mean \pm S.E.M.

Table 1
Effect of PB treatment at doses of 1 and 3 mg/kg/day on central zone activities using open field testing

Central activities	Controls (<i>n</i> = 11)		PB, 1 mg/kg/day (<i>n</i> = 12)		PB, 3 mg/kg/day (<i>n</i> = 9)	
	Basal	Day 7	Basal	Day 7	Basal	Day 7
Distance (cm) ^a	652 ± 238	430 ± 161	716 ± 276	451 ± 225	587 ± 167	421 ± 155
Time in zone (s)	62 ± 27	63 ± 55	66 ± 22	45 ± 19	61 ± 25	48 ± 16
Rest time (s)	5.9 ± 4.7	19.9 ± 5.4	5.9 ± 5.8	4 ± 4.3	3.9 ± 4.5	5.2 ± 4.3
Number of entries ^a	32 ± 9	21 ± 7	35 ± 13	23 ± 12	28 ± 8	20 ± 7
Number of pokes ^a	29 ± 10	19 ± 9	25 ± 8	19 ± 9	25 ± 9	20 ± 7

Values are mean ± S.E.M.

^a Main effect of time was significant.

followed by Duncan's test. ChE, AChE, and BChE were analyzed using two-way ANOVA (Group × Day of Experiment) and Tukey HSD test. Behavioral data were analyzed by means of two-way ANOVA (Group × Day of Treatment) followed by Duncan's post-hoc test. Values were considered to differ significantly if the *P* value was < .05. Statistical analyses were performed using Statistica, 1999 Edition (StatSoft, Tulsa, OK, USA). The results are presented as a mean ± S.E.M.

3. Results

3.1. Blood ChE activity

Average basal values of ChE, AChE, and BChE were 0.78 ± 0.05, 0.42 ± 0.10, and 0.36 ± 0.06 μmol/min/ml, respectively (Fig. 1). There was a significant main effect of PB treatment on ChE [$F(1,41) = 12.6$, $P < .001$] and AChE [$F(1,41) = 14.8$, $P < .001$] activity. PB treatment at the dose of 3 mg/kg/day significantly decreased ChE and AChE activity on Day 7 by 56% ($P < .03$ vs. basal value) and 85% ($P < .02$ vs. basal value), respectively. There were no significant differences in ChE, AChE, and BChE in mice treated with PB at the dose of 1 mg/kg/day.

3.2. Blood pressure and HR

Mean arterial pressure (MAP) and HR were analyzed using a program that compiles all of the data over the 12-h dark/light periods (3.7×10^6 samples per 12-h period). There was a significant main effect of the circadian rhythm

in MAP [$F(1,82) = 13.76$, $P < .0001$]. Average basal MAP in all groups was 108 ± 2 and 104 ± 2 mm Hg during dark and light periods (12-h averages), respectively (Fig. 2). There were no significant changes in MAP between controls and PB-treated animals. Average basal HR of mice was 510 ± 18 and 493 ± 19 beats/min during dark and light periods (12-h averages), respectively (Fig. 3). There were no significant differences in HR between control and PB-treated animals.

3.3. Open field test

PB treatment for 7 days did not affect the behavior of the mice in any of the parameters investigated. The two-way ANOVA revealed only a significant main effect of time on some behavioral variables. There was a significant main effect of time in the intensity of locomotor activity [$F(1,59) = 42.57$, $P < .0001$] and rearing [$F(1,59) = 17.86$, $P < .0001$]. Post-hoc test demonstrated significant habituation—a decrease in these behavioral activities on Day 7 compared to basal testing in all experimental groups ($P < .01$ for both activities) (Fig. 4).

Zone analysis revealed a significant main effect of time on distance traveled in the central zone [$F(1,59) = 16.69$, $P < .0001$], number of entries to the central zone [$F(1,59) = 16.34$, $P < .0001$], and number of head pokes in the central zone [$F(1,59) = 9.72$, $P < .003$; Table 1]. In the peripheral zone, time significantly affected the distance traveled [$F(1,59) = 19.45$, $P < .0001$], rest time [$F(1,59) = 4.72$, $P < .04$], and number of entries [$F(1,59) = 18.23$, $P < .0001$; Table 2]. Emotional reactivity, in the number of fecal boli, did not decrease after repeated exposure of mice to the open field (data not shown).

Table 2
Effect of PB treatment at doses of 1 and 3 mg/kg/day on peripheral zone activities using open field testing

Peripheral activities	Controls (<i>n</i> = 11)		PB, 1 mg/kg/day (<i>n</i> = 12)		PB, 3 mg/kg/day (<i>n</i> = 9)	
	Basal	Day 7	Basal	Day 7	Basal	Day 7
Distance (cm) ^a	2565 ± 836	1958 ± 599	2490 ± 429	1788 ± 609	2372 ± 539	1626 ± 644
Time in zone (s)	421 ± 118	452 ± 116	412 ± 59	415 ± 156	416 ± 105	373 ± 108
Rest time (s) ^a	71 ± 34	100 ± 49	65 ± 40	96 ± 66	64 ± 47	88 ± 67
Number of entries ^a	87 ± 18	69 ± 16	94 ± 20	72 ± 17	91 ± 19	75 ± 16
Number of pokes	57 ± 26	47 ± 17	54 ± 18	50 ± 26	52 ± 16	62 ± 24

Values are mean ± S.E.M.

^a Main effect of time was significant.

Two-way ANOVA did not reveal any significant effects of interaction of time and PB treatment in the behavioral parameters investigated.

4. Discussion

The present study examined the effect of chronic subcutaneous PB treatment on CV and behavioral parameters in mice. The results show that while PB inhibited blood ChE and AChE activity, it had no effect on MAP, HR, or open field behavior.

CV effects are observed with some AChE inhibitors. For example, the cholinergic stimulation produced by sarin or soman evoked hypertensive responses in rats and humans (McGee and Brezenoff, 1987; Letienne et al., 1999). On the other hand, Anzueto et al. (1990) found a decline in blood pressure and bradyarrhythmias in baboons after inhalation of these organophosphates. These effects were supposedly linked with central cholinergic stimulation (Letienne et al., 1999; Smith et al., 2001) even though peripheral effects may also be involved (Buccafusco and Brezenoff, 1979). PB, due to its lipophobic structure, is unlikely to cross the blood–brain barrier under normal conditions; thus, any effect should be related to peripheral rather than central actions.

The majority of studies that have looked for CV effects of PB have used acute treatments. A decrease in HR was observed after a single dose of PB in anesthetized dogs (Caldwell et al., 1989), while no alterations were observed in marmosets (Wolthuis et al., 1995) and rats (Bataillard et al., 1990). Blood pressure was either increased or unchanged after acute PB treatment (Caldwell et al., 1989; Bataillard et al., 1990; Chaney et al., 2002). On the other hand, PB pretreatment before central stimulation with L-glutamate blunted the pressor response (Grabe-Guimaraes et al., 1999).

In humans, a single oral dose of PB (30 mg) was well tolerated and caused no alterations in BP, but produced a drop in HR (de Pontes et al., 1999; Nobrega et al., 2001) and an increase in HR variability (Nobrega et al., 2001). A higher dose of PB (45 mg) caused no alterations in systolic or diastolic pressure (Nobrega et al., 1999), but reduced HR at rest (Serra et al., 2001). When the same dose of PB was administered before mental stress, the stress-induced increase of BP and tachycardia were blunted as compared to placebo (Nobrega et al., 1999).

There is little information on the CV effects of prolonged PB exposure. Wenger et al. (1993) and Cook et al. (1992) investigated the effect of a 7-day PB treatment (3×30 mg PB/day) on red blood cell ChE activity, BP, and HR in soldiers. After 4 days of exposure, ChE activity was reduced by approximately 28%. There were time-related effects of PB on HR during exercise. By the fourth day of treatment, exercise tachycardia was reduced in PB-treated soldiers as compared to the placebo group (Wenger et al., 1993). Resting diastolic pressure was also slightly reduced (-4

mm Hg) after PB treatment (Cook et al., 1992). In contrast, chronic PB administration to nonhuman primates caused no change in CV parameters (Avlonitou and Elizondo, 1988).

In our experiment, the high PB dose (3 mg/kg/day), administered via a slow infusion, produced no change in blood BChE, but caused a decrease in AChE activity after 7 days of treatment. No changes in AChE and BChE activity were observed in mice treated with PB at 1 mg/kg/day. The data are in agreement with the study of Somani et al. (2000), which showed no differences in AChE and BChE activity after 2 weeks of oral PB treatment in mice (1.2 mg/kg/day). It is of interest that blood BChE in mice was not affected by even higher dose of PB since plasma BChE activity is often used as a marker for AChE activity. Thus, the data raise a question as to whether BChE activity is always a reliable marker of AChE.

The decreases in blood ChE and AChE activity were not accompanied by alterations in MAP or HR. This is consistent with studies that show little relationship between peripheral ChE activity and CV parameters (Avlonitou and Elizondo, 1988; Caldwell et al., 1989). The data suggested that alterations in the peripheral cholinergic signaling mechanisms could be successfully compensated by other regulatory mechanisms in the face of ChE inhibition. Certainly, the method of drug delivery may be a factor in explaining the lack of changes. The continuous slow infusion of PB (0.125 mg/kg/h) by osmotic minipump may produce the gradual development of cholinergic tolerance by reducing sensitivity to cholinergic stimulation.

While there is much information on the behavioral effects of PB in the rat, there is little information on mice. Wolthuis and van Wersch (1984) determined that PB decreased two-way shuttle box avoidance efficiency, decreased open field locomotion, and produced a decrease in stepping activity. PB was also found to decrease locomotor activity in a dose-dependent manner with obvious gender differences (Hoy et al., 2000). Other studies showed that acute and repeated PB administration negatively affected learning paradigms, such as fixed-ratio, fixed-interval, and progressive-ratio performances (van Haaren et al., 1999). Despite the generally accepted notion that PB does not cross the blood–brain barrier, the behavioral alterations indicate that there may be interactions with central cholinergic systems. However, the exact mechanism by which this compound exerts behavioral effects in rats remains to be determined.

In contrast to the results in rats, chronic PB treatment did not have any effect on open field behaviors in mice. In both control and PB groups, there was a significant decline in the behavioral variables recorded when mice were repeatedly tested in the open field. This behavioral decline represents a normal physiological habituation to repeated testing. The lack of a PB behavioral effect may be related to the accommodation to the drug treatment (slow and accumulative infusion) as seen with the CV parameters. However, it may also be related to species and strain differences. For

example, the C57BL/6J mouse strain used in our study was found to be nonemotional and less fearful as compared to other strains (Van Gaalen and Steckler, 2000). Furthermore, other features of the laboratory environment—such as housing in isolation or in a group, and standard vs. enriched housing or handling procedure—may also affect the experimental results (Wahlsten, 2001).

Even though our results suggest that PB treatment does not affect CV or behavioral parameters in the laboratory environment, it is not possible to directly translate these results to the human condition. During the military deployment, the soldiers were treated with PB, but they were also exposed to other environmental conditions, such as, heat, physical and psychological stress, smoke, and chemical exposure. Investigators have postulated that it was the interaction of these various influences which led to the Gulf War Syndrome (Haley and Kurt, 1997; Haley et al., 1997). Recently, it has been shown that stress disrupted the blood–brain barrier and allowed PB penetration across the blood–brain barrier (Friedman et al., 1996) even though these results were not replicated by others investigators (Servatius et al., 2000; Kant et al., 2001). In rats, short-term treatment with PB (1.85 mg/kg twice daily for 4 days) induced a prolonged apoptotic response, which was evident in rat cortex up to 30 days after the last dose. These observations indicate that PB can initiate a prolonged neurodegeneration (Li et al., 2000). PB treatment in mice was also found to inhibit hypothalamic AChE activity and to decrease the peak intensity for hypothalamic peptide/protein profiles in mice (Ropp et al., 2002). Moreover, atypical genetic predisposition of some individuals resulting in the lack of BChE, a plasma scavenger of PB, may allow free PB to influence central cholinergic transmission (Loewenstein-Lichtenstein et al., 1995). Thus, the role of PB in Gulf War Syndrome is not satisfactorily understood and more experiments are needed to elucidate the cause of this illness.

Taken together, we believe that the finding of no CV or behavioral effects after chronic subcutaneous PB administration may have important clinical implications. Perhaps, chronic subcutaneous dosing may be a useful way of PB administration for the prophylactic treatment of military personnel. Development of cholinergic tolerance may provide protection against nerve gas poison. Moreover, it would eliminate the problems of underdosing as well as overdosing of soldiers. The methods of long-acting subcutaneous treatment in human have been developed and refined over the last 15 years, mainly as a part of conception control (Olsson et al., 1990; Pollanen et al., 2001). Similar methods could be developed for PB administration.

In conclusion, we tested the effect of chronic PB treatment on behavioral and CV parameters in mice. A comprehensive investigation using long-term blood pressure and HR monitoring, and behavioral evaluation revealed no changes in any parameters. This occurred concurrent with significant inhibition of blood ChE activity. The results

suggest that prolonged low-dose PB treatment itself does not represent a significant risk factor.

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Circadian Differences in Stress-Induced Pressor Reactivity in Mice

Iveta Bernatova, Mary P. Key, James B. Lucot, Mariana Morris

Abstract—The objective of this study was to determine the effect of chronic stress exposure on the circadian pattern of cardiovascular responses in mice. Using male C57BL6 mice with carotid arterial catheters, we tested the effect of 7 days of intermittent shaker stress on body weight, food intake, drinking activity, plasma corticosterone, mean arterial pressure (MAP), and heart rate. The stress was delivered automatically for 2-minute periods (150 cycles/min), 45 times/d for 7 days. Plasma corticosterone was significantly increased in acutely and chronically stressed mice, with a partial attenuation in the chronic condition. Stress increased water intake, produced no change in food intake, and significantly decreased body weight (5% change). MAP and heart rate were measured continuously on stress days 1, 3, and 7 and during the basal and recovery periods. Chronic stress did not produce a sustained increase in MAP; however, there was an increase in MAP during the first stress day and a decrease during the recovery period. There was a circadian pattern in the pressor responses, with greater increases seen during the light period (nonactive phase) than in the dark period (+24% versus +11% on stress day 3, light versus dark). The results suggest that a stress delivered during the nonactive phase represents a higher cardiovascular risk. (*Hypertension*. 2002;40:768-773.)

Key Words: blood pressure ■ heart rate ■ corticosterone ■ circadian rhythm

Lifestyle stress is a risk factor for human diseases, including cancer, stroke, psychological disorders, and heart disease. There is a circadian pattern in the incidence of cardiovascular pathologies, with a higher frequency of heart attacks, strokes, and arrhythmias during the morning hours.¹⁻³ Stressful conditions may also be a precipitating factor in the occurrence of cardiovascular accidents.

In animals, stress exposure produced by a variety of methods provokes a cascade of autonomic adjustments characterized by increased blood pressure (BP), increased heart rate (HR), and behavioral alterations. Most studies of stress biology have been conducted in rats, providing information on changes in cardiovascular function and eating and drinking behaviors.⁴⁻¹⁰ However, one complication of the experimental paradigms is that the stress effects can be enhanced by animal handling, noise, or pain, as seen in the forced swim test, air jet exposure, and physical restraint.^{4,8,11}

Shaker stress is a mild, pain-free stimulus that elicits reproducible changes in BP, HR, sympathetic activity, and stress hormone secretion.^{12,13} There is no information on the application of shaker stress in mice and no data on the long-term effects of shaker stress on the cardiovascular system or on drinking and eating patterns.

For investigations in mice, we developed a computerized system for chronic, continuous BP recording and combined this with electronic recording of licking activity for analysis

of circadian patterns.^{14,15} This methodology was applied to the investigation of the effects of stress on BP and HR responses and drinking behavior during the active (dark period) and nonactive (light period) phases in normotensive mice. For this purpose, we also set up a model for chronic stress exposure in mice, using shaker stress that was delivered remotely and automatically in the animal's home cage.

Methods

Animals

Male C57BL6 mice (Harlan Inc, Indianapolis, Ind), 10 to 12 weeks of age, were used. All animal experiments were approved by Wright State University's Laboratory Animal Care and Use Committee. The mice were housed at 22°C with a 12:12-hour dark-light cycle (5 AM to 5 PM, lights on). Animals were housed individually in clear cylindrical cages with a metal grid flooring, which covered standard bedding. Mice were maintained on a standard pellet diet (Harlan Teklad, 0.5% sodium by weight) and tap water ad libitum.

Shaker Stress

For shaker stress, a specially designed caging system was attached to a shaking platform (Model 5901; Eberbach Inc). The shaking device was programmed to provide intermittent shaker stress, 2 minutes of shaking (150 cycles/min; 2.86 cm stroke) followed by variable rest periods from 13 to 45 minutes. The variable rest periods between stress sessions were chosen with the aim of introducing an unpredictable stress stimulus. The same paradigm of 45 shaking sessions/24 h was used during all 7 days of stress.

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Plasma Corticosterone

Corticosterone levels were compared in mice exposed to acute or chronic stress. Seventy-four mice were divided into 3 groups. In the acute group, 28 mice were exposed to a single 2-minute shaking session. In the chronic group, 30 mice were exposed to chronic shaker stress for 7 days. Sixteen mice served as controls, with the same handling but without stress. Mice were killed 5, 30, or 60 minutes after the last shaking session ($n=9$ to 10/group) between 9 and 11 AM. After decapitation, trunk blood was collected in heparinized test tubes and centrifuged; plasma samples were stored at -30°C . Plasma corticosterone was determined with the ImmuChem double antibody corticosterone ^{125}I RIA kit (ICN Biochemicals Inc).

Body Weight, Food Intake, and Drinking Activity

Basal data were recorded for 4 days, followed by 7 days of intermittent shaker stress and 3 days of recovery. Body weight (BW), food intake ($n=10$), and water intake ($n=5$) were measured daily. Drinking activity was recorded with a drinkometer system¹⁵ (Columbus Instruments) interfaced with a computerized data acquisition system (Biopac Inc). Incidental contacts of mice with the water bottles, registered as single data points, were excluded from the analysis. Food intake was measured by daily weighing of the food.

Cardiovascular Measurements

Mice were prepared with chronic carotid arterial catheters according to the method of Li et al.¹⁴ After surgery, a heparinized saline solution (100 U/mL) was continuously infused into the catheter at 25 $\mu\text{L}/\text{h}$ with the use of a syringe pump (model 220, KD Scientific). The pressure produced by the infusion pump modified BP readings by ≈ 2.9 mm Hg. The catheter was covered with a metal spring that was attached to a fluid swivel at the top of the cage. The animals were allowed to recover from surgery for at least 5 to 6 days, by which time water and food intake had returned to normal. BP and HR were recorded continuously (24 hours) on stress days 1, 3 and 7 and on the day before and after stress (basal and recovery).

Stress responses of mean arterial pressure (MAP) and HR to individual shaking sessions were evaluated on stress days 1, 3, and 7 and on day 1 of the recovery period during the light and dark periods (11 AM and 11 PM). Responses were calculated as a percentage of the values during the 2 minutes preceding the stress.

Statistical Analysis

All results are presented as mean \pm SEM. One-way ANOVA and least significant differences tests were used for analysis of BW and food intake. Two-way ANOVA and Newman-Keuls tests were used for analysis of the stress effect on corticosterone levels and drinking activity. Differences in MAP and HR in the chronic experiment were evaluated by 2-way ANOVA followed by Duncan's test. Stress responses were evaluated by 3-way ANOVA followed by the Newman-Keuls test. Values were considered to differ significantly if the probability value was <0.05 . Circadian data of drinking activity, systolic BP, diastolic BP, and HR were recorded with a sampling rate of 85 samples/s and converted from digital to numeric form with the use of acquisition software. Data were processed by calculation of 10-minute means of the respective variable. These 10-minute means were averaged for calculation of the dark and light period means. Drinking data were then converted to text files, processed by Circadia software (Behavioral Cybernetics), and smoothed using 60-minute moving averages. Finally, data were converted to a circadian autogram and to the graph (MATLAB Graphics 5.2.1).

Stress responses of MAP and HR were recorded and analyzed in the same manner as circadian data of MAP and HR; however, data were processed by calculation of 2-minute averages that were used for calculation of MAP and HR values before, during, and after stress (divided into periods 1 to 8 minutes and 9 to 16 minutes after stress).

Results

Plasma Corticosterone

Corticosterone levels in control mice were 12 ± 2.7 ng/mL. Stress produced significant increases in plasma corticosterone

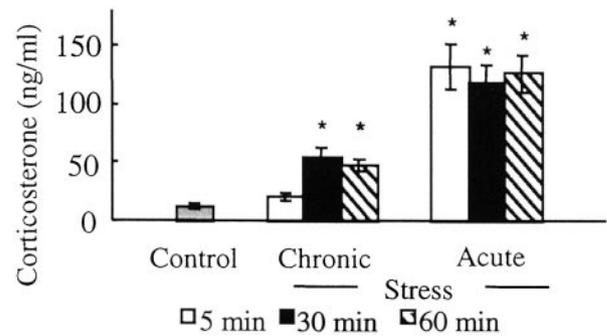


Figure 1. Plasma corticosterone response to acute and chronic shaker stress. All effects of stress on corticosterone levels were significant ($F[2,65]=65.8$, $P<0.0001$). Acute stress increased corticosterone concentrations significantly more compared with chronic stress ($P<0.0001$). $^*P<0.01$ vs control. Values are mean \pm SEM.

under acute and chronic conditions ($F[2,65]=65.8$, $P<0.0001$, Figure 1). The acute response was ≈ 2.5 -fold greater than the chronic response, with peak levels of 132 and 53 ng/mL, respectively ($P<0.0001$). There were no time-related differences in plasma corticosterone in the acute experiment between 5, 30, and 60 minutes after stress. In chronically stressed mice, a 4-fold increase of corticosterone was found 30 and 60 minutes after stress compared with the control levels ($P<0.01$).

Body Weight and Food Intake

BW differed during basal, stress, and recovery periods ($F[2,107]=3.21$, $P<0.05$, main effect of stress, Table). Post hoc analysis showed a marginally significant decrease of BW on the third day of stress versus the last day of basal recording ($P=0.06$, decrease of 6%). There were no observed differences in food intake (Table).

Drinking Activity

Drinking activity was measured by continuous recording of licking activity during the basal, stress, and recovery periods. On

Effect of Chronic Intermittent Shaker Stress on Body Weight and Food Intake

Day	Body Weight, g* (n=10)	Food Intake, g (n=10)
Basal 1	27.22 \pm 0.54	4.42 \pm 0.18
Basal 2	27.34 \pm 0.59	4.86 \pm 0.15
Basal 3	27.59 \pm 0.68	4.76 \pm 0.18
Basal 4	27.78 \pm 0.67	4.17 \pm 0.30
Stress 1	26.43 \pm 0.58	4.65 \pm 0.17
Stress 2	26.33 \pm 0.59	5.23 \pm 0.32
Stress 3	26.13 \pm 0.58	4.76 \pm 0.11
Stress 4	26.26 \pm 0.59	4.82 \pm 0.11
Stress 5	26.41 \pm 0.56	4.76 \pm 0.11
Stress 6	26.54 \pm 0.61	4.77 \pm 0.11
Stress 7	26.59 \pm 0.63	4.66 \pm 0.13
Recovery 1	26.86 \pm 0.70	4.85 \pm 0.22
Recovery 2	27.21 \pm 0.80	4.87 \pm 0.22
Recovery 3	27.41 \pm 0.74	4.50 \pm 0.21

*Stress decreased body weight significantly ($F[2,107]=3.21$, $P<0.04$ for main effect of stress) compared to basal values. Values are mean \pm SEM.

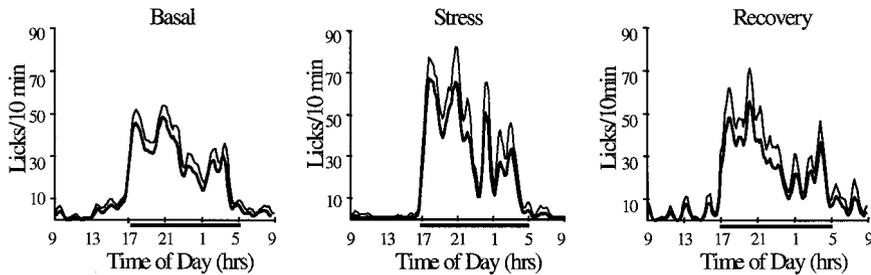


Figure 2. Circadian pattern of drinking activity, comparing basal (average waveform for 4 days of basal recording), the first day of stress, and the third day of the recovery period. Heavier part of x-axis represents dark period. Values represent average waveform (dark line) and average waveform positive error (light line).

the basis of these data, average waveforms were constructed that showed the diurnal pattern in drinking activity (Figure 2). Stress did not alter the diurnal pattern of drinking (Figures 2 and 3), which was concentrated in the dark period ($F[1,128]=345.3$, $P<0.0001$, main effect of circadian factor). Stress increased significantly drinking activity of mice in the dark period ($F[1,128]=5.90$, $P<0.02$, interaction stress \times circadian factor) as compared with nonstress periods (basal and recovery).

Blood Pressure and Heart Rate

MAP and HR were analyzed with the use of a program that compiles all of the data over the 12-hour dark/light periods (3.7×10^6 samples/12-hour period). Basal MAP in the stress group was 111 ± 0.98 and 104 ± 1.15 mm Hg during dark and light periods (12-hour averages), respectively (Figure 4). There was a significant light/dark rhythm in MAP ($F[1,54]=63.2$, $P<0.0001$, main effect of circadian factor), with highest levels noted during the dark period. Analysis of the time-related changes showed significant alterations ($F[4,54]=17.9$, $P<0.0001$, main effect of experiment day), with an increase in MAP (24-hour average) on the first day of stress ($P<0.008$) and a decrease during recovery ($P<0.001$ versus basal value). A significant increase in MAP occurred in the light period of stress day 1 (versus basal/light), whereas there were decreases in both light and dark periods of recovery (versus the appropriate basal period, Figure 4).

The basal HR of mice was 600 ± 13 and 565 ± 10 beats/min during dark and light periods (12-hour averages), respectively (Figure 4). Analysis of the time-related changes showed a decrease

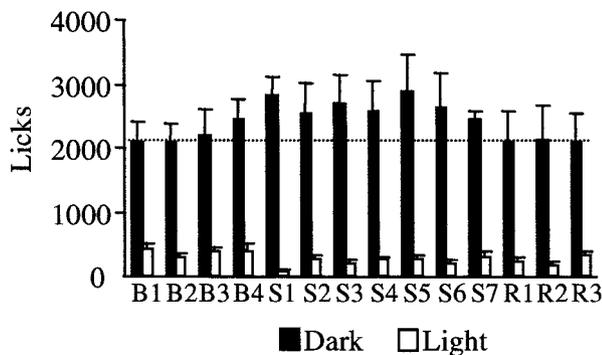


Figure 3. Effect of chronic intermittent shaker stress on drinking activity. B1 through B4, Days of basal recording; S1 through S7, days of stress; R1 through R3, days of poststress recovery. Drinking was significantly concentrated in the dark period ($F[1,128]=345.27$, $P<0.0001$). Stress significantly increased drinking activity of mice in the dark period ($F[1,128]=5.90$, $P<0.02$ for interaction of stress and circadian factor) compared with nonstress periods (basal and recovery) without alterations during the light period. Values are mean \pm SEM.

in HR (24-hour average) on the last day of stress ($P<0.003$) and during recovery ($P<0.007$) versus the basal day. Circadian analysis revealed that the significant drop of HR occurred in the dark period of day 7 of stress and recovery versus basal/dark (Figure 4).

Stress-Induced Cardiovascular Responses

To evaluate cardiovascular reactivity, we examined the pressor and heart rate responses during the light/dark periods on days 1, 3, and 7 of chronic stress. The results showed that there was a diurnal pattern in stress-induced pressor reactivity. Stress delivered during the light period increased MAP significantly more than stress delivered in the dark period ($F[1,162]=30.0$, $P<0.0001$, main effect of circadian factor; Figure 5). The immediate increases of MAP during the stress event in the light period were $\approx 26\%$, 24% , and 23% ($P<0.001$) on days 1, 3, and 7, respectively, as compared with basal. The immediate increases of MAP during the stress event in the dark period were 16% ($P<0.001$), 11% ($P=0.16$), and 21% ($P<0.01$) on days 1, 3, and 7, respectively. In both light and dark periods, MAP gradually decreased after stress ($F[2,162]=23.3$, $P<0.0001$, main effect of the time course, ie, stress period, 1 to 8 minutes and 9 to 16 minutes after stress).

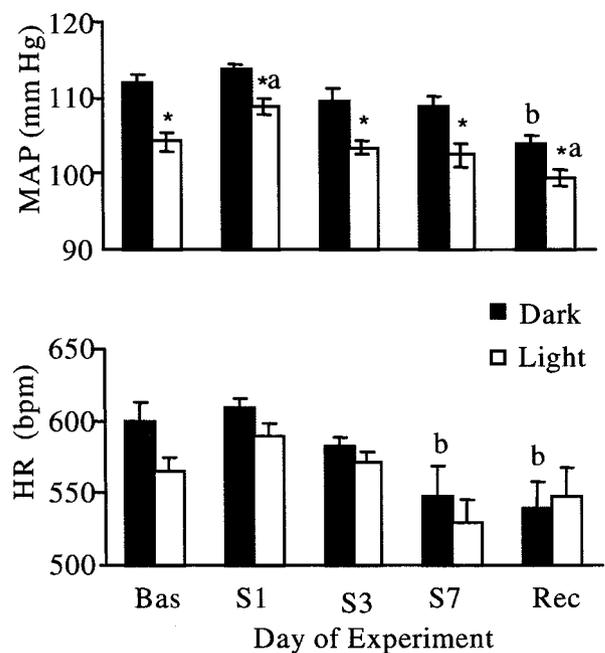


Figure 4. MAP and HR of stress-exposed mice. Bas indicates basal day; S1, S3, and S7, days of stress; Rec, day 1 of poststress recovery. $^*P<0.01$ vs dark period on the same day; $^aP<0.05$ vs basal/light; $^bP<0.05$ vs basal/dark. Values are mean \pm SEM.

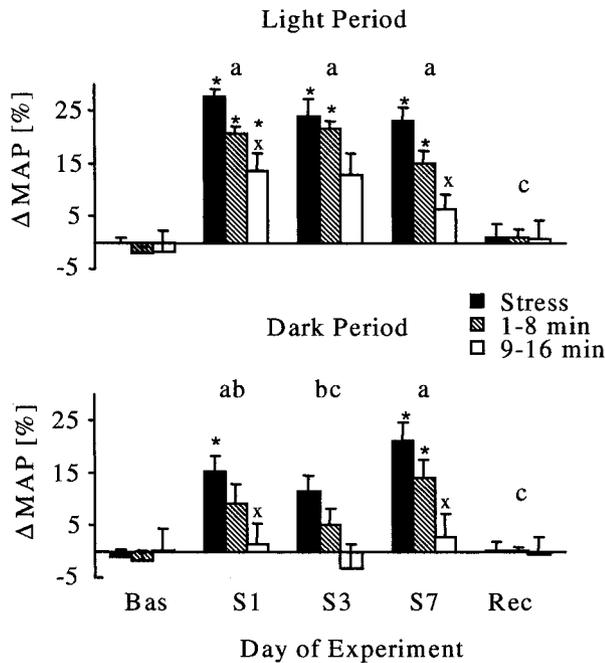


Figure 5. Time course of stress-induced BP changes on days 1, 3, and 7 of chronic stress. Bas indicates basal day; S1, S3, and S7, days of stress; Rec, day 1 of poststress recovery. Responses were measured during light and dark periods (11 AM and 11 PM) and calculated as a percentage of values during 2 minutes preceding stress. Results show MAP alterations during stress (2 minutes) and after stress (1 to 8 minutes and 9 to 16 minutes). ^a $P < 0.01$ vs basal day in the same period; ^b $P < 0.001$ vs light period on the same day; ^c $P < 0.002$ vs stress 7 during the same period, where a, b, and c represent a comparison of overall response (ie, average of stress, 1 to 8 minutes, and 9 to 16 minutes). ^{*} $P < 0.05$ vs time-related values on basal day; ^x $P < 0.03$ vs stress on the same day and period. Values are mean \pm SEM.

There were no differences in HR responses during the dark/light periods (Figure 6). The immediate increases of HR during the stress event were not different as compared with basal values. Significant differences between the dark and light period response were observed on day 3 ($P < 0.05$).

Discussion

The objective of the present study was to characterize a model of chronic shaker stress exposure in mice and to investigate the effect of shaker stress on the circadian pattern of cardiovascular responses. The key findings are (1) chronic shaker stress provides a viable model for the investigation of stress effects in mice; (2) stress produced alterations in MAP, with an increase noted on the first day of stress; and (3) there was a diurnal pattern in pressor reactivity with prominent differences noted between daytime and nighttime exposures.

Long-term shaker stress increased plasma corticosterone, although the activation of the hypothalamic-adrenal axis was partially attenuated as compared with the acute stimulus. There was a >4 -fold increase in plasma corticosterone after 7 days of stress (compared with controls), which suggests that shaker stress remained an effective stimulus suitable for long-term studies. Adaptation to stress is a normal response, since without physiological compensation there could be escalating, detrimental effects on the organism. Indeed, for repeated restraint

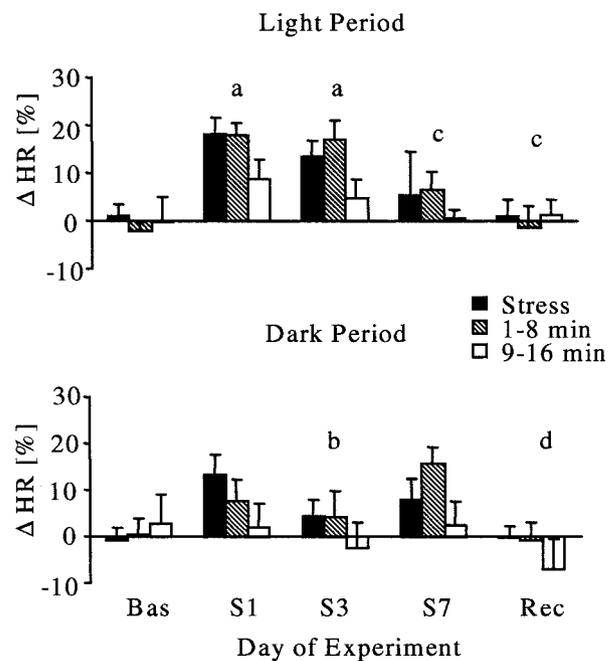


Figure 6. Time course of stress-induced HR changes on days 1, 3, and 7 of chronic stress. Bas indicates basal day; S1, S3, and S7, days of stress; Rec, day 1 of poststress recovery. Responses were measured during light and dark periods (11 AM and 11 PM) and calculated as percentage of values during 2 minutes preceding stress. Results show HR alterations during stress (2 minutes) and after stress (1 to 8 minutes and 9 to 16 minutes after stress). ^a $P < 0.02$ vs basal day in the same period; ^b $P < 0.05$ vs light period on the same day; ^c $P < 0.03$ vs stress 1 during the same period; ^d $P < 0.04$ vs stress 7 during the same period, where a, b, c, and d represent comparison of overall response (ie, average of stress, 1 to 8 minutes, and 9 to 16 minutes). Values are mean \pm SEM.

stress,^{16,17} social stress,¹⁸ noise stress,¹⁹ and forced swimming,²⁰ there is a habituation of corticosterone responses. However, in contrast to our results, a study in rats showed no attenuation of the corticosterone response to repeated shaker stress.¹³ The difference in the results may be related to the level of stimulation and/or the animal species. Hashiguchi et al¹³ tested the effect of daily shaking sessions delivered over 14 days, whereas in our experiment, mice were exposed to 315 shaking sessions over a 7-day period. Taking into consideration the number of stress sessions administered and the time course of the plasma corticosterone response, it is likely that there is a generalized increase in adrenal steroid secretion in the chronically stressed mice.

Various models have been used to study stress/cardiovascular interactions in experimental animals.^{4,6-9} In the majority of these experiments, subjects were exposed daily to a single stress event and the cardiovascular responses were determined over limited time periods after the stress session.^{5,7-9} However, chronic paradigms are more relevant to the human situation in which people are exposed to multiple small stressors over long periods of time. Another issue is that most of the experimental stress models include the possibility of exposure of the animals to other stressful stimuli, such as handling, noise, and vibration. We used shaker stress as the test paradigm because it elicits reproducible cardiovascular changes, and with our specialized caging system it is easy to administer to the conscious animal without

additional handling. In previous studies, 5-minute of acute shaker stress (150 cycles/min) in rats produced significant increases in MAP and HR that persisted 5 minutes after the end of the shaking.¹²

There is less information on the effects of chronic stress on cardiovascular parameters and no information on the effects of chronic shaker stress. In this study, MAP and HR were recorded continuously for 24-hour periods on selected days of the stress exposure. Intensive monitoring is important because it provides an accurate picture of acute cardiovascular responsiveness as well as long-term changes. The composite data show that there were no sustained increases in BP during chronic shaker stress. However, an increase of MAP was noted on the first day of stress and a decrease on the first day of recovery. It is interesting that the changes occurred during the initiation and termination of the stress, suggesting that a rapid adjustment in autonomic/endocrine outflow occurred at these times.

Several studies have addressed the problem of stress-induced hypertension. However, there are conflicting data as to the nature of the cardiovascular changes induced by these stressors. A social stress paradigm in which male rats were housed with different females produced no change in basal blood pressure or its circadian rhythm.²¹ A chronic multiple stress paradigm produced increases in HR, HR variability, and stress-induced tachycardia but no changes in BP.²² Other studies demonstrated interactions between stress and genetic background. Henry and colleagues²³ presented evidence that chronic social stress, produced by group housing, caused an increase in blood pressure in Long-Evans rats but not in Wistar-Kyoto rats. Similarly, in spontaneously hypertensive rats, restraint-induced tachycardia and pressor responses were greater than in the normotensive Wistar rats.⁸ Lawler et al²⁴ reported that borderline-hypertensive rats showed a greater pressure response to conflict stress as compared with normotensive strains. Thus, genetic factors may play a crucial role in cardiovascular regulation and the development of stress-induced hypertension.

In mice, psychosocial stimuli produced prolonged hypertension.²⁵ However, this study used a tail-cuff method for determination of blood pressure, which exposes the mice to additional "acute stressors" associated with the recording method (handling, noise, heat, restraint, and pain). In this situation, the pressor response of chronically stressed mice to "acute stress" during BP recording may be greater than in controls as the result of sensitization of cardiovascular responses.²⁶ Thus, an appropriate method of BP recording is necessary for accurate evaluation of cardiovascular responses to stress exposure. The chronic arterial catheter and data acquisition system used in the present study allows for recording BP and HR in undisturbed mice during stress sessions and rest periods. The catheter system also permits painless blood sampling for the evaluation of stress hormone levels (not available using radiotelemetry). This prevents any hyperresponsiveness caused by handling and novelty.

The analysis of the circadian pattern of stress responsiveness revealed some interesting findings. There was a marked increase in the pressor response when the stress was delivered during the light phase (nonactive, sleeping period). In fact, on the third day of stress, there were no significant changes in BP during the dark (active) period but a 24% increase during the light period. An examination of the behavioral activity associated with chronic

stress showed a similar circadian pattern.²⁷ Stress significantly decreased locomotor activity but only during the day. The reason for the difference in the day/night cardiovascular and behavioral responses is not known but may be related to sensory activation (waking from sleep), changes in regional hemodynamics, input from circadian pacemakers, or other factors. Certainly, in humans there is considerable evidence linking the circadian period to cardiovascular events such as myocardial infarctions and strokes. For example, there was a peak in cerebrovascular strokes in humans in the morning (6 AM to noon) and a decreased incidence during the night (6 PM to 6 AM).^{1,3} The morning onset of cardiovascular events may be associated with increased sympathetic activation as well as the stress from daily activities. These epidemiological studies were, however, focused on the resultant pathology rather than cardiovascular responsiveness, as investigated in our animal study.

The time course of BP alterations observed during shaker stress suggested sudden increases of BP during stress events, with a decline to basal or even below basal values during rest periods. Even though the pressor responses are of relatively short duration, these repeated fluctuations could have pathological consequences. On the one hand, changes in arterial pressure lead to corresponding alterations in shear stress that can increase nitric oxide (NO) release from the endothelium and at least partially buffer the pressure overload.²⁸ On the other hand, sudden pressure fluctuations could damage the endothelial monolayer and result in reduced endothelium-dependent NO release. Thus, a functional endothelium may be especially important during chronic stress. Indeed, chronic social conflict increased the rate of endothelial cell damage and reduced NO bioavailability in the coronary arteries of primates.^{29,30} In humans, brief episodes of mental stress induced transient endothelial dysfunction in healthy young adults,³¹ which may represent a link between stress and atherogenesis.

For evaluation of stress-induced metabolic changes, we studied water and food intake and BW. Despite the increase in water intake and the lack of change in food intake, chronic stress caused a small but significant loss of BW. This decrease in BW is presumably associated with increased metabolic demands, reduced digestion, and increased adrenal steroid secretion.³² There are various reports of a stress-induced alterations of BW: decrease in BW,^{33,34} no change in BW,¹⁰ and even an increase in BW.³⁵ Similarly, variable effects of stress on food and water intake were observed.^{36,37} In terms of the circadian pattern of water intake, the increase in drinking (recorded as increased licking activity) produced by chronic stress occurred primarily during the dark period. However, the stress did not change the diurnal rhythm of water intake, even though there were experimentally imposed disruptions in the sleep cycle. Thus, alterations of BW and food and water intake in the different experimental studies probably are dependent on the type of the stressor, its duration, and animal species and sex.

In conclusion, we developed and characterized a model for chronic stress exposure in mice that allows for the determination of BP and HR in the undisturbed state. The results showed that stress produced an activation of the hypothalamic-adrenal axis, which was partially attenuated in the chronic condition. There was no sustained increase of MAP, but there were important circadian changes in pressor responsiveness. The study provides evidence that stress

delivered during the nonactive phase of the day represents a higher cardiovascular risk than stress delivered during the active phase. This may have implications for the human condition, in which there are circadian patterns in the incidence of cardiovascular problems as well as links between stress and heart disease.

Perspectives

Stress, defined as a physical, chemical, or emotional factor that causes bodily or mental tension, is a risk factor in a variety of diseases. Because stressful conditions are often chronic, it is important to have animal models for examining the pathological processes. In this paper, we report on the characterization of a model for chronic stress exposure in mice. Intermittent shaker stress provides a unique pain-free model of emotional and physical stress. It can be administered remotely and is easily combined with data collection systems such as those for cardiovascular, ingestive, and endocrine function, allowing for a comprehensive evaluation of its effects. Studies in humans suggest that stress augments and/or initiates cardiovascular pathologies. There is also evidence for day/night rhythms in the incidence of myocardial infarctions and stroke, with greater occurrences during the early waking hours. We report that in mice, there are circadian variations in stress-induced cardiovascular responses. This is seen as increased pressor responses during the day period when the animals are sleeping. This data may be relevant to the human condition because it suggests that stress presented during the inactive period represents a greater cardiovascular risk.

Acknowledgments

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Experimental Physiology

Stress-induced pressor and corticosterone responses in oxytocin-deficient mice

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We used oxytocin knockout (OTKO) mice to investigate the role of oxytocin in regulation of blood pressure, heart rate and stress reactivity (pressure reactivity and plasma corticosterone). Male OTKO and control wild-type mice with carotid arterial catheters were exposed to intermittent shaker stress for 7 days (2 min stressors, 45 times per day). Mean arterial pressure (MAP) and heart rate (HR) were recorded continuously (24 h) before stress (basal), on stress days 1, 3 and 7 (S1, S3 and S7) and 1 day poststress (recovery). Plasma corticosterone (Cort) was measured before stress and 30 min after the last stress on day 7. Twenty-four hour averages of MAP and HR were lower in OTKO mice than in controls ($P < 0.0001$ and $P < 0.005$, respectively) with a significant diurnal rhythm. Chronic stress (S1 and S3) produced an increase in 24 h average MAP in OTKO mice, but not in controls. There were no stress-related changes in 24 h average HR values between control and OTKO mice. The immediate pressor responses were analysed during the dark and light periods (19.00 and 08.00 h). During the dark period, stress-induced pressor responses were observed only in OTKO mice (S1 and S3). In the light period, stress-induced MAP increases were seen on all days in OTKO mice and on days S1 and S3 in controls. There were no differences in baseline Cort between the groups; however, OTKO mice showed a reduced response to chronic stress (+298 versus +411%, OTKO mice versus controls, $P < 0.005$). In conclusion, oxytocin deficiency alters the endocrine and pressor responses to chronic stress, suggesting that the endogenous oxytocin system is important in regulating the stress-induced pressor response.

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The neurohypophyseal hormone oxytocin (OT) is synthesized in neurones located in the supraoptic and paraventricular nuclei of the hypothalamus and secreted from axonal terminals in the neurohypophysis into the systemic circulation. The 'classical' OT effects are related to female reproductive physiology (Richard *et al.* 1991). However, the fact that circulating levels of OT and the pattern of OT distribution in the central nervous system (CNS) are sex independent supports the idea that OT has roles outside reproductive biology (Carter & Lightman, 1986; Haussler *et al.* 1990). Indeed, there is evidence that OT is important in pain sensitivity (Agren *et al.* 1995), emotional behaviour (Winslow *et al.* 2000), exercise tolerance (Michellini, 2001), baroreflex activity (Higa

et al. 2002) and stress responsiveness (Morris *et al.* 1995; Uvnas-Moberg, 1998). There are also data showing that OT and its receptors are present in peripheral organs, such as the heart, blood vessels and kidney, with suggestions that it is important in the regulation of sodium excretion, salt appetite and cardiac function (Richard *et al.* 1991; Gutkowska *et al.* 2000). Oxytocinergic neurones innervate brain regions important in cardiovascular control, such as nucleus tractus solitarius, nucleus ambiguus, locus coeruleus, dorsal motor nucleus of the vagus, dorsal raphe nucleus and intermediolateral cell column of the spinal cord (Buijs, 1978; Higa *et al.* 2002). There is evidence that OT has effects on blood pressure and heart rate although the results are often contradictory (Petty *et al.*

1985; Tan & Tsou, 1985; Petersson *et al.* 1996; Mack *et al.* 2002).

One way in which to investigate the function of oxytocin is by using OT-deficient mice (Young *et al.* 1996). These mice are able to procreate, but not to nurse their young. They are characterized by changes in social behaviour and salt appetite (Winslow *et al.* 2000; Puryear *et al.* 2001). Cardiovascular studies in oxytocin knockout (OTKO) mice indicate that there are changes in autonomic balance, seen as enhanced baroreflex function, and greater responses to cholinergic blockade (Michelini *et al.* 2003). OT is also a stress-reactive hormone. Elevated plasma and central levels of OT have been found in a variety of stress models (Lang *et al.* 1983; Callahan *et al.* 1992; Jezova *et al.* 1995; Nishioka *et al.* 1998) and there are diurnal changes in stress responsiveness (Carter & Lightman, 1986; Key *et al.* 2003). There is also evidence for a role of central OT in the mediation of cardiovascular and endocrine responses to stress. Hypothalamic lesions or treatment with OT peptide antisense oligonucleotides resulted in attenuation of the heart rate response to immobilization and shaker stress (Callahan *et al.* 1992; Morris *et al.* 1995).

The objective of this study was to use the oxytocin gene deletion model to study the role of OT in the cardiovascular and endocrine responses to stress. We employed shaker stress because it provides a mild, reproducible stimulus, which activates the cardiovascular system and adrenal axis (Nakata *et al.* 1993; Bernatova *et al.* 2002). Experiments were conducted in male OTKO mice with chronic carotid arterial catheters for continuous cardiovascular recording. Since previous studies had shown that there was a diurnal variation in stress-induced blood pressure changes and oxytocin secretion (Bernatova *et al.* 2002; Key *et al.* 2003), we examined the time course of the cardiovascular responses during the light (sleeping) and dark (active) periods of the day. In addition, we examined the effect of chronic stress on adrenal corticosterone (Cort) secretion, water and food intake and body weight.

Methods

Animals

Adult male control (wild type, OT+/+) and OTKO (OT−/−) mice with baseline body weights of 24 ± 1 g were used for this study ($n = 6$ – 7 per group). Colony founders were produced by W. S. Young III and colleagues (NIH, Bethesda, MD, USA; Young *et al.* 1996). Mice were bred in our animal facility using heterozygous (OT+/-) parents. Thus, the experimental animals have the same genetic and environmental background. Mice

were genotyped using DNA prepared from tail extracts with a polymerase chain reaction (Young *et al.* 1996). Mice were housed individually at 22°C on a 12 h–12 h dark–light cycle (05.00–17.00 h lights on, 300–350 Lx). Mice were maintained on a standard pellet diet (Harlan Teklad, 0.5% sodium by weight) and tap water, *ad libitum*. All experiments were approved by Wright State University's Laboratory Animal Care and Use Committee.

Mice were prepared with chronic catheters that were inserted into the left common carotid artery according to the method of Li *et al.* (1999) under ketemine–xylazine anaesthesia (5:1 mg kg⁻¹, i.m.). A ventral mid-line skin incision (~2 cm long) was made in the neck and the submaxillary glands were separated. The left common carotid artery was isolated under a binocular surgical microscope and a catheter was inserted into the carotid artery. After surgery, a heparinized saline solution (80 U ml⁻¹) was continuously infused into the catheter at 25 μ l h⁻¹ using a syringe pump (Model 220, Kd Scientific, Boston, MA, USA). The catheter (Micro-Renathane, MRE-025, Braintree Scientific, Inc., Braintree, MA, USA) was covered with a metal spring that was attached to a fluid swivel at the top of the cage. Mice were allowed to recover from surgery for at least 6 days before experimentation, by which time water and food intake had returned to basal levels.

Shaker stress

The animals' home cages were inserted into a specially designed cage rack, which was attached to the shaking platform (Model 5901; Eberbach Inc., Ann Arbor, MI, USA). The device was computer activated to provide intermittent shaker stress, 2 min of shaking (150 cycles min⁻¹; 2.8 cm stroke) followed by variable rest periods from 13 to 45 min (30 min average). The variable rest periods between shaking sessions were chosen with the aim of introducing unpredictability to the stress exposure. The animals were exposed to 45 shaking sessions per 24 h for 7 days. A previous study in C57BL mice showed that this stress protocol produced only small changes in body weight and the diurnal pattern of water consumption (Bernatova *et al.* 2002).

Plasma corticosterone

Plasma corticosterone (Cort) levels were compared in control and OTKO mice before stress exposure and after 7 days of stress (30 min after the last stress session, ~09.30 h). Blood samples (40 μ l) were collected

directly from the carotid arterial catheter into heparinized haematocrit tubes. After centrifugation (10 min, 1000 g, 4°C), plasma samples were stored at –30°C until assays were conducted. Plasma Cort was measured using the ImmChem™ double antibody corticosterone ¹²⁵I RIA kit (ICN Biochemicals, Inc. Costa Mesa, CA, USA). The assay required less than 5 µl of plasma.

Body weight, food intake and drinking activity

Body weight (BW), food intake and drinking activity were measured before stress exposure (Basal), on stress days 1, 3 and 7 (S1, S3 and S7) and on day 1 of recovery. Food intake was measured by daily weighing of the food. Drinking (licking activity) was recorded using a drinkometer system (Columbus Instruments, Columbus, OH, USA) interfaced with the computerized data acquisition system (MP 150, Biopac Systems Inc., Santa Barbara, CA, USA; Puryear *et al.* 2001). Circadian data of licking activity were recorded with a sampling rate of 85 Hz and converted from digital to numeric form using acquisition software. Data were processed by calculation of 10 min means, which were averaged for calculation of the dark and light period means. Incidental contacts of mice with the water bottles, registered as single data points, were excluded from the analysis.

Cardiovascular measurements

BP and HR were recorded continuously (24 h) on S1, S3 and S7 and on the day before and after stress (Basal and Recovery) using the chronic carotid arterial catheter that was connected to a pressure transducer (DCX III, Maxxim Medical, Athens, TX, USA). Systolic BP and diastolic BP (for calculation of mean arterial pressure, MAP) and HR were recorded (sampling rate 85 Hz) and converted from digital to numeric form using acquisition software (MP 150, Biopac Systems Inc., Santa Barbara, CA, USA). Data were processed by calculation of 10 min means of the respective variables. These 10 min means were averaged for calculation of the dark and light period means.

Pressor reactivity

Immediate responses of MAP during individual shaking sessions were evaluated before initiation of stress (Basal, without stress), on S1, S3 and S7 and during Recovery (day 1 after cessation of stress). The tests were made during the light and dark periods (08.00 and 19.00 h, respectively). This experimental design (automatic stress delivery and continuous cardiovascular recording) allows for easy

measurement during the day–night cycle. Responses were calculated as the values during baseline (2 min preceding shaking), during shaking (2 min) and 16 min after shaking (divided into two 8 min periods).

Statistical analysis

All results are presented as means ± s.e.m. Multifactorial ANOVA and Duncan's test were used for evaluation of differences. Values were considered to differ significantly if $P < 0.05$.

Differences in plasma Cort were evaluated using 2-way ANOVA (treatment × genotype). BW and food intake were analysed by 2-way ANOVA (genotype × day of experiment). Drinking activity was analysed using 3-way ANOVA (genotype × treatment × day phase). Alterations of 24 h MAP and 24 h HR were analysed using 3-way ANOVA (genotype × day of experiment × day phase). Pressor responses were analysed using 4-way ANOVA (genotype × day of experiment × day phase × time course).

Results

Plasma corticosterone

Basal Cort concentrations in control and OTKO mice were not different (29 ± 4.5 versus 18 ± 1.5 ng ml⁻¹, respectively; $P = 0.64$, Fig. 1). The main effect of treatment showed that stress produced significant increases in plasma Cort ($F_{1,20} = 26.0$, $P < 0.0001$). There was also a significant effect of genotype, with a lower plasma Cort response in OTKO mice ($F_{1,20} = 6.9$, $P < 0.02$). The Cort

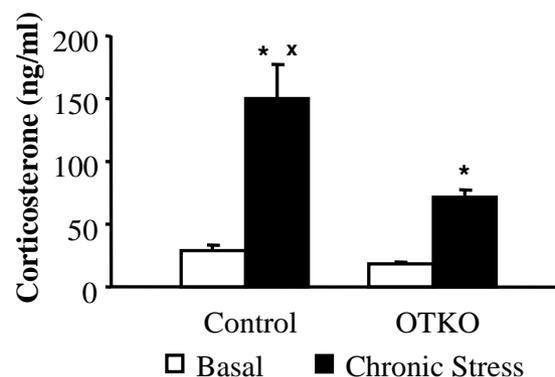


Figure 1. Plasma Cort response to chronic shaker stress

Main effects of stress and genotype were significant: stress ($F_{1,20} = 26.0$, $P < 0.0001$) and genotype ($F_{1,20} = 6.9$, $P < 0.02$).

* $P < 0.05$ versus basal, * $P < 0.005$ versus OTKO chronic stress. Values are means ± s.e.m.

Table 1. Effect of chronic shaker stress on body weight, food intake and drinking activity

	Basal	Day 1	Day 3	Day 7	Recovery
Body weight (g)					
Control	24.2 ± 0.7	NR	22.9 ± 0.7	22.9 ± 0.8	23.3 ± 0.8
OTKO	23.7 ± 1.3	NR	23.8 ± 1.2	24.3 ± 1.1	25.5 ± 1.2
Food intake (g)					
Control	5.1 ± 0.2	4.0 ± 0.3	4.5 ± 0.2	3.8 ± 0.6	4.4 ± 0.2
OTKO	4.9 ± 0.4	4.2 ± 0.4	4.2 ± 0.2	4.0 ± 0.1	4.3 ± 0.3
Drinking activity (licks × 10 ⁻³ per day)					
Control	2.2 ± 0.36	1.37 ± 0.36*	1.39 ± 0.28*	1.43 ± 0.27*	1.59 ± 0.32
OTKO	2.12 ± 0.36	1.45 ± 0.42*	1.57 ± 0.39*	1.47 ± 0.33*	1.98 ± 0.49

Stress reduced drinking activity in mice ($F_{4,114} = 9.8$, $P < 0.0001$, main effect of day). * $P < 0.03$ versus basal drinking of the same genotype. NR, not recorded. Values are means ± S.E.M.

increase in controls was ~400%, compared to ~300% in OTKO mice after 7 days of stress ($P < 0.005$).

Body weight, food intake and drinking activity

There were no observed differences in body weight and food intake between the groups (Table 1). Stress significantly decreased drinking activity ($F_{4,114} = 9.8$, $P < 0.0001$), but, there were no genotype-related differences (Table 1). Drinking activity was elevated during the dark period ($F_{1,114} = 651.1$, $P < 0.0001$, main effect of day phase). Average licking activity was more than 5 times greater in the dark than in the light period (2779 ± 84 versus 506 ± 52 licks per 12 h; $P < 0.0001$).

Blood pressure and heart rate

In order to analyse the effect of stress on cardiovascular function, we used 24 h MAP and HR recordings, data pooled for dark and light periods. Basal MAP in controls was 111 ± 2 and 104 ± 3 mmHg during dark and light periods, respectively (Fig. 2). In OTKO mice, basal MAP was 106 ± 2 and 100 ± 1 mmHg during dark and light periods, respectively. The MAP of OTKO mice was significantly lower than that of controls ($F_{1,102} = 24.55$, $P < 0.0001$, main effect of genotype). There was a significant diurnal difference in MAP in both genotypes ($F_{1,102} = 98.2$, $P < 0.0001$, main effect of day phase) with higher MAP during the dark period ($P < 0.0001$). Twenty-four hour MAP was elevated significantly in OTKO mice on S1 and S3 ($P < 0.05$), while there were no changes in controls.

Basal 24 h HR of control mice was 595 ± 9 and 563 ± 6 beats min⁻¹ during dark and light periods, respectively (Fig. 3). In OTKO mice, basal HR was 581 ± 7 and 548 ± 12 beats min⁻¹ during dark and light periods,

respectively. The HR of OTKO mice was significantly lower than that of the control group ($F_{1,92} = 7.9$, $P < 0.005$, main effect of genotype). There were significant diurnal differences in HR ($F_{1,92} = 46.24$, $P < 0.0001$, main effect of day phase) with higher HR during the dark period in both experimental groups ($P < 0.01$). There were no overall effects of stress on HR in either group.

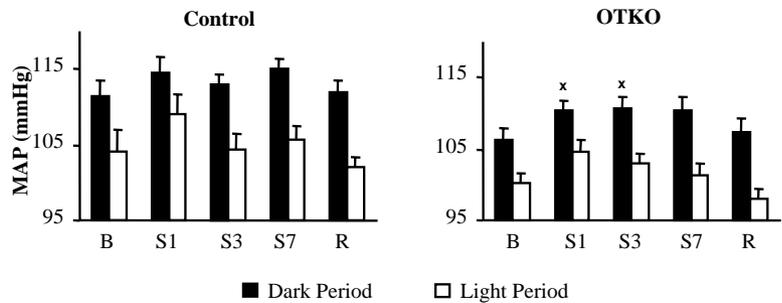
Blood pressure reactivity

For analysis of cardiovascular stress responsiveness, we measured MAP changes produced by shaker stress delivered during the dark and light periods (08.00 and 19.00 h, Figs 4 and 5, respectively). The analysis confirmed that MAP was lower in OTKO mice ($F_{1,392} = 12.25$, $P < 0.0006$, main effect of genotype). There were significant increases in MAP ($F_{4,392} = 17.04$, $P < 0.0001$, main effect of day of experiment) on stress days 1, 3 and 7 versus basal values ($P < 0.01$ during all days). A diurnal pattern in MAP was observed in both groups ($F_{1,392} = 140.48$, $P < 0.0001$, main effect of day phase) with higher blood pressure noted during the dark period ($P < 0.0001$ versus light period). Analysis of the main effect of time course ($F_{3,392} = 30.98$, $P < 0.0001$) revealed significant pressor responses during the shaking event ($P < 0.0001$, shaking versus baseline) with a gradual decrease in MAP after cessation of shaking. However, MAP in the time period 9–16 min postshaking was still significantly higher than baseline ($P < 0.04$).

The time course showed an immediate shaking-induced increase in MAP in OTKO mice in the dark period on day 1 (+20%, $P < 0.001$ versus baseline) with no changes seen in controls (Fig. 4). Evaluation of the overall pressor response (composite of MAP, before, during and after shaking) also

Figure 2. Twelve hour MAP averages for the dark and light periods under basal and stress conditions

Blood pressure was measured continuously. B, basal; S1, S3 and S7, stress days 1, 3 and 7; R, poststress recovery. The MAP of OTKO mice was significantly lower than that of the controls ($F_{1,102} = 24.55$, $P < 0.0001$, main effect of genotype). * $P < 0.05$ versus basal (24 h average). Values are means \pm S.E.M.



revealed changes only in OTKO mice ($P < 0.05$, S1 and S3 versus basal).

In the light period, the immediate pressor responses during shaker stress were similar in both genotypes on all days (+23–31% versus baseline, $P < 0.001$; Fig. 5) and the pressor responses were maintained through day 7. Overall pressor responses (composite of MAP, before, during and after shaking) were seen in OTKO mice on all stress days ($P < 0.005$, all stress days versus basal) and even during the recovery period ($P < 0.01$ versus basal). Overall pressor responses in controls were elevated only on S1 and S3 ($P < 0.03$ versus basal).

Discussion

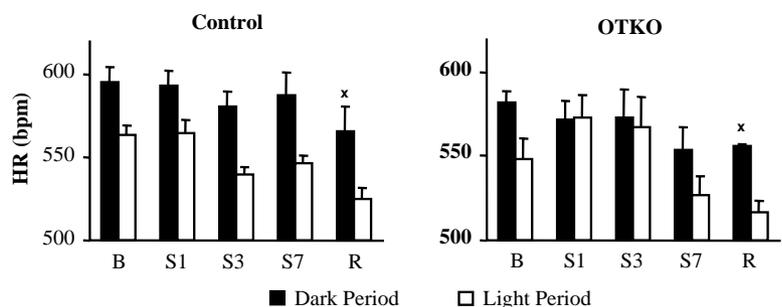
This study provides evidence that oxytocin is involved in the pressor as well as the endocrine responses to chronic stress. A role for OT in the regulation of blood pressure was seen under basal conditions and in response to chronic stress. There was a sustained reduction in BP in OTKO mice at rest and an increased BP response to chronic stress. With regard to the hypothalamo–pituitary–adrenal (HPA) axis, there was a reduction in stress-induced Cort release in chronically stressed OTKO mice. Thus, data provide evidence for a dissociation of pressor and endocrine activation in mice lacking the OT peptide.

There is increased interest in the idea that OT acts as a cardiovascular hormone. This has been spurred by studies which show that OT and its receptors are present in the heart and vasculature and that OT

has effects on blood pressure and salt intake (Uvnas-Moberg, 1998; Gutkowska *et al.* 2000; Michelini, 2001; Puryear *et al.* 2001). Our studies have focused on the use of OTKO mice for evaluation of the role of OT in cardiovascular function. Results show that OTKO mice have reduced baseline blood pressure, enhanced baroreflex gain and increased pressor response to OT infusion (Rigatto *et al.* 2003; Michelini *et al.* 2003). The data suggest that endogenous OT functions as a vasopressor peptide, similar to its complimentary peptide, vasopressin. This is supported by a study which showed that microinjection of OT into the rostral ventrolateral medulla produced a marked hypertension (Mack *et al.* 2002), in contrast to the lack of change produced by intracisternal OT injection (Petty *et al.* 1985). Peripheral injection of OT also affects blood pressure, although the responses are variable, with evidence for both pressor and depressor responses (Petty *et al.* 1985; Petersson *et al.* 1996; 1999). Baroreflex function, controlled by brainstem pathways, is modulated by oxytocinergic input. Higa and coworkers reported that OT and OT antagonists injected into the nucleus of the solitary tract and dorsal motor nucleus of the vagus of conscious rats produced opposite effects on baroreflex activity, accentuation or inhibition, respectively (Higa *et al.* 2002). In contrast, OT injected intracisternally produced a decrease in baroreflex index (Petty *et al.* 1985). Our studies in OTKO mice also suggest that OT inhibits baroreflex function, assuming that there is a direct relationship between the change produced by OT deficiency and the physiological action of the peptide

Figure 3. Twelve hour HR averages for the dark and light periods under basal and stress conditions

Heart rate was measured continuously. B, basal; S1, S3 and S7, stress days 1, 3 and 7; R, poststress recovery. The HR of OTKO mice was significantly lower than that of the controls ($F_{1,92} = 7.9$, $P < 0.005$, main effect of genotype). * $P < 0.05$ versus basal (24 h average). Values are means \pm S.E.M.



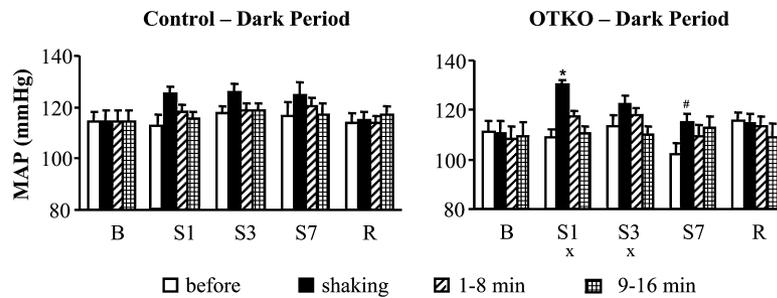


Figure 4. Time course of the BP response to individual shaker stresses administered during the dark period (19.00 h)

B, basal; S1, S3 and S7, stress days 1, 3 and 7; R, poststress recovery. Basal recordings were performed 1 day before initiation of the chronic stress (no shaking) and recovery recordings were made after cessation of chronic stress (no shaking). The immediate shaking-induced pressor responses were measured at the same time of day. Results show MAP before shaking (2 min preceding shaking), during shaking (2 min) and after shaking (1–8 min and 9–16 min postshaking). * $P < 0.001$ versus before shaking (\square) on the same day, # $P < 0.03$ versus S1 shaking (\blacksquare). The overall effect of stress exposure (average of all bars) was significant versus overall basal values before initiation of chronic stress (B) if denoted by 'x' under day ($P < 0.03$).

(Higa *et al.* 2002). The mechanism(s) by which a deficit in OT secretion affects blood pressure and heart rate are not known. While OTKO mice have lower blood pressure than controls, there is not a generalized decrease in BP sensitivity to pressor and depressor stimuli, suggesting that vascular responsiveness is not altered (Michellini *et al.* 2003). Sodium/osmolar balance affects cardiovascular status; however, there are no demonstrable changes in haematocrit, osmolality or sodium balance in OTKO mice (Amico *et al.* 2001; Puryear *et al.* 2001; Goldstein *et al.* 2002). There is evidence for an enhancement in salt appetite under basal conditions and in response to volume depletion (Puryear *et al.* 2001; Rigatto *et al.* 2003), suggesting that there are changes in the central sensing

mechanisms. The effects of OT on the heart may also play a role in its cardiovascular actions. OT stimulates cardiac atrial natriuretic peptide (ANP) secretion, resulting in natriuresis (Verbalis *et al.* 1991; Gutkowska *et al.* 2000), and elicits direct effects to cause bradycardia and decreased strength of cardiac contraction (Gutkowska *et al.* 2000; Mukaddam-Daher *et al.* 2001). However, if OT is critical in the maintenance of ANP secretion, one would predict decreased levels of ANP in OTKO mice, resulting in volume expansion and hypertension, and increased heart rate. These effects were not observed in the OTKO strain. The finding of reduced 24 h MAP and HR in OTKO mice suggests that the central actions of OT may outweigh its peripheral actions. This is supported by a study

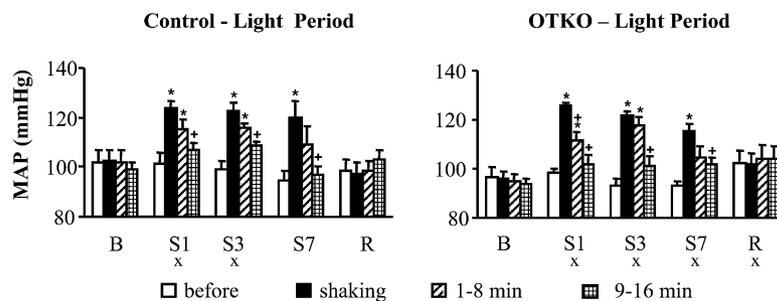


Figure 5. Time course of the BP response to individual shaker stresses administered during the light period (08.00 h)

B, basal; S1, S3 and S7, stress days 1, 3 and 7; R, poststress recovery. Basal recordings were performed 1 day before initiation of chronic stress (no shaking) and recovery recordings were made after cessation of chronic stress (no shaking). The individual stress responses were measured at the same time of day. Results show MAP before shaking (2 min preceding shaking), during shaking (2 min) and after shaking (1–8 min and 9–16 min postshaking). * $P < 0.05$ versus before shaking (\square) on the same day, + $P < 0.05$ versus shaking (\blacksquare) on the same day. The overall effect of stress exposure (average of all bars) was significant versus overall basal values before initiation of chronic stress (B) if denoted by 'x' under day ($P < 0.01$).

which showed that microinjection of OT into the rostral ventrolateral medulla produced an increase in heart rate (Higa *et al.* 2002).

Interactions between the renin–angiotensin system and OT were suggested by the results of studies testing the effect of haemorrhage in dogs. OT treatment prevented haemorrhage-induced hypotension, an effect that was associated with increased plasma renin activity (Brooks *et al.* 1984). We found that intracerebroventricular injection of angiotensin produced pressor responses in OTKO mice, which were equivalent to those in controls (Rigatto *et al.* 2003). This indicates that increased sensitivity of central angiotensin signaling cannot explain the blood pressure status. Analysis of cardiovascular stress responsiveness revealed a marked increase in the pressor response when the stress was delivered during the light period (nonactive, sleeping phase for rodents). This diurnal rhythm in stress reactivity confirms the results of a previous study which used C57BL6 mice (Bernatova *et al.* 2002). It is interesting that, in addition to the pressor rhythm, stress-induced OT secretion is also coordinated with the light cycle (Carter & Lightman, 1986; Key *et al.* 2003). We found that shaker stress increased plasma OT only when it was administered during the light period. The HPA axis was still active, since stress stimulation of Cort was similar during the light and dark periods (Key *et al.* 2003). It is thought that the differential changes in stress responsiveness may be associated with changes in the autonomic nervous system. In the light period, when sympathetic outflow is depressed, the pressor response to the same stressor may be higher than during the dark period, when sympathetic drive is active and basal BP is higher. These time-related changes in stress reactivity may have clinical implications related to the timing of cardiovascular pathologies (Muller, 1999).

Although both groups showed diurnal differences in BP reactivity, OTKO mice showed an enhanced BP response to stress. Stress-induced pressor responses during the light period were seen in OTKO mice on all stress days, continuing into the recovery period. In the dark period, a time at which stress reactivity is at a nadir, OTKO mice showed pressor responses on stress days 1 and 3. In controls there were no stress-induced blood pressure changes during the dark period. Similarly, studies in nursing mothers, with physiologically increased OT levels, demonstrated that high plasma OT levels were associated with a reduced BP response to stress (Light *et al.* 2000). Together, these data support the idea that OT may act as an antistress hormone with regard to the cardiovascular axis.

Investigation of the HPA axis showed that plasma Cort levels were similar under basal conditions, while OTKO mice were less responsive to chronic stress. Previous studies also showed interactions between the OT and HPA axes. In rats, central OT administration blunted noise stress-induced Cort release (Windle *et al.* 1997; Lightman & Young, 1989), while there was an enhanced CNS effect of acute restraint stress in OTKO mice (Nomura *et al.* 2003). These studies using OT supplementation and genetic deficiency indicate that OT may inhibit the endocrine stress response. In our studies, there was a marked decrease in stress-induced Cort release in mice lacking OT. However, one difference between these studies is the use of acute *versus* chronic stress stimulation. Assuming that OTKO mice are hyper-responsive to acute stressors (Nomura *et al.* 2003), it is feasible that chronic stress exposure (315 sessions per 7 days) may produce HPA exhaustion.

There is other evidence which supports a positive effect of OT on the HPA axis. *In vitro* studies showed that OT may augment the effect of corticotrophin-releasing hormone on ACTH secretion. There was a reduction in stress-induced ACTH levels when OT was neutralized with a specific antiserum, a type of pharmacological knockout (Gibbs, 1985). These data fit with our findings of lower stress-induced Cort responses in OTKO mice. We assume that the lack of OT may result in attenuation of ACTH-dependent Cort release. However, additional studies are needed to determine the mechanisms by which the endocrine systems interact to regulate stress responsiveness.

In conclusion, deletion of the OT gene and protein altered the endocrine and cardiovascular responses to chronic stress in mice. Shaker stress-induced pressor responses were more pronounced in OTKO mice despite a lower basal MAP and reduced Cort responses. We hypothesize that endogenous OT may play a protective role by attenuation of stress-induced cardiovascular changes.

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Short communication

Extraction of oxytocin and arginine–vasopressin from serum and plasma for radioimmunoassay and surface-enhanced laser desorption–ionization time-of-flight mass spectrometry

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Abstract

Oxytocin and arginine–vasopressin (AVP) are secreted into the blood in low concentrations. To analyze these peptides, we investigated two common extraction procedures, acetone–ether precipitation and C₁₈-SepPak columns. Recovery from both procedures approached 70–80% of the spiked amount, though the SepPak columns were more efficient. C₁₈-SepPak columns were used to sequentially separate oxytocin from AVP by eluting oxytocin first with 98% acetone followed by elution of AVP with 80% acetonitrile. Surface-enhanced laser desorption–ionization time-of-flight mass spectrometry (SELDI-TOF MS) was used to analyze oxytocin and AVP extracted with C₁₈-SepPak columns from an autistic patient's plasma sample. We conclude that C₁₈-SepPaks provide more consistent and efficient peptide extraction from serum or plasma that augments both quantitative and qualitative analysis by radioimmunoassay and SELDI-TOF MS.

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Keywords: Oxytocin; Arginine–vasopressin

1. Introduction

Oxytocin and arginine–vasopressin (AVP) are peptide hormones composed of nine amino acids, derived from the intracellular cleavage of related precursor prohormones and released into the blood to regulate endocrine and neuroendocrine responses throughout the body [1–5]. Due to the small concentrations secreted and their short half-life, peptide hormones, such as AVP and oxytocin, are normally found in the blood in relatively low concentrations

(<20 pg/ml) [6–9]. Furthermore, the small sample volumes often obtained from animal experiments (<300 µl of plasma) are not sufficient for multiple analyses of peptides from the same sample and are often irreplaceable. Thus, it would be advantageous to extract and separate peptides of interest from the same sample for independent analysis. While the extraction can be accomplished using acetone–ether precipitation or C₁₈-SepPaks, the separation of the two peptides is made difficult because oxytocin and AVP have nearly identical primary amino acid sequences, i.e. CYFNPCRG for AVP (1084.25 daltons) vs. CYINPCLG for oxytocin (1009.22 daltons). We provide evidence that these two peptides can be separated from each other using C₁₈-SepPaks and a

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multiple solvent system in preparation for quantitative analysis by radioimmunoassay (RIA).

In addition to quantitation of peptide levels, qualitative analyses may also be required for more in depth studies of plasma peptide hormones, e.g. mass. We used surface-enhanced laser desorption–ionization time-of-flight mass spectrometry (SELDI-TOF MS) to analyze plasma from an autistic child for the oxytocin peptide. SELDI-TOF MS (Ciphergen, LaJolla, CA, USA), utilizes chemically treated metal chips that specifically bind peptides and proteins based on their chemical characteristics [10,11]. Following a wash step, peptides and proteins retained on the chips are analyzed by time of flight mass spectrometry. The aim of the present paper was to compare the extraction qualities of two protein extraction methods, C_{18} -SepPaks and acetone–ether, followed by peptide analysis using RIA and SELDI-TOF MS ProteinChip technology.

2. Experimental

2.1. Oxytocin and AVP radioimmunoassay

All chemicals are of reagent grade. Acetone, acetonitrile and trifluoroacetic acid were HPLC grade. Oxytocin (Bachem) and AVP (Bachem) were iodinated by the chloramine T method and separated from free ^{125}I by C_{18} -SepPak (New England Nuclear). [^{125}I]Oxytocin or [^{125}I]AVP was diluted in a buffer containing $1\times$ PBS, 1% BSA and 1 mM phenylmethylsulfonyl fluoride (PMSF) (RIA buffer). Unlabeled ligand inhibition of binding and displacement curves were generated using oxytocin antibody concentrations (1:30 000) or AVP antibody concentrations (1:25 000) that yielded 20–30% [^{125}I]oxytocin or [^{125}I]AVP binding. Separation of bound and free [^{125}I]oxytocin or [^{125}I]AVP was achieved by addition of 20 μl horse serum followed immediately by 1 ml of 18% polyethylene glycol 8000. After centrifugation and removal of the supernatant, the pellet was counted for 1 min in a MicroMedia 4/200 Plus automatic gamma counter. The resulting cpm were analyzed by a four parameter method [12]. For oxytocin, the average effective dose (ED) ED_{20} was 15 ± 0.13 pg/ml, the average

ED_{50} was 3.8 ± 0.4 pg/ml, the average ED_{80} was 0.62 ± 0.25 pg/ml. For AVP, the ED_{20} was 21 ± 7 pg/ml, the ED_{50} was 5.3 ± 1.9 pg/ml and the ED_{80} was 0.74 ± 0.3 pg/ml. The limit of quantitation by RIA for oxytocin and AVP was determined to be 0.49 ± 0.1 and 0.66 ± 0.18 pg/ml, respectively.

2.2. C_{18} -SepPak and Accubond peptide extraction

SepPak (Millipore) C_{18} and Accubond (Fisher) columns were pre-conditioned with 1 ml methanol, followed by 1 ml of distilled water. Various concentrations of oxytocin were added to heat-inactivated horse serum and passed through the columns. The columns were washed with 6 ml of water followed by 3 ml of 3% acetone and fractions collected. The peptides were eluted by 3 ml of 98% acetone followed by 3 ml of 80% acetonitrile containing 0.1% TFA (v/v) and 1 ml fractions were collected. All samples were evaporated to dryness on a Savant Speed Vac and reconstituted in 0.2 ml of RIA buffer.

2.3. Acetone–ether peptide extraction

Ice-cold acetone was mixed with 1 ml of heat-inactivated horse serum containing different concentrations of oxytocin and centrifuged in a Beckman J-6B centrifuge at 3500 rpm for 20 min. The upper, acetone layer was transferred to a clean glass tube and 1 ml of petroleum ether (ether) added, mixed and allowed to sit for 5 min. The upper ether layer was discarded and the lower layer was evaporated to dryness on a Savant Speed Vac. The dried residue was resuspended in 0.2 ml RIA buffer for RIA analysis.

2.4. Ciphergen ProteinChip SELDI-TOF MS

For analyzing plasma oxytocin from an autistic child, 1 ml of plasma was extracted with a C_{18} -SepPak as outlined above and eluted with 80% acetonitrile. The samples were evaporated to dryness and each tube was reconstituted in 25 μl 0.1 M HCl. From this, 1 μl was spotted onto a weak cation-exchange (WCX2) protein chip for 30 min in a moist chamber followed by washing with distilled water. Matrix, alpha-cyano-4-hydroxy cinnamic acid

(CHCA) in 50% acetonitrile containing 0.1% TFA (0.5 μ l) was added to the spots and allowed to dry. The chip was analyzed with a spot protocol that ionized each spot four times over 20 different areas. The spot was initially ionized one laser hit at 200 intensity, with the sensitivity set at 10, followed by four laser hits at 195 intensity. The source voltage was set at 20 000 V and detector voltage at 1900 V. The 80 laser hits were averaged for each spot and the resulting protein profile analyzed to determine the mass of each peak. An aliquot of the eluate from the autistic patient sample was analyzed by RIA for oxytocin. Oxytocin and AVP standards (20 pg/ μ l) were also analyzed by SELDI-TOF MS in serum, were spotted on WCX2 chips and analyzed with the same spot protocol used for analysis of the plasma from the autistic child.

3. Results and discussion

3.1. Comparison of acetone–ether with C_{18} -SepPak and Accubond columns

The acetone–ether procedure was used to extract [125 I]oxytocin or [125 I]AVP from serum (1 ml each). Addition of acetone to serum causes precipitation of larger proteins while smaller peptides remain in the aqueous phase. Approximately 15% of the [125 I]oxytocin and 13% of the [125 I]AVP cpm were precipitated following addition of the acetone to the spiked serum samples (Fig. 1A). The aqueous layer was transferred to another tube, ether added and mixed. After 5 min, the upper ether layer was removed to a clean tube. Less than 8% of the [125 I]oxytocin and 12% of the [125 I]AVP were removed with the ether layer. The aqueous layer contained the majority of the [125 I]oxytocin or [125 I]AVP cpm (~75–77% for both).

C_{18} -SepPaks were loaded with an aliquot of the same serum (1 ml) containing [125 I]oxytocin or [125 I]AVP. Less than 3% of the [125 I]oxytocin or 7% of the [125 I]AVP cpm were observed in the flow through and water wash fractions, indicating a high efficiency of retention on the column (Fig. 1B). In addition, less than 4% of the [125 I]oxytocin cpm were found in the 3% acetone wash fraction. Approximately 92% of the cpm were eluted with 98%

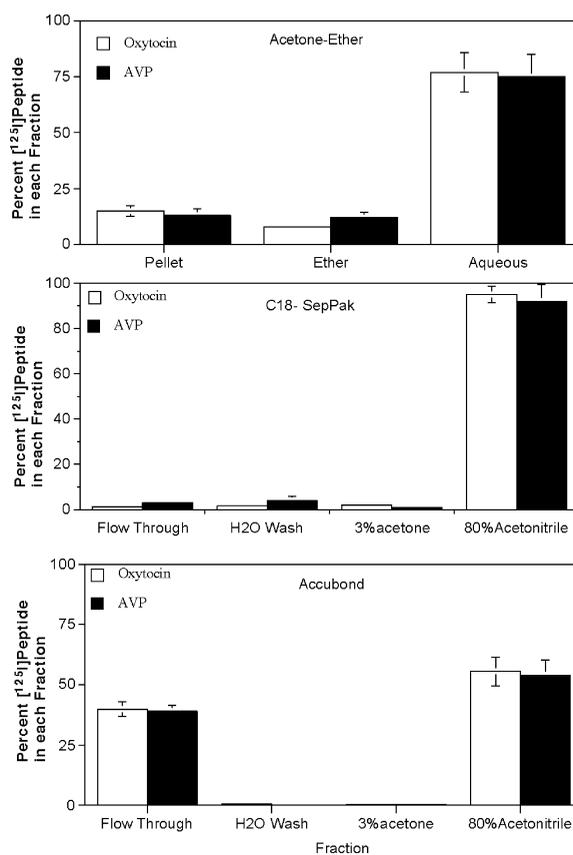


Fig. 1. Relative amount of [125 I]oxytocin ([125 I]OT) or [125 I]AVP recovered using acetone–ether, C_{18} -SepPak or Accubond Column extraction procedures. The cpm added were ~5000–6000 per assay. (A) Pellet, represents the percent of [125 I]oxytocin or [125 I]AVP remaining in the pellet after addition of acetone. Ether represents the percent cpm removed from the aqueous phase which contains the majority of the iodinated peptides. (B) Extraction of [125 I]oxytocin or [125 I]AVP using C_{18} -SepPak columns. (C) Extraction of [125 I]oxytocin or [125 I]AVP using Accubond columns. H₂O wash indicates washing the column with distilled water. The data represent the mean \pm SEM of duplicate measurements from three experiments.

acetone (Fig. 1B). Less than 1% remained bound to the SepPak columns. Accubond C_{18} columns yielded a profile, in which ~40% of the cpm ([125 I]oxytocin or [125 I]AVP) were found in the flow through (Fig. 1C). The results suggest that less [125 I]oxytocin or [125 I]AVP was lost during the initial wash steps from the C_{18} -SepPaks than from the Accubond columns or in the precipitation with acetone–ether.

3.2. Comparison of acetone–ether with SepPak and Accubond columns

Preliminary studies using iodinated oxytocin and iodinated AVP suggested that a higher recovery could be obtained using the SepPak extraction procedure. The extraction efficiency of acetone–ether, SepPaks and Accubond columns was compared by extracting serum (1 ml) containing; 0, 1, 2.5, 5, 10 and 20 pg of unlabeled oxytocin. The amount recovered for each concentration was determined by RIA. In Table 1, acetone–ether extraction gave values higher than the absolute amount spiked through 2.5 pg oxytocin. From 5 to 20 pg, the recovery decreased from 78 to 62% of the spiked values, respectively. The percent recovery from C₁₈-SepPak columns ranged between 65% at 1.25 pg/ml to a high of 80% at 10 pg/ml spiked oxytocin (Table 1). Accubond columns were less efficient, yielding only 21–54% recovery at each concentration (Table 1). In addition, a salt crystal was often found in the tube after evaporating the aqueous phase to dryness. When the sample with the salt was resuspended for RIA analysis, the increased salt concentration was found to affect the RIA. Using the standard error of the mean (SEM) as an indicator of the variability in the three assays; the C₁₈-SepPak columns provided the least variability among the three methods. The percent recovery using the C₁₈-SepPaks was consistently higher than acetone–ether precipitation. Together, these results suggest that the C₁₈-SepPaks are more efficient and more accurate than acetone–ether precipitation or accubond columns and are the preferred method for extracting oxytocin from serum or plasma samples.

3.3. Extraction and separation of oxytocin and AVP using C₁₈-SepPaks

Oxytocin and AVP, 20 pg of each, were mixed with serum (1 ml) and loaded onto a single C₁₈-SepPak column. The column was washed with H₂O and 3% acetone. The column was eluted with 98% acetone followed by elution with 80% acetonitrile. Fractions were collected, evaporated to dryness and reconstituted in 200 µl of RIA buffer and analyzed by RIA. Water and 3% acetone washes caused a negligible release of either peptide (<15%). Elution with 98% acetone resulted in release of 80% of the oxytocin and 15% of the AVP (Fig. 2A and B, respectively). Elution with 80% acetonitrile caused the remainder of the oxytocin to be released from the SepPak as well as AVP (>80%) (Fig. 2A,B). These results are similar to those reported previously [13]. Since acetone–ether precipitation is not capable of separating the oxytocin and AVP peptides, SepPak extraction is the better method for extracting oxytocin and AVP from the same sample for separate analysis. Microliter pipet tips containing C₁₈-SepPak material have been found to provide similar separations, though in much smaller volumes, i.e. <50 µl.

3.4. Qualitative SELDI-TOF MS analysis of oxytocin and AVP

SELDI-TOF MS is a relatively new mass spectrometric technique in which proteins and peptides are applied to chemically treated metal chips, the chips washed with a buffer and the proteins that are retained on the chips analyzed by time-of-flight mass

Table 1
Percent recovery of oxytocin using acetone–ether, C₁₈-SepPaks or Accubond columns

Method	Oxytocin spiked (pg/ml) ^a				
	1.25	2.5	5.0	10.0	20.0
Acetone–ether	143±29 ^b	110±11	79±7	62±4	61±8
C ₁₈ -SepPak	65±5	73±3	73±3	80±3	76±7
Accubond	54±22	21±14	28±12	24±3	45±7

^a Increasing concentration of oxytocin were added to 1 ml of serum and extracted using acetone–ether, C₁₈-SepPak columns and Accubond columns. The amount of oxytocin recovered was determined by RIA.

^b Results represent the average percent recovered±standard error of the mean (SEM) for three separate experiments.

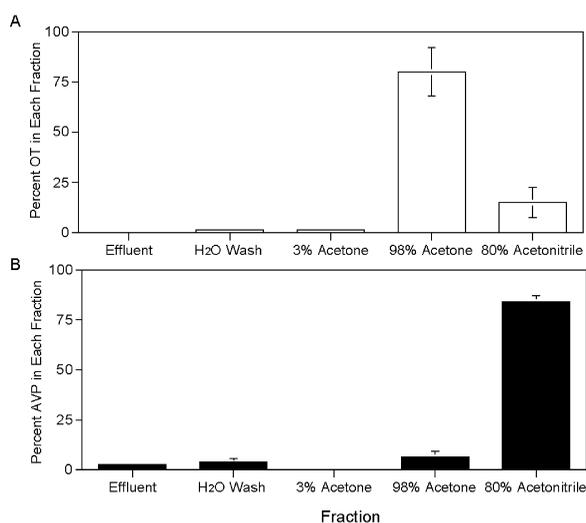


Fig. 2. Extraction and separation of oxytocin (A) and AVP (B) using C_{18} -SepPaks. The SepPak column was loaded with 20 pg of oxytocin and AVP in 1 ml of serum. The columns were washed with 6 ml of distilled water followed by 3 ml of 3% acetone. The peptides were eluted from the SepPak with 3 ml of 98% acetone and with 3 ml 80% acetonitrile. Fractions were collected and the amount of peptide determined by RIA. The results represent the mean \pm SEM for three experiments.

spectrometry. Oxytocin and AVP standards (20 pg each) were analyzed on WCX2 chips (weak cation-exchange) using SELDI-TOF MS (Fig. 3A). The mass determined for each peptide following SELDI-TOF MS was 1009.22 M_r for oxytocin and 1030.76 M_r for its sodium peak. Likewise, AVP was seen as a single peak of 1084.24 M_r with its sodium peak at 1105.3 M_r . Limitations to SELDI-TOF MS are similar to comparable MALDI-TOF MS systems, i.e. low concentration of peptides, high salt concentrations, detergents and other contaminants that can cause low sensitivity and even suppression of the peptide peak intensity [14–16]. Therefore, prior to attempting SELDI-TOF MS, it was first necessary to extract the peptides from the plasma using C_{18} -SepPaks.

Plasma (1 ml) from the blood of a 7-year-old male diagnosed with autism was extracted and concentrated using a C_{18} -SepPak that was washed and eluted with 80% acetonitrile. When the eluate was analyzed by SELDI-TOF MS using a WCX2 chip,

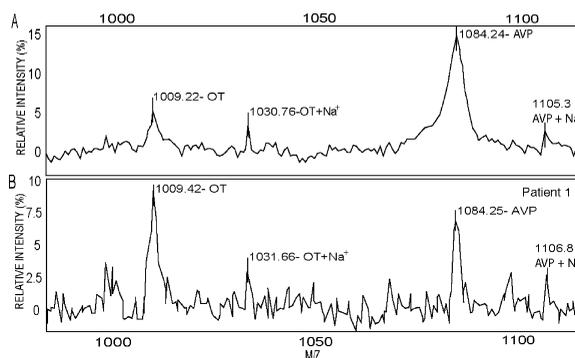


Fig. 3. SELDI-TOF MS ProteinChip analysis of oxytocin and AVP. (A) Serum containing 20 pg/ μ l of oxytocin (OT) and AVP was analyzed by SELDI-TOF MS on a WCX2 proteinchip. The spectrum revealed peaks for oxytocin (1009.22 M_r), oxytocin+sodium (1030.56 M_r), AVP (1084.21 M_r) and AVP+sodium (1105.56 M_r). These peaks were absent when oxytocin and AVP were omitted from the serum (data not shown). (B) Plasma from the blood of a child diagnosed with autism was extracted by C_{18} -SepPak and analyzed by SELDI-TOF MS. The peaks for OT (1009.22 M_r), OT+sodium (1031.66 M_r), AVP (1084.25 M_r) and AVP+sodium (1106.80 M_r) were observed.

peaks corresponding to oxytocin, AVP and their corresponding sodium ions, were identified (Fig. 3B, patient sample). The observation of oxytocin at 1009.42 M_r and its sodium adduct at 1031.66 M_r suggests that in this patient, the correct processing of oxytocin was not altered as had been proposed by other researchers [8,17].

The concentration of oxytocin in the autistic patient sample was determined by RIA to be 4.16 pg/ml. This value is higher than previously published values that were obtained using the acetone-ether method for extracting patient plasma [8], but consistent with other studies on plasma oxytocin levels [6,7].

4. Conclusions

The methods outlined in this paper show that C_{18} -SepPak columns provide better efficiency of recovery and accuracy for peptide hormone extraction from serum than acetone-ether precipitation or Accubond columns. This paper is the first to describe the analysis of the peptide hormones, oxytocin and

AVP, in blood samples using SELDI-TOF mass spectrometry.

5. Nomenclature

AVP	arginine–vasopressin
OT	oxytocin
SELDI-TOF MS	surface-enhanced laser desorption–ionization time-of-flight mass spectrometry
RIA	radioimmunoassay
ELISA	enzyme-linked immunosorbant assay

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C-Terminal sequencing of peptide hormones using carboxypeptidase Y and SELDI-TOF mass spectrometry

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The identification and precise analysis of peptides expressed and processed under different conditions is an integral part of the new and growing field of proteomics (1–5). Of particular interest is the proper identification of the C termini of peptide hormones. Prohormones are cleaved at the C termini of paired-basic residues. Thus, the N-terminal amino acids may be the same, whereas the C-terminal amino acids may be modified or extended and cause altered bioactivity or even inhibit the cleavage of the prohormone by endoproteases (6–11). Recently, a method was described in which peptides were digested with the enzyme carboxypeptidase Y (CPY) on the sample plate and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to determine the amino acid sequence of the peptides (12). Here, we describe further development of this method for sequencing peptides in both simple and complex mixtures using the unique properties of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) (1,4).

For this analysis, we first sequenced the peptide hormone, adrenocorticotrophic hormone (ACTH₁₋₃₉) (Phoenix Pharmaceuticals, Belmont, CA, USA). ACTH₁₋₃₉ is endogenously expressed in the anterior pituitary and is the precursor to other peptide hormones [e.g., α -melanocyte stimulating-hormone (α -MSH)]. ACTH₁₋₃₉ (1 μ g/ μ L) was diluted in deionized water to 100 ng/ μ L. From this, 1 μ L was applied to a spot on an H4 (hydrophobic) ProteinChip[®] array (Ciphergen Biosystems, Fremont, CA, USA) and air-dried. The array was placed in a “moist chamber” (i.e., pipet tip box containing a wet paper towel).

For the sequencing reaction, CPY (30 U/ μ L; Pierce, Rockford, IL USA) was diluted to 2.4×10^{-3} , 1.2×10^{-3} , and 8×10^{-4} U/ μ L in 50 mM MES, pH 6.5. One microliter of each dilution was added to the peptide on the array, and the moist chamber was placed in a 37°C incubator for 5 min. After incubation, 1 μ L of matrix, α -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile containing 0.1% trifluoroacetic acid, was added to each spot to stop the reaction. The array was analyzed using a PBSII ProteinChip reader (Ciphergen Biosystems).

The C-terminal amino acid sequence of ACTH₁₋₃₉ was derived using this protocol (Figure 1A). For the 2.4×10^{-3} dilution of CPY, the spectra revealed a “ladder” of 11 ion peaks, beginning with the original ion peak for ACTH₁₋₃₉ [mass/charge (M/Z) of 4578.7]. Each successively smaller ion peak represents the removal of an amino acid from the C terminus of the peptide. The difference in mass for each peak represents the mass of an amino acid. The single nomenclature amino acid sequence identified by the Ciphergen Biosystems 3.1 software was ESAEAFPi/IEF (Figure 1A). Since isoleucine and leucine have the same mass (approximately 113.1 Da), the software identified the third amino acid from the C terminus as either isoleucine or leucine. The known sequence for ACTH₁₋₃₉ is: ESAEAFPLEF, which represents a 100% homology with the sequence generated on the ProteinChip. Further dilution of CPY yielded similar results, the only exception being that only 9 and 5 amino acids could be easily identified at

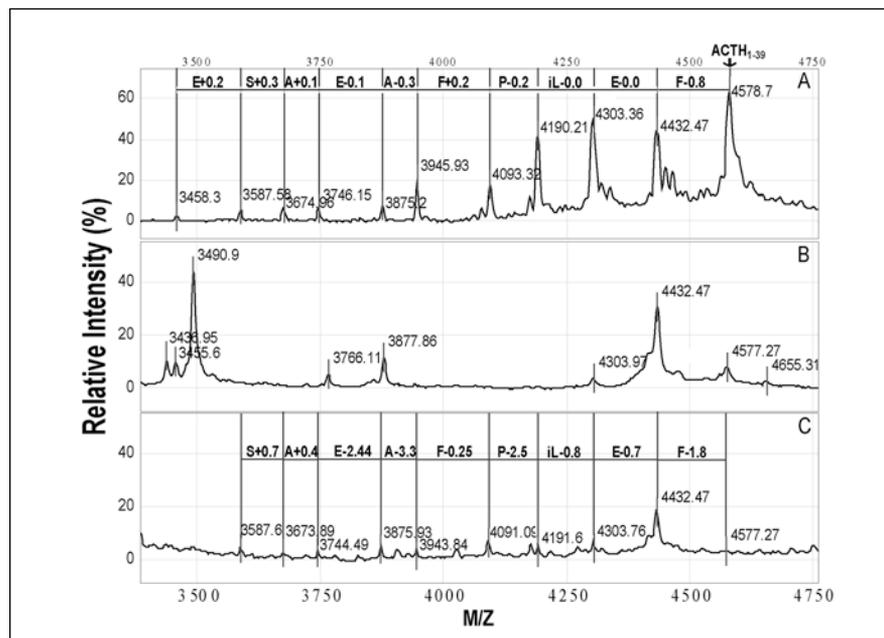


Figure 1. On-chip C-terminal sequencing of simple and complex mixtures of peptides by enzyme digestion. M/Z indicates mass/charge and is represented as daltons in the spectra. Representative spectra generated by digestion of synthetic adrenocorticotrophic hormone (ACTH₁₋₃₉) with carboxypeptidase Y (CPY). (A) ACTH₁₋₃₉ (4578.7 Da) is labeled by an arrow. Each ion peak is labeled with the mass for the peptide and on the line between ion peaks with the single amino acid code for the amino acid derived from subtracting the smaller peak from the larger peak and comparing with a table of the mass for each amino acid. The number beside each amino acid indicates the difference in daltons between the derived amino acid mass and the mass of the true amino acid. (B) The spectrum represents the region around 4578 Da (ACTH₁₋₃₉) from the anterior pituitary prior to treatment. (C) After treating the sample with CPY, the same area was scanned, and new ion peaks were identified. Note that after the incubation, ACTH₁₋₃₉ was completely absent, and ACTH₁₋₃₈ was diminished in intensity.

Table 1. Expected Versus Observed Ion Peaks for α -MSH

Sequence	des-acetyl- α -MSH		α -MSH		di-acetyl- α -MSH		Amino Acid
	Exp	Obs	Exp	Obs	Exp	Obs	
SYSMEHFRWGKPVG	1679	1679.72	1721	1721.93	1763	1766.66	—
SYSMEHFRWGKPV	1622	1621.81	1664	1664.1	1706	1706.05	G
SYSMEHFRWGKVP	1523	1523.13	1565	1565.77	1607	1607.57	V
SYSMEHFRWGK	1426	—	1468	1470.98	1510	1511.43	P
SYSMEHFRWG	1298	—	1340	—	1382	1379.08	K
SYSMEHFRW	1241	1243.57	1283	1282.66	1325	1323.47	G

All ion peaks are in daltons. The single amino acid code is used for the amino acid sequence. Exp indicates expected ion peak and Obs indicates observed ion peak. Not all ions were observed (i.e., 1426 and 1298 Da). The amino acid column indicates the amino acid removed from the C terminus. α -MSH, α -melanocyte stimulating-hormone; G, glycine; V, valine; P, proline; K, lysine.

1.2×10^{-3} and 8×10^{-4} U/ μ L dilutions, respectively. Longer incubation times or a higher concentration of CPY resulted in the generation of smaller peptide fragments (i.e., 20–26 amino acids long) (data not shown).

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 adjacent ion peaks, important sequence data can be obtained. Mouse anterior pituitary tissue was suspended in 30 μ L 0.1 M

HCl and homogenized with a plastic 0.5-mL microcentrifuge homogenizer, centrifuged at 15,000 \times g for 5 min, and the soluble protein was adjusted to 1 μ g/ μ L. One microgram of protein was applied to an H4 ProteinChip, dried, washed with 5 μ L distilled water, dried again, and then 1 μ L of CPY (0.0024 U/ μ L) was added in the moist chamber. After 15 min, CHCA was added to stop the reaction, the chip was air-dried and scanned in the ProteinChip reader. A separate spot was analyzed without CPY digestion to determine the origi-

nal ion peaks for comparison (Figure 1B). As an internal calibration, after the entire ProteinChip had been analyzed, ACTH₁₋₃₉ (10 ng) was added to each spot, the ProteinChip was analyzed again, and the spectra was calibrated to the new ACTH₁₋₃₉ ion peak.

Based on the known mass for ACTH₁₋₃₉ (4578 Da), initial examination of this region of the mass spectra revealed several ion peaks. In contrast to the synthetic ACTH₁₋₃₉ used in Figure 1A, the intensity of the ion peak representing endogenously expressed ACTH₁₋₃₉ in the anterior pituitary was very small, while the ion peak representative of ACTH₁₋₃₈ was larger, indicating that it was the major form of ACTH in this sample. After digestion with CPY, eight ion peaks were formed that were identified as ACTH₃₀₋₃₈, respectively (Figure 1C), as compared with the ion peaks generated from CPY treatment of synthetic ACTH₁₋₃₉. The identity of the amino acids was determined manually, rather than relying entirely on the software package. The C-terminal amino acid sequence was similar to the sequence derived with the standard in

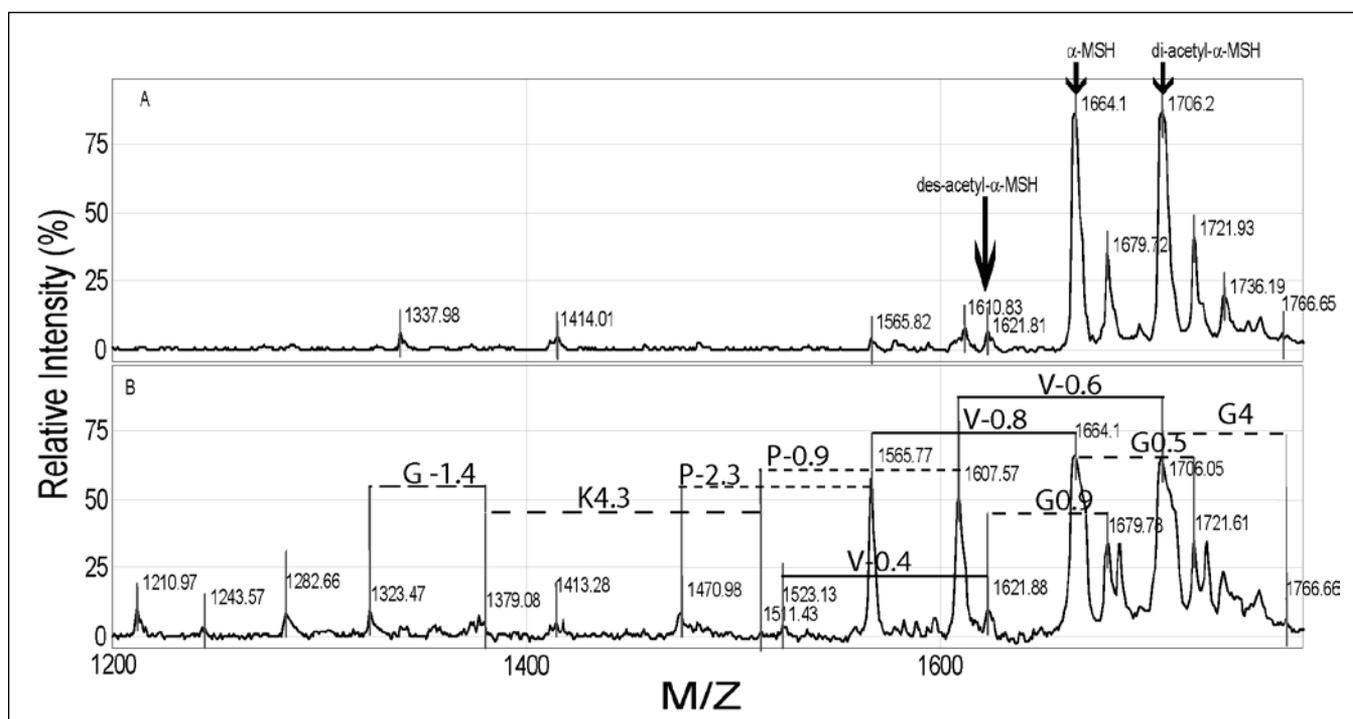


Figure 2. On-chip C-terminal analysis of peptides in the region representative of α -MSH. (A) The spectrum indicates three peptide peaks tentatively identified based on mass as des-acetyl- α -MSH, α -MSH, and di-acetyl- α -MSH. (B) Following treatment with carboxypeptidase Y (CPY), a complex ladder of ion peaks was generated. From the right, wide dashed lines indicate a glycine (G) residue (from ACTH₁₋₁₄) was removed, and solid lines connecting peaks indicate a valine (V) residue was removed. Other dashed lines indicate the removal of proline (P) and lysine (K) residues. ACTH, adrenocorticotropic hormone; α -MSH, α -melanocyte stimulating-hormone. M/Z, mass/charge.

Figure 1A (i.e., SAEAFPi/LEF).

To show that this technique can be used to sequence other anterior pituitary peptides on the same chip simultaneously, we examined the anterior pituitary peptide hormone, α -MSH, which represents the first 13 amino acids of ACTH. α -MSH is interesting because it is acetylated but can exist in the nonacetylated form (i.e., des-acetyl- α -MSH). Acetylation adds 42 Da to the peptide mass. α -MSH is predicted to be 1664 Da, and when the spectra were reviewed, a major peak at M/Z of 1664.17 was identified (Figure 2A). A minor ion peak representative of des-acetyl- α -MSH, at M/Z of 1621.91, was also identified. Another major ion peak was observed at M/Z of 1706.31, which potentially represented a di-acetylated form of α -MSH. When the ProteinChip spot containing anterior pituitary extracts was incubated with CPY, the three original ion peaks were diminished, and a new series of peaks was observed (Figure

2B and Table 1). The difference in mass for each of the largest of the new ion peaks was nearly the same (i.e., 99.1 Da less than the original, representing a valine residue). The last amino acid of α -MSH, des-acetyl- α -MSH, and di-acetyl- α -MSH is a valine. Analysis of the CPY digests revealed smaller peaks, representing further amino acid removal from the des, mono-, or di-acetylated forms of α -MSH (Figure 2B and Table 1). The entire sequence generated by CPY digestion is: GKPVG. This is 100% homologous with the corresponding sequence for α -MSH and clearly suggests that these ion peaks represent α -MSH in its acetylated, di-acetylated, or des-acetylated form.

Advanced qualitative and quantitative 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 the CPY enzyme followed by SELDI-TOF MS analysis, we provide evidence that important peptide amino acid sequence data can be derived from complex mixtures of peptides and proteins.

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Endocrinomic profile of neurointermediate lobe pituitary prohormone processing in PC1/3- and PC2-Null mice using SELDI-TOF mass spectrometry

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Abstract

Pro-vasopressin and pro-oxytocin are prohormones processed in the neurointermediate lobe pituitary to form the biologically active peptide hormones, arginine vasopressin (AVP) and oxytocin. Neurointermediate lobe pituitaries from normal (+/+), heterozygous (+/-), PC2-Null (-/-), PC1/3-Null and oxytocin-Null mice were analyzed by SELDI-TOF mass spectroscopy for the peptide hormone products, AVP, oxytocin and neurophysin I and II. Molecular ion species with masses characteristic of oxytocin, AVP, neurophysin I and II, i.e. 1009.41, 1084.5, 9677 and 9679 daltons respectively, were identified in all but the oxytocin-Null mice by comparison with synthetic standards or by C-terminal sequence analysis. Other ion species were found specifically in PC2-Null, heterozygote or normal mice. The results indicate that, in mice, both PC1/3 or PC2 enzyme activity are capable, but not required to correctly process pro-vasopressin or pro-oxytocin to their constituent active peptide hormones.

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Introduction

The investigation of the proteome is beginning to provide new and exciting insights in the field of endocrinology. To facilitate endocrine proteomic studies (endocrinomics), new and diverse methods for mass spectrometry have been developed that offer greater sensitivity and resolution to more accurately analyze and identify small peptides (van Strien *et al.* 1996, Jimenez *et al.* 1997, Slominski *et al.* 2000). One of the newer mass spectrometric techniques is surface enhanced laser/desorption ionization-time of flight mass spectrometry or SELDI-TOF MS (CIPHERGEN, Palo Alto, CA, USA) (Hutchens & Yip 1993). SELDI-TOF MS utilizes chemically treated metal chips, ProteinChips, that specifically bind peptides and proteins based on their chemical characteristics (Hutchens & Yip 1993, Merchant & Weinberger 2000). Following a wash step, peptides and proteins retained on the chips are analyzed by time of flight mass spectrometry. Thus, using SELDI-TOF MS, proteins and peptides in crude tissue extracts can be systematically and quantitatively analyzed at a greater resolution and specificity than by traditional techniques e.g. SDS-PAGE, Western blots or

RIA. By coupling SELDI-TOF MS techniques with analysis of endocrine tissues from genetically modified mice, prohormone processing and peptide hormone synthesis can be studied in greater detail than before.

Biologically active neuroendocrine peptide hormones are generated through the coordinated action of intragranular conditions and specific enzymes that cleave prohormones at paired-basic residues (Loh 1988, Darby & Smyth 1990, Lindberg 1991, Seidah *et al.* 1992, Zhou *et al.* 1999). Two enzymes from the subtilisin-like enzyme family have been identified that are proposed to be involved in nearly all neuroendocrine prohormone processing, PC1/3, also known as SPC3, and PC2, also known as SPC2 (Steiner *et al.* 1992, Seidah *et al.* 1993). (At the 6th Gordon Research Conference on Proprotein Processing, Trafficking and Secretion (2004), the leading researchers agreed to use the terminology, PC1/3, to describe the identical PC1 and PC3 prohormone convertase.) The two prohormones found in the posterior lobe of the pituitary, pro-oxytocin and pro-vasopressin, were proposed, based on co-localization studies, to be processed to their biologically active peptide hormones, oxytocin and arginine vasopressin (AVP), by one or both of these enzymes (Dong *et al.* 1997). Previous *in vitro*

studies have suggested that PC1/3 can cleave pro-vasopressin and pro-oxytocin to AVP and oxytocin respectively (Coates & Birch 1998), while the processing of these prohormones by PC2 has not been examined. However, in humans, pro-vasopressin and pro-oxytocin processing is impaired when there is a putative deficit in processing by the PC2 enzyme, such as in patients with Prader-Willi syndrome or Wolfram's syndrome (Gabreels *et al.* 1994, 1998, Swaab *et al.* 1995).

In this study, genetically modified mice, deficient in the processing enzyme PC1/3 (Zhu *et al.* 2002*b*) or PC2 (Furuta *et al.* 1997), were analyzed by SELDI-TOF MS. Analyses of the neurointermediate lobe pituitaries from PC1/3-Null or PC2-Null mice indicate that the lack of each of these enzymes had no effect on the efficient processing of pro-vasopressin and pro-oxytocin, while the processing of other peptides was affected.

Materials and methods

PC1/3-, PC2- and oxytocin-Null mice

For the PC1/3-Null mice, a neomycin cassette was inserted in the first exon of the PC1/3 gene (Zhu *et al.* 2002*b*). For the PC2-Null mice, a neomycin cassette was inserted in the third exon of the PC2 gene (Furuta *et al.* 1997). Both lines of mice were bred to generate wild-type, heterozygote and null offspring. Oxytocin-Null mice (Nishimori *et al.* 1996) were generously provided by Dr Mariana Morris, Wright State University, Dayton.

Ciphergen ProteinChip SELDI-TOF mass spectrometry

For direct analysis of mouse neurointermediate lobe pituitary peptides, the pituitaries were homogenized in 0.1 M HCl containing 1 mM PMSF and the cell debris removed by centrifugation (5 min at 15 000 × g). One microgram of each sample was spotted onto a weak cation exchange (WCX2) ProteinChip (Ciphergen) for 30 min, washed with 5 µl distilled water and allowed to dry. For comparison and quantitation, WCX2 ProteinChips were spotted with different concentrations of reconstituted oxytocin and AVP standards (Bachem, Torrance, CA, USA). Matrix, alpha-cyano-4-hydroxy cinnamic acid (CHCA) in 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA) (0.5 µl), was added to the spots and allowed to dry. The ProteinChips were analyzed on a Ciphergen SELDI Protein Biology System II (Ciphergen) with a spot protocol that ionized each spot four times over twenty different areas. The spot was initially ionized with 1 laser hit at an intensity of 200, with the sensitivity set at 10, followed by 4 laser hits at 195 intensity. The source voltage was set at 20 000 volts and the detector voltage at 1900 volts. The 80 laser hits

were averaged for each spot and the resulting protein profile analyzed to determine the mass of each peak. Ciphergen ProteinChip software was used to integrate the area under each peak for use in quantitative analysis. For the AVP and oxytocin standards, the integrated area under each peak was plotted against the corresponding concentration of peptide and the best-fit line was analyzed by linear regression.

On-chip C-terminal peptide sequencing

Neurointermediate lobe tissue lysates (1 µg protein) were spotted on an H4 ProteinChip and air-dried, as previously described (Cool & Hardiman 2004). The ProteinChip was placed in a 'moist chamber' - a pipette tip box (with lid) containing a wet paper towel. For the sequencing reaction, carboxypeptidase Y (CPY) (Pierce, Rockford, IL, USA) (30 units/µl) was diluted to 2.4×10^{-3} units/ml in 50 mM MES, pH 6.5. One microliter of each dilution was added to each spot on the ProteinChip, the lid closed and the chamber placed in a 37 °C incubator for 5 min. After incubation, 1 µl of a supersaturated solution of matrix, CHCA in 50% acetonitrile containing 0.1% TFA, was added to each spot to stop the reaction. The ProteinChip was then analyzed in a Ciphergen PBSII ProteinChip reader.

Results

Proteomic analysis of neurointermediate lobe pituitaries by SELDI-TOF MS

SELDI-TOF MS analysis of the neurointermediate lobe pituitaries from the nine mice (PC2-Null, PC2-heterozygote and PC2 wild-type) showed four distinct profiles between 1000 and 12 000 daltons. In the first profile, the ion species between 1600 and 1750 daltons were identified as being present in the heterozygote and normal genotype, but absent from or greatly diminished in the PC2-Null mice (a in Fig. 1). These peaks have previously been identified as des-acetyl- α -melanocyte stimulating hormone (MSH), α -MSH and di-acetyl- α -MSH (~1620, 1660 and 1702 daltons respectively) (Cool & Hardiman 2004). In the second region, ion species between 2350 and 2506 daltons were also identified in the heterozygote and normal genotype, but were absent from the PC2-Null genotype (b in Fig. 1). These ion peaks were identified by matrix-assisted laser/desorption ionization (MALDI)-TOF/TOF as the CLIP region of adrenocorticotropin (2505 daltons for CLIP and 2350 daltons after loss of the C-terminal Phe-residue) (S M Leung and D R Cool, unpublished data). In the third region, a series of ion peaks between 2700 and 3500 daltons, representing acetylated and non-acetylated forms of β -endorphin₁₋₂₇ through β -endorphin₁₋₃₁, were identified as different in the

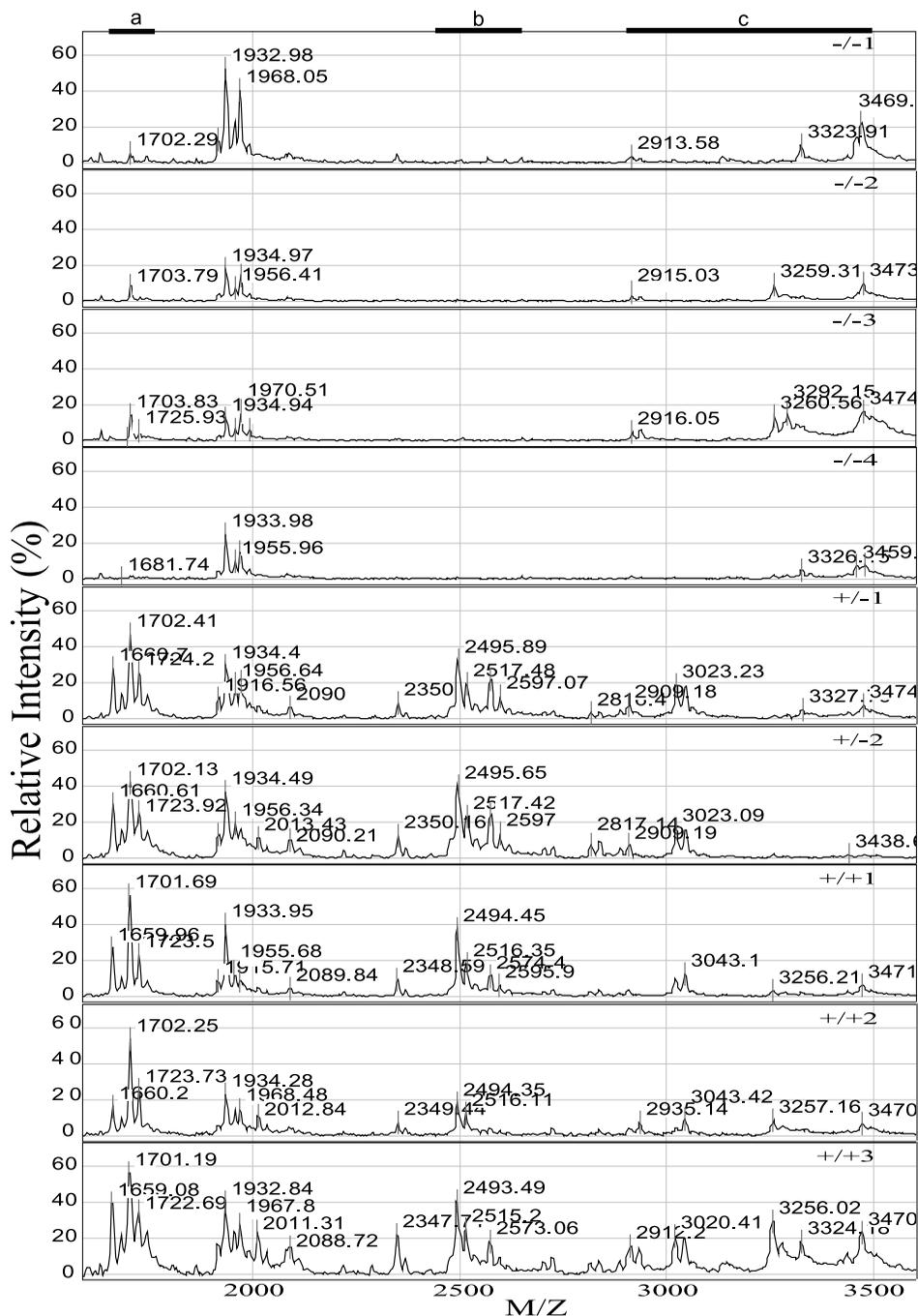


Figure 1 SELDI-TOF mass spectrometric profiles of neurointermediate lobe pituitaries from PC2-Null (-/-), heterozygote (+/-) and normal mice (+/+). Neurointermediate lobe pituitary lysates were applied to each spot on a Ciphergen SELDI-TOF WCX2 ProteinChip. The ProteinChip was analyzed on a Ciphergen PBSII SELDI-TOF mass spectrometer using version 3.0 of the Ciphergen software. The relative intensity (%) plotted for each peptide/protein (mass/charge, M/Z) is shown for the region between 1500 and 3600 daltons. Regions where peptides are noticeably changed in the PC2-Null mice compared with heterozygote and wild-type mice are indicated by a black bar with the letter a, b or c above.

PC2-Null compared with heterozygote and normal mice (c in Fig. 1). The results clearly show differences in the proteomic profile between PC2-Null, heterozygote and normal mice. The differences between these spectra represent the first step in characterizing the effect of 'knocking out' a major processing enzyme in a mouse model.

Analysis of oxytocin and AVP processing by SELDI-TOF MS

Oxytocin and AVP are the two predominant peptide hormones present in the neurointermediate lobe pituitary. Fully processed and amidated AVP (1086.5 daltons and AVP+sodium, 1105.92 daltons) and oxytocin+sodium (1031.23 daltons) appeared in the spectra generated from all the PC2 mice (Fig. 2). A small amount of oxytocin (1009.22 daltons) was observed in only one PC2-Null mouse (Fig. 2). Subsequent analysis of other neurointermediate lobe pituitaries showed varying amounts of the 1009.22 dalton form of oxytocin. The predominant peak for oxytocin was the sodium peak at 1031 daltons. AVP and oxytocin were definitively identified by comparison with AVP and oxytocin synthetic standards analyzed by SELDI-TOF MS, as previously shown (Cool & DeBrosse 2003). The identity of oxytocin was confirmed by the presence of ion peaks of the expected mass (1009.22 and 1031.23 dalton) in pituitaries of wild-type mice compared with the absence of these two peaks in spectra generated from a neurointermediate lobe of oxytocin-Null mice (OT-Null) (Fig. 2). In addition, this spectrum was compared with spectra generated from PC1/3-Null mice (Fig. 3), where it was apparent that AVP and oxytocin were generated in the absence of PC1/3.

The relative amount of oxytocin and AVP in the samples was determined by integrating the corresponding ion peak areas (Table 1). There was no significant difference ($P>0.05$) in the integrated area of the peaks for oxytocin or AVP (and their sodium adducts) between PC2-Null, heterozygote and normal mice (Table 1).

Neurophysin I and II

The peptides, oxytocin and AVP, are generated by cleaving pro-oxytocin and pro-vasopressin at the junction between oxytocin or AVP and their respective neurophysin regions (neurophysin I from pro-oxytocin and neurophysin II from pro-AVP), followed by removal of the basic residues and amidation. Therefore, the neurophysin regions of the two prohormones should also be present if oxytocin and AVP were correctly processed. When the profiles for each of the mice were analyzed, a series of ion peaks was found between 9000 and 10 000 daltons (Fig. 4). The predicted masses for mouse neurophysin I and neurophysin II were

determined using DNA Strider software (Marck 1988) and are shown in Tables 2 and 3 respectively. In the PC2-Null, heterozygote and wild-type mice, an ion peak at ~9677 daltons was identified that correlated with the predicted size of neurophysin I and II (Tables 2 and 3 respectively). This peak was not observed in tissues that do not make oxytocin or AVP, e.g. anterior pituitary (bottom plot in Fig. 4). Finally, an ion peak at 8150 daltons was identified as being specific to the PC2-Null genotype (Fig. 4). Antibody capture analysis using antibodies to neurophysin failed to identify this peak as part of the neurophysin molecule. Thus, this peak is not neurophysin related.

On-chip peptide sequencing

To more accurately determine the presence and identity of the neurophysin peptides, pituitary lysates were dried on Ciphergen hydrophobic H4 ProteinChips and incubated with CPY, a protease that systematically removes amino acids from the C-terminus of peptides and proteins. Preliminary analysis revealed ion peaks of sizes 9676, 9519, 9369, 9304, 9153 daltons (Fig. 5A and Tables 2 and 3). This multiple peak (or ragged end) pattern has been observed in neurointermediate lobe pituitaries from nearly 1000 mice (D R Cool, unpublished results) and represents the naturally occurring C-terminal amino acid(s) truncated state for neurophysin. Analysis of the ion peaks and the difference in their mass indicates an amino acid sequence that corresponded to the C-terminal amino acid sequence for neurophysin I, FSER (Fig. 5B). Upon treatment with CPY, the major ion peak shifted, to generate an additional two amino acids, SAFSER (Fig. 5B, Table 2). The ion peak for neurophysin II was nearly identical in size to the predicted peak size for neurophysin I and could not be distinguished from neurophysin I (Fig. 5A). After CPY treatment of another sample, several peaks were identified (sizes 9585, 9473, 9314.8, 9166.2 daltons), yielding the amino acid sequence, FRLT, that corresponds to the last four C-terminus amino acids of neurophysin II (Fig. 5C, Table 3). The ion peak at 9094 in Fig. 5C, was 8 daltons from the 9086 peak of neurophysin I, and thus could not be positively identified as coming from neurophysin I.

The oxytocin-Null mouse was expected to lack ion peaks for neurophysin I. When neurointermediate lobe pituitary tissue was analyzed by SELDI-TOF MS, two ion peaks, 9519 and 9389 daltons that correspond to neurophysin I, were missing (Fig. 5D). Three ion peaks remained, 9677, 9471 and 9315 daltons, that correspond to neurophysin II, providing further proof that this series of ion peaks represent the neurophysin peptides.

In addition to the neurophysin region, pro-vasopressin contains a 39 amino acid C-terminal region, the glycopeptide region. This glycopeptide is the only

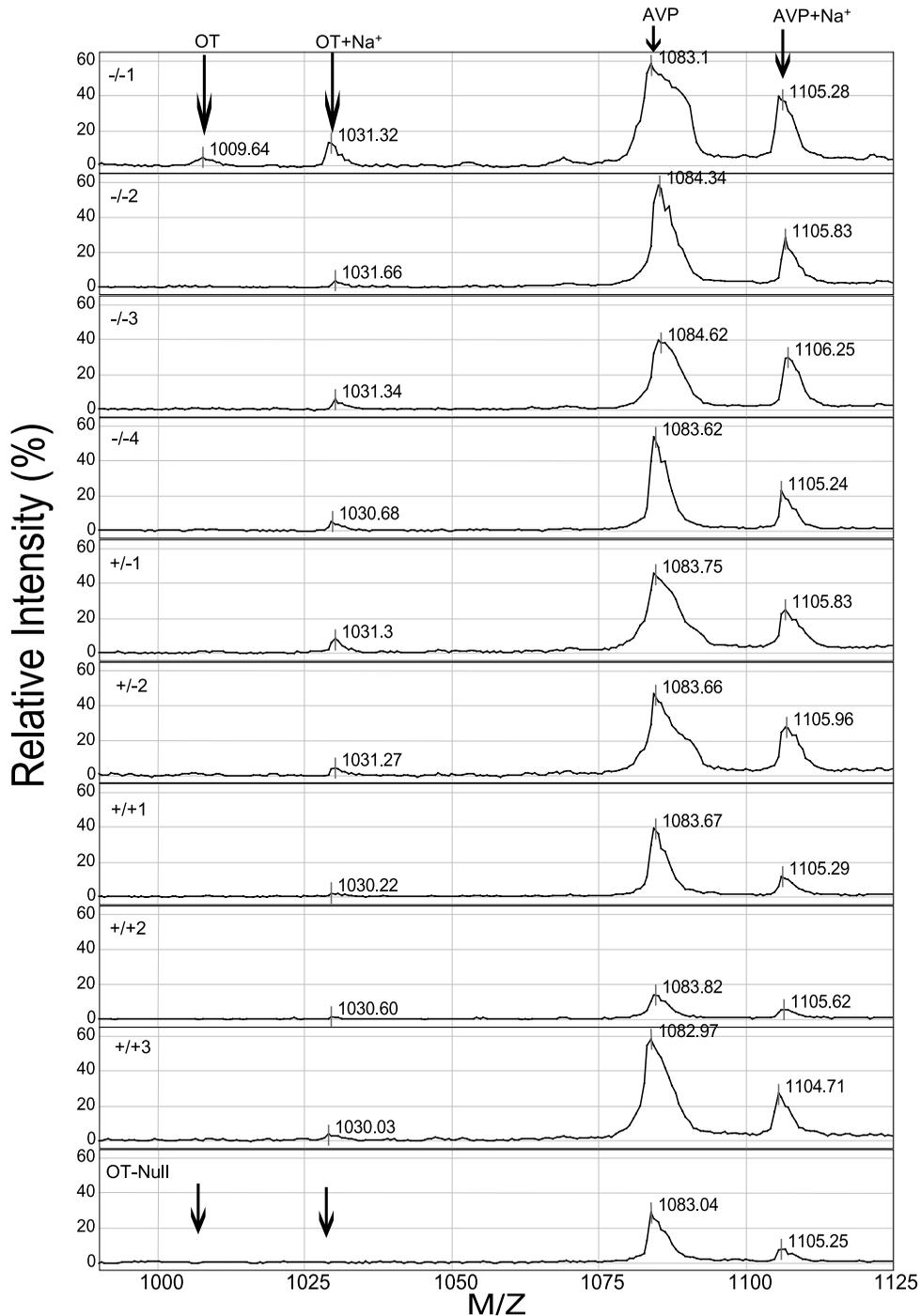


Figure 2 SELDI-TOF mass spectrometric profiles of oxytocin (OT) and AVP in PC2-Null (-/-), heterozygote (-/+), wild-type (+/+) mice or OT-Null mice. Oxytocin is seen as a small ion peak at 1009.64 daltons with a sodium ion at 1031.32 daltons in the first -/- spectra. Sodium adds approximately 22 daltons to the mass. The 1009.64 dalton ion is absent from the other animals. For AVP, a larger 1083.1 dalton ion was present in all samples with the sodium ion appearing at 1105.28 daltons. In the OT-Null profile, the arrows indicate the lack of an ion peak for oxytocin or its sodium salt.

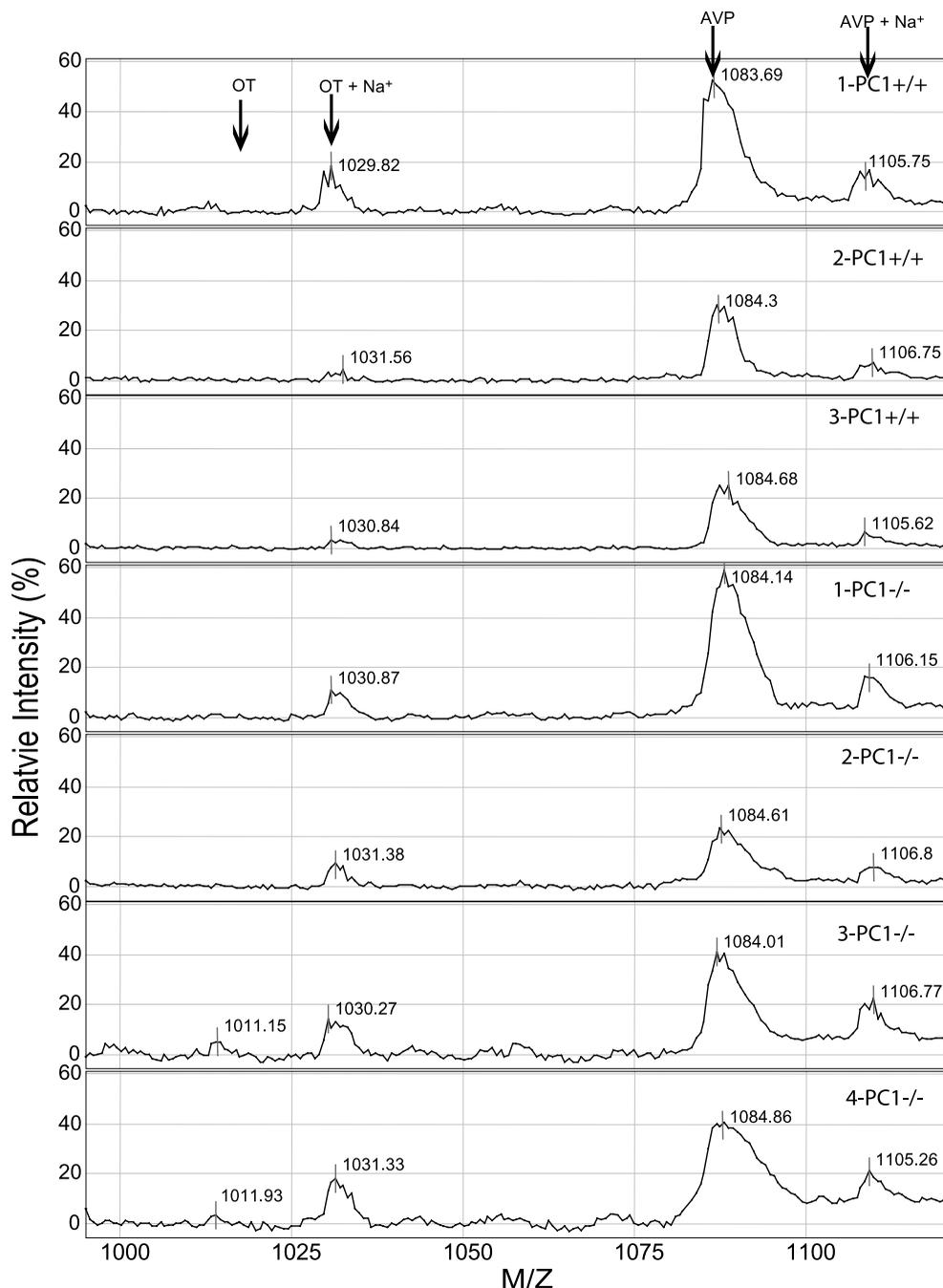


Figure 3 SELDI-TOF MS profile of oxytocin (OT) and AVP in wild-type (+/+) and PC1/3-Null (-/-) mice. Neurointermediate lobe pituitaries from four PC1/3-Null mice were analyzed by SELDI-TOF MS using a 0.1 M HCl buffer, the same as for mice in Fig. 2. Ion peaks of 1009, 1031, 1084 and 1105 daltons were identified and correspond to oxytocin, oxytocin+Na⁺, AVP and AVP+Na⁺.

portion of pro-vasopressin that is glycosylated, and thus its mass will be determined by the amount and type of glycosylation. The expected mass of glycopeptide is 4349 daltons without glycosylation. In a recent study of rat

vasopressin, glycosylated glycopeptide was determined by mass spectrometry to be 5494 daltons (Jimenez *et al.* 1997). In the present study, two peptides have been found in the range between 5000 and 6000 daltons.

Table 1 SELDI-TOF analysis of relative oxytocin and AVP amounts. The mean±S.E.M. was reported for the PC2-Null, heterozygote and wild-type groups (n=3)

M/Z	PC2			PC1	
	-/- PC2	-/+ PC2	+/+ PC2	-/- PC1	+/+ PC1
1009	4.5±3	1.04±1	0.24±0.03	5±3	2±0.6
1030	16.6±6	14.8±8	5±1.5	47±11	24±14
1084	319±58	321±16	196±75	319±70	219±72
1105	124±23	129±7	61±24	83±21	42±17

The data represent the average integrated peak area for the peptide and its sodium adduct for each ion peak.

However, neither ion peak has been positively identified as glycopeptide using C-terminal sequencing.

Discussion

The processing of prohormones to active hormones is a complex event that requires the coordinated action of specific processing enzymes such as PC1/3 and PC2, carboxypeptidase E and peptidylglycine α -amidating monooxygenase (PAM) (Zhou *et al.* 1999). Some prior studies on prohormone processing have concentrated on the use of synthetic peptides and purified processing enzymes for *in vitro* analysis (Cawley *et al.* 1996, Coates & Birch 1998, Olsen *et al.* 1998). Other studies have used over-expression of substrate and enzyme in a cell system (Rouille *et al.* 1992, Cool *et al.* 1996, Wang *et al.* 1998, Min *et al.* 1999). The drawback to these methods is the lack of regulation and biological relevance as found *in vivo*. For example, endogenous inhibitors and activators of processing enzymes may be present in intact cells, but absent from purified preparations. Over-expression of substrate and enzyme also lacks physiological relevance. In order to study the actual activity of prohormone processing enzymes, mice have been genetically altered, i.e. one of the processing enzymes (PC1/3 or PC2) has been 'knocked out'. The results presented here show the effects of deleting the PC2 processing enzyme on the formation of oxytocin/AVP. The implication from this data is that PC2 is not required for processing pro-oxytocin or pro-vasopressin. Analyses of PC1/3-Null mice (Zhu *et al.* 2002a,b) were studied to further assess the role of PC1/3 in pro-oxytocin or pro-vasopressin processing and it was shown that PC1/3 is also not required for pro-oxytocin or pro-vasopressin processing. We interpret these results to show that both PC1/3 and PC2 can process the precursors, and if one is 'knocked out', the other enzyme 'takes over'. Proof of this will require double null mice.

For this paper, a relatively new, yet powerful, mass spectrometry technique was used to identify and quantitate the smaller peptide hormones, oxytocin and

AVP. SELDI-TOF MS was able to resolve oxytocin and AVP in extracts of neurointermediate lobe pituitary glands from all the mice analyzed. The results clearly show that pro-oxytocin and pro-vasopressin were correctly processed in the PC2-Null, heterozygote and wild-type mice. Furthermore, quantitative analysis revealed that there was no difference in the amount of AVP or oxytocin present in these mice. This is consistent with the results of Coates and Birch (1998) who concluded, based on studies with purified PC1/3, that PC1/3 enzyme was capable of (although not necessarily required for) processing pro-vasopressin to AVP. However in our study, when PC1/3-Null mice were analyzed for oxytocin and AVP, it appeared that both AVP and oxytocin were still present. These results can be compared with clinical conditions in which the lack of pro-oxytocin or pro-vasopressin processing is attributed to the putative absence of active PC2, for example in Prader-Willi syndrome and Wolfram's syndrome. The main hypothesis to explain these processing differences is that an enzyme redundancy may exist in mice, i.e. both PC1/3 or PC2 are capable of processing mouse pro-oxytocin and pro-vasopressin.

If PC1/3 or PC2 are both capable of processing pro-oxytocin and pro-vasopressin, then why are they not processed in humans with Prader-Willi syndrome or Wolfram's syndrome? The most plausible explanation is substrate specificity. That is, structural differences at or near the cleavage site in pro-oxytocin or pro-vasopressin would dictate the processing by a particular enzyme. When the amino acid sequences for mouse and human pro-oxytocin or pro-vasopressin were compared, the homology was 100% at the actual cleavage site, i.e. GKRA (Fig. 6). The region in mouse pro-oxytocin beginning with PLGG on the N-terminal side of the paired basic residue cleavage site represents a β -turn (see Fig. 6). The region on the C-terminal side of the paired basic residues with the sequence KRAVLDDVVR, represents an α -helix (Brakch 2000). Both secondary structures appear to be required for proper processing (Fig. 6). However, for both human pro-vasopressin and pro-oxytocin, there are significant amino acid differences

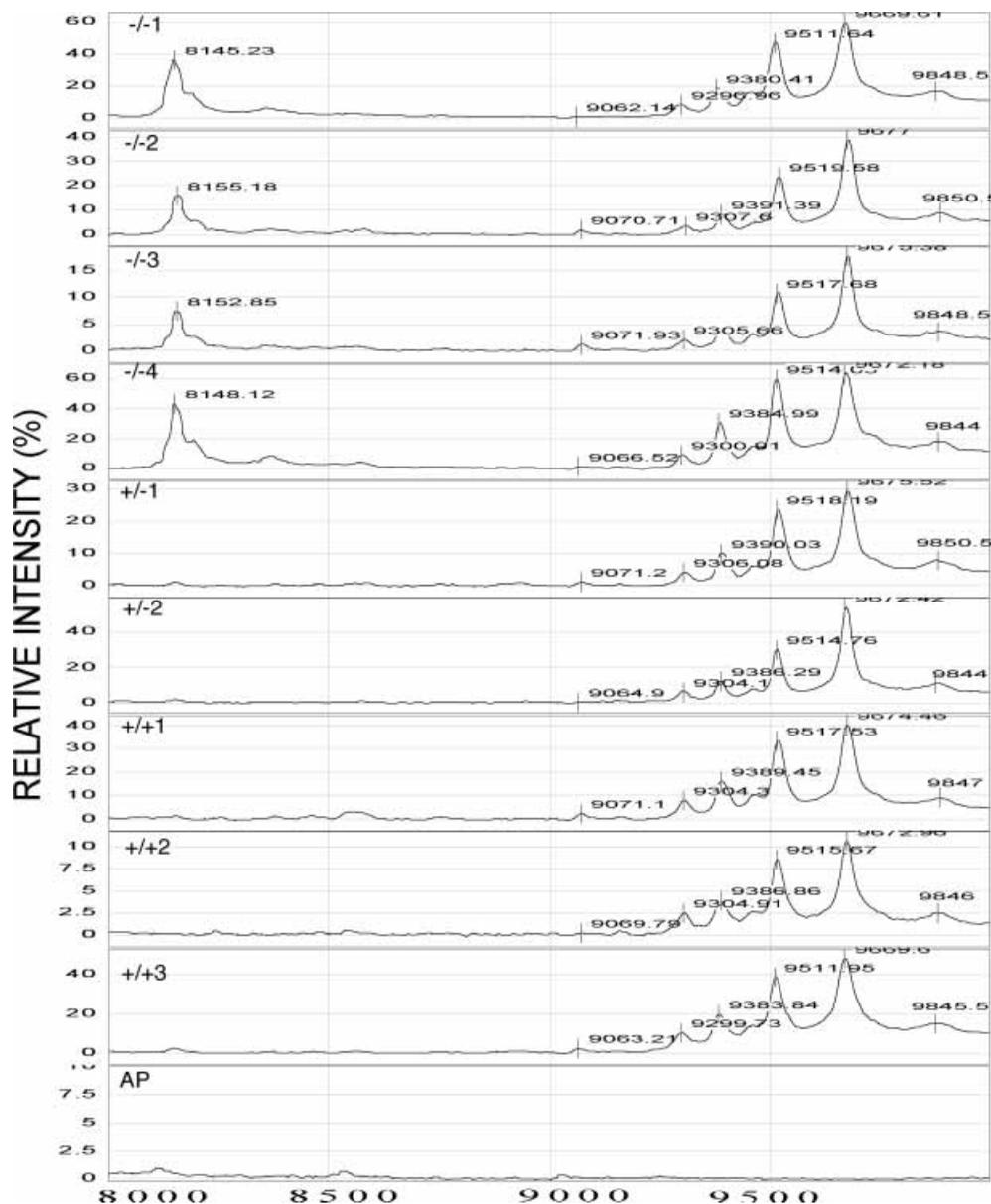


Figure 4 SELDI-TOF MS profiles of ion peaks in the 8000 to 10 000 dalton range from PC2-Null (-/-), heterozygote (+/-) and normal mice (+/+). Neurointermediate lobe pituitary lysates were analyzed using a WCX2 ProteinChip. The relative intensity (%) was plotted for each peptide/protein (mass/charge, M/Z). A peak at ~8100 daltons was identified in the PC2-Null mice, but not in the heterozygote or wild-type mice. The bottom profile resulted from SELDI-TOF MS analysis of an anterior pituitary (AP) from a normal (+/+) mouse.

in the same position within this α -helix region, only 2 amino acids from the C-terminus of the processing site. In human pro-vasopressin, a Met-residue replaces the Ile-residue (Fig. 6A). In human pro-oxytocin, Ala-Pro replaces Val-Leu, in this position (Fig. 6B). In both cases, a more hydrophobic amino acid is present in the mouse sequence compared with the human. The presence of

the proline residue in human oxytocin is especially important for breaking the α -helix structure. In addition, a Leu-residue is substituted for a Met-residue at the +5 position in human pro-vasopressin, and a Val-residue is substituted for a Met-residue at the +7 amino acid position in human pro-oxytocin (Fig. 6A and B). In a recent article on oxytocin processing by PC1/3,

Table 2 Expected versus observed ion peaks for neurophysin I

Neurophysin I				
	<u>Expected daltons</u>	<u>Observed daltons</u>	<u>Δ Mass</u>	<u>Amino acid removed</u>
Sequence				
- DPACDPESAFSER*	9677	9676.34	—	—
- DPACDPESAFSE	9521	9519.62	156.72	R
- DPACDPESAFS	9392	9389.95	129.67	E
- DPACDPESAF	9305	9304.10	85.85	S
- DPACDPESA	9157	9158.33	145.77	F
- DPACDPES	9086	9086.26	72.07	A
- DPACDPE	9000	9000.10	86.16	S

*Only the last 13 amino acids of neurophysin I are shown. All ion peaks are in daltons and represent the monoisotopic single charge form of the peptide. The single amino acid code is used for the amino acid sequence. Expected indicates expected ion peak and Observed indicates observed ion peak. The amino acid column indicates the amino acid removed from the C-Terminus.

substitution of an alanine or valine residue on the C-terminal of the KR cleavage site with an amino acid that would block either the β -turn or the α -helix caused disruption of the processing of pro-oxytocin by PC1/3 (Brakch 2000). Thus, the case for processing redundancy by PC1/3 or PC2 in mice based on structural specificity is strongly supported by the data presented here compared with the findings for Prader-Willi syndrome and Wolfram's syndrome. That is, amino acid differences in the human pro-oxytocin or pro-vasopressin sequence near the processing site cause fundamental changes in the secondary structure that is required for correct processing.

An alternative hypothesis is that a third enzyme exists that is specific for processing pro-vasopressin and pro-oxytocin in mice but not in humans. This is supported by our results in which the processing of other prohormones, i.e. pro-opiomelanocortin, was perturbed in the PC2-Null mice, while pro-oxytocin and pro-vasopressin were processed normally. For this to occur, the enzyme would have to be expressed only in the posterior pituitary. Recent evidence suggests that another enzyme capable of processing pro-vasopressin

and pro-oxytocin may be present in the paraventricular nucleus, PC5, although it was only observed in vasopressinergic but not oxytocinergic neurons (Dong *et al.* 1997).

Of further interest were the results obtained from the oxytocin-Null mice, since this is also the first analysis of the oxytocin-Null mouse by mass spectrometry. As was expected, oxytocin was not present in the neurointermediate lobe pituitary. However, of note was that two of the ion peaks in the neurophysin range, 9389 and 9519 daltons, were also missing, suggesting that these are neurophysin I-related peptides derived from the pro-oxytocin molecule. This is significant, because the lack of two of the peaks associated with neurophysin I further confirmed the sequence data. Another observation of note is that in virtually every neurointermediate lobe sample examined using SELDI-TOF MS, a series of 3–4 ion peaks in this region prevail, regardless of the preparation or use of proteolysis inhibitors. The observation of multiple peaks for the neurophysins is consistent with reports by others using MALDI-TOF MS to study rat AVP-related peptides and may represent the natural state of the peptides in this tissue (Jimenez *et al.* 1997).

Table 3 Expected versus observed ion peaks for neurophysin II

Neurophysin II				
	<u>Expected daltons</u>	<u>Observed daltons</u>	<u>Δ Mass</u>	<u>Amino acid removed</u>
Sequence				
-VAEPECHDGGFFRLT*	9679	9676.34	—	—
-VAEPECHDGGFFRL	9585	9585.00	91.34	T
-VAEPECHDGGFFR	9473	9473.20	111.8	L
-VAEPECHDGGFF	9315	9315.81	158.39	R
-VAEPECHDGGF	9168	9166.16	148.65	F

*Only the last 13 amino acids of neurophysin II are shown. All ion peaks are in daltons and represent the monoisotopic, single charge form of the peptide. Expected indicates expected ion peak and Observed indicates observed ion peak. The amino acid column indicates the amino acid removed from the C-terminus.

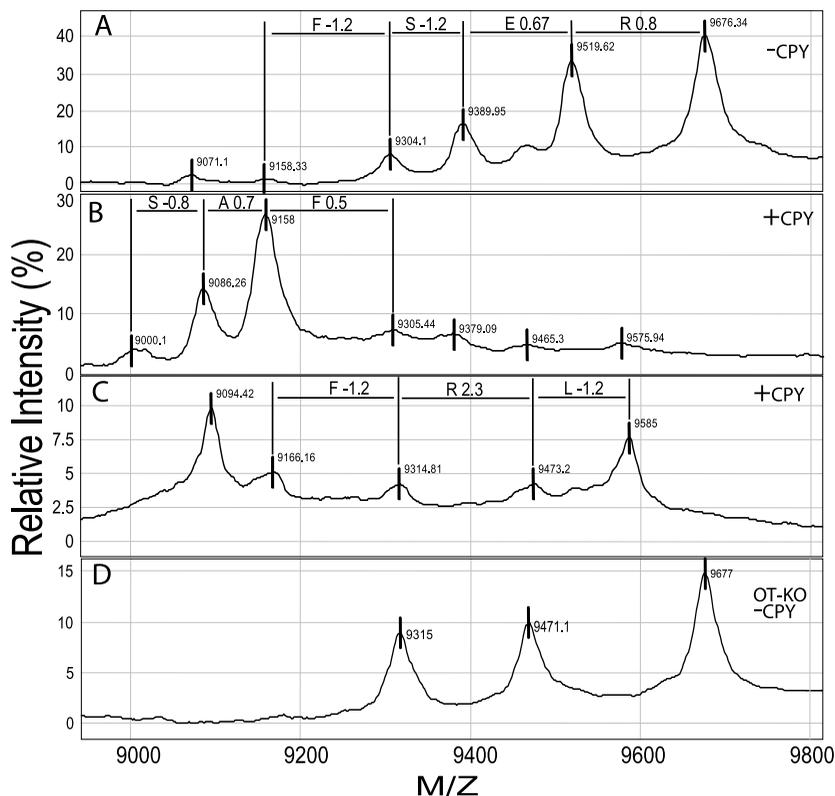


Figure 5 On-chip C-terminal sequence analysis of peptides in the 9000 to 10 000 dalton range from PC2-Null mice. Both neurophysin I and neurophysin II have a predicted mass of ~9677 daltons. Naturally occurring ion peaks present before CPY treatment were 9676, 9519, 9389 and 9304 daltons (-CPY, A). Additional ion peaks characteristic of neurophysin I (+CPY, B; Table 2) and neurophysin II (+CPY, C; Table 3) were observed after treatment of the ProteinChips with CPY. Neurointermediate lobe pituitaries from oxytocin-Null mice were also analyzed (OT-KO -CPY; D) and were found to contain only three peaks characteristic of neurophysin II. Noticeably absent were the 9519 and 9389 peaks of neurophysin I. The single amino acid code is shown above the lines between peaks. The difference in daltons between the exact amino acid mass and the observed amino acid mass for each sequenced amino acid is also shown, i.e. R0.8, E0.67, etc.

Mass spectrometry represents a major advancement over RIAs and SDS-PAGE Western blots for analyzing peptide hormones in tissues such as the pituitary. The major advantages of mass spectrometry are: (1) the ability to analyze peptides that are too small to be resolved by SDS-PAGE, (2) the ability to quantitate the amount of the peptide and (3) the ability to confirm a peptide by its mass. Mass spectrometry, especially SELDI-TOF MS and MALDI-TOF MS, are excellent for analyzing peptides in the range between 500 and 10 000 daltons. Another advantage of mass spectrometry is its level of sensitivity. For the Ciphergen SELDI-TOF MS used in this study, sensitivity in the attomole range has been reported (Hutchens 1993, Merchant & Weinberger 2000). Another benefit of mass spectrometry compared with RIAs is the knowledge of the exact mass

of the peptide being analyzed. The ability to quantitate the peptides is similar to that of an RIA in that both require a standard curve with the known peptide. As used here, SELDI-TOF MS represents a new step in mass spectrometric peptide analysis by allowing 'on-chip' peptide concentration and selective retention-based purification (Hutchens 1993, Merchant & Weinberger 2000). For traditional MALDI-TOF MS systems, the peptides must be desalted and peptides/proteins purified prior to spotting on the metal MALDI targets. While similar results can be obtained, the on-chip method provides a much faster method for preparing the samples. The resolution and sensitivity is more than adequate for comparative analysis of peptides and proteins in a wide range of samples, e.g. tissues from knockout-animals, cells in culture, or synthetic peptides.

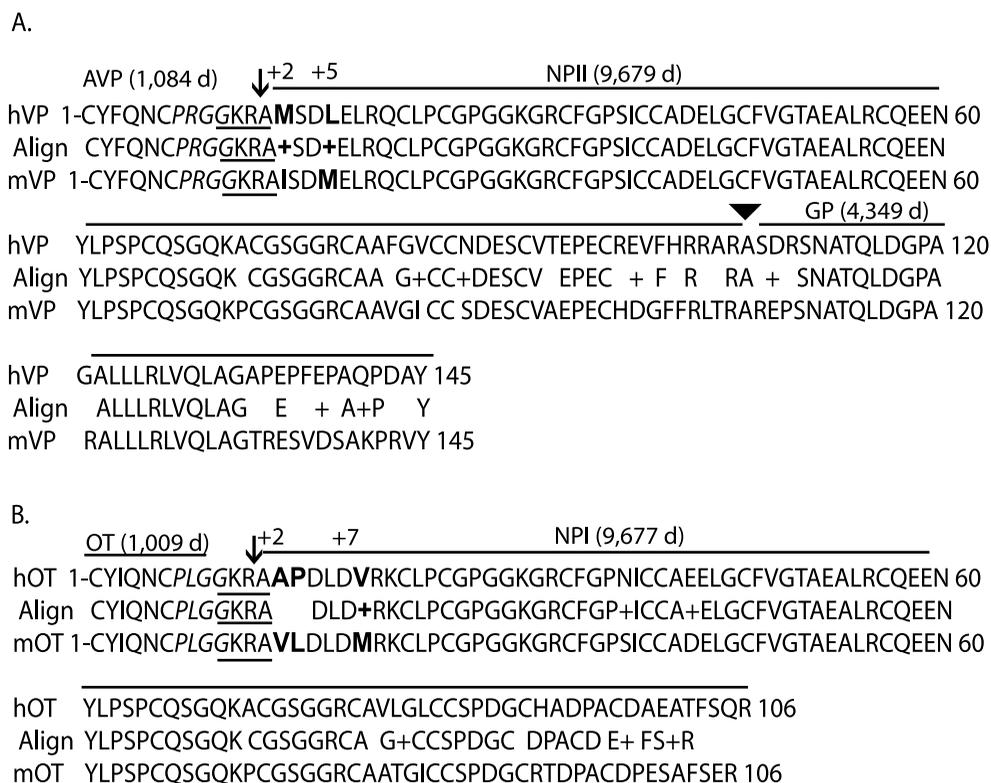


Figure 6 Blast amino acid sequence alignment for human and mouse pro-vasopressin (A) and pro-oxytocin (B). The single-letter amino acid sequences for human and mouse pro-vasopressin (hVP or mVP respectively) and pro-oxytocin (hOT or mOT respectively), lacking the signal peptide region, were obtained from GenBank and analyzed by Blast sequence alignment (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Accession numbers were: hOT #AY082910; mOT #AF483509; hVP #NM000490; mVP #NM009732. The amino acid sequence homology is displayed between the human and mouse sequence (Align). The homology was >82% for pro-vasopressin (119/145 amino acids) and >85% for pro-oxytocin (91/106 amino acids). Numbering before and after each line indicates the number of amino acid residues in that line. Spaces in the Align row indicate no homology, (+) indicates similar charge substitutions. The peptide hormones AVP, oxytocin (OT), neurophysin (NPI and NPII) and the glycopeptide region (GP), along with their predicted mass in daltons, e.g. 1084 d, are indicated by the bar above their corresponding amino acid sequence. The arrows indicate the paired-basic residue prohormone cleavage site in the two prohormones. The arrowhead indicates the cleavage site between NPII and glycopeptide in pro-vasopressin. The GKRA cleavage site is underlined in each sequence. The PRGG conserved sequence is in italics and the +2, +5 and +7 amino acid changes are indicated in bold type.

Thus, the SELDI-TOF MS method presented here provides a unique and powerful way for the investigator to quickly analyze numerous and complex biological samples.

In conclusion, we have analyzed the neurointermediate lobe pituitary proteome from a prohormone processing enzyme null mouse using a specialized mass spectrometric technique, SELDI-TOF MS. The results clearly show that the two neurointermediate pituitary prohormones, pro-oxytocin and pro-vasopressin, do not require the activity of either PC1/3 or PC2, suggesting that the enzymes are redundant and one enzyme can

process the precursor in the absence of the other. The results suggest that a proteomic examination of the spectra from a specific tissue from wild-type and null mice can yield useful information that can help identify and characterize the effect of an enzyme on prohormone processing. The proteomic analysis of endocrine and neuro-endocrine tissues and peptides is part of a multifaceted and comprehensive analysis of the endocrine system called 'endocrinomics', and techniques such as SELDI-TOF MS will play a unique and vital role in developing the endocrinomic profile of a cell, tissue or animal.

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Y. Chen, L. F. Joaquim, V. M. Farah, R. B. Wichi, R. Fazan Jr., H. C. Salgado and M. Morris

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Enhanced heart rate variability and baroreflex index after stress and cholinesterase inhibition in mice

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Joaquim, Luis F., Vera M. Farah, Iveta Bernatova, Rubens Fazan, Jr., Robert Grubbs, and Mariana Morris. Enhanced heart rate variability and baroreflex index after stress and cholinesterase inhibition in mice. *Am J Physiol Heart Circ Physiol* 287: H251–H257, 2004. First published February 26, 2004; 10.1152/ajpheart.01136.2003.—Experiments tested the effect of stress coupled with cholinesterase inhibition on blood pressure, heart rate, baroreflex index, and variability in time and frequency domain in conscious mice. The objective was to determine whether cholinergic systems interact with stress to alter cardiovascular responses. Male C57BL/6J mice with arterial catheters were exposed to 3-day treatments: 1) intermittent shaker stress, 2) pyridostigmine (10 mg·kg⁻¹·day⁻¹); or 3) combined pyridostigmine and stress. Pyridostigmine reduced blood cholinesterase (–33%) with no added effects of stress. Twenty-four-hour blood pressure recordings showed that there were no differences in blood pressure and heart rate with the treatments. Pulse interval standard deviation was greatly increased in the pyridostigmine/stress group compared with stress or pyridostigmine groups (11.0 ± 1.4, 5.0 ± 0.9, and 7.5 ± 0.9 ms, respectively). Spectral analysis showed two distinct components for pulse interval variability (low and high frequency). Variability in the low-frequency range was greatly enhanced in the pyridostigmine/stress group, seen as a doubling of the power (9.5 ± 1.7, 3.3 ± 0.9, and 5.0 ± 0.6 ms for pyridostigmine/stress, stress and pyridostigmine groups, respectively). Baroreflex sensitivity was also increased in the pyridostigmine/stress group (3.6 ± 0.5 compared with 1.8 ± 0.3 and 1.7 ± 0.5 ms/mmHg in the stress and pyridostigmine groups, respectively). There was no difference in blood pressure variability or its spectral components. Results demonstrate that there are potent interactions between a mild stressor and cholinesterase inhibition seen as an accentuation of low-frequency variability in pulse interval time series, probably associated with baroreflex input and autonomic drive.

cardiovascular; spectral analysis; cholinergic; stress biology

PYRIDOSTIGMINE (PB), a reversible inhibitor of acetylcholinesterase (AChE), is used clinically for the treatment of autoimmune disease and prophylactically against organophosphate poisoning (9, 32, 36). Generally, it causes few overt symptoms and is thought to be a safe drug (8, 19, 40). However, there are questions as to whether PB may have central interactions under stressful conditions or may have access to brain areas lacking a sufficient barrier. Forced swimming or immobilization disrupted the blood-brain barrier (BBB) and allowed access of PB into brain tissue (17, 48). Chronic PB exposure in mice led to a decrease in AChE activity in the hypothalamus, a brain region with a reduced BBB (45). PB also modified the response

to novelty stress in rats and mental stress in humans (28, 40, 47). These observations suggest that the combination of operational/environmental stress and PB exposure might cause unexpected health effects, raising questions about a possible role of PB in the etiology of the symptoms collectively termed Gulf War illness (25).

Reduced heart rate (HR) variability (HRV) is associated with a variety of cardiovascular pathologies, from myocardial infarction to heart failure (18, 34). The overall magnitude of HRV can be easily quantified by measurement of standard deviation of the beat-by-beat series of pulse interval (PI). However, spectral analysis provides more detailed and specific information on the frequency-domain characteristics of PI variability, including data on sympathetic and parasympathetic modulation of cardiac function (38). There is evidence from studies in patients with congestive heart failure that reduced low-frequency power (LF) was an independent predictor of sudden cardiac death (18, 34). Even spectral analysis of short-term electrocardiogram measurements were useful in identifying patients at greatest risk who might benefit from further treatment (34).

Stress, defined as any physical or emotional influence that causes bodily or mental tension, results in a cascade of cardiovascular, endocrine, and immune changes. Clinical studies suggest that mental stress is associated with adverse cardiovascular events such as myocardial ischemia, arrhythmias, infarction, and stroke (14, 20, 43, 44, 53). In an extensive clinical study, cardiac patients were exposed to exercise or psychological stress (49). Results showed that mental stress-induced ischemia was a predictor of sudden death. With the use of an actual lifestyle stress, a medical school examination, Lucini et al. (37) found that stress was associated with impairment in cardiac and enhancement in vascular responses. The results of this study verified the importance of measurement of a spectral profile rather than simple time-domain variance.

To extend the results of clinical studies, animal models have been developed to investigate the pathophysiological effects of stress. In many cases, the stressors are physical insults, such as restraint, exhaustive swimming, or electric footshock. We used a chronic stress model that combines shaking (a physical stimulus) with novelty (intermittent exposure) (7). It produces consistent, repeated increases in blood pressure (BP), HR, and corticosterone secretion (7, 33).

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Experiments were designed to explore the interactions between psychosocial stress exposure and cholinesterase (ChE) inhibition on the 24-h pattern of BP and HR and its respective spectral components in mice. The idea was to emulate some of the conditions of the Gulf War in which soldiers self-medicated with PB tablets while being exposed to environmental stressors. The hypothesis is that the dynamic sensitive indexes of HRV and pressure variability might be predictive of the problems associated with this syndrome.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice (Harlan, Indianapolis, IN), 26–27 g, were housed individually at 22°C on a 12:12-h light-dark cycle. They were given a standard diet (Harlan Teklad, 0.4% sodium by weight) with tap water ad libitum. The Laboratory Animal Care and Use Committee of Wright State University approved all experimental protocols.

Surgery. Under ketamine-xylazine anesthesia (120:20 mg/kg im), mice were prepared with chronic carotid arterial catheters according to methods previously described (7, 35). Heparinized saline (100 IU/ml) was continuously infused intra-arterially (25 μ l/h) to maintain catheter patency. Animals were allowed at least 6 days to recover from the cardiovascular surgery before experimentation. Osmotic minipumps, filled with saline or PB (Alzet model 1007D, flow rate 0.5 μ l/h, DURECT; Cupertino, CA), were inserted subcutaneously (ketamine-xylazine anesthesia) after completion of the basal cardiovascular recordings (Basal).

Cardiovascular recordings. All recordings were made in conscious mice while in their home cages. The catheter was connected to a flow-through pressure transducer (model 041–500503A, Argon; Athens, TX), which was connected to a computerized data-acquisition system (model MP100WSW, BIOPAC Systems; Santa Barbara, CA). Arterial BP (AP) was sampled at different rates for 24-h recording and spectral analysis (80 and 4,000 Hz, respectively). HR was derived from AP data.

In the groups exposed to shaker stress (Stress and PB/Stress), cardiovascular recordings were made under basal conditions (Basal) and after 3 days of stress (*day 3*). The latter recording was begun 30–40 min after the last stress exposure. This protocol was based on the time course of cardiovascular changes produced by 2 min of shaker stress. There was an immediate increase in BP and HR, which returned to baseline 20–30 min later (data not shown). For 24-h recordings, BP and HR were continuously recorded before minipump implantation (Basal) and on *day 3* of drug treatment and/or stress. The data were processed by calculation of 10-min means that were averaged for calculation of the dark-light levels. For spectral analysis, the pressure recordings were made for 20 min (0900–1100 hours) under Basal conditions and on *day 3* of drug treatment and/or stress as described above.

The experimental groups ($n = 5–6$ mice/group) were as follows: 1) stress with saline infusion, 2) stress with PB infusion, and 3) PB (10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) infusion without stress. The stress paradigm (7) used intermittent shaker stress delivered in the home cage for 3 days (2 min stress, 150 cycles/min, 45 times/day). The interstress rest periods were variable (13–45 min) with the goal of adding unpredictable timing to the stress.

Spectral analysis. A Windaq Waveform Browser (Dataq Instruments; Akron, OH) was used to process AP data to extract beat-by-beat time series of PI and mean AP (MAP). The overall variabilities of the PI and MAP series in time domain were calculated and expressed as the standard deviation (SD) of the entire time series. PI and MAP fluctuations were assessed in the frequency domain using autoregressive spectral analysis (software provided by A. Porta, Milan, Italy). The theoretical and analytic basis for autoregressive modeling of oscillatory components has been described previously (3, 38).

Briefly, the PI and MAP series were divided in segments of 300 beats, overlapped by 50%. The spectra of each segment were calculated via the Levinson-Durbin recursion, and the order of the model was chosen according to Akaike's criterion, with the oscillatory components quantified in the LF (0.1–1.0 Hz) and high-frequency (HF; 1.0–5.0 Hz) ranges (29). Spontaneous baroreflex sensitivity (BRS) was calculated using the α -index within the LF range (α -index = square root of the $\text{LF}_{\text{PI-to-LF}_{\text{MAP}}}$ power ratio). The calculation of the α -index requires the presence of significant coherence between PI and MAP time series at the LF range. Therefore, to evaluate the coherence, a bivariate autoregressive analysis was performed between PI and MAP time series (42, 46). In the LF range, the coherence between PI and MAP is an expression of the baroreflex control of HR (38, 42, 46). The α -index was calculated in all cases because the coherence value was significant ($k^2 > 0.5$).

Blood ChE. Total blood ChE, AChE, and butyrylcholinesterase (BChE) activities were determined before treatment (Basal) and on *day 3* of treatment. ChE activity was determined by a modified colorimetric method (6, 13). Measurements were made in whole blood, which was collected from the arterial catheter. Blood samples were stored at 4°C, and enzyme activities were determined within 4 h of collection. AChE activity was determined by inhibiting BChE activity with 25 μ M tetraisopropylpyrophosphoramidate (Sigma Chemical; St. Louis, MO). BChE activity was calculated by subtracting AChE activity from total ChE activity.

Statistical analysis. Results are expressed as means \pm SE. ChE activities were compared using one-way ANOVA. Multi-way ANOVA was used to determine differences in day-night variations of MAP and HR (day cycle, group, and time as sources of variation). Two-way ANOVA for repeated measures was performed on all MAP/HR baseline values as well as their respective parameters of variability and baroreflex index data. There was no violation of circularity assumptions, as required for the use of two-way ANOVA for repeated measures. Tukey test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

MAP and HR were measured over 24-h periods with calculation of the day-night averages (Fig. 1). There was a significant effect of diurnal circadian cycle with increases noted during the dark/active period [$F_{(1,60)} = 6.97$ and $P = 0.01$ for HR; $F_{(1,60)} = 16.82$ and $P = 0.001$ for MAP]. There was no overall effect of stress or PB on the circadian patterns of MAP and HR; there were, however, individual stress responses over the 24-h period as shown in a previous publication (7).

For evaluation of PI variability, pressure was sampled over a shorter period of time (~ 20 min). There were significant treatment [$F_{(3,81)} = 6.56$ and $P = 0.024$] and interaction [$F_{(3,81)} = 5.90$ and $P = 0.015$] effects for PI variability in the time domain (SD). There were no differences between stress (5.0 ± 0.9 ms) and PB (7.5 ± 0.9 ms), but the combined treatment produced up to a twofold increase in SD (11 ± 1.4 ms, PB/Stress).

Figure 2 shows a representative PI time series and respective spectrum from each group in Basal and *day 3* of treatment and/or stress (Stress, PB, and PB/Stress). Spectral analysis showed two distinct oscillations of PI, one in the LF and another in the HF band. The central frequency of the LF oscillation was 0.39 ± 0.06 , 0.36 ± 0.06 , and 0.32 ± 0.05 Hz in Basal and 0.44 ± 0.04 , 0.30 ± 0.02 , and 0.26 ± 0.02 Hz in *day 3* recordings for the Stress, PB, and PB/Stress groups, respectively. The central frequency of the HF oscillation was 3.4 ± 0.2 , 3.0 ± 0.3 , and 3.5 ± 0.1 Hz in Basal and 3.6 ± 0.2 , 3.6 ± 0.3 , and 3.3 ± 0.2 Hz in *day 3* recordings for the Stress,

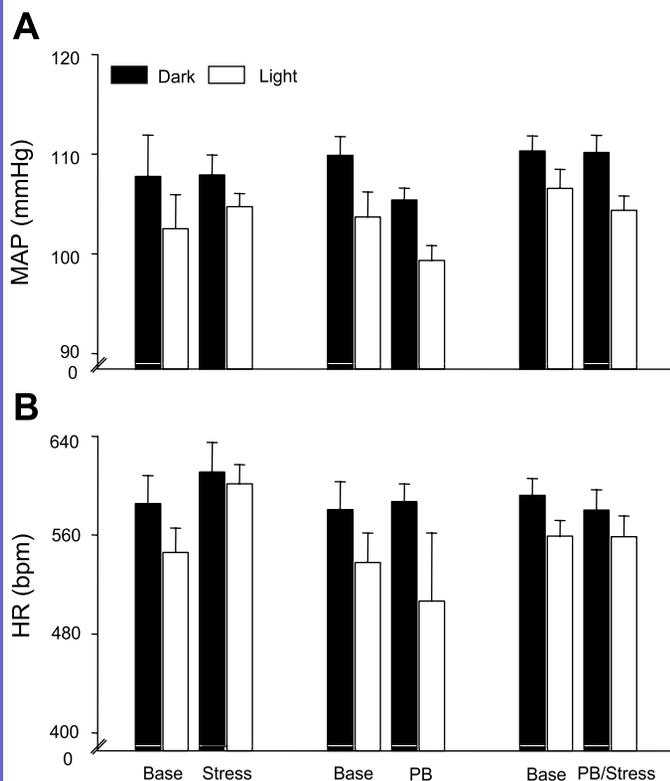


Fig. 1. Day-night average values of mean arterial pressure (MAP; A) and heart rate [HR, in beats/min (bpm); B] recorded over 24 h during basal (Base) and after 3 days of stress, pyridostigmine (PB) treatment, or PB treatment coupled with stress. There were no significant effects of treatment.

PB, and PB/Stress groups, respectively. The frequencies of the oscillations were similar among groups in Basal as well as *day 3* recordings. Shaker stress alone or PB treatment did not alter the frequency or power of either LF or HF oscillatory components. On the other hand, spectral analysis showed a marked increase in the power, but not frequency, of the LF oscillations of PI when PB treatment was coupled with chronic stress (Figs. 2 and 3). The LF peak in the example reached a level of $>4,000 \text{ ms}^2/\text{Hz}$ (power), which required a graphic *inset* with an expanded y-axis. When all group values of LF power were compared (Fig. 3), there were significant effects of treatment [$F_{(3,81)} = 4.87$ and $P = 0.046$] and interaction [$F_{(3,81)} = 4.93$ and $P = 0.025$], and a difference was found only for the PB/Stress group (9.5 ± 1.7 compared with 3.3 ± 0.9 and $5.0 \pm 0.6 \text{ ms}$ in the Stress and PB groups, respectively).

The spectral analysis of MAP revealed two distinct LF and HF oscillatory components at frequencies similar to the PI oscillations (Table 1). There were no differences in the power of MAP oscillations in the groups (Table 1).

Bivariate spectral analysis between PI and MAP revealed high coherence values (0.67–0.96) in both LF and HF range. The α -index calculated in the LF range was not affected by stress ($1.8 \pm 0.3 \text{ ms/mmHg}$) or PB treatment ($1.7 \pm 0.5 \text{ ms/mmHg}$), but it increased tremendously when PB treatment was combined with chronic shaker stress ($3.6 \pm 0.5 \text{ ms/mmHg}$; Fig. 4).

Blood ChE and AChE activities were reduced in the mice treated with PB (Fig. 5), but there was no effect of stress. BChE was not altered by the treatment.

DISCUSSION

The idea that psychosocial stress can modify the effects of pharmacological agents, resulting in enhanced or reduced effects, has received much attention. In retrospective analysis of the “Gulf War syndrome,” there was speculation that stress interacts with the ChE inhibitor PB to accentuate the pathology (23–25). While there is experimental information on the behavioral, enzymatic, and receptor alterations associated with stress and PB treatment, there are less data on the cardiovascular axis (1, 4, 17, 51). We approached the problem by testing the effect of stress and PB exposure on the 24-h pattern of BP and HR and the associated variability in the time and frequency domains. Results were surprising in that there were no changes in absolute BP or HR levels in any of the groups, but there were dramatic increases in variability when PB treatment was coupled with stress exposure. This was seen as an increase in the HR spontaneous variations in both time and frequency domains and an increase in baroreflex index. The changes in the HRV were specific for the LF range, which is associated with autonomic and baroreflex function in mice. These results demonstrate 1) the utility of a detailed statistical analysis of cardiovascular oscillatory patterns for functional evaluation and 2) evidence for potent interactions between environmental stressors and cholinergic systems, raising questions about the global use of PB.

There was increased interest in PB and its physiological effects after the conclusion of the 1992 Persian Gulf War (9, 23–25, 32). This was a situation in which large numbers of healthy, young people were given PB chronically. The military personnel were required to take PB (45 mg po 3 times daily) as a prophylactic against chemical warfare agents. With the appearance of the so-called Gulf War syndrome in the years after the military engagement, there was speculation that PB had toxic side effects that were not observed under normal conditions (23, 24). Experimental studies explored the idea that stress, a normal condition of military deployment, might alter the effects of PB. Friedman et al. (17) showed that swim stress in mice increased the entry of PB into brain tissue and activated neuronal systems. Later, they verified that the treatment produced long-lasting changes in cholinergic neuronal expression (31). However, these findings were not universally accepted because others failed to find significant interactions between PB and stress as measured by behavior and ChE activity (21, 30, 51). One investigation even reported an enhancement, rather than a reduction, in AChE activity after PB/stress (50). We studied the effect of chronic PB infusion in mice and found that there were regional changes in brain ChE activity (45). AChE was reduced or increased in the hypothalamus but unchanged in the cerebral cortex. There were no changes in behavioral parameters as related to locomotion and no changes in BP or HR (6). Beck et al. (4) reported a small change in cortical AChE activity in animals subjected to a PB/electric shock combination. PB may also interact with the central nervous system (CNS) via effects on afferent nerve traffic as demonstrated by a study that showed that PB produced rapid changes in stress-induced hyperthermia (28).

Investigations in humans have focused on the idea that PB may have beneficial effects, related to HRV, and might be used as a treatment for heart disease. There is evidence that autonomic dysfunction, associated with increased sympathetic

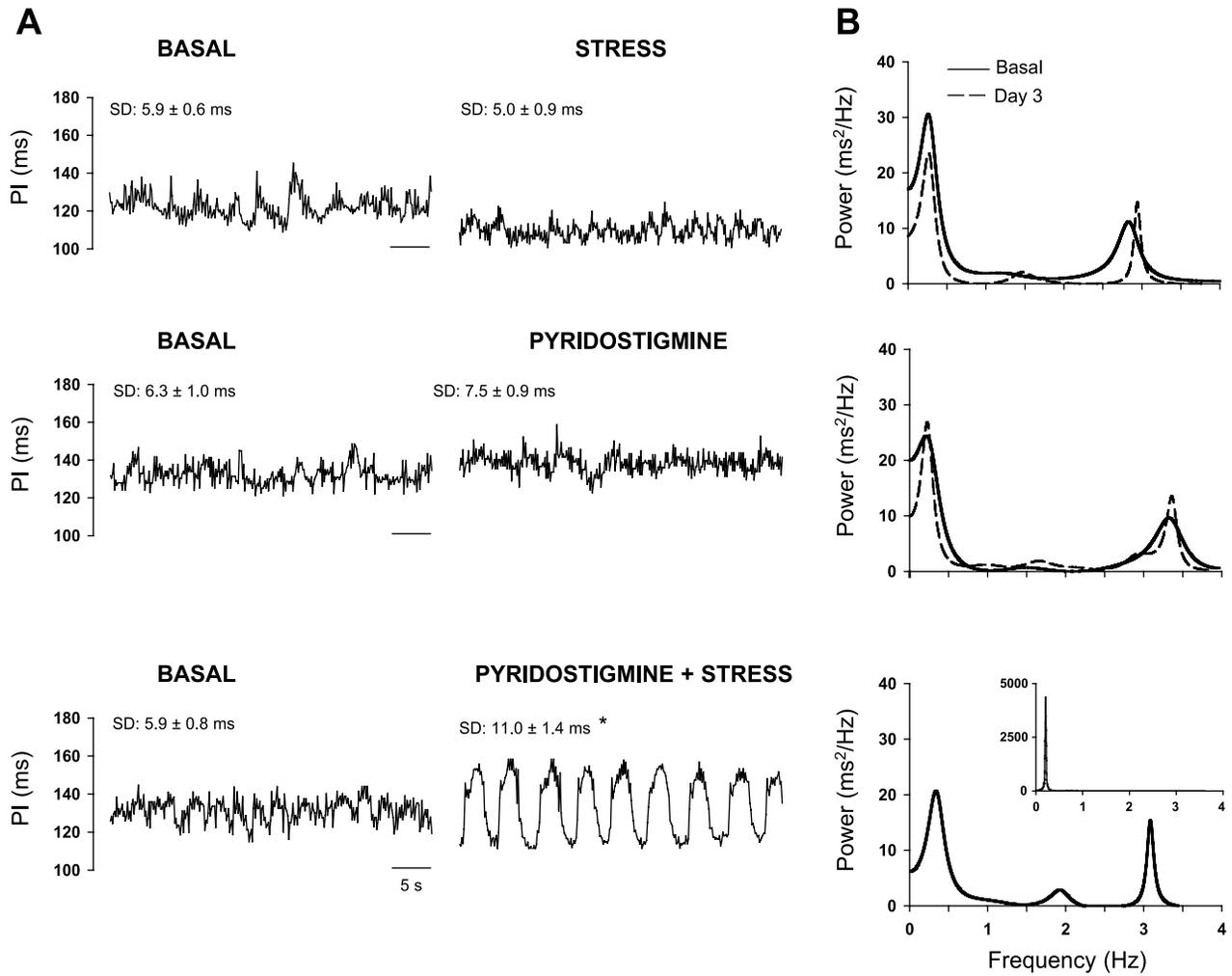


Fig. 2. *A*: time series of pulse interval (PI; in ms) for one representative mouse of each experimental group during basal conditions and 3 days after stress, PB, or PB/stress. The respective spectra are shown in *B*. The standard deviation (SD; in ms) of PI variability is shown above the time series (means ± SE). The graphic *inset* with an expanded y-axis was necessary for the PI spectra of the PB/stress mouse on *day 3* because of the large low-frequency (LF) power. **P* < 0.05 vs. basal in the PB/stress group.

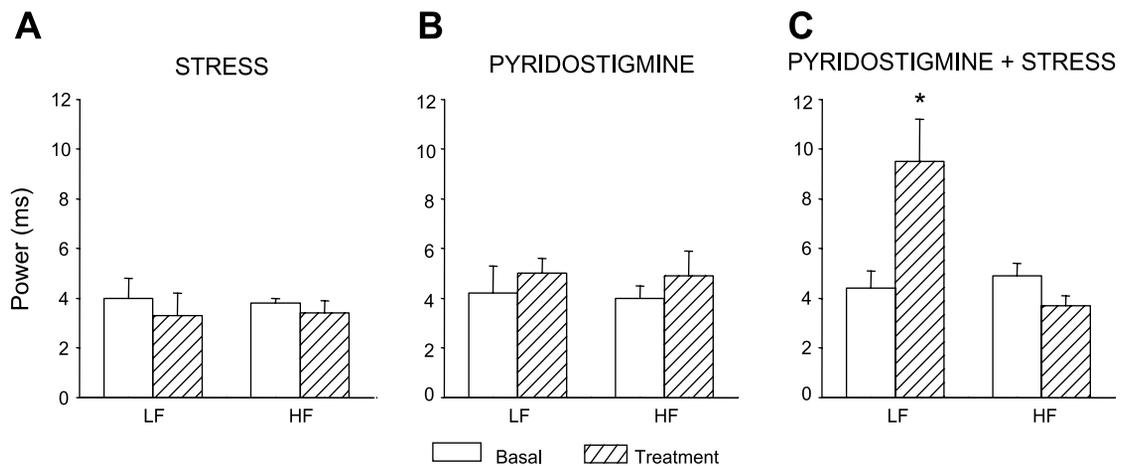


Fig. 3. Power spectral density (in ms) of LF (0.1–1.0 Hz) and high-frequency (HF; 1–5 Hz) components of PI variability calculated by spectral analysis for each experimental group [stress (*A*), PB (*B*), and PB/stress (*C*)] during basal and after treatment. **P* < 0.05 compared with basal.

Table 1. MAP, and respective variability in time and frequency domains under basal and treatment conditions

	Stress		PB		PB/Stress	
	Basal	Day 3	Basal	Day 3	Basal	Day 3
MAP, mmHg	103±5	99±2	101±4	96±4	95±1	94±3
SD, mmHg	3.2±0.5	2.3±0.2	2.9±0.3	3.3±0.5	2.5±0.2	3.1±0.3
LF, mmHg	2.8±0.5	1.8±0.2	2.7±0.3	3.0±0.5	2.2±0.2	2.8±0.4
HF, mmHg	1.3±0.2	1.1±0.1	0.9±0.1	1.1±0.1	1.0±0.1	1.2±0.1

Data are expressed as means ± SE. LF, low-frequency power; HF, high-frequency power; PB, pyridostigmine. Under basal conditions, the central frequencies in the three groups were LF MAP, 0.41 ± 0.05 , 0.32 ± 0.01 , and 0.32 ± 0.04 Hz; and HF MAP, 3.4 ± 0.2 , 3.0 ± 0.3 , and 3.5 ± 0.1 Hz. The treatments produced no changes in LF or HF central frequencies.

drive and reduced vagal modulation, may play a role in heart failure. Indeed, reduced HRV is a predictor of increased mortality risk in patients with heart disease (18, 34). Behling et al. (5) tested the effect of PB treatment in patients with heart failure. They reported a 65% reduction in ventricular ectopic activity and an increase in HRV and suggested that long-term clinical trials were warranted. Studies in healthy subjects showed that PB treatment increased HRV (41) and interacted with mental stress to produce increased left ventricular outflow velocity (47). Thus results indicate that PB may act in the periphery, on nerve endings, to stimulate acetylcholine release and modulate cardiac function.

Spectral analysis techniques have been applied to the study of cardiovascular function in humans and rodents. The basic premise is that BP variability and HRV studied in the frequency domain provides information on autonomic modulation of cardiovascular system and may be used to calculate spontaneous baroreflex activity (2, 3, 12, 26). In humans, the LF-to-HF ratio is used to quantify sympathetic/parasympathetic balance; however, in mice this relationship is not so clear (26, 29). Both sympathetic and vagal modulations of the heart have significant roles in the genesis of LF oscillations in mice (26). Cholinergic blockade attenuates LF of PI variability as does genetic enhancement of β -adrenergic input or treatment with dobutamine (26, 29, 52, 54). On the other hand, the opposite effect (an increase in HR LF power) was achieved by β_1 -adrenergic blockade with metoprolol (54). Nevertheless, the choice of mice in the present study was justified by the

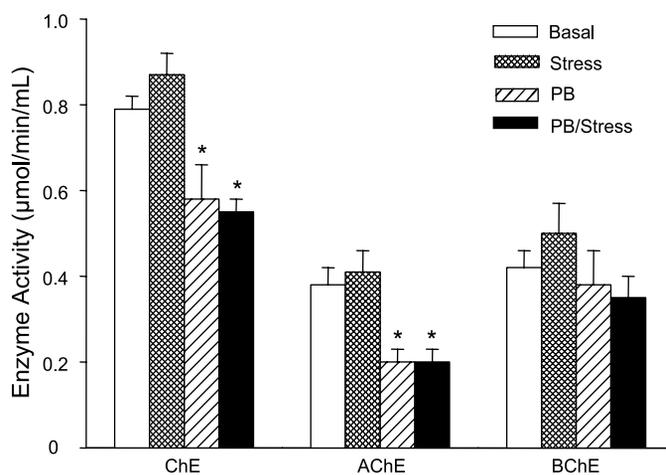


Fig. 5. Blood cholinesterase activities (in $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$) during the basal period and 3 days after treatment. * $P < 0.05$ compared with basal. ChE, total ChE activity; AChE, acetylcholinesterase; BChE, butyrylcholinesterase.

increasing reliance on the use of this species for physiological genomic studies.

In addition to studies of HRV, spectral analysis has been applied to spontaneous fluctuations of AP (26, 29, 52). In humans and rats, it is well established that slow rhythms of BP (Mayer waves) are modulated by sympathetic activity, which is physiologically under the control of the baroreceptor reflex (3, 42, 46). In mice, spectral analysis has revealed cumulative variability between 0.08 and 1.0 Hz, characterizing the LF range in this species (26). Peripheral autonomic blockade with prazosin, a specific α_1 -adrenoreceptor antagonist, abolished the LF peak of BP spectra in mice, whereas inhibition of endogenous nitric oxide (NO) formation consistently enhances BP variability in the LF range (26, 52). This latter result suggests that endogenous NO could function as a buffer for such fluctuations. It is noteworthy that cholinergic or β -adrenergic blockade seems to have little effect on BP variability and its spectral parameters (26).

Even considering the limitation of transferring data regarding HRV from mice to other species, the usefulness of spectral analytic methods is clearly evident in the present study. Continuous measurement of BP and HR failed to reveal any effect of PB, stress, or the combination in the steady-state values of

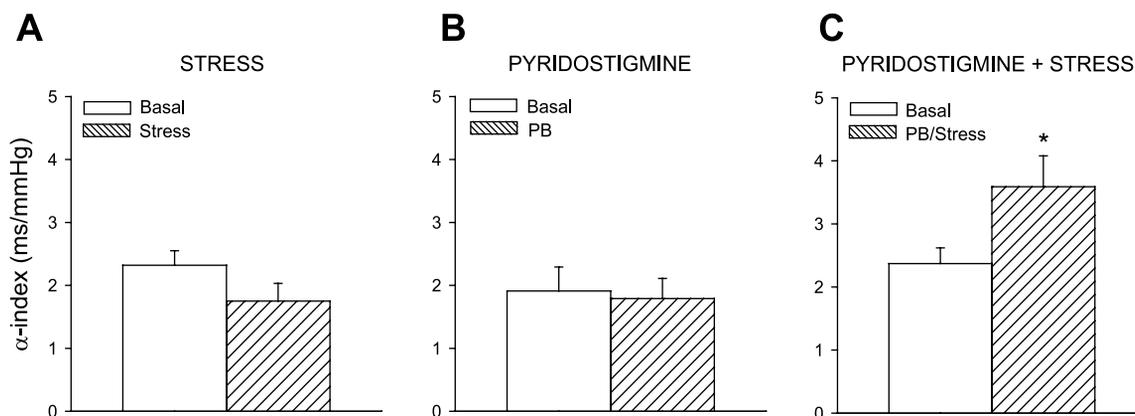


Fig. 4. Index of baroreflex sensitivity (α -index; in ms/mmHg) calculated for all experimental groups [stress (A), PB (B), and PB/stress (C)] during the basal period and 3 days after treatment. * $P < 0.05$ compared with basal.

these cardiovascular parameters. In contrast, spectral analysis showed that there were marked changes (up to 2-fold) not only in PI variability in the time and frequency domain but also in the baroreflex index, when PB was associated with stress. Several studies have shown that HR and AP variabilities in the LF range are associated to the autonomic modulation of the cardiovascular system, because these oscillations are generated in the CNS and transferred to the periphery through autonomic nerves (2, 3, 10, 27, 39). Even though the LF oscillations could be differentially transmitted to the heart and vessels, HR fluctuations should not be totally disconnected from AP fluctuations because the baroreflex (10, 11, 46). In this way, changes in BRS could strongly affect the power of the HR oscillations in the LF range (16), which could explain, at least in part, the results found when combining PB treatment and stress exposure.

Studies in humans also failed to show changes in baseline hemodynamic parameters after PB treatment; changes were evident only when PB was combined with mental stress (40). Thus it appears that the ChE effects on HR are somehow dependent on possible alterations in the autonomic drive to the heart induced by 3 days of shaker stress. This is supported by data that suggest that autonomic drive input is critical in the mediation of variability, because treatment with autonomic blockers reduced LF variance and attenuated the response to stress (15).

The mechanism responsible for the enhanced PI variability and baroreflex index found in the PB/Stress group could involve afferent baroreflex input, autonomic drive to the circulatory system, or the complex integration of the baroreflex at the level of the CNS. Regarding the CNS, it is well known that the brain is protected from many classes of pharmacological substances by the BBB, including PB itself. This is not the case for the peripheral nervous system. Thus we would expect PB to manifest its pharmacological and toxicological actions primarily in the periphery. However, one cannot discount central actions because the BBB was modified in specific brain regions under stressful conditions (17, 48). The probable mechanism underlying BBB permeability in these conditions is speculative but may involve neurochemical mediators, such as serotonin (48). Infusion of this amine in small amounts increased BBB permeability within 15 min after administration. In another study, swim stress in mice increased BBB permeability, reducing the PB dose required to inhibit mouse brain AChE activity by 50% (17). This coincided with a >10-fold increase in brain penetration of an albumin-binding dye. Because we found the PB effect only in the presence of stress, it is reasonable to speculate that the effects observed are due to CNS interactions.

In line with other species, baroreflex control of HR seems to have an important role in the genesis of slow oscillations of HR in mice (26, 29). Therefore, it is not surprising that the marked increase in the power of LF oscillations of PI is associated with an increase in the α -index, a measure of spontaneous baroreflex. In humans, there was a relationship between vagal control of heart and BRS (22). Patients with low vagal tone had reduced BRS and vice versa for patients with high tone. Once again, the mechanism(s) responsible for the enhanced BRS associated with PB administration and stress could involve either of the mechanisms listed above (baroreflex afferent and efferent branches and the complex integration at the CNS).

In conclusion, spectral analysis of PI and BP variability revealed a hidden cardiovascular effect of the combination of PB treatment and stress exposure. Extended treatment with PB and stress resulted in dramatic changes in HRV and baroreflex function, likely associated with alterations in sympathetic/parasympathetic balance in the heart.

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Behavioral changes after acetylcholinesterase inhibition with physostigmine in mice

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Abstract

The effect of the central and peripheral acetylcholinesterase (AChE) inhibitor, physostigmine (PHY), was examined on spatial memory using a water maze, motor activity as well as acoustic startle response (ASR) and prepulse inhibition (PPI) in C57BL/6J mice. PHY was administered intraperitoneally (IP) at doses of 0.0, 0.01, 0.03, 0.1 and 0.3 mg/kg and the mice were tested 30 min after injection. Administration of PHY reduced motor activity in the open field in a dose-dependent fashion, with notable decreases in activity observed at 0.1 and 0.3 mg/kg. The results also showed that animals receiving 0.1 mg/kg spent more total time in the peripheral zone than in the central zone. The water maze data showed impairment of acquisition and performance of the task, accompanied by a reduced swimming time and enhanced thigmotaxis at a dose of 0.1 mg/kg. We also found that the ASR was significantly decreased after 0.03 and 0.1 mg/kg with no change in PPI. These results indicate that central plus peripheral cholinesterase inhibition (ChEI) decreased ASR, which is contrary to our previous experiments with the peripheral ChEI pyridostigmine bromide (PB), suggesting different involvement of cholinergic systems in modulating ASR in mice.

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Keywords: Acoustic startle response; Locomotor activity; Water maze; Physostigmine; Mice; Cholinesterase

1. Introduction

Cholinesterase inhibitors (ChEI) of carbamate, organo-phosphorus and aminoacridine structures are widely used and intensively studied agents. Prior work from this laboratory examined the behavioral effects of the peripherally active acetylcholinesterase (AChE) inhibitor pyridostigmine bromide (PB). The present study is focused on the evaluation of behavioral changes after central and peripheral AChE inhibition by physostigmine (PHY). The rationale for this study was to determine whether differences exist between ChEI with combined peripheral/central and peripheral only AChE action on different types of behavior. The

results of these experiments will be useful in comparing the data from studies employing chemical warfare agents.

Physostigmine (PHY) is an old drug, isolated from Calabar beans by Jobst and Hesse in 1864. The first therapeutic use of this drug was in 1877 by Laquer, in the treatment of glaucoma, and is one of its clinical uses today (Hardman et al., 1995). PHY has recently regained prominence due to its use in the clinical trials of Alzheimer's disease and to its potential as a potent prophylactic antidote to chemical warfare agent poisoning, providing protection for both central and peripheral cholinesterase (ChE; Leadbeater et al., 1985). PHY is a reversible cholinesterase inhibitor and has a short duration of action. Since it is a tertiary amine, PHY is lipid soluble and hence crosses the blood–brain barrier (BBB) to produce central actions (Somani et al., 2001).

The measurement of movement and locomotion is central to obtaining qualitative and quantitative information about an animal's general behavior. Changes in an animal's spatial

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location and/or changes in the spatial relationship of the animal's limbs and body have consequences for the expression and measurement of all aspects of behavior. PHY increased rat exploratory activity after intracerebroventricular injection of a limited dose range (Sienkiewicz-Jarosz et al., 2003). PHY administered subcutaneously decreased the distance traveled in open field in a dose-dependent fashion (Wang and Fowler, 2001). The dose of 0.5 mg/kg PHY administered intraperitoneally (IP) decreased rat locomotor activity as well (Sienkiewicz-Jarosz et al., 2000); similarly in C57BL/6J mice, PHY administered IP decreased locomotor activity (Retz et al., 1987).

The acoustic startle response (ASR) is a behavioral reflex evoked by abrupt, intense sound that is readily elicited in a wide variety of animal species, including humans (Landis and Hunt, 1939). In rodents, it is a sensitive method for determining how different neurotransmitter systems modulate sensorimotor activity (Davis, 1980). Despite its relatively simple, reflex-like appearance, the startle response magnitude can be modulated by a variety of external and internal variables. Under appropriate experimental conditions, startle has a nonzero baseline and can be enhanced or attenuated (Koch, 1999). The ASR magnitude is reduced if a distinctive nonstartling tactile (Pinckney, 1976), visual (Campeau and Davis, 1995), or acoustic (Hoffman and Ison, 1980) stimulus is presented 30–500 ms prior the startling stimulus. This phenomenon is termed prepulse inhibition (PPI) and is used as an operational measure for sensorimotor gating mechanisms (Hoffman and Ison, 1980). Studies using PHY report different results in rats and guinea pigs (Jones and Shannon, 2000; Philippens et al., 1997). While the influence of PHY on ASR and PPI in mice has not been reported, we found in our previous experiments that pyridostigmine bromide (PB) increased ASR and reduced PPI in mice (Lucot et al., 2002).

The aim of this study was to determine the effect of PHY on locomotor activity, spatial memory and ASR and PPI in C57BL/6 mice in order to compare its central plus peripheral action with that of peripherally active PB, and to establish normative data in mice for ongoing studies with chemical warfare agents.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (HARLAN, body weight 26–28 g, aged 3 months) were individually housed in plastic cages with wood shaving bedding in a temperature-controlled room ($T=70^{\circ}\text{F}$) with 12:12-h light/dark cycle (lights off from 1700 h). Standard pellet diet and tap water were provided ad libitum. After 10 days of acclimatization, the mice were subjected to 3 days of handling before behavioral testing. All experiments were in compliance with the National Institutes of Health Guide for Care and Use of

Laboratory Animals and the protocol was approved by the Institutional Laboratory Animal Care and Use Committee.

2.2. Treatment

Physostigmine (eserine sulfate, control no. 6864, Nutritional Biochemicals, Cleveland, OH) was dissolved in 0.9% sodium chloride (saline) and administered intraperitoneally (IP) 30 min before testing (10 ml/kg).

2.3. Behavioral protocols

2.3.1. Water maze protocol

The water maze apparatus (Videomex-V, Columbus Instruments Int., Columbus, OH) consisted of a circular pool 110 cm in diameter, which was filled with water to a depth of 50 cm and had a circular plastic platform 8 cm in diameter placed 0.5 cm below the surface of the water. The water temperature was 75°F and colored with white tempera paint to hide the platform. The pool was placed in a room surrounded with fixed spatial cues (cabinet, table, computer, pictures, camera, sink, etc.). For descriptive data collection, the pool was subdivided into four equal quadrants formed, which intersect in the center of the pool at right angles and into two concentric circles subdividing the pool into peripheral and central (60 cm in diameter) zones. The protocol for the water maze was previously described in Dell'Anna et al. (1997). Testing consisted of placing the mouse in one of the randomly chosen quadrants facing the pool wall and permitting it to swim for 2 min. If it failed to find the platform, it was gently guided to the platform and stayed there for 20 s before the next trial. If it found the platform, it was permitted to remain for 20 s before the next trial. All mice received four consecutive trials, each randomly started in a different quadrant. The water was cleared of any debris with a scoop. The mouse was patted dry, placed in a home cage, allowed to rest on a heating pad until dry and then returned to the colony. The mice ($n=18$) were acclimated to the facility for 10 days and then handled 3 min per day 3 days before testing. Prior to the test, each mouse was placed in the water without the platform and observed for 60 s to verify that it was able to swim. Mice were injected IP with either saline or 0.1 mg/kg physostigmine 30 min before testing. On Monday through Friday, the platform was hidden in the SE quadrant and the latency time to find the platform was recorded (Place Navigation—phase I of spatial memory acquisition). The following Monday to Wednesday, the platform was moved from SE to NW quadrant, 1 cm above the water surface and the mice tested using the same protocol as the first week (Cue Navigation). On Thursday and Friday, the platform was hidden in the NW quadrant and testing was conducted (Place Navigation—phase II of spatial memory acquisition). The initial dose 0.1 mg/kg of PHY was selected based on Symons et al. (1988), who reported that this dose improved spatial learning in C57BL/10J mice.

2.3.2. Open-field test

Locomotor activity was evaluated in an automated open-field system with infrared photo-beams (Motor Monitor, Version 3.11, 2000, Hamilton Kinder, Poway, CA). The open field was 16×16 in. (40.6×40.6 cm), and it was divided into central (12×12 in.) and peripheral zones. The mice ($n=18$) were 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 and head pokes in individual zones were also recorded. As in the case of the water maze experiment, all animals were regularly handled before individual tests in order to minimize handling-related stress. The animals were assigned to the groups (Control vs. PHY group) according to their basal locomotor activity which was evaluated before any injections. The mice received saline or PHY injections weekly, beginning with the lowest dose. One day before every administration, we observed motor activity in open field to screen out possible effects of habituation or cumulative drug effects. Thirty minutes after IP administration of PHY at 0.0, 0.01, 0.03, 0.1 or 0.3 mg/kg, mice were placed in the open field for 15-min sessions. After the session, the number of fecal pellets (defecation) was noted for assessment of emotional reactivity and the open-field arena was cleaned with 70% alcohol solution and allowed to dry.

2.3.3. Acoustic startle response and prepulse inhibition

Mice were tested in the SM100 Startle Monitor System Version 4.0 (Hamilton Kinder, 2001) for acoustic startle response (ASR) and prepulse inhibition (PPI). The system was programmed for six types of white-noise burst stimulus trials: no stimulus (background, 60 dB), prepulse (70 dB), pulse (100 or 120 dB), prepulse plus pulse (70+100 dB or 70+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 intertrial interval varied from 9 to 16 s. 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 response to 100 and 120 dB. In the chamber, 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 follows: 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 IP administration of PHY at 0.0, 0.01, 0.03 or 0.1 mg/kg. The values in the figures are presented as a peak startle amplitude [Newtons] during a 250-ms recording window.

2.4. Blood collection

For basal ChE activity measurements prior to treatment, blood was collected (~50 μ l) in heparinized cap tubes from cut tails and stored on ice in 1.5 ml microfuge tubes. The mice ($n=24$; 8 for each dose group) were sacrificed 30 min after PHY administration (0.0, 0.01 or 0.1 mg/kg) by decapitation and trunk blood was collected in 1.3 ml microfuge tubes with heparinized beads (Sarstedt, Newton, NC).

2.5. Blood cholinesterase activity

Cholinesterase (ChE) activity was determined by a modified version of the colorimetric assay of Ellman et al. (1961) using a Packard Fusion™ Microplate Analyzer. Fresh whole blood was diluted 1:100 with 0.1 M NaPO₄ pH 7.4 buffer. Total ChE activity was measured in diluted whole blood samples. Blood acetylcholinesterase (AChE) activity was determined at 77 °F by inhibiting butyrylcholinesterase (BChE) with 100 μ M *iso*-OMPA (tetraiso-propylpyrophosphoramidate). BChE activity was then calculated by subtracting AChE activity from total ChE activity.

2.6. Statistical analysis

The data were analyzed by means of one-way or two-way analyses of variance (ANOVAs) followed by a Duncan post hoc test to assess statistical significance. For the water maze data, ANOVA for repeated measures was conducted with the drug (PHY vs. Control) as the between-subject factor, and day (repeated measure: average value per trial per day) as the within-subject factor. These tests were run using the statistics program, STATISTICA 6.1 (StatSoft, Tulsa, OK). The results are presented as means \pm S.E.M. The confidence limit of $p<0.05$ was considered as statistically significant.

3. Results

3.1. Morris water maze

Administration of 0.1 mg/kg PHY increased latencies to locate both the hidden and the visible platform during testing [$F(1,16)=5.57$, $p<0.03$; Fig. 1]. We found an influence of drug administration on swimming speed [$F(1,16)=10.67$, $p<0.005$], which was probably caused by decreased locomotor activity after PHY administration (see below). Circle zone analysis of the water maze revealed a preference for the periphery over the center circle (PHY_{periphery} 90.6 \pm 1.4% vs. Control_{periphery} 75.5 \pm 1.6%) [$F(1,16)=9.13$, $p<0.008$], which we confirmed using zone analysis in the open field (below). The dose of 0.03 mg/kg physostigmine did not change spatial learning and there were no significant

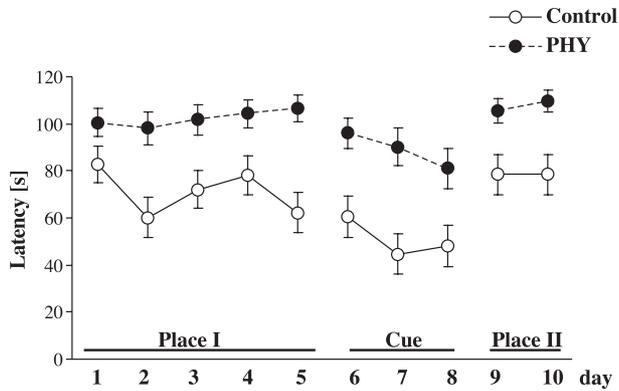


Fig. 1. Latency to reach the platform in Morris water maze exhibited by saline- or physostigmine (PHY—0.1 mg/kg)-treated mice. Main effect of drug administration ($p < 0.03$). Place I phase represents latency to locate hidden platform in the SE quadrant, Cue phase represents latency to locate visible platform in the NW quadrant and Place II phase represents latency to locate hidden platform in the NW quadrant. Values are presented as means \pm S.E.M.

differences in latency time to locate the platform [$F(1,12) = 0.42$; $p = 0.53$; data not shown]. The average speed and time spent in the peripheral circle in both groups were equal (data not shown).

3.2. Open field

Administration of physostigmine affected open-field motor activity in a dose-dependent fashion. The two-way ANOVA demonstrated a significant main effect of treatment [saline vs. PHY; $F(1,59) = 228.3$, $p < 0.001$] and dose [$F(3,59) = 29.84$, $p < 0.001$] on the basic movements. There was a significant effect of treatment \times dose interaction and a post hoc test revealed a significant decrease in activity at 0.03, 0.1, 0.3 mg/kg ($p < 0.001$; Fig. 2). Rearing behavior was also affected significantly. There was a main effect of treatment [$F(1,59) = 63.21$, $p < 0.001$] and dose [$F(3,59) = 4.73$, $p < 0.005$] and post hoc testing revealed an impairment

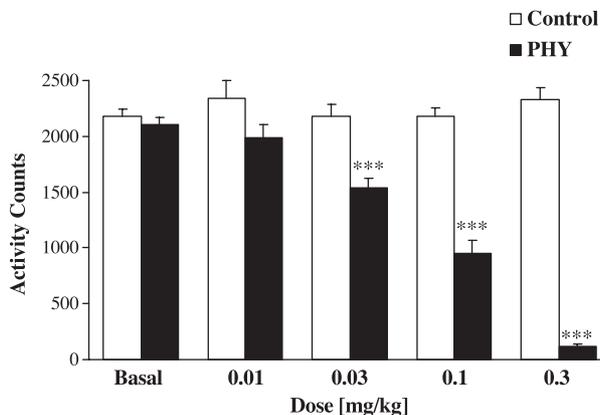


Fig. 2. Locomotor activity (basic movements) in the open field observed for saline-treated (Control) or physostigmine (PHY)-treated mice at the various doses. Data are presented as means \pm S.E.M. ($n = 9$ for each dose; *** $p < 0.001$ —significant difference compared to Control).

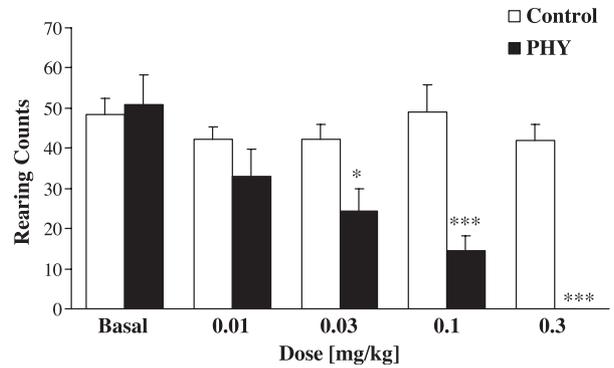


Fig. 3. Exploratory behavior (rearing) in the open field observed for saline-treated (Control) or physostigmine (PHY)-treated mice at the various doses. Data are presented as means \pm S.E.M. ($n = 9$ for each dose; * $p < 0.05$; *** $p < 0.001$ —significant difference compared to Control).

at 0.03 ($p < 0.05$), 0.1 and 0.3 mg/kg ($p < 0.001$; Fig. 3). The doses of 0.1 and 0.3 mg/kg increased the total time in the peripheral zone and decreased it in the central zone [$F(1,59) = 12.21$, $p < 0.001$; Table 1].

3.3. Acoustic startle response and prepulse inhibition

We found that the ASR elicited by the 100-dB stimulus was significantly decreased at 0.03 mg/kg PHY [$F(1,13) = 7.17$, $p < 0.019$] and 0.1 mg/kg [$F(1,13) = 4.75$, $p < 0.048$], as was the ASR elicited by the 120-dB stimulus at 0.03 mg/kg [$F(1,13) = 5.29$, $p < 0.039$] and 0.1 mg/kg [$F(1,13) = 8.12$, $p < 0.014$; Figs. 4 and 5]. The PHY dose 0.01 mg/kg was ineffective. There were no significant effects on the PPI (data not shown).

3.4. Blood cholinesterase activity

There was a significant drug effect on blood total ChE and AChE activity after PHY administration [$F_{\text{ChE}}(1,21) = 5.95$, $p < 0.009$; $F_{\text{AChE}}(1,21) = 8.16$, $p < 0.002$]. The post hoc test revealed a significant decrease in activity at 0.1 mg/kg ($p < 0.05$; Fig. 6). The total ChE and AChE activity were

Table 1
Zone preference in the open field observed for saline-treated (Control) or physostigmine (PHY)-treated mice at the various doses

Dose	Peripheral zone		Central zone	
	Control (n=9)	PHY (n=9)	Control (n=9)	PHY (n=9)
Basal	429.7 \pm 30.5	474.3 \pm 32.2	470.3 \pm 30.5	425.7 \pm 32.2
0.01 mg/kg	574.7 \pm 12.1	658.9 \pm 11.8	325.3 \pm 34.3	241.1 \pm 31.2
0.03 mg/kg	623.2 \pm 13.1	672.3 \pm 9.4	276.8 \pm 37.1	227.7 \pm 26.6
0.1 mg/kg	523.2 \pm 9.8	714.7 \pm 15.9*	376.8 \pm 29.3	185.3 \pm 47.6*
0.3 mg/kg	613.0 \pm 11.7	769.5 \pm 31.1*	287.0 \pm 35.2	130.4 \pm 93.2*

Data are presented as means of time spent in the zone \pm S.E.M.

* $p < 0.05$ —significant difference compared to Control.

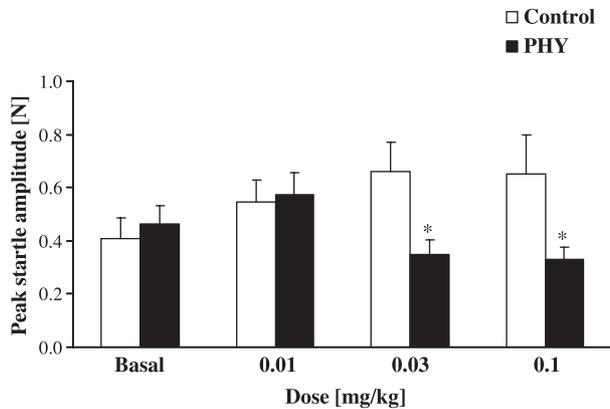


Fig. 4. Acoustic startle response (ASR) after 100 dB stimulus observed for saline-treated (Control) or physostigmine (PHY)-treated mice at the various doses. Data are presented as means \pm S.E.M. (* p < 0.05—significant difference compared to Control).

also significantly decreased compared to the dose of 0.01 mg/kg (p < 0.05).

4. Discussion

Our results show that IP administration of PHY did not improve spatial memory in mice. 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, but it also 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, thus offering an alternative explanation for the failure to acquire and perform the learning task. Furthermore, PHY impaired locomotor activity in a dose-dependent fashion with a robust decrease at the dose of 0.3 mg/kg, and significantly decreased ASR to the 100- and 120-dB stimuli at the doses 0.03 and 0.1 mg/kg. The cholinesterase assay

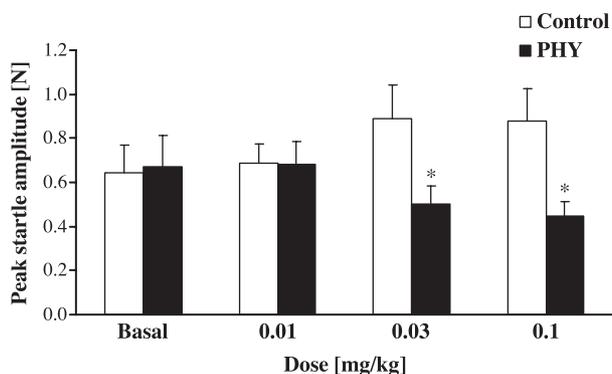


Fig. 5. Acoustic startle response (ASR) after 120 dB stimulus observed for saline-treated (Control) or physostigmine (PHY)-treated mice at the various doses. Data are presented as means \pm S.E.M. (* p < 0.05—significant difference compared to Control).

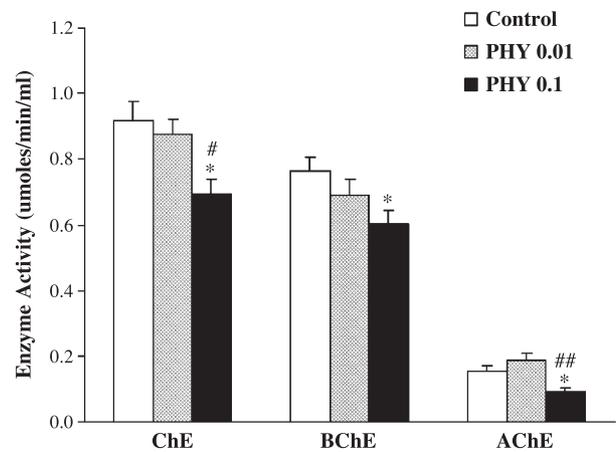


Fig. 6. Blood cholinesterase activity observed for saline-treated (Control) or physostigmine (PHY)-treated mice at the doses of 0.01 and 0.1 mg/kg (ChE—cholinesterase, BChE—butyrylcholinesterase, AChE—Acetylcholinesterase; * p < 0.05—significant difference compared to Control; # p < 0.05; ## p < 0.01—significant difference compared to the dose 0.01 mg/kg).

results showed a significant decrease in enzyme activity at the dose 0.1 mg/kg but not at 0.01 mg/kg.

PHY works by inhibiting the chemical destruction of acetylcholine (ACh) in the synapses, causing increased ACh transmission. Being a tertiary ammonium, PHY effectively crosses the blood–brain barrier (Smith and Swash, 1979) and alters central cholinergic pathways. Central cholinergic systems are implicated in many brain functions, such as learning and memory, habituation, locomotor activity, drug dependence and emotional reactions. Moser (1995) discovered that the profiles of the acute effects of cholinesterase inhibitors (aldicarb, carbaryl, parathion, DFP, chlorpyrifos, fenthion and diazinon) were similar.

It is well known that the central cholinergic systems play an important role in learning and memory and it is generally assumed that in the adult organism, the hippocampus interacts with the neocortex during memory “consolidation” so as to enable information to be permanently stored in cortical sites. Symons et al. (1988) suggest that the hippocampus plays an important role in the acquisition and performance of spatial memory tasks, such as the water maze. Morphological differences in hippocampal cell count and volume exist in selectively inbred strains of mice (Wimer et al., 1980; Abusaad et al., 1999), providing the opportunity to study hippocampal involvement in learning and memory in intact mice. Symons et al. (1988) found that the acquisition deficit of C57BL/10J mice, caused by a low level and volume of the hippocampal cells, can be overcome by increasing cholinergic function with PHY at the dose of 0.1 mg/kg. However, we observed no improvement in acquisition. The dose of 0.1 mg/kg of PHY impaired acquisition and performance of the task, reduced swimming speed and enhanced thigmotaxis while the dose of 0.03 mg/kg had no effect. Although both C57BL/6J and C57BL/10J mice have a similarly low hippocampal cell number and volume with respect to the genetically defined divisions of the pyramidal

layer (Wimer et al., 1980; Abusaad et al., 1999), increased stimulation of the cholinergic system by administration of PHY did not enhance spatial memory in C57BL/6J as it does in C57BL/10J (Symons et al., 1988). Drug-induced improvements in learning tasks are generally seen when the subject is impaired by an experimental manipulation or genetic abnormality. The dose of 0.1 mg/kg produced extensive locomotor inhibitory effect, which may interfere with learning.

The largest magnitude of change produced by ChEI occurs in autonomic (cholinergic stimulation), neuromuscular (weakness) functions and motor activity. Loizzo et al. (1996) observed significant decreases in motor activity in C57BL/6 mice 15 min after 0.1 mg/kg IP of PHY, but not after 0.025 and 0.05 mg/kg, whereas we also observed changes at 0.03 mg/kg after 30 min. One possible explanation for this apparent discrepancy is that we measured responses 30 min after injection rather than 15 min. Xia et al. (1981) reported that ChE inhibition with PHY in vivo reaches peak levels at 30 min. In the rat, PHY increased exploratory activity after intracerebroventricular injection of a limited dose range (Sienkiewicz-Jarosz et al., 2003) while a dose of 0.5 mg/kg administered IP decreased locomotor activity (Sienkiewicz-Jarosz et al., 2000). PHY administered subcutaneously decreased the distance traveled in the open field in a dose-dependent fashion (Wang and Fowler, 2001). These data suggest that PHY has a similar locomotor activity decreasing effect in both rats and mice.

The startle response is composed of a constellation of reflexes elicited by sudden, relatively intense stimuli. It offers many advantages as a behavioral measure of central nervous system (CNS) activity and can be measured in numerous species, including humans. Anxiogenic drugs, such as yohimbine (Morgan et al., 1993; Fendt et al., 1994), and drugs that reduce inhibitory neurotransmission in the CNS, such as the glycine receptor antagonist strychnine (Kehne and Davis, 1984; Koch and Friauf, 1995), enhance the ASR, whereas drugs that reduce overall excitability of the CNS, such as ethanol or diazepam, attenuate the ASR (Berg and Davis, 1984; Grillon et al., 1994). Although the ASR is a sensitive method for examining modulation of CNS activity in rodents, there are still different interpretations as to how cholinergic systems influence the startle response and PPI in different rodent species. In guinea pigs, PHY dose-dependently enhanced the startle reflex with the maximally effective dose around 0.3 mg/kg, which produced AChE inhibition comparable to our results at the dose of 0.1 mg/kg (Philippens et al., 1996, 1997; Fig. 6). In rats, doses of PHY from 0.01 to 0.1 mg/kg administered subcutaneously had no effect on the ASR or PPI (Jones and Shannon, 2000). However, tacrine (10 mg/kg) significantly decreased startle amplitude (Jones and Shannon, 2000). In our study with mice, PHY caused a significant decrease in the ASR at doses of 0.03 and 0.1

mg/kg, in contrast to what was observed in other species. These discrepancies confirm the finding of Davis (1980), that the role of the cholinergic system in modulating ASR is unclear. Possible explanations for the species differences are differences in body weights and different direct or indirect interactions of PHY with other neurotransmitter systems. In the case of cholinesterase inhibition by organophosphorus agents, differences in carboxylesterase activity among the species also need to be considered (Maxwell et al., 1987).

The caudal pontine reticular nucleus (PnC) is one of the key elements of the primary ASR circuit; it mediates the ASR and is also the recipient of ASR-modulating inputs from a variety of other brain regions that enhance the ASR by aversive states or which reduce the ASR by prepulses (Koch, 1999). The attenuating effect of acoustic prepulses on the ASR probably affects the primary ASR pathway at the level of the PnC (Lingenhöhl and Friauf, 1994; Willott et al., 1994; Carlson and Willott, 1998) by activation of inhibitory cholinergic projections from the pedunculopontine tegmental nucleus (PPTg) to the PnC (Koch et al., 1993; Swerdlow and Geyer, 1993). Systemic administration of muscarinic agonists and microinfusion of the muscarinic/nicotinic agonist carbachol into the PnC decreases the ASR in rats demonstrating that ACh is inhibitory in this brain region. Microinfusion of scopolamine produces an increase in the ASR (Fendt and Koch, 1999), suggesting that this inhibitory circuit is tonically active. Thus, it is reasonable that PHY would decrease the ASR as it would augment tonic inhibitory action. While nicotine produces a small increase in the ASR of C57BL/6J mice (Lewis and Gould, 2003), the muscarinic effects of the excess ACh made available by PHY were of greater magnitude than that of its nicotinic effects. The decrease in ASR produced by PHY may arise from its action on the same neural pathway that underlies PPI. In this case, the PHY may have maximally activated this modulatory input, precluding any other effect from the prepulse itself.

In a previous study, we focused on the effect of chronic administration of pyridostigmine bromide (PB) on ASR, PPI and locomotor activity (Lucot et al., 2002). These results showed that chronic exposure of mice to PB (10 mg/kg/day) resulted in an exaggerated ASR, reduced PPI and a nonsignificant trend to decreased locomotor activity. These behavioral changes were apparent only during exposure to PB and returned to control values when the minipumps ran out of drug. These results indicate an opposite effect of peripheral cholinergic activation on ASR than we observed with PHY, which is a central ChEI as well. However, because PB is a quaternary ammonium carbamate, the increase in the ASR could be due to a direct action on the skeletal neuromuscular junction, since there is a direct action of neostigmine and other quaternary ammonium anti-ChE agents on skeletal muscle. For instance, the intraarterial

injection of neostigmine into chronically denervated muscle, or into normally innervated muscle in which essentially all the AChE has been inactivated by prior administration of diisopropyl fluorophosphate, evokes an immediate contraction, whereas PHY does not (Hardman et al., 1995).

In our study, we found that administration of PHY decreased the locomotor activity in a dose–response manner, suggesting that the influence of combined central plus peripheral AChE inhibition is the same as peripheral only inhibition. However, peripheral cholinesterase inhibition alone increased ASR whereas central plus peripheral cholinergic enzyme inhibition decreased the ASR in mice. While studies with other rodent's species showed similar effect of PHY on the locomotor activity, the effects of PHY on the ASR varied, suggesting species differences in cholinergic modulation of that response.

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Characterization of steady-state activities of cytochrome *c* oxidase at alkaline pH: mimicking the effect of K-channel mutations in the bovine enzyme

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Abstract

The cytochrome *c* oxidase activity of the bovine heart enzyme decreases substantially at alkaline pH, from 650 s⁻¹ at pH 7.0 to less than 10 s⁻¹ at pH 9.75. In contrast, the cytochrome *c* peroxidase activity of the enzyme shows little or no pH dependence (30–50 s⁻¹) at pH values greater than 8.5. Under the conditions employed, it is demonstrated that the dramatic decrease in oxidase activity at pH 9.75 is fully reversible and not due to a major alkaline-induced conformational change in the enzyme. Furthermore, the *K_m* values for cytochrome *c* interaction with the enzyme were also not significantly different at pH 7.8 and pH 9.75, suggesting that the pH dependence of the activity is not due to an altered interaction with cytochrome *c* at alkaline pH. However, at alkaline pH, the steady-state reduction level of the hemes increased, consistent with a slower rate of electron transfer from heme *a* to heme *a*₃ at alkaline pH. Since it is well established that the rate of electron transfer from heme *a* to heme *a*₃ is proton-coupled, it is reasonable to postulate that at alkaline pH, proton uptake becomes rate-limiting. The fact that this is not observed when hydrogen peroxide is used as a substrate in place of O₂ suggests that the rate-limiting step is proton uptake via the K-channel associated with the reduction of the heme *a*₃/Cu_B center prior to the reaction with O₂. This step is not required for the reaction with H₂O₂, as shown previously in the examination of mutants of bacterial oxidases in which the K-channel was blocked. It is concluded that at pH values near 10, the delivery of protons via the K-channel becomes the rate-limiting step in the catalytic cycle with O₂, so that the behavior of the bovine enzyme resembles that of the K-channel mutants in the bacterial enzymes.

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1. Introduction

Cytochrome *c* oxidase (COX)(EC 1.9.3.1) is the terminal enzyme in the respiratory chains of mitochondria and aerobic bacteria, which oxidizes ferrocycytochrome *c* and reduces molecular oxygen to water [1,2]. The enzyme conserves the free energy of the reaction by pumping protons vectorially across the membrane from the mitochondrial matrix to the intermembrane space [3]. For each electron transported from ferrocycytochrome *c* to dioxygen (four in total), two protons are taken up by COX from the mitochondrial matrix. One proton is used in the reduction of

Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CD, circular dichroism; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; COX, cytochrome *c* oxidase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); (H)EPPS, *N*-(2-hydroxyethyl)piperazine-*N'*-(3-propanesulfonic acid); LM, dodecyl β-D-maltoside (lauryl maltoside); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMPD, *N,N,N',N'*-tetramethyl-1,4-phenylenediamine dihydrochloride; UV, ultra-violet

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dioxygen to water, and the other is vectorially translocated across the inner mitochondrial membrane.

Bovine heart COX consists of 13 polypeptide subunits in a 1:1 stoichiometry [4]. COX contains four redox-active metal centers, three of which (heme *a*, heme *a*₃, and Cu_B) are located in subunit I, with the remaining redox center, the dinuclear Cu_A, residing in the solvent exposed portion of subunit II [5]. The active site is the heme *a*₃/Cu_B binuclear center which is the binding site of the substrates, oxygen or hydrogen peroxide [6,7]. The primary binding site for ferrocyanochrome *c* is located on subunit II and electrostatic interactions between amino groups of lysine from the substrate and carboxyl groups from subunit II are thought to align the substrate for maximum electron transfer activity [8,9].

The routes of proton uptake through the enzyme are clearly defined [5]. Two channels, the “K-channel” [10] and the “D-channel” [11–13], are involved in proton uptake from the mitochondrial matrix during specific steps of the COX catalytic cycle. The K-channel appears to be required only for electron transfer steps prior to the interaction with dioxygen, in which the fully oxidized enzyme is reduced by two reducing equivalents. The D-channel is required for electron transfer steps after formation of an oxygenated enzyme intermediate (for a review, see Ref. [14]). Two oxygenated intermediates have been characterized, species P and F. The sequence observed is O→R₂→P→F→O, in which the oxidized enzyme (O) is reduced by two electrons (R₂), reacts with O₂ to yield the oxygenated intermediate P, which is converted by two one-electron transfer reactions to the F state and, finally, back to the O state. This is shown schematically in Fig. 1.

When the enzyme uses hydrogen peroxide (rather than dioxygen) as a substrate, the initial “pre-reduction” to form the R₂ state is bypassed, since the H₂O₂ itself is two electrons reduced compared to O₂ [15]. As shown in Fig. 1, this “peroxide shunt” eliminates the steps in the reaction which utilize the K-channel. This was demonstrated by

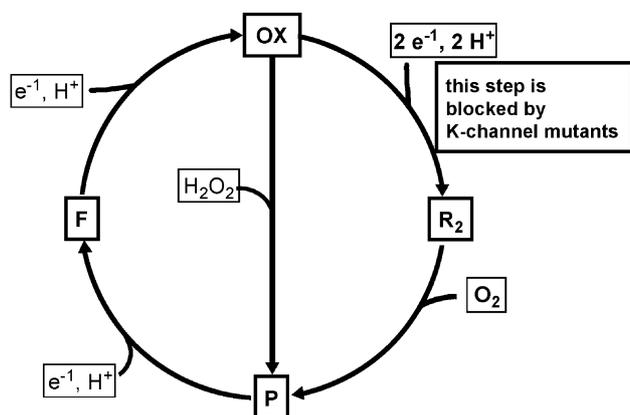


Fig. 1. Schematic diagram of the catalytic cycles of cytochrome *c* oxidase. The catalytic cycles of the enzyme for oxygen and hydrogen peroxide as substrates are presented. Intermediate E (formed by a one-electron reduction), which occurs between intermediates O_x (O), was omitted for simplicity.

analysis of site-directed mutants that block the K-channel in the *Rhodobacter sphaeroides* oxidase [16–18]. In contrast, mutations that block the “D-channel” completely eliminate peroxidase activity as well as the reaction with O₂ [19].

It has been shown previously that the oxidase activity of the bovine enzyme exhibits a steep decrease above pH 8.0, and it was proposed [20,21] that this is due to an increase in the *K_m* of ferrocyanochrome *c*. In the current work, it is demonstrated that the decrease in steady-state activity is not due to a change in the interaction between the oxidase and ferrocyanochrome *c*, but is due to a slow rate of electron transfer from heme *a* to heme *a*₃. Since no decrease in the peroxidase activity of the enzyme is observed in the same range of pH values, the most likely explanation is that at very alkaline pH, proton delivery through the K-channel becomes rate-limiting for the reaction with O₂. This step is not required for the reaction with H₂O₂, so there is no significant pH-dependent drop in the peroxide activity.

2. Materials and methods

2.1. Enzyme preparations

Mitochondria were isolated from bovine heart using the method of Azzone et al. [22] and the oxidase was prepared as described previously [23]. This preparation has been previously demonstrated to be in a monomeric form [24]. Monomeric enzyme has been shown to have distinct kinetic features in single turnover experiments [25], but has unaltered steady-state electron transfer and proton pumping activities [26]. Purity was assessed as described previously [26], protein concentration was determined by the method of Lowry et al. [27], and heme *aa*₃ concentration was determined using a reduced minus oxidized extinction coefficient of 24 mM⁻¹ cm⁻¹ at 605 nm [28].

Horse heart ferrocyanochrome *c* (Sigma, type VI) was prepared as described previously [29]. Reduction and elution were performed at the intended pH and ionic strength of the subsequent assays. An extinction coefficient of 29.5 mM⁻¹ cm⁻¹ at 550 nm was used for ferrocyanochrome *c* concentration [30]. The alkaline form of ferricytochrome *c* at pH 9.75 was monitored at 695 nm [31].

Phospholipid vesicles containing the oxidase were prepared by sonication in 0.1 M CHES NaOH, pH 9.5, as described by Nguyen et al. [32]. Respiratory control ratios of these preparations measured spectrophotometrically at pH 9.75 were routinely 3–5 and the orientation of the cytochrome *c* binding domain facing the outside was approximately 85% [32].

2.2. Steady-state kinetics of ferrocyanochrome *c* oxidation by bovine oxidase

COX was incubated for 1 h on ice in lauryl maltoside (LM) [5 mg LM/mg protein] and the dependence of the rate

of oxidation of ferrocycytochrome *c* on the concentration of cytochrome *c* was measured under four conditions of pH and ionic strength. At pH 7.8 and an ionic strength of 10 mM, both assay and solubilization buffers consisted of 100 mM sucrose, 1.7 mM KCl, 1 mM LM, and 10 mM HEPES NaOH, pH 7.8, while at pH 7.8 and 250 mM ionic strength, these buffers were 100 mM sucrose, 212.5 mM KCl, 1 mM LM, and 45 mM HEPES NaOH, pH 7.8. At pH 9.75 and 10 mM ionic strength, the buffers consisted of 100 mM sucrose, 1.3 mM KCl, 1 mM LM, and 10 mM CHES NaOH, pH 9.75, while at pH 9.75 and 250 mM ionic strength, the buffer was 100 mM sucrose, 210.9 mM KCl, 1 mM LM, and 45 mM CHES NaOH, pH 9.75.

For each individual assay, the initial absorbance level of ferrocycytochrome *c* was determined at 550 nm, and the assay was initiated by the addition of LM-solubilized oxidase to a final concentration of 40 nM. Ferrocycytochrome *c* oxidation was monitored at 550 nm at 25 °C for 2 min on a Hewlett-Packard model 8453 diode array spectrophotometer. Ferrocycytochrome *c* concentration was varied from 0.4 to 33 μM.

The pH dependence of cytochrome *c* oxidase and peroxidase activities was measured at 550 nm using oxidase reconstituted in phospholipid vesicles in a buffer containing 100 mM sucrose, 100 mM KCl, 25 μM ferrocycytochrome *c* and either 10 mM (H)EPPS, NaOH, pH 8.5; 10 mM CHES, NaOH, pH 9.0; 10 mM CHES, NaOH, pH 9.5; or 10 mM CHES, NaOH, pH 10.0. 10 μM valinomycin and 5 μM CCCP were included in the medium to dissipate any gradients formed during the assay. 150 mM H₂O₂ was included as a substrate for the peroxidase assays and all peroxidase rates were corrected for non-enzymatic oxidation of ferrocycytochrome *c* (usually 25% of the total rate). At pH values below 9.0, the non-enzymatic oxidation rates of cytochrome *c* were greater than the oxidase-catalyzed rate.

The steady-state kinetics of hydrogen peroxide interaction with COX in liposomes was monitored at 550 nm using 25 μM ferrocycytochrome *c*, 100 mM KCl, 100 mM sucrose, 10 μM valinomycin, 5 μM CCCP and various concentrations of H₂O₂ (10–500 mM). All assays were corrected for non-enzymatic oxidation of ferrocycytochrome *c* (25–38% of the rate). Hydrogen peroxide concentration was determined by using an extinction coefficient of 40 mM⁻¹ cm⁻¹ at 250 nm [15].

2.3. Steady-state heme *a* reduction levels

Heme reduction levels were monitored at 444–460 nm for hemes (*a+a*₃) and at 605–620 nm for heme *a* [32]. At pH 7.8, the buffer contained 100 mM sucrose, 1 mM LM, 7 mM ascorbate, 0.2 mM TMPD, 1.25 nM cytochrome *c*, and 10 mM HEPES, NaOH, pH 7.8, while at pH 9.75, the buffer was comprised of 100 mM sucrose, 1 mM LM, 7 mM ascorbate, 0.2 mM TMPD, 2 nM cytochrome *c*, and 10 mM CHES, NaOH, pH 9.75.

For individual assays, the substrates and buffer were added to the cuvette and then LM-solubilized oxidase was

added to a concentration of 0.6 μM. Absorbance at 444–460 nm was followed spectrophotometrically for 5 min. The reduction level of the hemes is expressed as a percentage of the ratio of the 444–460- or 605–620-nm absorbance under aerobic steady-state conditions to the fully (dithionite) reduced absorbance at this wavelength. The turnover number of the oxidase is defined as moles of ferrocycytochrome *c* oxidized per mole of oxidase per second.

2.4. Circular dichroism spectroscopy

COX was first incubated in LM [5 mg LM/mg protein] and then diluted to 5 μM COX in one of the following buffers: either 25 mM HEPES, NaOH, pH 7.0, or 7.4; 25 mM (H)EPPS, NaOH, pH 8.0; 25 mM CHES, NaOH, pH 9.0, or 9.75; or 25 mM CAPS, NaOH, pH 10.0 or 11.0, supplemented with 1 mM LM and either 90 or 95 (at pH 11) mM KCl.

Measurements were performed in quartz CD cuvettes with path lengths of 1 mm (for protein) and 10 mm (for heme Soret). All CD spectra were recorded at 18 °C as single scans (10 nm/min) using a Jasco J-500A spectropolarimeter.

3. Results

3.1. The effect of pH on the peroxidase and oxidase activities

Using the assay conditions described above, as the pH of the medium was raised from 8.5 to 10.0, with substrate concentration and ionic strength held constant, cytochrome *c* oxidase activity (which is 650 s⁻¹ at pH 7.0) decreased by one order of magnitude, from 37 to 3 s⁻¹, while the peroxidase activity remained relatively constant (30–40 s⁻¹) (Fig. 2). The dependence of the reaction rate on the concentration of ferrocycytochrome *c* was monitored spectro-

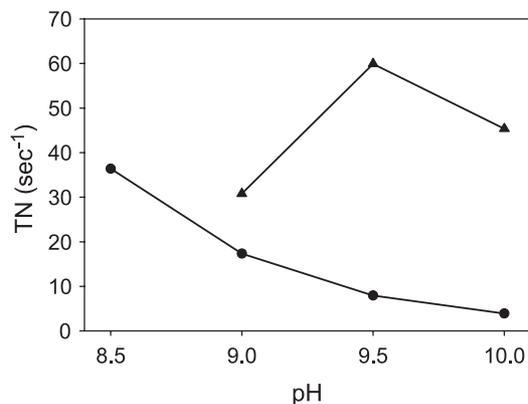


Fig. 2. The pH dependence of COX-catalyzed oxidase and peroxidase activities. Ferrocycytochrome *c* (29 μM) oxidation by COX in phospholipid vesicles (2.5 nM) at different pH values was assayed using either oxygen (circles) or hydrogen peroxide (150 mM, triangles) as substrates.

photometrically at 550 nm at pH 7.8, at which extensive studies have been performed previously [33], and at pH 9.75.

At pH 7.8 under low ionic strength conditions (10 mM), the rate of ferrocycytochrome *c* oxidation by bovine oxidase was biphasic on Eadie–Hofstee plots (Fig. 3A), as shown previously by Ferguson-Miller et al. [33]. The apparent high affinity site had an apparent K_m value of 0.66 μM and a V_{max} value of 100 s^{-1} , while the combined low and high affinity sites had an apparent K_m value of 7.2 μM , with a V_{max} value of 194 s^{-1} in qualitative agreement with Ferguson-Miller et al. [33] and Sinjorgo et al. [21].

At pH 9.75, low ionic strength conditions produced a qualitatively similar biphasic Eadie–Hofstee plot as that obtained at pH 7.8 (Fig. 4A). The high affinity site yielded an apparent K_m of 1.0 μM and a V_{max} value of 5.9 s^{-1} , while the combined high and low affinity sites had an apparent K_m value of 10.7 μM and a V_{max} value of 15 s^{-1} . The similarity of the biphasic Eadie–Hofstee plots and the K_m values observed at pH 7.8 and pH 9.75 under low ionic strength conditions suggests that at low ionic strength, the ability of cytochrome *c* to interact with bovine COX is unaffected by pH, but the rate of electron transfer is reduced.

At pH 7.8 under high ionic strength conditions (250 mM), the Eadie–Hofstee plot was monophasic (Fig. 3B),

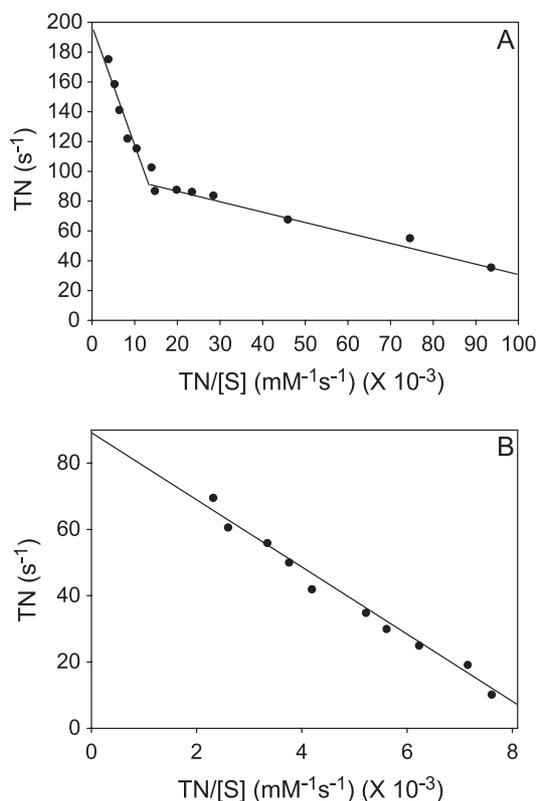


Fig. 3. Eadie–Hofstee plots of steady-state cytochrome *c* oxidation by bovine COX at pH 7.8 under low (A) and high (B) ionic strength conditions. Panel A indicates assays performed in a low ionic strength buffer ($I=0.01$) and Panel B indicates assays performed in a high ionic strength buffer ($I=0.25$) at pH 7.8. Each plot represents the results of three separate experiments. For each data point, three assays were performed per experiment. Values for K_m and V_{max} were estimated by linear regression.

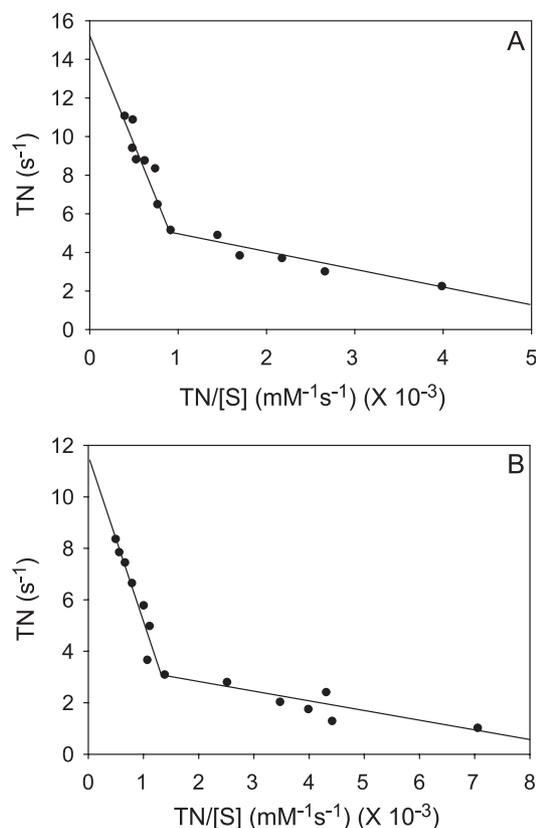


Fig. 4. Eadie–Hofstee plots of steady-state cytochrome *c* oxidation by bovine COX at pH 9.75 under low (A) and high (B) ionic strength conditions. Panel A indicates assays performed in a low ionic strength buffer ($I=0.01$) and Panel B indicates assays performed in a high ionic strength buffer ($I=0.25$) at pH 9.75. All other conditions were as described in Fig. 2.

also in agreement with the findings of Ferguson-Miller et al. [27]. The K_m value was 10 μM , and the V_{max} was 89 s^{-1} . These results are comparable to those reported by Sinjorgo et al. [21] who obtained K_m of 22 μM and V_{max} of 210 s^{-1} .

High ionic strength conditions at pH 9.75 (250 mM) yielded a biphasic Eadie–Hofstee plot (Fig. 4B), with values for apparent K_m and V_{max} qualitatively similar to those observed under low ionic strength conditions at pH 9.75. The high affinity site had an apparent K_m value of 0.38 μM , with a V_{max} value of 4 s^{-1} , while the combined high and low affinity sites had an apparent K_m value of 6.1 μM and a V_{max} value of 11 s^{-1} .

Previous work [31] has shown that equine heart cytochrome *c* exhibits a pH-dependent conformational transition near pH 9.0 and that this alkaline conformational form of cytochrome *c* only exists in the oxidized form. The kinetics of the formation of the alkaline form of ferri-cytochrome *c* was monitored during our assays, and it was determined that neither the extent nor the rate of formation of the alkaline form correlated with oxidase electron transfer.

These results indicate that the low cytochrome *c* oxidase activity at pH 9.75 is not due to an effect on the conformation of cytochrome *c* or due to a change in the

strength of the interaction between cytochrome *c* and the oxidase.

3.2. Steady-state kinetics of cytochrome *c* peroxidase activity using hydrogen peroxide as a substrate

The steady-state kinetics of the peroxidase activity was examined as a function of the concentration of H_2O_2 at pH 9.75, monitoring the oxidation of ferrocycytochrome *c*. The oxidase was incorporated into phospholipid vesicles to stabilize the subunit composition of the enzyme at alkaline pH values, as this pH has been shown to dissociate subunits (III, VIa, and VIb) from the solubilized enzyme [26]. In addition, hydrogen peroxide has been shown to cause chemical modification of the enzyme during long term incubations [34]. The oxidase activity of the enzyme reconstituted in phospholipid vesicles exhibited a respiratory control ratio of 3–5, even at pH 9.75, showing that at pH 9.75, the oxidase can establish a transmembrane electrochemical potential. At pH 9.75, the cytochrome *c* peroxidase activity was at least 10-fold higher than the oxidase activity (Fig. 2).

Fig. 5 shows that at pH 9.75, the peroxidase activity yields a biphasic Eadie–Hofstee plot upon varying the concentration of H_2O_2 . This analysis results in apparent K_m values of 40 and 300 mM, and corresponding V_{\max} values of 30 and 110 s^{-1} . The origin of the biphasic behavior is unknown.

3.3. Steady-state reduction levels of hemes *a* and a_3 at pH 7.8 and pH 9.75

It is well known that the rate of electron transfer from heme *a* to the heme a_3/Cu_B binuclear center is pH-dependent [35] and decreases at alkaline pH. In order to investigate the possibility that this process might be rate-limiting under the assay conditions used in the current work,

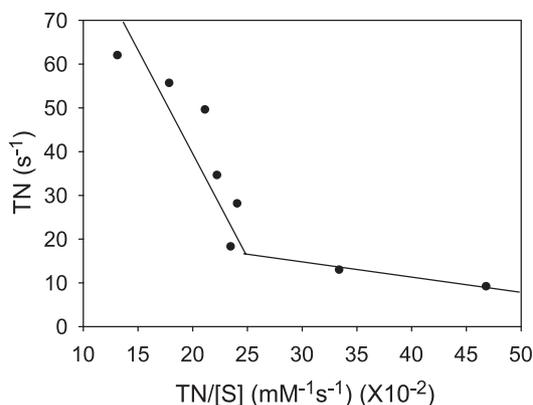


Fig. 5. Eadie–Hofstee plot of steady-state cytochrome *c* oxidation by bovine COX at pH 9.75 using hydrogen peroxide as a substrate. Ferrocycytochrome *c* (27 μM) oxidation by COX in phospholipid vesicles (2.5 nM) at pH 9.75 was assayed using various concentrations of hydrogen peroxide (20–500 mM) as the substrate.

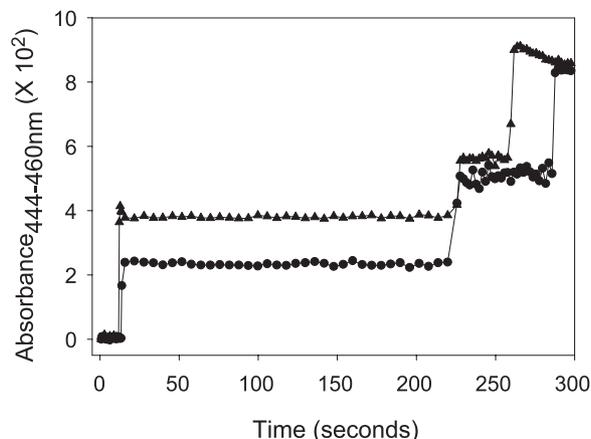


Fig. 6. Time course of the steady-state reduction levels of heme aa_3 of COX at pH 7.8 and pH 9.75. Heme reduction levels were monitored continuously at 444–460 nm [32], using oxidized COX as a spectrophotometric blank. The initial absorbance increase (at ~10 s) reflects the addition of COX, the second absorbance increase (at ~220 s) reflects the onset of the anaerobic state, and the final increase reflects the addition of dithionite in order to completely reduce the enzyme. The circles indicate COX assayed at pH 7.8, while the triangles indicate COX assayed at pH 9.75.

the steady-state reduction level of hemes *a* and a_3 was monitored by the absorbance at 444–460 nm at both pH 7.8 and 9.75 with COX undergoing a similar turnover number ($8\text{--}10 \text{ s}^{-1}$) at both pH values [36]. Fig. 6 shows that at both pH 7.8 and 9.75, the aerobic steady-state lasted approximately 210 s, at which point all dioxygen in the cuvette had been consumed and the reaction became anaerobic. At pH 7.8, the aerobic steady-state level of reduction of the heme $a+a_3$ was approximately 25%, whereas at pH 9.75, the reduction level of the hemes was 40% of the fully reduced enzyme. Similar results (approximately 30% and 60% reduction levels at pH 7.8 and 9.75, respectively) were obtained when the absorbance at 605 minus 620 nm (to correct for TMPD absorbance [48]) was monitored; the absorbance at 605 nm is a qualitative measure of heme *a* reduction levels (data not shown). The data shown in Fig. 6 most likely underestimate the steady-state reduction level of

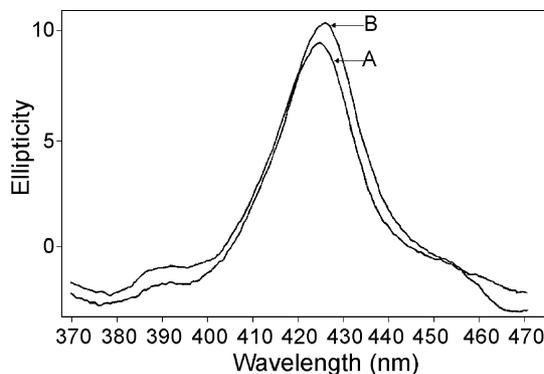


Fig. 7. Visible circular dichroism spectra of COX at pH 7.8 and pH 9.75. In trace A, the CD spectrum of COX (5 μM) in the Soret region was performed at pH 7.8, while in trace B, the CD spectrum was performed at pH 9.75. The units for ellipticity are mDeg.

heme *a* due to both heme *a* and *a*₃ contributing equally to the absorbance of dithionite reduced COX at 444 nm [28]. These results suggest that in the steady-state, the internal rate of electron transfer from heme *a* to heme *a*₃ is lower at alkaline pH and is rate-limiting for the oxidase reaction.

3.4. Circular dichroism spectroscopy

The increase in heme *a* reduction level at alkaline pH (Fig. 6) and the decrease in electron transfer rate (Fig. 2) suggest the possibility that alkaline pH induces a conformational change and possibly partially denatures the oxidase by changing protein–protein interactions or by removing subunits. This possibility was investigated by using circular dichroism spectroscopy in the UV region to detect conformational changes in the protein backbone, and also in the Soret (visible) region to detect changes in the heme environment.

The CD spectra in the far UV region of oxidized bovine COX solubilized in LM at pH 7.4 and pH 9.75 did not show any significant pH dependence (data not shown), indicating no large change in secondary structure at this pH. Also, SDS-PAGE showed that there was no loss of subunits from the enzyme at pH 9.75 (data not shown), emphasizing that no large change in the enzyme's tertiary structure occurred.

CD spectra of the oxidized enzyme in the Soret region are shown in Fig. 7. At pH 7.4, the CD spectrum of the oxidized enzyme has a strong positive transition at 426 nm, in agreement with the results obtained by Hill et al. [37]. After a 1-h incubation in LM at 9.75, the peak was red-shifted by 1.5 nm to 427.5 nm. Similar results were reported by Orii et al. [38]. Most important is that when the enzyme was returned to pH 7.4, the CD band returned to its position at 426 nm (data not shown). Hence, for incubation times up to 1 h, at least, the effect of pH on the heme environment is fully reversible, as is the effect of pH on the oxidase activity. The pH dependence of the red shift in the CD spectrum is shown in Fig. 8.

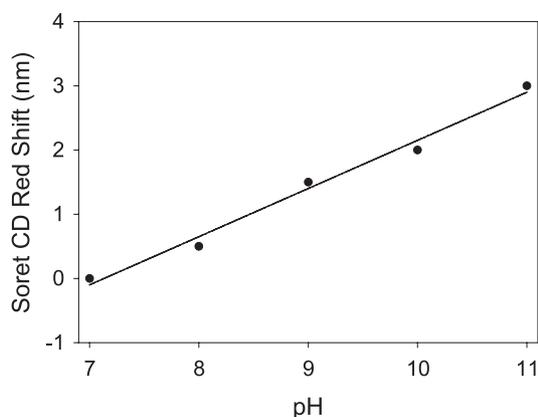


Fig. 8. The effect of pH on the position of the Soret maximum of the CD spectrum of COX. CD spectra were performed in buffers at different pH values as described in Section 2.

4. Discussion

The results presented show that the low oxidase activity of the enzyme at pH 9.75 is not due to any of the following: (1) the alkaline conformation of cytochrome *c*; (2) pH-induced denaturation of the oxidase; (3) decreased interaction strength between cytochrome *c* and the oxidase.

When assayed under low ionic strength conditions (10 mM) at a similar turnover number (8.0 s^{-1} to 10.0 s^{-1}), we found that the steady-state reduction level of heme *a* increased about 1.6- to 2-fold at pH 9.75 (~60%) as compared to pH 7.8 (~30%). Previous work with bovine heart COX solubilized in LM [39,40] or Tween-80 [41] has shown that electron transfer from heme *a* to heme *a*₃ is the internal rate-limiting step in COX turnover, and that electron transfer rates in both the bovine enzyme and the homologous bacterial (*R. sphaeroides*) oxidase are controlled by proton uptake in a tightly linked manner [42,43]. Hence, it is likely that the decreased electron transfer rate at pH 9.75 from heme *a* to heme *a*₃ in the current work is caused by a rate-limiting, redox-linked proton uptake by the enzyme.

The fact that the peroxidase activity of the enzyme is not reduced at alkaline pH strongly suggests that the limiting proton uptake is via the K-channel. Previous studies [15,16] have clearly demonstrated that the K-channel is not required for the reaction of the oxidase with H_2O_2 , shown schematically in Fig. 1. In contrast, decreased proton uptake via the D-channel would be expected to inhibit both the oxidase and peroxidase activities of the enzyme.

Previous reports have demonstrated pH-dependent change in the heme optical properties [44,45]. It is shown in the current work that the alkaline pH-induced 1.5-nm red shift in the Soret CD transition is reversible. This could be due to a change in the protonation state of an amino acid residue or residues (or the propionate groups of the heme itself) in the vicinity of one or both of the hemes. It is not known whether the changes in the heme environment are related to the observed slow rate of proton delivery through the K-channel to the active site.

It is important to note that our Yonetani preparation of the bovine oxidase [23] yields a monomeric enzyme in detergent solution [24]. Recent work has demonstrated that bovine oxidase which has been “monomerized” by either alkaline treatment or with high concentrations of Triton X-100 has distinct kinetic properties in single-turnover experiments [25]. However, monomeric enzyme retains high steady-state electron transfer activity [46,47] and exhibits normal proton-pumping activity [26,32] when reconstituted into phospholipid vesicles.

5. Conclusion

It is concluded that at pH 9.75, the activity of the “monomeric” bovine oxidase is severely inhibited primarily due to a limited ability to provide protons through the K-

channel. Proton flux through the D-channel may also be impaired, but under the conditions studied in this work, it is the proton transit through the K-channel that is rate-limiting. As a result, the rate of reduction of the fully oxidized binuclear center ($O \rightarrow R_2$ in Fig. 1) is inhibited. The characteristics of the bovine oxidase at this high pH, therefore, mimic the behavior of the bacterial oxidase with mutants that block the K-channel.

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Original article

Measurement of plasma catecholamines in small samples from mice

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Abstract

Introduction: A method is described by which it is possible to obtain measurements of plasma catecholamines in small samples from resting chronically catheterized mice. **Methods:** Standard alumina extraction procedures were systematically altered to maximize the recovery of catecholamines from 25- μ l samples. The technique used commonly available HPLC with electrochemical detection (EC) equipment which was optimized according to the manufacturers' guidelines. **Results:** The limit of detection is 40 pg/ml plasma noradrenaline and 20 pg/ml of plasma adrenaline and the resting levels were 300 pg/ml for noradrenaline and 80 pg/ml for adrenaline, both much lower than previously published. Comparison of resting levels from catheterized mice with those obtained by CO₂ plus decapitation and rapid decapitation reveal increases comparable to those reported following immobilization stress. The catecholamines adrenaline and noradrenaline were differentially increased by the two euthanasia methods. **Discussion:** The method was reliable, simple to perform and adequately sensitive. The resting levels of plasma catecholamines in mice are lower than previously published using a different sampling method. Differences in the norepinephrine and adrenaline increases produced by the different methods of euthanasia suggest caution in the selection of method in studies of sympathetic function.

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Keywords: Methods; Adrenaline; Noradrenaline; HPLC; Mouse

1. Introduction

The determination of the catecholamine concentration in the plasma is commonly used as a measure of the sympathetic nervous system's response to stress. In studies using rats, chronically catheterized animals are used to obtain samples to avoid handling stress and yield resting levels of 80 and 120 pg/ml plasma for noradrenaline and adrenaline by HPLC (Brimjoin, Hammond, Khraibi, & Tyce, 1994) to 510 and 390 pg/ml plasma for noradrenaline and adrenaline by radioenzymatic methods (Jezova, Ochedalski, Glickman, Kiss, & Aguilera, 1999). These amounts are readily measured employing common laboratory HPLC techniques given that the typical sample size is roughly 1 ml of blood. As plasma samples require prior extraction to eliminate constituents which co-elute with the

catecholamines, the final elution actually provides a concentration of the sample that offsets any loss due to the extraction procedure (Peaston & Weinkov, 2004), thus providing levels well within the limit of detection.

Due to the development of genetically altered mice, studies in this species are increasing. However, mice yield much smaller blood samples than the larger rats. The technique of fluorogenic derivitization prior to chemiluminescence, which can measure levels in samples of 10 μ l (Takezawa, Tsunoda, Murayama, Santa, & Imai, 1999), is not readily available in many institutions. Therefore, we developed a modification of the solid phase pre-extraction to provide adequate amounts of catecholamines for measurement by HPLC/EC in small samples. We compared catecholamine levels in blood collected from chronically catheterized mice (Bernatova, Key, Lucot, & Morris, 2002; Li, Sur, Mistlberger, & Morris, 1999) with blood collected after decapitation in conscious and CO₂ anesthetized mice.

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2. Methods

2.1. Animal use approval

The experiments were approved by the Wright State University Laboratory Animal Care and Use Committee.

2.2. Animals

Male C57BL/6J mice (Harlan Sprague–Dawley, Indianapolis, IN), 10–12 weeks of age, were used in the present study. The mice were housed at 22 °C with a 12:12-h dark–light cycle (0500–1700 h lights on). Animals were housed individually in plastic cages with wooden shavings. They were maintained on a standard pellet diet (Harlan Teklad, 0.5% sodium by weight) with tap water, ad libitum. After 10 days of acclimatization, the mice were randomly assigned to the three experimental groups: catheterized group (Cath; $n=6$), decapitated group (Decap, $n=20$) and CO₂+decapitated group (CO₂; $n=8$). To reduce the total number of animals used, the latter two groups were untreated controls of the same age from unrelated experiments. Mice in the Cath group were prepared with chronic carotid arterial catheters according to the method of Li et al. (1999). After surgery, a heparinized saline solution (100 U/ml) was continuously infused into the catheter at 25 μ l/h using a syringe pump (Model 220, Kd Scientific, Boston, MA). Blood samples (about 70 μ l) were collected from the catheter and placed into heparinized hematocrite tubes 12–13 days after the surgery. Because the blood was drawn while the animals were in their home cage, the mice were not handled during the procedure. Basal corticosterone levels measured in these mice are low, verifying that the mice are under resting conditions (Bernatova et al., 2002). Mice in the Decap group were removed from their cage and rapidly decapitated by guillotine. Mice in the CO₂ group were anesthetized by placing them in a CO₂ chamber for 15 s before decapitation as above. In the Decap and CO₂ groups, trunk blood was collected in eppendorf tubes with dilute heparin.

Blood samples for catecholamine determinations were collected between 9:00 and 12:00 a.m. and kept in ice. Within 15 min they were centrifuged for 10 min at 1000 \times g (4 °C) to separate plasma without lysing the cells. Plasma samples were kept at –80 °C until catecholamine determination in 25- μ l samples.

2.3. Sample preparation

The analysis of plasma catecholamines using electrochemical detection requires preliminary extraction over alumina to remove electroactive species that are not separable using reverse-phase liquid chromatography. Most procedures start with 1–2 ml of blood and the assay was initially based on this volume. Starting with the Bioanalytical Systems, Application Note 14, based on Hallman, Farnedo, Hamburger, and Jonsson (1978), we systematically reduced the

amount of alumina and volumes of liquids. Extractions were conducted in 1.5 ml eppendorf tubes using 10 mg of alumina, 125 μ l of 0.1 M HCl, 25 μ l of plasma, and 5 μ l of 5 mM sodium metabisulfite. It was acidified with 50 μ l of 0.2 N HClO₄ containing 1 ng/ml of dihydroxybenzylamine as an internal standard. Tris buffer was carefully added until pH 8.6. The resulting volume was the minimum that provided a good mixing action on a reciprocating shaker, which yielded a better percent (60%) recovery than that following vortexing (50%). After 10 min of shaking, they were centrifuged at 500 \times g for 5 min and the supernatant discarded. This was followed by two wash steps each consisting of vortexing with 1 ml of distilled water for 15 s, centrifugation at 500 \times g and discarding of the supernatant. The catecholamines were eluted with by vortexing with 100 μ l of 0.1 M HClO₄ containing 0.1 mM sodium metabisulfite for 15 s, centrifugation and aspiration of the supernatant into tubes for analysis.

2.4. Catecholamine determination

Chromatographic separation was conducted using a Coulochem II analyzer (ESA, Chelmsford, MA). Preliminary work led to the adoption of an ESA 80 \times 4.6-mm column packed with 3 μ m C18 resin and a 5014B coulometric detector optimized to 175 mV and –225 mV. These yielded better sensitivity than the 150 \times 2-mm column or the 5011 cell. The lesser performance of the thinner column may have resulted from not optimizing the mobile phase to fully exploit its chromatographic characteristics. The mobile phase consisted of a stock of 6.9 g sodium phosphate, 29.4 g of sodium citrate, 0.2 g of EDTA, 2.2 g of diethylamine HCl, and 0.5 g 1-octanesulfonic acid per liter, the pH adjusted to 3.1 using phosphoric acid. During analysis, 250 ml of stock was used plus an additional 120 mg of 1-octane sulfonic acid (varied with the age of the column), 12 ml acetonitrile, and 5.5 ml of dimethylacetamide. The flow rate was 0.4 ml/min and the injection was through a pre-measured 20- μ l loop.

2.5. Statistics

The data were analyzed by one-way ANOVA with Tukey's post hoc testing for each neurotransmitter (STATICA 6.1, Statsoft, Tulsa, OK). The significance level was set to $p<0.05$.

3. Results

Assuming an average of 50% recovery from the alumina extraction and specifying a minimum signal to noise ratio of 3:1, the limits of detection were 40 pg/ml plasma and 20 pg/ml plasma for noradrenaline and adrenaline, respectively.

Based on the successful analysis of six samples, we obtained resting levels of approximately 303 pg/ml and 82 pg/ml for noradrenaline and adrenaline, respectively (Fig. 1).

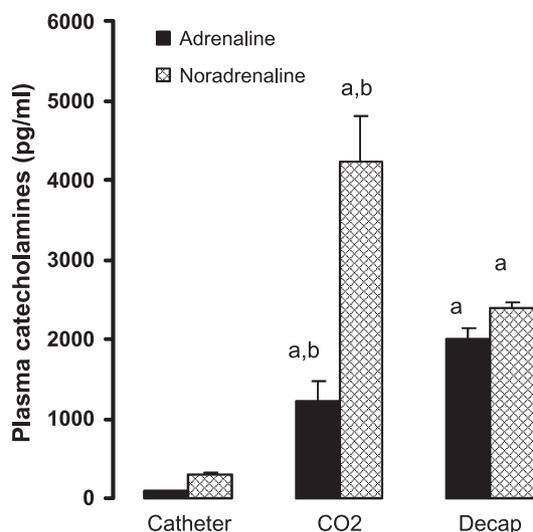


Fig. 1. Mean (SEM) plasma adrenaline and noradrenaline levels in the three groups. (a) Different from Catheter, $p < 0.005$. (b) Different from Decap, $p < 0.05$.

There were significant between group differences for noradrenaline ($F_{2,30}=47.66$, $p < 0.00001$) and adrenaline ($F_{2,29}=21.1$, $p < 0.00001$). Noradrenaline was higher in the CO₂ group than either the Decap group ($p < 0.0005$) or Cath group ($p < 0.0001$). Adrenaline was higher in the Decap group than either the CO₂ ($p < 0.02$) or Cath ($p < 0.0002$) groups.

4. Discussion

The method provides a means of measuring plasma levels of catecholamines from undisturbed catheterized mice using commonly available equipment. Key features included optimizing the volumes of the extraction constituents to improve recovery from small samples, use of shaking rather than vortexing to adsorb the catecholamines onto the alumina, and using vortexing at the other steps to minimize the time for the procedure. Optimization of the EC detection voltages was also important and was accomplished by following the manufacturer's recommendations. The use of the method made it possible to measure resting levels of plasma catecholamines in conscious mice which are lower than previously reported. There are potentially lower limits of detection possible by using thinner columns with optimization of the mobile phase and/or the use of larger injection volumes. Further, different methods of euthanasia appeared to produce different effects on the neuronal and adrenal components of the sympathetic system.

There is increased interest in studies using mice with the development of new genetically altered strains. Due to the technical challenges of blood vessel implantation in mice, it is common to obtain samples from the retro-orbital sinus, which can be done in less than 1 min and yields resting levels of 2800 pg/ml for noradrenaline and 4000 pg/ml for adrenaline (Jeong et al., 2000), well within the limits of detection using standard methods. However, the high rate at

which blood completes its circulation in mice provides ample time for a sympathetic response to the sampling procedure. The measurement of plasma catecholamines from catheterized mice becomes more challenging in part because the amount of plasma that can be removed is far less than is the case with rats. Published extraction procedures are not scaled optimally and the final elution dilutes the constituents rather than concentrates them. Using chronically implanted mice, preliminary studies in our laboratory found that the small volumes of plasma were beyond the limit of detection using the published techniques.

The resting levels of the catheterized mice in this study were lower than those reported following sample collection from the retro-orbital sinus, which were similar to those obtained in this study from trunk blood from mice euthanized by decapitation with or without CO₂ anesthesia (Jeong et al., 2000). These levels were well above the limit of detection obtained with the modified procedure.

An unexpected finding was the difference between noradrenaline and adrenaline in response to decapitation and CO₂ anesthesia followed by decapitation. Epinephrine rose to higher levels following CO₂ anesthesia and decapitation than after rapid decapitation, suggesting that the adrenal response was greater in the former method of sacrifice. In contrast, noradrenaline levels were higher after decapitation than after CO₂ anesthesia with decapitation, suggesting that the sympathetic neuronal activation was reduced by the CO₂ anesthesia. These differences are important in the selection of euthanasia method when sympathetic activity is under examination.

In conclusion, we modified the extraction procedure and HPLC determination of plasma catecholamine levels to enable measurements from chronically catheterized mice. Samples as small as 25 μ l can be measured with detection limits of 40 pg/ml of plasma noradrenaline and 20 pg/ml of plasma adrenaline. The differences between noradrenaline and adrenaline responses after the two different euthanasia methods suggest the importance in the selection of euthanasia method when sympathetic activity is being examined.

Acknowledgments

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Urinary Oxytocin as a Non-Invasive Biomarker for
Neurohypophyseal Hormone Secretion

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Abstract

The objective was to characterize the urinary oxytocin (OT) system with the goal of using it as a biomarker for neurohypophyseal peptide secretion. We studied urinary OT secretion in mice under three conditions: 1) in OT gene deletion mice (OT $-/-$) which lack the ability to produce the peptide; 2) after arterial vascular infusion of OT; and 3) after physiological stimulation with consumption of 2% sodium chloride. OT was measured by radioimmunoassay (RIA) and Surface-Enhanced Laser Desorption Ionization Time of Flight Mass Spectroscopy (SELDI TOF MS). In OT $-/-$ mice (n = 25), urinary OT levels were not detectable, while in OT $+/+$ mice (n = 23) levels were 250.2 ± 35.3 pg/ml. To evaluate blood/urine transfer, mice with chronic carotid arterial catheters were infused with saline or OT (5 or 20 pmol/min). Peak urine OT levels were 89 ± 11.5 and 844 ± 181 ng/ml in the low and high OT groups, respectively. Proteomic evaluation showed MS peaks, corresponding to OT (~1009 Da) and a related peptide (~1030 Da) with highest levels in mice infused with OT. Salt loading (5 days of 2% NaCl as drinking water) increased plasma osmolality (3.3%), increased plasma and urinary vasopressin (AVP), but caused no changes in OT. Thus, using non invasive urine samples, we document that urinary OT and AVP can be used to monitor changes in peptide secretion. Urinary OT and AVP, as well as other urinary peptides, may provide a viable biomarker for peptide secretion and may be useful in clinical studies.

Key Words: Sodium chloride, vasopressin, hypothalamus, genetic models, kidney, osmotic control, mouse

1. Introduction

Endocrine status is traditionally monitored by measuring blood levels of steroids, proteins and peptides. However, blood sampling is invasive, stressful and requires careful sample processing. Furthermore, in experimental animal studies, there are limitations in the volume of blood that can be collected. Thus, methods have been developed that utilize urinary measurements to monitor endocrine status [10,16,16,31,33-35,42,45,47].

Urine is a major excretory product, a water/electrolyte solution containing metabolic wastes. It is a complex mixture of salts, amino acids, peptides, carbohydrates and other solutes that is produced by renal filtration, reabsorption, and tubular secretion. Urine can be considered a window to the human body, useful for monitoring health and various medical conditions [12,32,44]. For example, in centuries past, urine was used to probe for diabetes. To test for the condition, the urine sample was poured onto the ground to see if it attracted ants and even tasted for sweetness. Urine is currently used to test for infections, immunological status and metabolic diseases as well as the presence of toxins and contraband drugs. The advantage of using urine samples is that they are collected easily and non-invasively as compared with blood, cerebrospinal fluid or tissue. For the endocrine axis, urine provides a chronic, integrated measure of hormone secretion, rather than information on acute, dynamic changes.

Our specific interest is in the utility of urinary peptide measurements as biomarkers of the neurohypophyseal axis. The two peptides, arginine vasopressin (AVP) and oxytocin (OT), share similar synthesis and secretory characteristics, varying by only two amino acids. They are synthesized by magnocellular neurons of the hypothalamus,

and the processed peptides are secreted from neurohypophyseal axonal terminals into the peripheral circulation [17]. The AVP and OT neurosecretory response is very rapid, related to the time course of release as well as the degradation by peptidases in plasma and target tissues [7,23,28]. The end result is urinary excretion of a mixture of metabolized and intact peptides.

The important issue is whether urinary peptide levels provide information on physiological function. Previous research has shown that changes in urinary AVP can indicate disease states in humans. For example, studies have suggested that measurement of urinary AVP levels may be used to diagnose polyuric and polydipsic syndromes [10,11,27]. Likewise, there are data that correlate changes in urinary angiotensin peptides and hypertension [16,52]. However, there is little information on urinary OT and whether it is physiologically regulated.

Experiments were performed to determine whether urinary oxytocin provides a good biomarker for peptide secretion in mice. We have taken advantage of the availability of gene deletion mice that lack the ability to produce OT [53], thus, providing null, peptide-deficient samples. For biochemical characterization of urinary OT, we applied a new mass spectrometric (MS) method that is especially useful for small molecular weight compounds [9,13,14,50]. Finally, we have examined peptide transfer from plasma to urine using a chronic infusion protocol and determined the effect of osmotic stimulation on peptide secretion.

2. MATERIALS AND METHODS

2.1 REAGENTS

OT and AVP were obtained from Bachem Inc. (Torrance, CA). All other reagents, unless stated otherwise, were obtained from Sigma Aldrich (St. Louis, MO).

2.2 Animals

Studies used male oxytocin gene deletion and wild type mice (25-27 gm) obtained from a WSU breeding colony. The gene deletion strain was developed by Young *et al* [53] and the original breeding pairs were obtained from Dr. Janet Amico (University of Pittsburgh, Pittsburgh, PA). Exons 2 and 3 of the OT gene were replaced with a vector containing the neomycin resistance gene, effectively disrupting their ability to produce OT. The breeding paradigm used heterozygous (OT +/-) parents, producing progeny with the same genetic and environmental background. Mice were genotyped using DNA prepared from tail extracts with a polymerase chain reaction (PCR) [53]. Mice were housed individually under conditions of constant temperature, humidity and lighting (12/12 hr of light/dark, lights on from 0500h) and had *ad libitum* access to standard mouse chow (0.4% NaCl) and tap water, unless stated otherwise. All experiments were approved by Wright State University's Animal Care and Use Committee.

2.3 Effect of systemic infusion of oxytocin

Mice with chronic carotid arterial catheters were housed singly in metabolic cages (Nalgene, Rochester, NY). The arterial catheter is threaded through a metal spring which is attached to the top of the cage with a fluid swivel, allowing the mice to move freely in their cages. The surgical procedure was performed as previously described [3,22]. Experiments were conducted after a recovery period of at least 7 days. Animals were

infused with saline (2 hr) followed by either saline (control) or a low (5 pmol/min) or high (20 pmol/min) dose of OT (2 hr). The infusion rate was 725 μ l/hr and urine samples were collected over 30 minute intervals.

2.4 *Effect of salt loading with 2% NaCl*

Adult male OT $+/+$ mice were housed singly in metabolic cages and assigned randomly into salt-loaded or control groups. Salt-loaded animals had *ad libitum* access to standard mouse chow (0.4% NaCl) and 2% NaCl as the sole drinking liquid. Daily fluid intake, urine output and body weight measurements were taken. After five days of salt consumption, animals were decapitated with collection of trunk blood and pituitaries.

2.5 *Urine and blood collections and measurement of creatinine clearance*

For the study of urinary OT in OT $-/-$ and OT $+/+$ mice, individual urine samples were collected (1100-1300 h). For the peptide infusion and salt loading studies, timed urine samples were collected on ice (30 min or 24 hr, respectively). Urine samples were transferred to 0.5 ml microcentrifuge tubes for storage. Blood was collected by decapitation and placed in tubes coated with lithium heparin. The blood was centrifuged at 4,800 rpm for 10 min at 4° C. Urine and plasma samples were stored frozen at -80° C until analyzed.

Creatinine clearance was measured to provide information on renal function. Creatinine was measured in 20 μ l of urine or plasma using the Jaffé method with a Dimension® RxL Max™ auto analyzer (Dade Behring, Deerfield, IL). In the reaction, creatinine combines with picrate in an alkaline solution to form a creatinine-picrate complex that can be measured by absorbance at 520 nm.

2.6 *Measurement of hematocrit and osmolality*

Heparinized blood was placed in microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) to measure hematocrit. The osmolality of each sample was determined using a Wescor vapor pressure osmometer, model 5500 (Wescor, Logan, UT). Undiluted plasma (10 μ l) or urine (10 μ l) that was diluted 1:4 prior to measurement.

2.7 Extraction of oxytocin and vasopressin from plasma and tissues

Plasma samples were extracted using C₁₈ Sep-Pak cartridges (Waters, Milford, MA) as previously described [8,43], with some modification. The method used a vacuum manifold (Waters, Milford, MA) to pull the samples and solutions through the columns. The steps were the following: 1) Wash C18 cartridge with 2 ml of 50% methanol followed by 6 ml of distilled water 2) apply plasma sample (100 μ l) to the cartridge, followed by 6 ml of distilled water 3) Wash column with 2 ml of 3% acetic acid 4) elute OT and AVP sequentially with 2 ml of 98% acetone and 80% acetonitrile containing 0.1% trifluoroacetic acid, respectively. This sequential solvent elution was shown to selectively elute OT and AVP with recovery greater than 76% [8]. Extracts were dried using a Speed Vac (Savant Instruments, Holbrook, NY) and dissolved in 225 μ l of assay buffer, containing: 62 mmol Na₂HPO₄, 13 mmol Na₂-EDTA, 0.1% bovine serum albumin (BSA), 0.02% NaN₃ (sodium azide) in deionized water at pH 7.4. Duplicate 100 μ l aliquots were used for RIA.

Posterior pituitaries were mechanically homogenized in 50 μ l of 0.1N HCl on ice. The homogenates were further diluted 1:100 in 0.1N HCl, followed by a final dilution of 1:10,000 in RIA buffer. An aliquot (200 μ l) of the diluted homogenate was used for RIA in duplicate.

2.8 *Oxytocin and vasopressin radioimmunoassay*

Plasma OT and AVP concentrations were determined as previously described [30], with some modification, using antisera specific for either OT or AVP. Briefly, 100 μ l of the plasma extracted, pituitary homogenate or urine (15 μ l urine with 85 μ l of RIA buffer) were assayed in duplicate and incubated with 100 μ l of RIA buffer and 100 μ l of antisera, diluted to 1:30,000 for OT or diluted to 1:60,000 for AVP, with 2% normal rabbit serum (Vector Labs, CA) for 24 h at 4° C. Then, 100 μ l (approximately 6000 cpm/100 μ l) 125 I labeled OT or AVP (New England Nuclear/PerkinElmer Life and Analytical Sciences, Torrance, CA) were added, and tubes were incubated for an additional 24 hr at 4° C. This yielded 25-30% binding of 125 I labeled OT or AVP. After incubation, 50 μ l of precipitating antibody (anti-rabbit gamma globulin) diluted 1/15 was added. The tubes were incubated for another 2- 4 hr at 4° C. 20% polyethylene glycol was added, and tubes were centrifuged at 3000 rpm at 4° C for 15 min to separate the free from the antibody-bound fraction. The supernatant was decanted and the pellet was counted using a gamma counter. The assay data was analyzed using a four parameter calculation [19]. For Plasma OT, the effective dose (ED) ED₂₀ was 293.63 pg/ml and the ED₈₀ was 16.65 pg/ml. For pituitary OT, the ED₂₀ was 130.5 ng/ml and the ED₈₀ was 7.4 ng/ml. For urinary OT, the average ED₂₀ was 1118.42 \pm 105.19 pg/ml and the average ED₈₀ was 55.42 \pm 7.33 pg/ml. For Plasma AVP, the ED₂₀ was 625.95 pg/ml and the ED₈₀ was 13.73 pg/ml. For pituitary AVP, the ED₂₀ was 278.2 ng/ml and the ED₈₀ was 6.1 ng/ml. For urinary AVP, the ED₂₀ was 1342.67 pg/ml and the ED₈₀ was 49.33 pg/ml.

2.9 *Characterization of urinary oxytocin by mass spectroscopy*

Urine samples were analyzed using Surface-Enhanced Laser Desorption Ionization Time of Flight Mass Spectroscopy (SELDI TOF MS). This method has been used extensively in our laboratory for evaluation of peptides and enzymatic peptide processing [13]. It is especially useful for measurement of low molecular weight peptides. Samples were desalted and purified with activated Millipore ZipTips® pipette tips containing a C₁₈ matrix. The ZipTips® were activated using 50% methanol followed by a rinse with deionized water. Urine samples (30 µl) were passed through the ZipTips® and the tips were rinsed with deionized H₂O. The samples were eluted with 80% Acetonitrile containing 0.1% trifluoroacetic acid. The eluent was spotted on a CIPHERGEN Weak cation exchange (WCX2) ProteinChips® (CIPHERGEN, Palo Alto, CA). Alpha-cyano-4-hydroxy cinnamic acid (CHCA) in 50% acetonitrile containing 0.1% TFA was applied as a matrix and allowed to dry. The WCX2 ProteinChips® were analyzed by CIPHERGEN SELDI Protein Biology System II (CIPHERGEN, Fremont, CA) and the resulting protein profile was the average of 80 laser hits over twenty different areas on each spot.

3.0 *Statistical analysis*

All results are reported as mean ± standard error of the mean (±S.E.M.). The results were analyzed using one-way ANOVA. Differences between the experimental and control groups were calculated using the two-tailed unpaired Student's t-test. GraphPad Prism 4 was used for statistical assessment of the data.

3. RESULTS

3.1 *Urinary oxytocin in oxytocin gene-deletion and wild-type mice*

To determine whether urinary OT levels are related to peptide synthesis, we tested urine samples collected from wild type and OT deficient mice (OT^{-/-}). In OT^{-/-} (n=25), OT levels were below the detectable limits of the assay. In OT^{+/+} mice (n=23), urine OT levels averaged 250.2 ± 35.3 pg/ml, ranging from 56.6-624.6 pg/ml.

3.2 *Effect of systemic oxytocin infusion on urinary oxytocin excretion*

To determine the precise relationship between plasma and urinary OT levels, we measured urinary OT after a 2 hr infusion of a low (5 pmol/min) and high (20 pmol/min) dose of OT in OT^{-/-} and OT^{+/+} mice. Since there were no significant differences in urinary OT levels between OT^{-/-} and OT^{+/+} infused with saline, the groups were combined. Infusion of OT produced a dose-related increase in urinary OT while saline infusion had no effect on OT levels (Figure 1). Peak urine OT levels in the low dose group were 89 ± 11.5 ng/ml, while peak OT levels in the high dose group were 844 ± 181 ng/ml ($P < 0.0001$) (Figure 2).

3.3 *Proteomic evaluation of urinary oxytocin*

To characterize the chemical identity of immunoreactive urinary OT, we used SELDI TOF MS with weak cation exchange ProteinChips®. Proteomic evaluation was performed on urine collected from OT^{-/-}, OT^{+/+} mice and mice infused with a high dose of OT (20 pM/min) (Figure 3). Results show the presence of peptide peaks with mass corresponding to OT and Na⁺ OT (~1009, ~1031 Da). The highest peak was in the form of Na⁺OT, related to the presence of salts in the sample. OT was highest in mice infused with OT, present at low levels in OT^{+/+} and not seen in OT^{-/-}.

3.4 *Salt loading on volume parameters and plasma/urinary oxytocin and vasopressin*

Consumption of 2% NaCl (5 days) was used to test osmotic responsiveness in mice. Salt loading had no significant effect on body weight, 24 hr urinary osmolality, plasma creatinine, 24hr urinary creatinine or creatinine clearance (Table 1). Under control conditions, 24hr urine osmolality was 3121 ± 88.20 mmol/L while in salt loaded mice (n=6) urine osmolality was 2611 ± 230.6 mmol/L. The lack of change in creatinine clearance indicates that GFR was not altered after the salt loading.

Salt loading had significant effects on urinary output, drinking, hematocrit, and plasma osmolality (Table 1). There was an increase in drinking and urinary output (~ 2 fold for each) as well as a volume depletion as indicated by the increase in hematocrit. Plasma osmolality was increased ~10 mM, suggesting that there was an osmotic response.

To determine if salt loading stimulated peptide secretion, we measured plasma, urinary and pituitary OT and AVP levels. Salt loading had no significant effect on the OT axis, no change in pituitary, plasma or 24hr urinary OT excretion (Figure 4). In contrast, salt loading increased plasma and urinary AVP, more than a 10 and 5 fold change, respectively.

4. DISCUSSION

Studies of peptide secretion usually rely on terminal or invasive blood sampling procedures. However, in mice, these methods are impractical because repeated blood

sampling produces hypovolemia and is stressful to the animal. Furthermore, terminal blood samples provide little information on dynamic neurosecretory changes. In our study, we used non-invasive urine samples to monitor peptide levels in mice. Key findings are: 1) there is a positive correlation between plasma and urinary OT in mice, as seen in OT gene deletion mice and in mice infused with OT; 2) proteomic evaluation using SELDI-TOF MS verified that urinary OT is structurally similar to the synthesized peptide; and 3) osmotic stimulation with salt loading activated the AVP system as seen by increases in urinary and plasma AVP, but had no effect on either plasma or urinary OT.

RIA methods for the measurement of OT and AVP were developed in the early 1970's [5,27]. These have been broadly applied to studies of plasma and tissue, but information is limited on urinary peptide excretion. Early studies showed that OT and AVP were present in human urine and that levels were correlated with plasma concentrations [1,5,27]. In view of the utility of mouse genetic models for physiological investigations, information on the use of urinary OT as a peptide biomarker is needed. Our results showed that immunoreactive OT was easily detectable in normal mouse urine with levels averaging 250 ± 35 pg/ml. The RIA method required no extraction and was rapid, sensitive and specific. However, it may underestimate circulating levels if there is aggregation of peptides as proposed by Kastin et al. [21]. In order to specifically verify OT's presence in urine, a negative assay control was required. We took advantage of the availability of an OT gene deletion mouse strain [53]. The OT $-/-$ strain, developed by two different groups, lacks the ability to produce OT [36,53]. The mice are able to reproduce, but not to lactate, and there are changes in social behavior, salt intake and

autonomic balance [4,15,26,38,40,53]. Our results showed that urinary OT was below detectable limits in the knockout mice, consistent with the genetic background. This documents a correlative relationship between plasma and urine levels. Previous studies in humans showed that urinary OT retained bioactivity and that OT levels averaged ~ 35 pg/ml [5].

To further characterize the plasma/urine relationship, we tested the effect of vascular infusion of OT on urinary OT excretion. The experiment used a chronically catheterized mouse model developed in our laboratory for cardiovascular studies [4]. Saline or saline containing OT (low and high dose) was infused into the carotid artery with concomitant collection of timed urine samples. Urinary OT levels increased rapidly after initiation of the infusion and began to decrease with termination of the peptide infusion. There was a clear dose-related increase in urinary OT. Peak urinary OT levels with the high dose were almost ten times that of the low dose even though the infused dose was only four fold difference (5 vs 20 pmol/min). This may be related to a saturation of the transport process or changes in peptide aggregation.

The next goal was to characterize the structural identity of urinary OT. For this study, we used SELDI TOF MS to measure peptide mass in urine of OT $-/-$ and $+/+$ mice and in urine of mice infused with the high dose of OT. Urine samples were extracted using C-18 zip-tips to concentrate the peptides and to remove excess salt. This is an important first step since peptides and proteins may be masked in un-extracted urine. Urine extracts and standards were applied to an ion selective metal chip (Protein Chip®) and analyzed by time-of-flight MS. Using SELDI TOF MS we characterized OT standards [8] and compared them to the urine profile. OT was detected as two peaks at

~1009 and ~1030Da with the higher MW peak representing the sodium adduct (Na^+OT). These results are consistent with previous studies of the hypothalamic/pituitary axis [8,20]. As expected, the MS OT peak heights were greatest in urine collected from the OT-infused mice., while the OT peaks were absent in urine from OT^{-/-} mice. The data supports the idea that OT measured in urine by RIA is consistent with the presence of the structurally intact OT peptide. This is the first paper to describe the characterization of urinary peptide using a SELDI TOF MS method and demonstrates a relationship between plasma and urinary OT. However, due to sensitivity issues, it is not possible to use this method to quantify basal urine or plasma levels.

The last objective was to determine the effect of physiological stimulation of the hypothalamic neurosecretory axis with an osmotic challenge. For this study, OT^{+/+} mice were given 2% NaCl as their sole drinking fluid for five days while the controls were given tap water. Previous studies showed that salt loading stimulates AVP release in rats, dogs and humans [29,39,41]. Our results in mice showed that salt loading produced no significant changes in body weight or GFR as measured with creatinine clearance. These results match earlier findings showing that salt loading did not alter GFR in rats or humans [6,25]. The mice drank large amounts of the salt solution (more than 6 ml/day), resulting in an osmotic load. This resulted in an increase in hematocrit and osmolality, systemic hypovolemia and hypertonicity.

Previous studies showed that salt loading stimulates OT secretion in rats, but not in humans [2,49]. Our results in mice are similar to the human in that osmotic stimulation did not alter plasma, urinary or pituitary OT concentrations. Central sodium stimulation also failed to increased plasma OT in mice while vascular stimulation

produced only a brief response [7]. Using a similar salt loading protocol, Ozaki, Y et al [37] reported that consumption of 2% NaCl increased hypothalamic OT mRNA in mice. However, there was no information on plasma or pituitary OT levels, making direct comparisons difficult. It is possible that the central mRNA response is not translated into a measurable secretory change or that the time course for the changes are different. For example in mice, the neuropeptide secretory response is rapid and transient, with peak plasma levels seen 1 min after peripheral osmotic stimulation [7]. In addition there is evidence that peptide mRNA levels are not always correlated with peptide secretion [18].

As expected, circulating AVP was significantly elevated after 5 days of drinking 2% NaCl. Other studies have shown that salt loading increases plasma AVP in animals and humans [24,46,48,49]. This is likely related to interactions with osmosensitive pathways, known to be connected to the AVP system [41,51]. The increase in plasma AVP is correlated with the increase in urinary AVP, suggesting that endogenous changes in peptide secretion can be monitored using urinary levels. This is supported by the lack of change in the urinary OT in the face of unchanged plasma levels. However, one must consider that changes in plasma and urinary levels may also be related to alteration in metabolism and not just a direct result of secretion.

The results of this study demonstrate a clear relationship between plasma and urinary OT, as documented using an oxytocin deficient animal model, systemic OT infusion and peripheral osmotic stimulation. Biochemical characterization suggested that the urinary peptide was the intact peptide form. Urinary OT, as well as other urinary peptides, may provide viable, non-invasive biomarkers for peptide secretion and may be useful in clinical studies.

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Figure 1

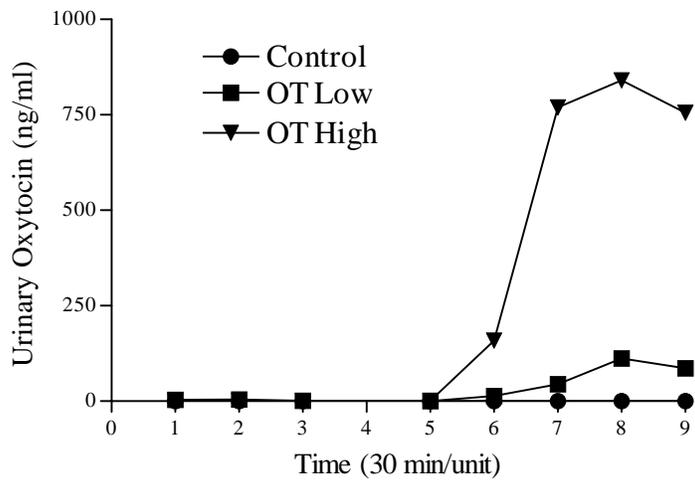


Figure 2

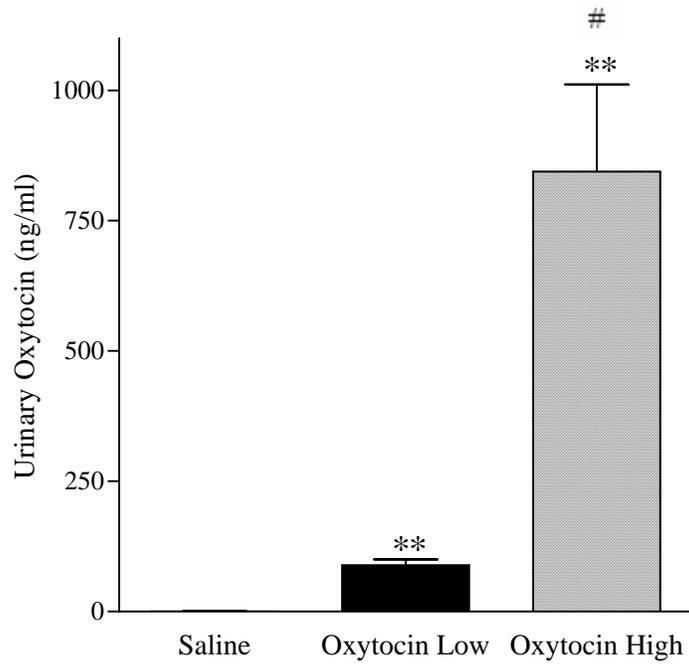


Figure 3

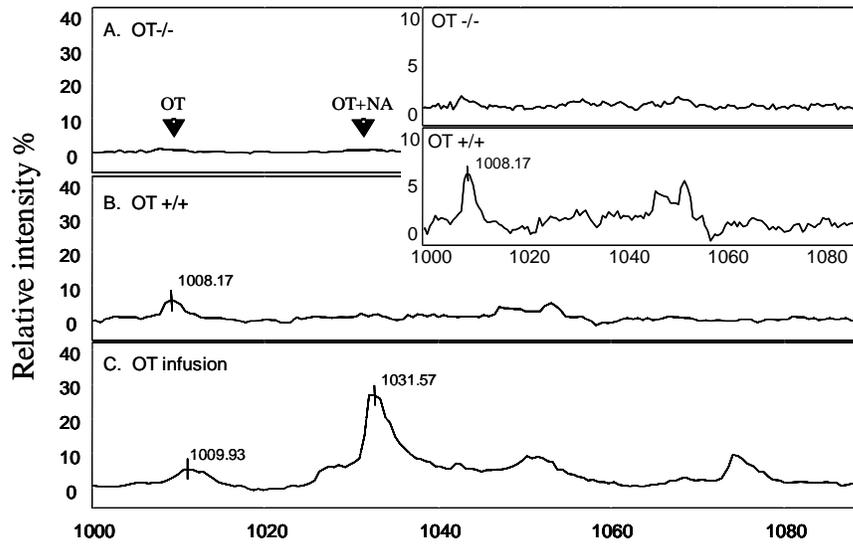


Figure 4

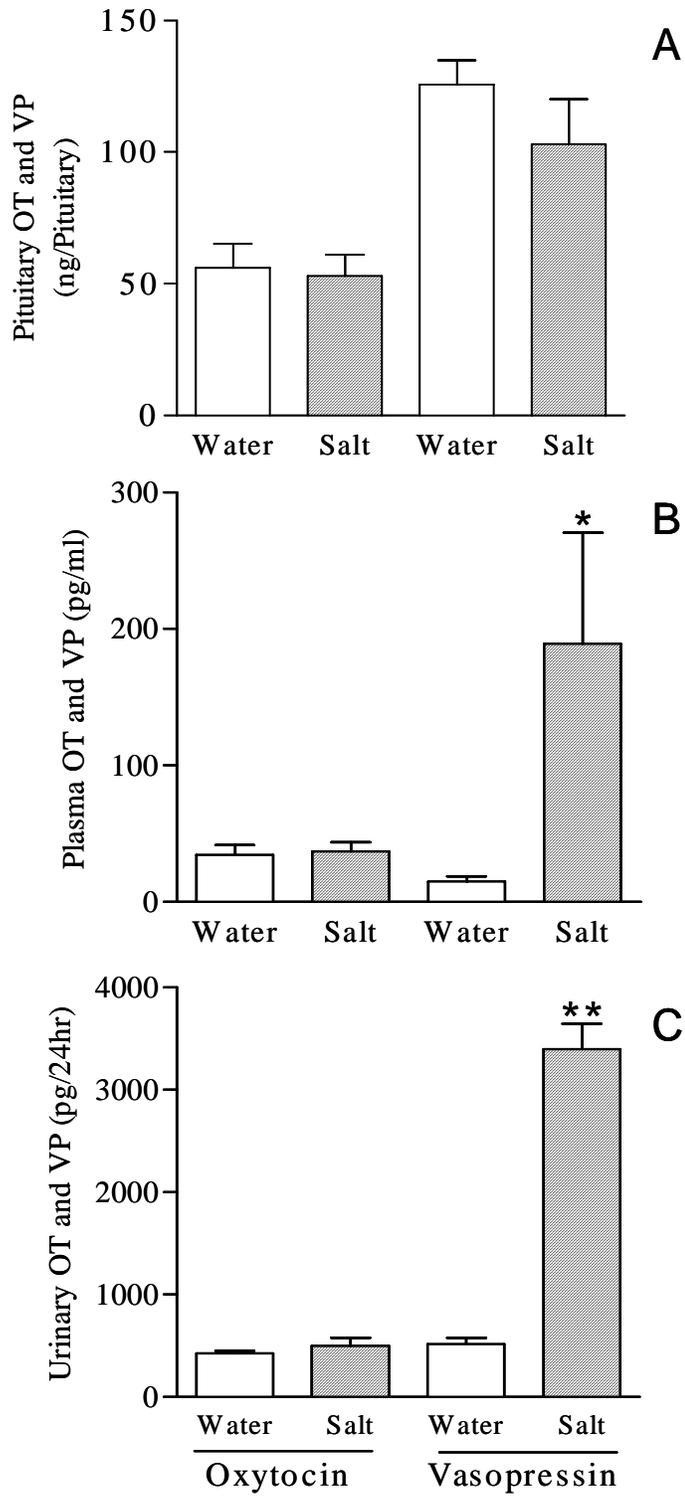


FIGURE LEGENDS

Figure 1. Effect of oxytocin infusion on urinary oxytocin levels. Mice were infused systemically with saline for baseline measurements (periods 1-3) and either saline (control) or a low (5 pmol/min) or high (20 pmol/min) dose of oxytocin (periods 5-8). The figure shows an individual animal through the three treatments.

Figure 2. Peak urinary oxytocin levels in mice infused with saline, a low (5 pmol/min) or high (20 pmol/min) dose of oxytocin. ** $P < 0.0001$ OT vs Saline # $P < 0.0001$ OT high vs OT low. N = 6 per group

Figure 3. Mass Spectrometric characterization (SELDI-TOF) of urinary peptides: A) Urine from OT $-/-$ mouse, B) Urine from OT $+/+$ mouse, and C) Urine from OT mouse infused with OT (20 pM/min). In (B) and (C) peaks (~1009, ~1031 Da) indicates the presence of OT and related peptides. In (A) these peaks are absent. Inset figure is a zoom-in on (A) and (B).

Figure 4. Effect of salt loading on A) Pituitary, B) Plasma, and C) 24hr urinary OT and AVP levels in mice. (N=5-6). Plasma AVP, salt loaded Vs control (* $P < 0.05$). Urinary AVP, salt loaded Vs control (** $P < 0.0001$).

Table 1. *Effect of salt loading on physiological parameters*

Parameter	Control	Salt	Significance
Body Weight (gm)	25.71 ± 1.0	26.37 ± 0.5	NS
Fluid Intake (ml/24hr)	3.742 ± 0.15	6.32 ± 0.63	** P=0.0025
Hematocrit (%)	45.20 ± 0.37	47.60 ± 0.51	** P=0.0053
Plasma Osmolality (mmol/l)	275.5 ± 3.16	284.5 ± 2.28	* P=0.0434
Plasma Creatinine (mg/dL)	0.175 ± 0.031	0.237 ± 0.021	NS
Urinary Output (ml/24hr)	1.49 ± 0.16	3.74 ± 0.65	** P=0.0074
Urine Osmolality (mmol/l)	3121 ± 88.20	2611 ± 230.6	NS
Creatinine Clearance (ml/hr)	19.87 ± 1.72	15.43 ± 1.71	NS
24hr Urinary Creatinine (mg/Vol)	0.79 ± 0.095	0.84 ± 0.068	NS

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